Nutrición Acuícola: Investigación y Desarrollo

L. Elizabeth Cruz Suárez, Denis Ricque Marie, Mireya Tapia Salazar, Martha G. Nieto López, David A. Villarreal Cavazos, Julián Gamboa Delgado, Martha Rivas-Vega, M. y A. Miranda-Baeza

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Índice

Improved High Soy Shrimp Feeds for the Pacific White Shrimp *Litopenaeus vannamei*

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Abstract

It could be suggested that, from a nutritional standpoint, one of the reasons for the popularity of Pacific white shrimp, *Litopenaeus vannamei*is the adaptability to a range of diets, tolerance of plant based feed ingredients and ability to utilize natural productivity. This species is very tolerant of alternative feed formulations and diets without fishmeal have been successfully implemented at the commercial level for a number of years. Despite the preponderance of information on plant based/high soy feed formulations, the wide spread acceptance of reduced fish meal feeds by the industry is still lacking. This review summarizes work we have accomplished with respect to low fishmeal or plant based/high soy feed formulations including the evaluation of new soy varieties, the assessment of SBM produced from alternative processing methods, incorporation of alternative protein

Key words: *Litopenaeus vannamei*, soybean meal, growth, digestibility, enzyme supplementation

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1. Introduction

The United States is one of the largest seafood markets in the world and shrimp is one of the most popular and valuable seafood products (Li *et al.*, 2014). Pacific white shrimp, *Litopenaeus vannamei*, is regarded as the most important cultured shrimp species worldwide and a top aquaculture commodity with a production of 3,314,447 t in 2013 (Bondad-Reantaso *et al.*, 2012, FAO, 2014). As shrimp consumption is expected to continue to increase, it is vital to develop sustainable alternative ingredients in the shrimp diets to support the rapid expansion of the shrimp industry (Achupallas *et al.*, In press).

Soybean meal (SBM) is often regarded as a cost-effective and nutritionally valuable protein source in shrimp and fish feeds. The popularity of SBM as a protein source is the result of a well-balanced nutrient profile, high digestibility, steady supply, expandable production and reasonable price (Amaya *et al.*, 2007, Davis & Arnold, 2000, Gatlin *et al.*, 2007, Lim & Dominy, 1990, Hardy, 2010, Samocha *et al.*, 2004). Although SBM is a proven alternative feed ingredient for shrimp feeds, it has a number of disadvantages including lower levels of some essential amino acid (e.g., methionine), the presence of several anti-nutritional factors (e.g. saponins, isoflavones, phytic acid, and trypsin inhibitor (Anderson & Wolf, 1995, Rackis, 1974, Rumsey *et al.*, 1994), and indigestible oligosaccharides (e.g. raffinose and stachyose) which may reduce performance of aquatic animals (Gatlin *et al.*, 2007, Knudsen, 1997, Parsons *et al.*, 2000).

Numerous studies have been conducted utilizing the soybean meal as a protein sources in aquatic animals. In a recent review, Sookying *et al.* (2013) summarized constraints when considering the move towards plant-based diets for *L. vannamei* with regard to balanced feed formulations. They concluded that the use of alternative feed formulations for *L. vannamei* is appropriate and warranted for commercial production. Over the years, many studies utilizing high soy shrimp feeds have been conducted by our laboratory at the E. W. Shell Fisheries Research Station (EWS), Auburn, AL, USA and the Claude Peteet Mariculture Center in Gulf Shores, AL, USA. A primary objective of these trials was to evaluate feed formulations that might overcome the disadvantages of conventional SBM. This review summarizes work we have accomplished in this respect

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including the evaluation of new soy varieties, the assessment of SBM produced from alternative processing methods, and the use of enzymes to improve digestibility.

2. New varieties/sources soybean meal used in diets of *L. vannamei*

Conventional SBM is available worldwide and is the dominant plant based protein source for aquatic feeds. However, as previously discussed, it generally has a moderate level of protein, relatively low levels of some essential nutrients and contains anti-nutritional factors (Herkelman *et al.,* 1992). To improve the nutritional quality of SBM one can chose to start by improving the characteristics of the soybean itself. As there is a rich history of genetic selection to improve agriculture characteristics or yield at the farm the same concepts can be applied to nutrient profiles of the soybean with the goal of producing a product of higher nutritional value.

Within our laboratory, a range of new non-genetically modified (NGM) soybean cultivars has been tested with the intent of improving SBM quality. Zhou *et al.* (2014) evaluated eight sources of SBM including six new NGM meals in practical diets formulated for *L. vannamei*. These new soy products contained higher levels of amino acids and protein as well as lower levels of some anti-nutritional factors. Results revealed significant difference in the final weight of shrimp reared on the various meals. Hence, some new lines of NGM soybean cultivars were promising and lead to improvement in the nutritional content of SBM and are suitable for inclusion in shrimp feed formulations. One of the primary advantages of these meals is an increase in protein content with meals reaching as high as 56% protein. Fang (2013) assessed thirteen soybean meal variants, which included one conventional soybean meal (FF), two commercially available NGM meals (Trifecta and 3010) and eight potential NGM varieties of which one was processed three ways (ingredients 13 processed as a meal, 14 boiled and processed as a meal, and 15 boiled and processed as press cake), in practical feed formulations for *L. vannamei*. Select meals were evaluated in an 8-week growth trial which was conducted using six replicate tanks per dietary treatment (10 shrimp per tank, initial weight 0.52 ± 0.04 g). The SBM-based

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reference diet was formulated using commercial SBM (45.3% diet), which was then completely replaced on an iso-nitrogenous basis with other experimental SBMs. As in the previous work, significant differences were observed in growth performance but not survival.

Fang (2013) also used principal component analysis to determine groupings of the meals based on biochemical composition. This was followed by the use of Pearson's correlation coefficients comparing growth performance of the shrimp and chemical content of the ingredients. A clear negative effect of a trypsin inhibitor on the shrimp's growth and weak correlations with stachyose levels and shrimp growth was observed. Both studies confirm that significant differences in growth are observed across a range of meals and that the level of trypsin inhibitor and possibly poorly digested carbohydrates in the meals are possible indicators of the growth performance of shrimp fed these meals.

3. Digestibility trials of different soybean sources

Digestibility data for feed ingredients is important to provide more precise information to balance feed formulations in shrimp feeds (Smith *et al.*, 2007, Zhou *et al.*, 2014). Determining digestibility of feed ingredients is widely used as an indirect assessment of nutrient availability in aquatic animals (NRC, 2011, Glencross *et al.*, 2007).

Within our laboratory, several studies of nutrient digestibility using different sources/varieties of soybean meal in *L. vannamei* have been documented. Many of these studies have utilized an equivalent reference diet and the same protocol for collection of feces and determination of digestibility coefficients (Zhou *et al.*, 2014, Fang, 2013). The apparent digestibility data for the reference diet and different soybean sources are presented in Table 1 and 2, respectively. Digestibility data for shrimp can often be quite variable compared to other aquatic species. To exemplify this we pulled data from several digestibility trials conducted over the years for which a similar basal diet was utilized. It is interesting to note that coefficients of variation across the data set (e.g. all individual data) ranged from 4.86 to 6.05% indicating globally there is about a 5% error associated with

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digestibility coefficients. Whereas the average coefficient of variation within the data sets (e.g. the average of the means for each set) was lower (2.0 to 2.9%). Clearly, there are differences between sets which are likely due to ingredient variability. However, it is our opinion this variability could also be due to differences between groups of experimental animals utilized in the trials. Overall, this indicates that the determination of digestibility coefficients within a set is quite comparable but between sets there is considerable variation and caution must be used when interpreting the data.

Table 1. Summary (mean and coefficient of variation in parenthesis) of apparent dry matter digestibility (ADMD), apparent protein digestibility (ADP), and apparent energy digestibility (ADE) of the basal diet used across a range of studies in our laboratory.

Reference	Replicates (n)	ADMD	AED	APD
1. Fang (2013)	3	68.24	74.52	85.74
		(2.47)	(2.20)	(5.02)
2. Fang (2013)	9	73.71	80.50	92.11
		(4.25)	(2.47)	(0.95)
3. Rhodes *et al.* (In press)	3	73.15	78.07	89.08
		(0.67)	(0.80)	(3.89)
4. Unpublished data	3	66.71	69.56	77.47
		(4.05)	(4.28)	(4.40)
5. Zhou *et al.* (2014)	4	72.25	77.92	88.95
		(0.84)	(0.71)	(1.09)
Average of mean CV	5	2.33	2.07	2.91
Average of all the data	22	71.67	77.39	88.26
CV		4.86	5.31	6.05

Coefficient of Variation (CV)

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Table 2. Apparent dry matter digestibility (ADMD), apparent protein digestibility (ADP), and apparent energy digestibility (ADE) of individual ingredients determined with juvenile shrimp using the reference diet technique (70:30 ratio).

	ADMD	ADP	ADE
Zhou *et al.* (2014)			
RD-A	85.2±4.9^{ab}	93.8±3.9^{b}	90.4±3.0^{a}
RD-B	71.3±5.3^{c}	96.9±4.6^{ab}	76.6±4.6^{b}
RD-C	88.3±2.2^{a}	99.8±1.3^{a}	90.6±1.1^{a}
RD-D	75.4±7.6^{bc}	94.2±2.3^{b}	83.2±6.4^{ab}
RD-E	76.8±13.6^{abc}	93.6±3.9^{b}	82.2±12.3^{ab}
RD-F	80.9±7.6^{abc}	95.7±2.1^{ab}	85.4±6.5^{ab}
RD-G	75.3±6.4^{bc}	95.6±1.3^{ab}	81.4±5.4^{ab}
RD-H	85.5±6.6^{ab}	97.7±1.9^{ab}	91.3±5.4^{a}
PSE[2]	1.31	0.52	1.12
P-value	0.0350	0.0750	0.0300
Fang (2013)			
Basal I	73.4±3.66^{bc}	80.2±1.39^{ab}	89.2±3.58 ^{a}
I-10	70.3±1.91^{bc}	76.2±4.38^{b}	91.9±1.27 ^{a}
I-11	77.9±2.17^{bc}	84.3±1.97^{ab}	96.3±1.67 ^{a}
I-12	63.4±5.43^{c}	72.5±4.46^{b}	90.4 ±3.34 ^{a}
I-13	64.8±4.16^{c}	73.0±2.28^{b}	80.3±2.13 ^{b}
I-14	76.6±7.69^{bc}	84.4±5.72^{ab}	83.1±5.40 ^{b}
I-15	78.0±2.02^{bc}	81.6±2.74^{ab}	93.7±1.58 ^{a}
I-16	78.5±13.74^{bc}	81.4±11.58^{ab}	92.7±1.88 ^{a}
I-17	82.1±11.69^{bc}	86.3±11.20^{ab}	96.3±3.59 ^{a}
I-18	95.4±4.43^{a}	96.1±2.63^{a}	98.0±0.69 ^{a}
I-19	73.4±3.66^{bc}	80.2±1.39^{ab}	89.2±3.58 ^{a}
Basal II			
FF	79.8±5.38^{bc}	83.0±3.96^{ab}	95.1±2.94^{a*}
Trifecta	77.8±6.27^{bc}	83.3±6.64^{ab}	90.4±3.56^{a}
3010	86.5±2.69^{ab}	88.7±2.27^{ab}	91.7±4.79^{a*}
PSE	1.81	1.58	0.84

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P-value	0.0003	0.0025	0.0001

[1] Mean of four replicates. Base on Duncan test, Number within the same column with different superscript are significant different (P <0.05). [2] Pooled Standard Error.

Zhou *et al.* (2014) assessed eight sources of soybean meal that include six new NGM soy varieties in *L. vannamei* feed formulations. In that study, to elucidate the interaction between apparent digestibility data and chemical characteristics of test ingredients, correction analysis followed by regression analysis was employed. Results from that study confirmed that protein dispersion index (PDI) and some antinutrient levels were decreased with increased processing temperature. These results are in agreement with previous studies by Francis *et al.* (2001), Genovese *et al.* (2006), and (Qin *et al.*, 1996). Additionally some ingredients with high levels of trypsin inhibition had reduced apparent digestibility coefficients (ADP and ADE) confirming the effect of a trypsin inhibitor.

In another study by Fang (2013), thirteen ingredients (as previously described) were assessed in feed formulations for *L. vannamei* using principle component analysis (PCA) to evaluate a wide range of variable and possible effects from multiple factors of chemical characteristics and processing. Results indicated that some ingredients had common characteristics while others were markedly different. Thus, different biological responses could results. Both studies indicated that some new lines of soybean meal could improve the digestibility coefficients in shrimp diets.

4. Validation in outdoor ponds and tank based systems.

There are numerous aquaria or clear water laboratory studies that have evaluated the use of soybean meals in shrimp feeds. However, until validated under commercial conditions, farmers are hesitant to pursue alternative diet formulations. Once alternative soybean meals are validated in indoor clear water systems where natural foods are not present the results can be validated outdoor tanks and research ponds. Within our laboratory, numerous pond based trials with parallel outdoor door tank studies have been conducted (list in Table 3 and 4, respectively). These studies, as well as those conducted by other laboratories, have demonstrated that *L. vannamei* can accept a wide range of

alternative feed formulations culturing under a variety of research condition (Achupallas *et al.*, In press, Sookying & Davis, 2011, Sookying & Davis, 2012, Yu *et al.*, 2013, Roy *et al.*, 2009).

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Table 3. Pond production trials of *Litopenaeus vannamei* fed experimental diets containing high levels soybean meal cultured in 0.1-ha ponds at Claude Peteet Mariculture Center in Gulf Shores, Alabama, USA.

Treatment	% inclusion SBM	SD (shrimp/m^2)	Period (wk)	IBW (g)	FBW (g)	Yield (kg/ha)	FCR	Survival (%)	Reference
9% FM	32.48	35	18	0.030	19.6	5847	1.24	87.2	Amaya *et al.* 2007
6% FM	34.82				18.4	5363	1.38	84.0	
3% FM	37.17				19.8	6548	1.12	94.0	
0% FM	39.52				20.7	6347	1.14	87.4	
10% PBM	55.12	35	18	0.038	16.0	5187	1.33	93.7	Sookying and Davis 2011
10% FM	53.71				16.9	5054	1.35	86.6	
10% DDGS	58.01				16.3	5265	1.32	92.2	
10% PM	58.00				16.2	5194	1.37	88.6	
17shrimp/m^2	53.24	17	16	0.015	25.3	2660	1.17	61.5	Sookying *et al.* 2011
26shrimp/m^2	53.24	26			20.7	3052	1.50	58.0	
35shrimp/m^2	53.24	35			22.0	4612	1.54	59.5	
45shrimp/m^2	53.24	45			21.9	6149	1.35	65.1	
0% SPC	58.01	35	18	0.013	13.5	4190	1.54	86.7	Sookying and Davis 2012
4% SPC	52.01				15.7	5051	1.28	89.5	
8% SPC	46.01				13.5	4508	1.45	92.9	
12% SPC	39.67				13.5	4479	1.44	93.3	
Fish oil	53.24	38	17	0.034	18.9	5254	1.40	74.0	Silva 2013
Soybean oil	53.24				18.0	5141	1.43	75.4	
Poultry grease	53.24				21.6	5070	1.45	65.6	
Flaxseed oil	53.24				21.0	5363	1.37	68.2	
0% CPC	46.69	38	16	0.023	20.5	5007	1.38	64.9	Yu *et al.* 2013
4% CPC	46.63				17.5	5190	1.34	77.6	

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8% CPC	46.48				17.2	5420	1.27	83.6	
12% CPC	46.32				18.2	5440	1.29	75.9	
0% GDDY	53.56	30	16	0.038	21.4	5527	1.03	86.5	Achupallas *et al*. in press
5% GDDY	48.17				19.8	5292	1.09	89.4	
10% GDDY	42.80				23.1	4760	1.23	69.6	
15% GDDY	37.51				21.7	5606	1.02	86.9	

Stocking density (SD), Initial body weight (IBW), Final body weight (FBW), Fish meal (FM), Poultry by product meal (PBM), Distillers dried grains with solubles (DDGS), Pea meal (PM), Soy protein concentrate (SPC), Grain distillers dried yeast (GDDY).

Table 4. Outdoor tank production trials (800 L/tank) of *Litopenaeus vannamei* fed experimental diets containing high levels soybean meal cultured in semi-recirculating system in Claude Peteet Mariculture Center in Gulf Shores, Alabama, USA.

Trt	% inclusion SBM	SD shrimp/m^2)	Period (wk)	IBW (g)	FBW (g)	Biomass (g/tank)	FCR	Survival (%)	Reference
9% FM	32.48	37.5	11.6	0.74	18.5	586.2	1.13	85.0	Amaya *et al.* 2007
6% FM	34.82				18.6	624.0	1.12	89.2	
3% FM	37.17				18.8	622.5	1.11	88.3	
0% FM	39.52				17.4	564.4	1.20	86.7	
10% PBM	55.12	37.5	12	2.13	18.7	553.0	1.32	98.7	Sookying and Davis 2011
10% FM	53.71				18.8	557.7	1.31	98.7	
10% DDGS	58.01				18.5	548.3	1.33	98.7	
10% PM	58.00				19.0	554.4	1.31	97.3	
15 shrimp/m^2	53.24	15	10	2.82	16.1	193.5	1.15	100.0	Sookying *et al.* 2011
25 shrimp/m^2	53.24	25			15.7	286.2	1.26	96.1	
35 shrimp/m^2	53.24	35			14.1	376.3	1.39	99.1	
45 shrimp/m^2	53.24	45			14.6	461.3	1.41	93.4	
55 shrimp/m^2	53.24	55			13.4	543.6	1.52	96.4	
65 shrimp/m^2	53.24	65			13.6	637.2	1.54	95.4	
0% SPC	58.01	37.5	10	1.00	13.5	398.9	1.28	98.0	Sookying and Davis 2012
4% SPC	52.01				13.7	397.2	1.28	96.7	
8% SPC	46.01				13.9	416.8	1.22	100.0	
12% SPC	39.67				15.0	411.5	1.23	98.7	
Fish oil	53.24	37.5	12	0.25	14.7	502.4	1.05	96.7	Silva 2013
Soybean oil	53.24				13.8	473.8	1.11	97.5	
Poultry grease	53.24				14.8	483.5	1.07	92.5	
Flaxseed oil	53.24				14.0	481.9	1.09	98.3	
0% GDDY	53.56	37.5	12	3.05	18.5	535.8	1.25	96.7	Achupallas *et al.* in press
5% GDDY	48.17				18.1	534.6	1.25	98.3	
10% GDDY	42.80				19.0	530.6	1.29	93.3	
15% GDDY	37.51				18.3	534.8	1.26	97.5	

Stocking density (SD), Initial body weight (IBW), Final body weight (FBW), Fish meal (FM), Poultry by product meal (PBM), Distillers dried grains with solubles (DDGS), Pea meal (PM), Soy protein concentrate (SPC), Grain distillers dried yeast (GDDY).

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Given that SBM is one of the primary protein sources in aquaculture feeds and the fact that it is widely available will likely increase the use of SBM in shrimp feeds (Sookying *et al.*, 2013, Lim *et al.*, 1998). Within our laboratory, the first indications that high levels of soybean meal could be used in shrimp feed was actually the result of work with a co-extruded wet poultry waste product (Davis & Arnold, 2000). This product was primarily soybean meal and thus indirectly demonstrated a high tolerance to soybean meal. The efficacy of these diets was later demonstrated in outdoor tanks (Samocha *et al.*, 2004). A range of studies identifying limiting nutrients and validating the response to nutrient limitations when natural foods were present in outdoor or green water systems have been conducted. This included evaluating the response of shrimp reared in outdoor tanks to: reduced levels of marine oils and highly unsaturated fatty acid (HUFA) supplements (Gonzalez-Felix *et al.*, 2010, Samocha *et al.*, 2011, Patnaik *et al.*, 2006, Samocha *et al.*, 2010) cholesterol supplements (Morris *et al.*, 2011); as well as phospholipids, phosphorus (see below) and various attractants such as squid liver meal (Morris, 2008). These studies confirmed that nutrient limitations induced under laboratory conditions can also be observed under field conditions when natural foods are present.

These studies complimented a series of studies to demonstrate the removal of fishmeal with poultry by-product meal (Amaya *et al.*, 2007) followed by the removal of poultry by product meal to produce a plant based feed formulation (Sookying & Davis, 2011). Over the years we have looked at a range of high soy feed formulations in combination with a number of proteins sources including fishmeal, poultry by-product meal, distillers dried grains with solubles, pea meal, soy protein concentrate, and corn gluten meal. Results of these studies are summarized in (Table 1 and 2).

Our most recent work, Achupallas *et al.* (In press), demonstrated that the high inclusions of SBM combined with different levels of grain distillers dried yeast can provide adequate growth performance in *L. vannamei* reared in outdoor tanks and production ponds. While pond based data provides a demonstration in the most widely utilized culture unit by the commercial industry, these results still needed to be validated using more statistically robust methods. Hence, these studies were all confirmed in parallel using outdoor tanks where water quality and natural

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foods are equalized. The validity of these trials are confirmed by excellent growth rates, adequate survival and good FCRs (Table 4). These data confirm that high soy shrimp feeds can be successfully used in diets for *L. vannamei*.

5. Enzyme supplementation – phytase

The presence of anti-nutrients in plant-based feed ingredients has been a challenge for the commercial feed mills. Thus, a number of studies have been conducted to evaluate the potential of reducing the adverse impact of anti-nutrients in plant-based feed formulations in shrimp. Phytate is one anti-nutrient found in most seeds and present in high enough levels in soybean meal to negatively impact growth performance of shrimp.

Phytate can produce a negative impact on nutrient digestibility and mineral availability (Kumar *et al.*, 2012). Fifty to eighty percent of the total phosphorus (P) content in plant seeds is found in the form of phytate (Ravindran *et al.*, 1995). Phosphorous in this form is generally not bioavailable to shrimp due to the lack of the intestinal digestive enzyme, phytase which is required to release P from the phytate molecule (Jackson *et al.*, 1996). Phosphorus is the most commonly supplemented macro-mineral which not only adds to the cost of the diet but also contributes to pollution loading of the culture system and the surrounding environment.

Phosphorus requirements of shrimp have been relatively well studied under laboratory conditions Davis *et al.* (1993). Few studies have been carried out under field conditions where natural foods could supplement the requirement. To determine if P is limiting in plant based diets under field conditions, three diets were formulated 1) without a P supplement, 2) nutritionally adequate P supplement and 3) P in excess of the requirement (Table 5).

Zhou, Y. and Davis, A. 2015. Improved High Soy Shrimp Feeds for the Pacific White Shrimp *Litopenaeus vannamei*. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 1-22.

Table 5. Ingredient composition (g 100 g^{-1} as is) of three experimental diets designed to contain 35% protein and 6.4% lipid using primarily plant-based ingredients. The three diets included a basal diet without a P supplement (0.59 % P), a diet with P supplement designed to meet the requirement (1.07% P) and a diet in excess of the requirement (1.53 % P).

	0.59% P*	1.07% P	1.53% P
SE Soybean meal	60.5	60.5	60.63
Corn Gluten meal	8.0	8.0	8.0
Menhaden Fish Oil	6.4	6.4	6.4
Wheat starch	3.93	0.93	0.0
Whole wheat	18.0	18.0	15.8
Trace Mineral premix	0.5	0.5	0.5
Vitamin premix	1.8	1.8	1.8
Choline chloride	0.2	0.2	0.2
Stay C 250 mg/kg	0.07	0.07	0.07
CaP-diebasic	0.0	3.0	6.0
Lecithin	0.5	0.5	0.5
Cholesterol	0.1	0.1	0.1

*P is the analyzed value as determined by New Jersey Feed Laboratory.

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The test diets were then offered to replicate groups of shrimp under standardized conditions. The methods used in this study were similar to those that have been used in numerous published studies from the AgriLife facility. The study was initiated with juvenile shrimp (0.59 ± 0.17 g) and conducted over an 84-day period in outdoor tanks (650 L working volume and 0.85 m^2 bottom area) stocked at 30 shrimp m^{-2}. The shrimp were feed twice daily throughout the study. The response of shrimp to plant based diets containing various levels of P supplements is summarized in Table 6. At the conclusion of the trial, average weight, growth, FCR and yield of the shrimp were significantly improved by the addition of P to meet the dietary requirement. These results confirm that even in the presence of natural productivity, shrimp reared on diets without animal meals are deficient in P. Furthermore, the addition of P in excess of the requirement does not improve growth and would simply contribute to increased costs and pollution loading. An alternative to supplementing high levels of P to plant based diets is to increase the biological availability of P in the feed.

Table 6. Mean final weight, survival, yield and FCR values of the Pacific white shrimp, *Litopenaeus vannamei*, reared for 84 days on diets with different levels of phosphorus in an outdoor tanks system. Values represent the mean of five replicates. Within a column, values with different letters are significantly different based on Student-Newman-Keuls multiple range test.

Total Phosphorus	Biomass (g)	Final Weight (g)	% Survival
0.59%	488.3[a]	18.8[a]	96.2
1.07%	515.4[b]	19.9[b]	99.2
1.53%	534.9[b]	20.6[b]	97.7
P value	0.0023	0.0008	0.5199

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Phytases (myo-inositol hexakiphosphate phosphohydrolase) are a group of enzymes that can catalyze the hydrolysis of phytate, and reduce the negative effects of phytate on nutrient availability in monogastric animals (Mullaney *et al.*, 1999, Mitchell *et al.*, 1997). Several studies have documented that phytase can help improve P bioavailability, growth performance, P digestibility when feeding phytase-supplemented feeds, and the efficacy of top spraying diets with phytase in fish (Zhu *et al.*, 2014, Papatryphon *et al.*, 1999, Sajjadi & Carter, 2004, Sugiura *et al.*, 2001, Yoo *et al.*, 2005, Sugiura *et al.*, 1999, Liu *et al.*, 2012, Liebert & Portz, 2005, Cain & Garling, 1995, Vielma *et al.*, 2004, Jackson *et al.*, 1996, Eya & Lovell, 1997, Rodehutscord & Pfeffer, 1995, Vielma *et al.*, 2000, Debnath *et al.*, 2005) and shrimp (Biswas *et al.*, 2007, Fox *et al.*, 2006). A review of the application of microbial phytase in fish feed was reported by Cao *et al.* (2007). However, limited data on the use phytase in *L. vannamei* are available to date.

Qiu (2015) conducted a series of growth and digestibility trials on the response of *L. vannamei* fed a high soy based diet with graded levels of phytase supplementation. Results revealed that shrimp growth performance, P and protein retention, and P whole body content were not significantly influenced by dietary phytase supplementation in the high soy based diet. However, protein digestibility was improved by phytase inclusion at both 500 and 2000 IU/kg. P digestibility was also improved by 2000 IU/kg phytase supplementation. Based on these results, we demonstrated that the phytase supplementation can improve the P and protein digestibility in *L. vannamei*.

With the exception of phytase, other enzyme supplements are not widely utilized and/or there is limited data. There are several proteinases and carbohydrates, which could be added to aquatic animal feeds when high inclusions levels of alternative ingredients are utilized (Hardy, 2000). Hence, more studies for other enzyme supplements should be pursued.

6. Conclusions

It is abundantly clear that a high soy shrimp diet is accepted by Pacific white shrimp as long as the diets have balanced levels of nutrients. Imbalances in nutrition, the use of poor quality ingredients and or sensitivity to some components of soybean meal can lead to poor

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performance. However, given the cost and benefits of soy based feed formulations the industry is strongly encouraged to reduce animal protein and in particular fish meal, to levels that provide the best economic returns.

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"Floc" Story

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Abstract

What makes the effectiveness of the "floc" on growth of tropical shrimps and its reproductive capacities? The "floc" as called it 40 years ago also named "particulate" biomass came from the design of sludge from that purify the water on the one hand and consideration on the power mode of the other bivalves. When we come to these concepts we arrive at the concept of floc which produces a kind of shrimp "external rumen". What are the benefits that the animals retrieve? This article gives a review of chains of explanations in terms of environmental quality and the nutritional physiology of shrimp lead to what is now the most popular growing breeding model. This model represents a future opportunity to shrimp farming in the world by saving water, increased stocking densities and lower surfaces used and probably a freedom from direct use of the coastal zone. Will therefore be considered in this article nutritional aspects, changes in phytoplankton and microbial population and the interactions between nutrients, dissolved organic matter that give this chaotic culture medium and difficult aspect to control in any case and renderers very difficult to model. But some aspects of the shrimp physiology as its food/feed supplement behavior seem fixed in this farming system called "moulinettes" and recently resumed in the acronym "BFT biofloc" technology sometimes slightly obscuring the entire history of research in this area.

Key words: floc, bacteria, plankton, water quality, sludge, intensive culture, juveniles, breeders

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Parte de la historia del “floc”

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Resumen

¿Cuál es la eficacia del "floc" sobre el crecimiento de los camarones tropicales y sus capacidades reproductivas? El "floc" como se denominó hace 40 años la “biomasa partículada", provino del diseño de recuperación de los lodos a partir de los sistemas de purificación de agua por un lado y la consideración del modo de alimentación de los bivalvos por otro lado. Cuando llegamos a estos conceptos se llega al concepto de “flóc” que se define como un "rumen externo" para las especies como los camarones. ¿Cuáles son los beneficios que los animales obtienen? Este artículo ofrece una revisión en términos de calidad del medio ambiente y la fisiología nutricional de camarones a lo que hoy es el modelo de cultivo en crecimiento más popular. Este modelo representa una futura oportunidad para el cultivo de camarón en el mundo por el ahorro de agua, el aumento de las densidades de población, superficies más bajas utilizadas y, probablemente, una libertad por evitar el uso directo de la zona costera. Por lo tanto, serán considerados en este artículo los aspectos nutricionales, cambios en el fitoplancton, la población microbiana y las interacciones entre nutrientes, materia orgánica disuelta que dan a este medio de cultivo caótico un aspecto difícil de controlar en cualquier caso y es muy difícil de modelar. Sin embargo, algunos aspectos de la fisiología de camarones como su comportamiento con suplemento alimenticio, pienso que parecen fijos en este sistema de cultivo llamado "moulinettes" y recientemente se reanudaron con el acrónimo tecnología biofloc "BFT " a veces oscurece parte de la historia de la investigación en esta área

Palabras clave: floc, bacteria, plankton, calidad de agua, lodo, engorda intensiva, juveniles, repr

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Sommaire

Gaxiola, G. and Cuzon*, G. 2015. "Floc" Story. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Univers idad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 23-50.

Introduction

When swimming in polynesian lagoon it can be seen through the mask some small whitish elements suspended in clear water poor in phosphorus that is trapped by zoothanthelles from coral reef; this is what is called a "light floc" support for bacteria and potential food for fish larvae, crustaceans and molluscs in addition to dissolved organic fraction that can be assimilated by oysters.

Long time without knowing, it promoted bacterial growth. Moreover originally when the COP met the Ralston Purina team in Crystal River (USA) early results in floc were due only to bacteria, bacteria of nitrification that transformed ammonia into nitrites and nitrates and then medium stood at equilibrium.

Up to 1977, COP/Tahiti used 12m^2 flat bottom circular tanks in fiberglass. Results obtained with *P.vannamei* and *L.stylirostris* were consistent, allowing to get in final 2.3kg per m^2,av. weight 22g with 108 per m^2 of shrimp in very good health. In theory, it yielded about 46mt/ha/year with a 10% daily water renewal. In 1977, CNEXO/cop launched a program on intensive shrimp farming to 100 individuals per m^2 based on permanent agitation and aeration of the medium and low turnover, the pumping cost being an important factor in the livestock economy. Results evidenced the role of bacteria in the system that produced together with organic matter (feed, leftovers, feces) in constant suspension, the formation of «activated sludge» (Ecotron, 1980). This compound was already found beneficial for *M japonicus* and incorporated up to 12% in a compounded feed (Shigeno, 1972). Actually there is a regain of single cell protein to replace fishmeal (Ali, 1992).

The shrimp were receiving so rich carbohydrate feed, low protein that favored the bacteria development; working in dark condition, it was a way to simplify the model turning into pure bacterial mode. Shrimp such as *P.vannamei* or *P.stylirostris* get accommodated perfectly. But we had to move to another level and this is where the system is complicated with the contribution of microalgae. We had to cope together to achieve significant production scale. Studies were then focused on the balance between bacteria and phytoplankton (Ecotron, 1980-85). Phytoplankton contributes to the formation of

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exopolysaccharides after the phyto ‘s phase of senescence. That’s provide in the culture medium a support for bacteria, filamentous microalgae, protozoans and nematods,.. nominated as “particulate biomass”. Obligatory scaling experienced some success, then the Sopomer farm used the "floc" technique but the management of culture medium faced little to small problems that took over and the farm switched to management of “green water” which failed to make this shrimp farm sustainable.

Material and methods

-The first technique was initiated by RP in Crystal River in 200L indoors tank
-Ecotron experiment in 5L, and to $10m^3$
-on initial biomass,
-transfer from previous preformed floc,
-adding salts or sugar as carbon source
-mastering floc in small tanks, or floc+phytoplankton in outdoors volumes

“Five different aspects of the impact of bacterial communities in the cultures were studied: (i) the quantitative and qualitative development of bacterial communities (biomass, production, species diversity, distribution); (ii) estimation of the metabolic potential of isolated bacteria (catabolic potential, nutritional requirements, auxotrophy/prototrophy); (iii) potential for liberating telemediaters by bacteria, vitamins B_{12}, thiamine, biotine, antibacterial substances, anti-algal substances; (iv) the zoopathogenic power of certain strains and (v) particulate or dissolved primary production and an analysis of feces with heterotrophic activity” (Martin *et al.*, 1978).

The following scheme summarizes main steps of shrimp life cycle where floc is commonly set to enhance weight gain, health status and potential for reproduction in clear water (CW) compared to floc.

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Pl's	juveniles	comercial size	ponds	maturation area	*eyestalk ablation*	spawn
	5g	20-25g	35-40g	CW+ff		
feed+meoifauna						
feed+floc			floc+feed			
4-5months			4months	2-3weeks		

-managing as in SE Asia (Vietnam, Indonesia, Malaysia, Philippines… with aerators and enough water depth

-managing back to its initial i.e. in hyper-intensive system indoors (Poulain, com. pers. 2014).

Results

1972, Shigeno formulated diets with activated sludge for juveniles *P.japonicus.* Reported by those authors that could be considered as a pioneering work in direction of further floc utilization to participate to the supply of single cells protein source.

1972 Ralston Purina and Aquacop (1976): the first trials were conducted in a system called “moulinettes” that involved a bacterial floc. This work done in collaboration with Ralston Purina was conducted on wild specimen and shrimp fed on marine ration MR^{30} or MR^{25} showing a possibility for a low requirement for protein under floc conditions.

Table 1-Trial in “moulinettes” with *L.vannamei* for 27days.

feed	shrp/m^2	IW	FW	wt gain%	survival%	FCR
MR^{30}	40	3.3	6	81	100	1.7
MR^{25}	40	3.2	5.8	78	95	1.7
18.1.1.0	40	3.2	4.9	53	100	2.2
MR^{30}	150	3.1	5.5	77	91	-
MR^{30}	260	3.3	5.6	70	91	-

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Reported results on *L. stylirostris* were similar to those observed on *L.vannamei* (Table1) and shrimp placed in 170l tanks were fed at 4%biomass daily. The set up of "moulinettes" as in Ralston Purina (13m^3 round tanks let achieve stocking densities approaching 3.5kg/m^2 at end (Aquacop, 1981).

1975-80 Ecotron program: In 1978, two circular concrete tanks 700m^2 around 0.8m depth with compacted coral in the bottom were built up at COP to change scale of production. Preliminary trials raised problems such as N-ammonia increase after 35d and no nitrification therefore nitrates did not form. Inhibition of nitrifying bacteria could be due to sterilized action of solar UV and a poor re-suspension of bacterial floc. The apparition of toxic dinoflagellates provoked massive mortalities.

In 1980 results and analysis evidenced the role of bacteria in the system and nitrifying bacteria will transform N-ammonia excreted in nitrate; the process of total nitrification lasted 29d without water renewal. Those bacteria definitely were implicated in the feeding of shrimp. Floc composition was surveyed with proximate analysis, but only few data exists on lipid classes indicative of a presence of mono and tri-acylglycerids and free cholesterol, 2.6% total lipids (Galois, 1980).(Table 2) and phospholipids remained

Table 2. floc composition for acylglycerids and sterols (Galois, 1980).

	mg/g dry wt.	%TL
acylglyc.		
mono	0.77	3.39
di	0.16	0.71
tri	0.6	2.64
sterols		
free	0.59	2.6
esterif.	0.03	0.13

largely to be taken into account because of presence of live microorganisms in constant supply.

Gaxiola, G. and Cuzon*, G. 2015. "Floc" Story. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Univers idad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 23-50.

Three stages were described by Sohier and Bianchi (1985) in a closed system with interrelationships between autotrophic nitrifying bacteria and the related heterotrophic community (Fig 1). During the first few days the bacterial community derived from seawater, shrimp and feed. Both ammonia and nitrite increase and the chemical composition of the water select a low diversity community requiring free amino acids and organic C plus energy. 20d later, the bacterial community reaches equilibrium in a so-called "non-stressed environment. The heterotrophic community on the contrary possessed a high diversity for metabolites. Few months later, there is a dominance of bacteria from feces and feed/food.

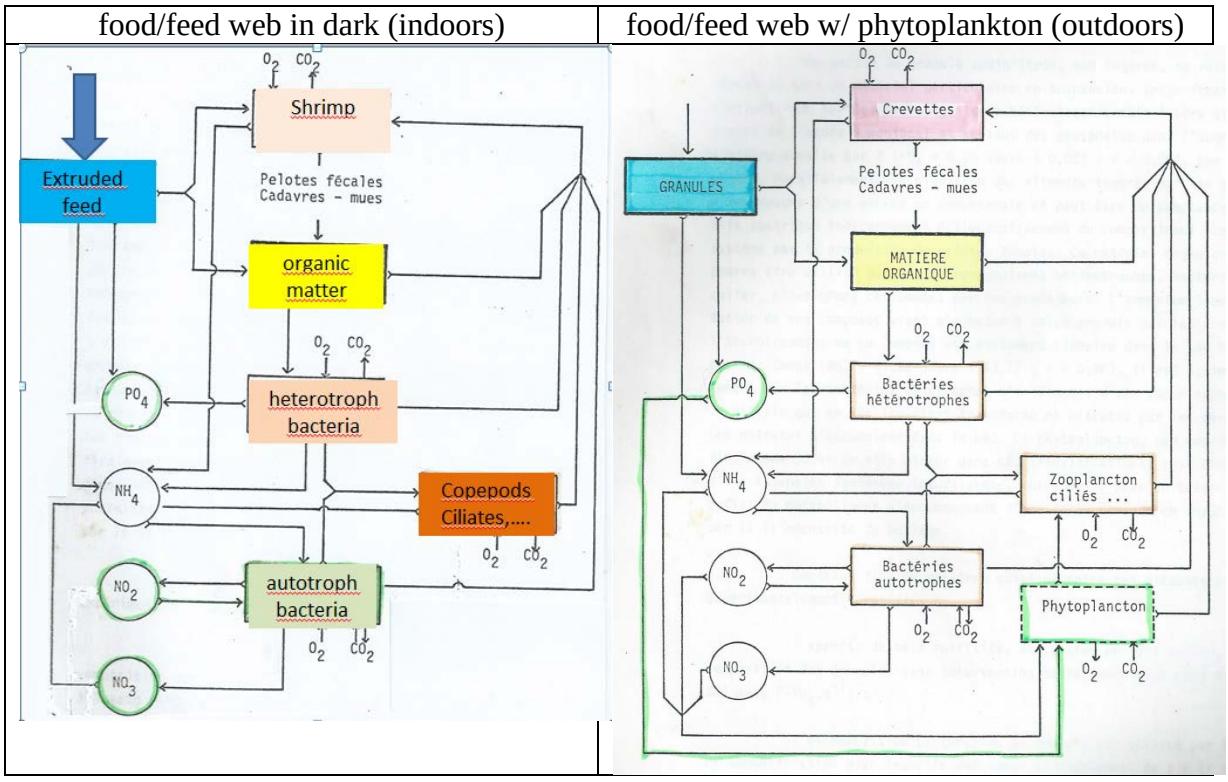

Fig 1. Ecotron, 1980

-sludge: the floc system can be managed without too much problem and with a minimum of microalgae. Yet, in routine, there is a need for flushing the tank that liberates dark black water, made of liquid sludge to be eliminated each day. In this context, the «zero water exchange» will produce foam on water surface. In practice, at lab level, during the

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Ecotron program short term experiments trials at 10, 30 and 50% water renewal in "moulinettes" K, L and M produced the following:

Table 3. Effect of water renewal on survival in floc with *P.monodon* juveniles (Ecotron, 1980).

	units	K		L		M	
water renewal		0		10		50	
		t_0	t_{35}	t_0	t_{35}	t_0	t_{35}
av. wt	g	5,4	6,3	3,9	**5,8**	3,6	5,3
av. wt gain	g		0,9		1,9		1,7
daily wt gain	% j_{-1}		0,5		1,4		1,3
feed input	g d_{-1}		280		197		167
biomass	g	1036	476	1002	800	1003	770
yield	%		0		12		9
survival	%		46		**80**		77

NB. «moulinettes» is a term called to describe a tank system where water depth is enough (>1m) to allow convection movements in the medium and maintain particulate biomass in suspension all the time, shrimp can remain quite on the bottom ready to filtrate particles in suspension and catch pieces of compounded feed before it desintegrate in the water, «re-invested» in mocrobiota (heteroptophic bacteria) or/and in zooplankton when present.

On the basis of Table 3 water renewal had a significant impact on weight gain and survival. In spite of low % in moulinette K, final weight is slow taking into account its initial weight due to cannibalism probably exacerbated by water quality. Then, the low biomass was drastically low in final. In regard of such experimental result, the best compromise with juveniles *P .monodon* would have been observed in moulinette. In practice, one would consider a survey with a minimum of water renewal to maintain water level. In fact, «zero water exchange» can provoke temperature fluctuations (day/night) in outdoors conditions that could impact the weight gain. This being, in Vietnam (2014) for example, it is observed liner ponds 300-500m^2 equipped with paddle wheels that produced in floc mixed with microalgae. Therefore when a pure floc is operated, the amount of sludge is substantial but the association floc+microalgae could purify the environment to a certain extent.

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In the end, the intrinsic contribution of the feed to the energy level is much higher than the "floc". The feed's energy density is unrelated to that of floc from its composition (energy density of about 15kJ g^{-1} while "floc" with its 70% water is almost 5 times less dense. The problem persists at intake that is difficult to assess. In fact, the feed on the one hand undergoes a leaching due to water movements caused by strong bubbling, and so a part of the daily shrimp ration escapes but not included heterotrophic bacteria especially during the last months of culture. On the other hand, floc appears to be ingested within 1% of body weight (Ecotron, 1980), but the data obtained in small volume (5L) remains questionable due to an extrapolation compared to shrimp in % per day. Assuming floc ingested quasi-continuously, this biomass seen its water content does not represent a substantial contribution gravimetrically compared to dry feed.

Fig 2. Bioenergetics in clear water (Gauquelin *et al.*, 1996) and in floc (2010)

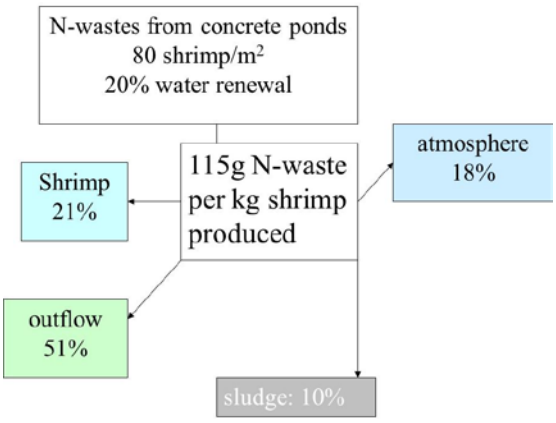

Fig 2. N partitioning from a diet containing 7g N (Burford *et al*., 2004)

Energy content of MOD was established based on mollusks research (Février *et al.*, 1975) and pointed out values near 7,5J L^{-1} of culture medium that made in terms of digestible energy an input far from negligible aside from other nutritional sources such as phyto, bacteria and feed. Feed for example brought according to formulation something like 12kJ g^{-1} and feces were re-colonized by bacteria then ingested like a well nutritive complement thanks to a double digestion but this is not quantified at the moment (Fig 2).

However, perspectives indicate that this is a very significant contribution because it reflects a bit what the animal can find in its natural environment. It complements this way

the compounded feed. Stable isotopes give comprehensive information about the contribution of each fraction (Cam *et al.*, 1999; Magaña-Gallegos et al, 2015) and allow discriminating between particle sizes of those to be absorbed preferentially on laboratory short cycles. With the feed on which one can play with the delta^{15}C it is possible to make its composition very negative (-40% ◦) with the addition of fossil ingredients such as ICI yeast and thereby obtain consistent differences in relation to the natural productivity or floc. In fact the contribution of the feed can range from 30% to 65% between the beginning and the end of a grower period (several months). In such a scheme, it is possible to infer the proportion of live microorganisms (meiofauna) actually more abundant during early rearing (Réquillart, 2004, pers com.). But also which reduces to nothing near harvest when this meiofauna practically disappeared with increasing shrimp biomass.

Recent work suggests, in floc tanks or in earthen ponds, a contribution of up to 60% for shrimp, perhaps the consumption of floc and its structure at the particle level can make it the most important while ingested in a row, isotopic labeling of shrimp muscle gave more intense signature when actually translating the greatest contribution of floc what can come from the feed. Again, further precisions on these measures will help to maintain a coherent description on the relative contributions but the parallel (Fig. 1) between feed and floc made from the work of Gauquelin *et al.* (1999) highlighted the level of energy retention (RE) with higher values based on floc.

Sopomer era (1987-2003)

This first shrimp farm operated on floc was created in 1999 and the technical background of Aquacop served to design and set up the operations at a significant scale (1ha) the culture of *L. vannamei* and *L. stylirostris*. The owners faced a few setbacks at start and then were able to run productions at two cycles per year; he even reached a world record with *L. vannamei* (24mt a year in two crops). But in the long run, some mortality appeared, the quality larvae decreased, the medium originally to evolve with a predominance of phytoplankton bloom to lead after more than 10years the farm to collapse. Reasons were technical (not enough water depth, no shaded area to limit UV action, phytoplankton bloom, feed with inconsistent quality,…and the shift from one species to

another did not really improved the situation, then attempts to mixed seawater and fresh water did not bring positive trend. But this was the first attempt to transfer results from a small or pilot scale to production.

Cuzon *et al.* (2008) produced a synthesis on Ecotron program with an emphasis on results obtained at COP during 80's leading to comprehensive interrelationships between communities and the exchange of salts by means of bacteria.

1999-2012 Avnimelech & Taw, (2012) " found that N was converted into microbial protein and using ^{15}N tagging of biofloc that more than 20% protein eaten by shrimp at harvest comes from biofloc (28-49% CP); protein utilization rose from 15-25% in conventional ponds to 45% in BFT (a save-up in feed cost up to 31-36% for fish and 50% for shrimp cultivation).

2011 MaB-floc (van den Hende et al., 2011) demonstrated the association of µalgae plus bacteria that improved by a balanced C/N and used fish waste water to maintained the system feeding animals with a feed MaB-floc enough to get animals grow-up to a commercial size.

2013 in SE Asia, shrimp culture development started following the floc method and a use of green pellet to cope with benefits of particulate biomass and microalgae in ponds with liner (Emerenciano *et al.*, 2012-13).

2014. Utilization of "floc" under dry form to be incorporated in a diet for peneids-to drastically replace fish meal in formulations (Glencross, 2014).

2010-15: **UNAM-UMDI Sisal: recent results on "floc"**

A research program started at UNAM in 2012 leading to several thesis. The originality of the work was the comparison of shrimp responses to clear water compared to floc conditions (Arevalo, Valenzuela, 2010). Moreover, this approach was possible not only with *L. vannamei* but with several others native species from the Gulf of Mexico (*F. brasiliensis*, *F. duorarum*). The main trials concerned weight gain performances in

"moulinettes" located outdoors with a shading zone to demonstrated the feasibility of the technique on site.

Arevalo *et al.* 2013 «Failure to improve performances of males *F.duorarum* in "floc". *F.duorarum* males were raised in "floc" or in clear water tank, fed with or without fresh food. Spermatophores formation, spiked sperm, spermatozoids (sptz) motility were not modified with the rearing conditions or feeding regime. Metabolites from (total protein, triglycerids, cholesterol), in hemolymph, hepatopancreas (HP) and gonads were measured during grow-out and maturation phases. Total soluble protein in gonad varied from 62 to 73mg g^{-1} with fresh food (eviscerated squid) or a commercial feed and in HP from 37 to 44mg g^{-1} ($p>0.05$). Neutral lipids varied from 8.to 11mg g^{-1} in "floc" conditions and clear water respectively ($p>0.05$). Such contrast in food/feed composition had incidence on fatty acids profile (% total fatty acids) in HP and gonads respectively with LOA (3*vs*7), LNA (2*vs*2), ARA (1*vs*11), EPA (2*vs*17), DHA (2*vs*11) in all rearing conditions. Similarly as with females there was a transfer of lc-PUFA from HP to gonads. Native protein+free amino acids+lc-PUFAs displayed there essentiality in the maturation phase more explicitely than during grow-out phase. "Floc" definitely provided better nutrition status than clear water but additional fresh food reinforced health status for reproductive phase.

Then a comparative study was conducted to propose an economic approach for two species as indicated in table 5 with two types of feed rations, traditional or optimal (Arbelaez *et al.*, 2013).

Simultaneously, it was examined to which extent future breeders could benefit from floc compared to floc plus fresh food (Emerenciano *et al.*, 2013; 2014) as displayed in table 5.

Table 4. Bioenergetics in clear water and in floc. Values with one digit are in kJ $shrimp^{-1}$ d^{-1} (up) or J/shrimp/day (below).

Gauquelin *et al.*, 1996	GE=7.5 100	DE=6 80	N-NH_4=0.4 5	ME=5.6 75	SDA=1.4 19	NE=4.2 56	~HeE=1.8 24	RE=2.4 32
floc, 2010	GE=100	DE=100 100	N-NH_4=5 5	ME=94 75	SDA=89 19	NE=89 56	~HeE=69 24	RE=69 69

Gaxiola, G. and Cuzon*, G. 2015. "Floc" Story. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Univers idad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 23-50.

A 40-day trial was performed to evaluate the effect of short-term fresh food (ff) supplementation twenty days prior to ablation in *L. vannamei* broodstock raised under biofloc conditions. Changes in biochemical composition and fatty acids (FA) profile were used as indicators of nutritional condition. Females that received ff supplementation (floc+ff) achieved better eggs production, spawned more promptly and presented higher levels of HUFA in eggs as compared to those ones that did not received ff (floc alone). Proximate analysis in biofloc and microorganisms assessment showed a higher crude protein and lipid content from(floc+ff) tanks (26.3 and 0.7% respectively) as compared to floc tanks (18.4 and 0.3%) and also demonstrated a higher concentration of filamentous cyanobacteria and nematodes.

Table 5 Comparative performances of *L.vannamei* and *F.duorarum* pre-breeders set in two different conditions (rations traditional or optima in floc or clear water CW) for further reproduction in "floc"

L.vannamei					*F.duorarum*			
	floc		CW		floc		CW	
rations	optima	tradition	optima	tradition	optima	tradition	optima	tradition
IBW	9.4	9.5	12	12	1.7	1.8	2.4	2.5
FBW	17	19	14	15	5	4	4	4
wt gain	8	10	1	3	3.2 ^{a}	2.7 ^{a}	1.6 ^{b}	1.7 ^{b}
g wk^{-1}	1 ^{a}	1 ^{a}	0.2 ^{b}	0.4 ^{b}	0.4	0.3	0.2	0.2
biomass	89 ^{a}	97 ^{a}	66 ^{b}	66 ^{b}	25	22	20	21
survival	86 ^{a}	89 ^{a}	66 ^{b}	66 ^{b}	63	69	66	71
FCR	1.3	2.0	1.3	2.0	1.3 ^{b}	2.0 ^{a}	1.5 ^{b}	2.1 ^{a}

The better outcomes obtained with females that received short-term ff supplementation justified its employ in *L. vannamei* broodstock. Effect of short-term fresh food on reproductive performance, biochemical composition and FA profile of *L. vannamei* (Boone) reared under biofloc conditions (Emerenciano *et al.*, 2011).

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Table 6. *L.vannamei.* and reproduction in "floc"

	floc	floc+FF	*p*
female weight (g)	35.0 (±3.0)	36.2 (±2.9)	0.2
mortality (%)	11.1	8.9	
total spawns	50	50	
spawns between ablation & day 20	6	12	
spawns between day 21 and 40	44	38	
unfertilized spawns (%)	88	60	
Nb of spawn/ablated female	2.8	2.8	
Nb of spawn/spawning female	3.1	2.9	
Maximum spawn order	5	7	
latency period (days)	22 (±5)	23 (±8)	0.8
females that spawn at least once (%)	88.9	94.4	
Nb of eggs per spawn ($\times 10^3$)	94.1^B (±33.3)	111.2^A (±36.4)	0.03
fertilization rate (%)	73.1 (±13.1)	79.5 (±17.0)	0.4
Nb of nauplii per spawn ($\times 10^3$)	50.3 (±33.3)	62.1 (±36.0)	0.6
hatch rate (%)	51.5 (±21.7)	61.8 (±18.2)	0.4

The theme of comparative situation in floc and in clear water was maintained in order to examine the potential effect when an exogenous probiotic was incorporated into the feed. An explanation came from the poor level of adaptation while shrimp shifted directly from "floc" to CW, impacting severely on its microflora. Adding probiotics increase the level of complex interactions; at this stage, out of bioreactor this medium will remain favorable to shrimp for nutrition, immune response, activation of quorum sensing, therefore health status and general aspect (hard shell, long antennae, good pigmentation) and the variety of *Vibrios* (common to all organic rich media) even though some of then turn to pathogens would not affect animals because of bacteria ecology aspect, leading to few symptoms when compared to animals remained in clear water (Aguilera *et al.*, 2014). "floc" was a chaotic ecosystem sometimes called a "black box" with a fragile equilibrium between two communities. On the contrary CW abruptly changed environmental conditions and increase daily variations unfavorable to some bacteria encountered an osmotic shock; animals became more susceptible to infection form opportunist bacteria (Fig 3).

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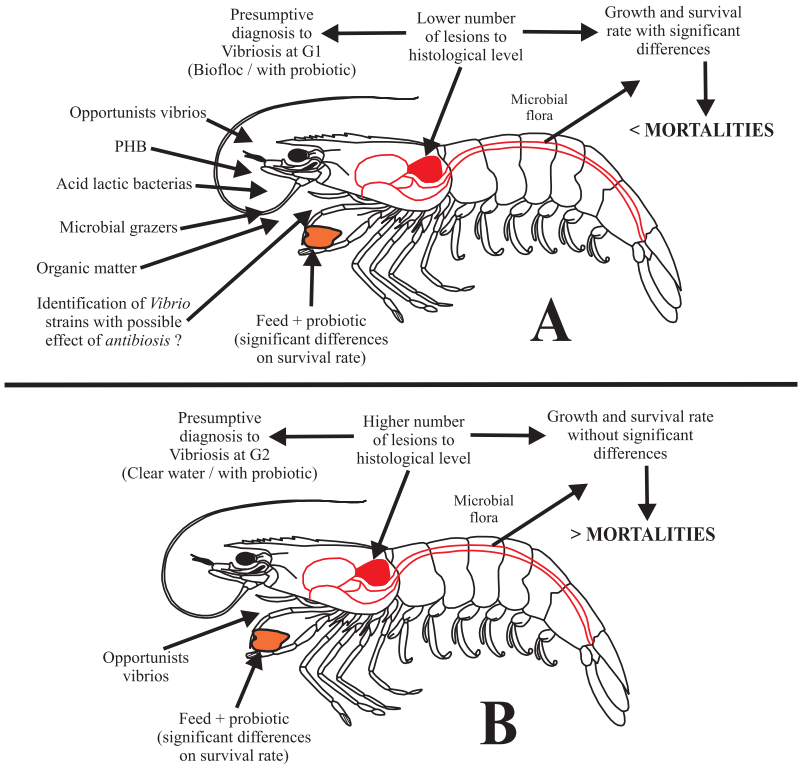

Fig 3. Interactions related to floc-probiotic effect (A) in comparison with results obtained in CW system (B) in the second study (after Aguilera *et al.*, 2014).

Finally, and according to the fact that each site of culture could be different, and various management especially with such a chaotic system, it was useful to examine once again the contribution of floc in two distinct species and in comparison with clear water as well as the incidence not only during the growing period but also when animals entered in pre-maturation (Magaña-Gallegos *et al.* 2015). Stable isotopes indicated some distinct situations according the type of fresh/frozen food aside from a compounded feed. Concerning *F.brasiliensis* it was evidenced a distinct situation when considering F^1 generation and wild specimen (Fig. 4).

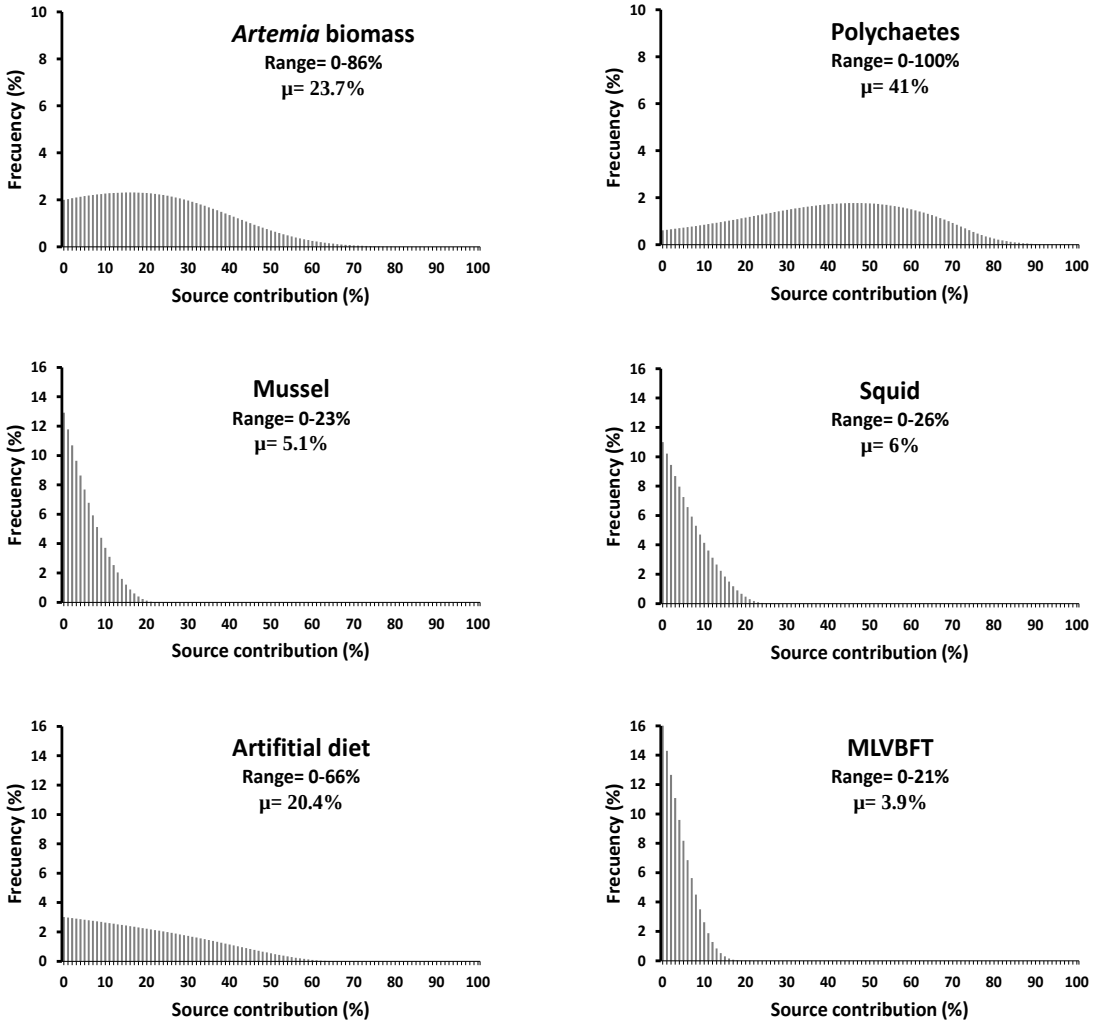

Fig 4 Contribution of 5 foods/feed sources from isotopic sign to *L. vannamei* eggs and muscle breeders raised in floc (mLv/BFT). (µ=media)

2015: back to original "floc" in dark (indoors) conditions with an example of an area of production near Madrid using artificial seawater, and indoors in order to benefit only from the bacterial community of "floc" plus a regular feed, and close to a great market place. Interestingly this approach derived from original work conducted in early 70's.

Discussion

The share of single food can be low, however leaching helping to fuel the medium (bacteria) so the feed plays a "double trigger" role and carbon intake is significant due to

carbohydrates ($IME_{glucides}$ around 48%) then and the end of a rearing cycle floc dynamics integrates feces production as it is another food source for shrimp (coprophagy) plus contribution of native protein originally single cell (heterotrophic bacteria). Finally secondary production is established only rich microorganisms (protozoans). It is without doubt a key to the explanation of the nutritional benefits associated with floc. One could say that the feed supplies not only shrimp but it enriches the environment at the same time boosting the formation of these micro-ecosystems (particulate biomass) shrimp will filter at will. In fact shrimp raised in "floc" find conditions of the natural environment in which animals are in constant feeding activity, recovering these tiny amounts of particles that ultimately provide all nutrients that animals molt and grow so near their genetic potential, close to shrimp in the wild can express weight gain and reproductive performances. Also, always in these conditions, animals do not attack each other (survival>80%) thus almost no cannibalism in such stress-free environment despite high stocking density. 40 years later, floc rediscovered gives the best chance to respond a request quality shrimp, presentable, and have an analytical composition probably close to that of farmed shrimp, unlike what one has seen for farmed fish such as trout for example. Let's say that feed distributed 2 or 3 days time will not produce the same effects that a tiny contribution of small particles and highly digestible "a release of energy by small change" as wrote Szent-Georgyi, A. (1957). In addition to floc, one is facing a triphasic diet supply [bacteria+protozoaires+nematodes+copepods] plus a dry pelleted feed and finally soluble substances (MOD) source of amino acids, vitamins, glucose, lipid, minerals, nucleotides... In fact, the simultaneous contribution of these groups that brings the best supply to meet the demand for weight gain as well as breeding. This suggests that such needs are not so different. However, if growth with dry feed must be supplemented via "floc" for example, during maturation phase adding fresh products (mussels, squid, ...) will need less than floc contribution give less need to floc intake which allowed in its time (Kawahigashi, 2010-15) and advocate reproductive penaeid in clear water. Note that these authors have recently revised their position and support the idea of conditioning floc before the entry into maturation.

-Nutritive value

Aside from protein content of floc (30-40%DW), carbohydrates min. or lipid (1-2%) primarily it derives from native aspects of those compounds compared to processed ones delivered by a regular dry pellet. The focus on minerals and energy appears of great interest to cover up. Based on floc composition indications, that can help to explain some aspects of its nutritive value.

There is one point on which we can return on shrimp nutrition in floc is that of mineral requirements, based on the observation that a shrimp (50g live weight) for example will release at molt its exoskeleton that makes ~20g representing approx. 40% fresh weight. This shows how the synthesis of new body tissues will be active and the intake of essential minerals necessary. Or precisely, the floc is an environment that is characterized by a very substantial mineral content; it is only to see the data on dry matter composition of floc that is around 40%. And paradoxically, shrimp feed trend in downward; in the 70's indeed, there were formulations or nutritional data on extruded feed bags that showed up to 19% ash while today it is more in the range of 11-12% that is a substantial decrease but that would not be harmful to the shrimp "floc" while in clear water or cage culture that could be limiting. One would not return to the form of phosphorus in the feed that was present (via dicalphos) with a low level of assimilation for shrimp while mono-P form would have-been much more digestible. Again "floc" will play its full role to meet the shrimp requirements.

But there exists again a point to elucidate, the one of energetic value of floc? What is its contribution in terms of energy supply daily and in final the retention (RE) for growth?

-energetic values:

It is a key point with floc. There exists a large contrast between dry feed supply (kJ) and live preys (J) due to respective water content. Intake cannot precisely be determined but an attempt is made to explain the energy intake that sounds plausible taking into account a putative intake but especially when considering the exceptional weight gain

correlated directly to RE (retained energy); that is one of key points to explain performances in floc.

The characterization of the media is important. Indeed it is in the presence of particulate biomass MOD (dissolved organic matter) and live and dead microorganisms. The particle is the essence of the floc we say literally and figuratively because that environment is well established all the positive properties of the "floc". However, we have long forgotten the component MOD. This component was described long ago for mollusks (Février *et al.*, 1975) and the parallel between what was described and what one is studying is striking and illuminates the concept of scope for "floc" as applied today at penaeid shrimp culture. This is MOD that provides nutritive elements we had not sufficiently considered by focusing too much on particulate fractions, yet it is a compartment of the environment and its contribution is probably greater than one could imagine including prior moulting (*see next paragraph*) or spawning. It is a permanent contribution since comes from degradation of phytoplankton and bacterial communities. MOD (dissolved organic matter) based on mollusks studies (Février *et al.*, 1975) produced data such as 7,5J L^{-1} of medium representing in terms of DE a substantial input aside from other sources such as phyto, bacteria, and compounded feed. Feed, for example, brought according to the formulation around 12kJ g^{-1} and feces re-colonized by bacteria then ingested with a beneficial effect of a double digestion but no data exist at this date.

The energy efficiency of a diet based "floc" should at least be excellent with a digestibility ($ADC_{nutrients}$) close to 100% DE, and less than 6% excretion. a minimum extra heat because of the low solicitation digestive enzymes on native nutrients, a BMR protected from disturbances due to a bit stressful environment. Finally retention (RE) should be good to excellent; however, it remains very difficult to make an assessment in metabolic chamber. What one can say is that *a priori* energy expenditure would be reduced in "floc" compared to clear water on one hand and reducing stress on the other by the simple fact that shrimp landing on the bottom of the tank can thanks to water movement (in “moulinette”) continuously received food/feed: pellets or collets, suspended particulate biomass, water (a ”forgotten nutrient”) rich in dissolved organic substances. This is true for

small scale basins (see the “moulinette” of 700m^2 at COP) of sufficient height (1.20^m) but perhaps less true in tanks with liner where the water will flow in a horizontal plane under the action of paddle wheels but probably that environmental conditions remain favorable in such modules like raceways.

-Antibiotics in floc and antibiosis effect of some bacteria (Ecotron). Bacterial diversity increased in the stomachs of white shrimp *L. vannamei* adminstered oxytetracycline via feed, although diversity decreased in the hepatopancreas and digestive tract. *Vibrio* and *Achromobacter* genus bacteria persisted in the presence of OTC, but species in the order Lactobacillales, and *Citrobacter koseri* dissappeared. The bacteria present in the clear water and floc system differed, although OTC exposure had similar effects in shrimp cultured in either system It seeks to determine if the antibiotic had a detrimental influence on the bacterial community structure of *L.vannamei* under different culturing subtropical conditions. By comparing clear water and biofloc-reared shrimps microbial load may have played a role in determining the extent to which the bacteria community responded to a perturbation of the medium (Arena *et al.* 2015).

Then after years of R&D and taking account of advances in knowledge about ecosystems, one can just repeat an adaptation of such system allowing (i) a reduction of water utilization (ii) a possibility to use artificial seawater (iii) intensification of shrimp culture up to super-intensive and hyper-intensive (iv) fitting such medium for one species in particular, *L .vannamei* (v) less pressure on the quality of compounded feed whether pellet or “collet” (vi) a microbiota favorable with the settlement of various bacteria ecotypes and then less chance for pathogens to grow (vii) a depuration of toxic compounds such as nitrites in a medium in its stable form (viii) in low volume, floc can settle in after 20d. (ix) an opportunity to work in dark area with a pure bacterial floc or in a mix bacteria+µalgae in large outdoors ponds (x) a large advantage to produce shrimp not only along seashore but inland near food markets, while reducing the carbon footprint (xi) in an ideal situation (i.e. indoor+artificial SW+pure floc) where the risk of diseases would be largely reduced (xii) the principle of water treatment plants and microbiology mix well at

least for primitive non-grooved species well adapted to this culture medium, where a set of fundamentals to manage it would change radically production methods with a care for coastal environment.

In practice it takes a lot of vigilance to maintain a floc respecting the basic principles namely, water depth, ventilation, convection cells, sieving the light, blackish discharge of sludge daily ...). However, one speaks of "zero water exchange" but in practice it seems that we compensate for the less evaporation and a central purge can be made.

Conclusion and perspectives

These studies on “floc” with an identification of size particles help understand about the point of floc qualification on a basis of its nutritive value. “Floc” particle sizes of course will depend of the intensity of aeration, the best being when convection circulation, keeping shrimp on the tank bottom positioned to filtrate with a minimum of energy expenditure, these kind of micro-ecosystems. But one needs to keep in mind the dynamic aspects of “floc” and its main characteristic as a chaotic medium (May, 1976). Because phytoplankton when present and bacteria followed a cyclic development, sometimes with a prevalence of one community, the ideal being a majority of bacterial flora that attract protozoa, copepods,…such situation is fully nutritive for juveniles. Shrimp gut will be colonized by bacteria from “floc”, and a hypothesis would be tract would contain generalist bacteria (Bolnick *et al.*, 2014). But the medium of culture is so rich (10^6) that shrimp cope with a diversified flora. And in final, after 2-3months rearing, shrimp in high stocking density produce large amount of feces, colonized by heterotrophic bacteria, which will be re-ingested by shrimp (coprophagia) and in a sense the evolution of the culture medium is such that one can figured out that heterotrophic bacteria will prevail in gut flora.

The level of interpretation of “floc” is quite complex, including a chaotic aspect, exemplified by a succession of communities, and the most simple situation will be to have shrimp in “floc” without light to avoid microalgae and to have a successive of autotrophic bacteria (nitrification) that will stabilize the system, followed by heterotrophic bacteria

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during the ultimate two or three months when the production of feces reaches a maximum. Would we consider C/N, “floc” in its stable status will contain large particles with 250µm (this paper) and aeration will be strong enough to let convection inside the medium and keep particles expanding to form a kind of ecosystem more attractive, surrounded by live organisms such as copepods bearing a microbiota rich in *Vibrios* (Bianchi, 1976). This complex medium not only can be assimilate to an external rumen but possess a capacity of auto-depuration to a certain extent that can limit wastes. Understanding “floc” in its complexity will remain a hard exercise but species such as *L.vannamei* adapted quite readily to such medium during grow-out as well as pre-maturation; next step could be to examine the transcriptome in “floc” compared to clear water and identify those genes from intermediary metabolism that could be up or down regulated under conditions of feeding on a basis of a triphasic diet live food+bacteria, extruded feed+bacteria, MOD.

Perspectives du “floc”

The evolution of techniques of "floc" after the concept water treatment plants was long and discontinuous. We had to face lot of settings, rather strict breeding conditions, and we faced a chaotic system as described previously by May (1976) about the copepods populations. The launch of the program Ecotron (1980) with the participation of a group of enthusiast’s researchers in microbiology portrait in itself a lot of hope for modeling the "floc" system but it was not counting the levels of variation and interactions water - microorganisms-shrimp-waste. Technological constraints remained to meet. The gestation was long because in 70-80 years the aquaculture was rather oriented semi-intensive. Indeed intensification of attempts in Taiwan for example, with the species *P. monodon* had proved catastrophic in terms of results with densities that reached more 44 shrimp/m^2 in 1988. The water level was an important factor to hold a phytoplankton layer in the first 50cm below the surface.

This was the time when Greenpeace was opposed to the massive destruction of mangrove areas along the Pacific coast, Ecuador, Baja California who presided over the construction of huge basins for the establishment of semi-intensive technique. Over the

years, these techniques have proved catastrophic showing episodes of severe mortality related to the occurrence of pathogens (viruses and *Vibrios*) and it took a long hard realize awareness for the limitations of these productions (often to export so with a high carbon footprint) despite hopes to resistant strains (Super-shrimp with Persyn, H.O., SPR[43] at COP) or antibiotics treatments prove risky (Arena *et al.*, 2015).

The industry has taken the lead ultimately and expert help to make quite substantial investments giving possibility to bring intensive or super-intensive shrimp farming with much better biosecurity conditions, low water utilization and fishmeal in the grower feed therefore a path to sustainability of the sector (sustainable aquaculture).

It is clear that the shrimp are in a "on-stressful environment", rich in specific nutrients and convener for shrimp physiology (possible isosmoticity, digestion from native proteins, provision of long-chain fatty acids , vitamins, ...) leading to optimal nutrition, leading to a limitation of cannibalism (high survival), less energy expenditure devoted to immune .response so strong retention for growth. In this environment, the approach to the formulation of an artificial diet will change, simplify proteins including a low rate (20-25%CP), but also carbohydrates which will also contribute to maintaining a C:N ratio ad hoc (around 6), less vitamins, minerals load to adjust, but with inputs such hydrolysates (from fisheries co- products) and final basic corn-wheat-soya-alfalfa that will be transformed by shrimp. Incidentally it may be interesting from an economic standpoint, while maintaining FCR which may surprise while on conventional bases was difficult to see changes in composition to meet relative's values 1:1. In such a set, do not forget the organoleptic characteristics of shrimp and maintaining an abdominal muscle texture similar to that of wild shrimp. Will I need to check on the bright pigmentation or staining after cooking? ... without having an absolute use of synthetic carotenoïds, the presence of alfalfa showed a positive effect in the past.

Going further down, will one take into account aspects of organic (BIO)? Currently there is a farm in the world that relies on BIO production, based in Madagascar, the OSO farm. There will be then to distinguish between high-quality products and feed a population in staggering growth (9MM by 2052).

It's probably like a little revolution that is brewing in the world of industrial production of shrimp near the markets where consumption of large cities exist in order to reduce the carbon cost in the process. The vow of Addison Lawrence was to make shrimp the "chicken of the sea" and with support from the food industry and investors, perhaps we are heading slowly towards the realization of this dream.

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Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case

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Abstract

The demand for safety seafood is increasing globally year-by-year. On the other hand, the low productivity and recent diseases outbreaks lead the scientists to search for an alternative system to improve efficiently the aquaculture growth. Biofloc system, also called as biofloc technology (BFT), has the advantage to allow high production with limited or no water exchange. BFT has gained popularity because it offers a practical solution to maintain water quality and recycle feed nutrients. The continuous availability of natural food source in a form of microbial biomass lead the decrease of the feed conversion ratios and the possibility of employ alternative low protein diets, as well as alternative feed ingredients. More efforts have been done in penaeid shrimp nutrition as compared to fish nutrition under biofloc conditions.

Keywords: BFT, nutrition, microbial community, alternative ingredients

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Introduccion

The Biofloc Technology (BFT)

In recent years, studies approaching the production of fish and shrimp in biofloc system desired great attention (Avnimelech, 2015). The low productivity, recent diseases outbreaks and the increasing need of a production with an "environmental friendly" approach lead the scientists to search for an alternative system to improve efficiently the aquaculture growth. At the same time, the demand for safety seafood is increasing in a rate of ~10% per year (FAO, 2015).

According to Emerenciano *et al.* (2013), in 70's, Ifremer-COP Tahiti started R&D with enclosed limited water exchange biosecure system with different penaeid species. In connection with Aquacop and Ralston Purina, biofloc system was applied to grow-out *L. stylirostris* and *L. vannamei* both in Crystal River (USA) and Tahiti; and first considerations on benefit of such system for shrimp culture emerged. In 80's BFT was applied to grow-out tilapia and other fish species in Israel. Currently, BFT have being applied successfully in large-scale commercial farming around the world.

Biofloc system, also called as biofloc technology (BFT), has the advantage to allow the production of a great amount of fish/shrimp biomass per area with limited or no water exchange. This provides better biosecurity for the production, especially if the farm is situated in areas with high concentration of aquaculturists using the same water source. In this context, BFT has gained popularity because it offers a practical solution to maintain water quality and recycle feed nutrients simultaneously (Xu and Pan, 2012). BFT has become a popular nursery and grow-out technology, although interest in larviculture and husbandry is increasing day-by-day.

Other advantage of the biofloc system is the possibility to use alternatives low protein diets and consequently decrease the production costs (Ballester *et al*., 2010; Scopel *et al*., 2011); mainly due to the continuous availability of natural food source in a form of

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bacteria, protozoa, nematodes, microalgae, rotifers and copepods (Azim and Little, 2008; Ray *et al*., 2010). Although a diverse microbial community is present in BFT, many factors could modify the microorganism profile in the cultured ambience such as the C:N ratio, total organic load, source of organic carbon, water exchange ratio (minimum or zero), mix intensity in the water column and light intensity. Water quality parameters such as total suspended solids, temperature, salinity, dissolved oxygen and pH also could affect the microbial community (Martínez-Córdoba *et al* 2014).

BFT and Nutrition

Some components in aquafeed are focus of intensive research mainly due to its effects on improvement of animal growth, health and also aiming to decrease the production costs. Fishmeal is one of the most expensive and unsustainable ingredient used in aquaculture diets (Naylor *et al*., 2009). Therefore, the replacement or reduction of fishmeal presents a great interest for the aquaculture industry. On the other hand, fishmeal posses an excellent digestibility and amino acids profile (Cruz-Suárez *et al*., 2007). Problems related to the fishmeal replacement by alternative ingredients such as deficiency of some essential amino acids, presence of anti-nutritional factors, palatability and digestibility have been identify (Forster *et al*., 2003; Naylor *et al*., 2009).

Although problems exist, many cases of success have been reported replacing fishmeal by alternative protein sources such as vegetable grains and terrestrial animal industry by-products. In the specific case of penaeid shrimp, Forster *et al*. (2003) and Suarez *et al*. (2009) recommended levels until 75% and 80% of fishmeal replacement using cattle by-product and a mixture of canola and soya, respectively. Amaya *et al*., (2007) and Hernández *et al*., (2008) concluded that is possible to replace 16% and 35% of fishmeal by poultry and swine by-product, respectively, without shrimp performance losses. Samocha *et al*. (2004) and Cruz-Suárez *et al*., (2007) achieved success replacing 100% and 80% of fishmeal by soya meal and poultry by-product in *L. vannamei* diets. Paripatananont *et al*., (2001) achieved 50% of replacement using soya protein concentrate in *Penaeus monodon*

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diets.

In the nutrition of animals in BFT, microorganism present in the system might help by three ways: i) reducing commercial feed ingestion by microbial biomass consumption and lead the decrease of the feed conversion ratios (Wasielesky *et al.,* 2006); ii) employment of alternative low protein content diets (Ballester *et al*., 2010) and iii) reduce or replace fishmeal by alternative feed ingredients (Scopel *et al* 2011). It is important to note that in systems with natural productivity (i.e. semi-intensive pond systems) natural biota plays a key role. For instance, it has been estimated that only 29.7% of the carbon and 33.8% of the nitrogen comes from the inert feed in penaeid culture (Nunes *et al*., 1997; Miranda *et al*., 2009). In water column, biofloc available 24 hours per day can be utilized for fingerlings (Ekasari *et al.,* 2015), shrimp larvae (Lorenzo *et al.,* 2015) and postlarvae (Mishra *et al* 2008; Emerenciano *et al.,* 2011 and 2012a), juveniles (Wasielesky *et al.,* 2006; Azim and Litle 2008) and for the first stages of broodstock's gonads formation and ovary development (Emerenciano *et al.,* 2013).

The microorganisms present in BFT could be a rich source of native protein and amino acids for aquacultural organisms (Ballester *et al.,* 2010). The concept of "native protein" is related to protein source without previous treatment mainly including live food (Emerenciano *et al*., 2012b). Also, the microbial community is a continuously source of lipids (Maicá *et al*., 2012), vitamins and essential aminoacids (Ju *et al*., 2008). Rojas-López, 2015 evaluated the aminoacid profile of tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*), cultured in BFT with four experimental diets (fishmeal replacement by vegetable meals); in the diet with total replacement found, that lisina, an aminoacid limiting in the vegetal meals, appear in the tilapia tissue profile in similar concentration that commercial diet, as a contribution of the microbial biomass (Table 1). According to Emerenciano *et al*.,(2013), protein, lipid and ash content in biofloc particles could vary substantially (12 to 49, 0.5 to 12.5 and 13 to 46%, respectively). The same trend occurs with fatty acids (FA) profile. Essential FA such as linoleic acid (C18:2 n-6 or LA), linolenic acid (C18:3 n-3 or ALA), arachidonic acid (C20:4 n-6 or ARA), eicosapentanoic

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acid (C20:5 n-3 or EPA) and docosahexaenoic acid (C22:6 n-3 or DHA), as well as sum of n-3 and sum of n-6 differ considerably between 1.5 to 28.2, 0.04 to 3.3, 0.06 to 3.55, 0.05 to 0.5, 0.05 to 0.77, 0.4 to 4.4 and 2.0 to 27.0% of total FA, respectively.

For broodstock, BFT can enhance the shrimp reproductive performance as compared to the conventional pond and tank-reared systems. Moreover, fresh food items (i.e. squid, mussels, polychaetes, artemia biomass, etc.) combined with BFT helped to improve reproductive performance in terms of shorter latency period, higher spawning activity and higher number of eggs per spawn (Emerenciano *et al* 2012b and 2013b). In tilapia broodstock, Ekasari *et al.,* (2015) evaluating *Oreochromis niloticus* breeders (85±5 g) suggested that the application of BFT effectively enhanced reproductive performance and therefore *in situ* biofloc production can be suggested as a way to increase tilapia seed production. In addition, previous promising results also have been showed over-wintering tilapia in BFT (Crab *et al* 2009). In *Macrobrachium rosenbergii,* Perez-Fuentes *et al*.,(2013) evaluated during six months two rearing systems: biofloc and traditional water-exchange cultivation. The results suggested that survival rate was similar in both treatments (>85%), but final size was significantly higher in BFT. Protein (51.19%) and lipid (13.84%) content in harvested prawns was also higher in BFT. With this result in mind, BFT seems to be an efficient tool for *M. rosenbergii* broodstock production and maintenance.

Table 1. Amino acid profile (g/100 protein) of tilapia tissue (*Oreochromis mossambicus* x *Oreochromis niloticus*) cultured with different diets in BFT (C = commercial diet, 0, 10, 20 and 30 = experimental diet containing 0%, 10%, 20% and 30% of fishmeal, respectively). Different letters in the same row indicate significant differences (P<0.05); modified from Rojas-Lopez (2015).

Amino acid	C	0%	10%	20%	30%
Isoleucine	2.58 ± 0.47^{bc}	3.52 ± 0.70^{abc}	4.54 ± 0.48^{a}	4.41 ± 0.21^{ab}	2.02 ± 0.45^{c}
Leucine	7.53 ± 2.38^{a}	5.58 ± 0.25^{a}	7.27 ± 1.57^{a}	8.03 ± 0.23^{a}	4.09 ± 0.03^{b}
Lysine	5.24 ± 0.59^{ab}	9.10 ± 2.23^{a}	7.42 ± 1.14^{ab}	8.20 ± 0.77^{ab}	4.18 ± 0.35^{b}

Methionine	7.71±4.40[a]	2.40±0.26[b]	2.35±0.26[b]	2.05±0.22[b]	1.73±0.22[b]
Phenylalanine	1.61±0.46[a]	2.28±0.48[a]	2.76±0.51[a]	2.89±0.16[a]	1.52±0.04[a]
Threonine	1.51±0.21[bc]	1.95±0.15[ab]	2.17±0.18[a]	1.41±0.13[bc]	0.98±0.12[c]
Tryptophan	12.41±5.48[a]	2.84±0.49[b]	2.78±0.57[b]	2.70±0.13[b]	1.23±0.09[c]
Valine	3.25±0.57[b]	6.17±0.91[a]	5.37±0.71[ab]	5.27±0.19[ab]	3.79±0.30[ab]
Histidine	12.01±5.37[a]	2.25±0.20[b]	2.40±0.29[b]	2.46±0.50a[b]	1.24±0.01[b]

Regarding to nutrition of juveniles, Scopel *et al*. (2011) evaluating the replacement of fishmeal (0, 12.5 and 21.0%) by a combination of soya and animal terrestrial by-products in BFT found that 12.5% of replacement did not affect shrimp growth. Recently, Camaño (2014) indicated that fishmeal and fish oil could replaced by vegetable sources at a level of 75% without growth losses in BFT systems. Bauer *et al*. (2012) suggested that a mixture of soy protein concentrate and microbial floc meal can be utilized as a substitute for fishmeal in diets for *L. vannamei* juveniles. Microbial floc meal can be generated in bioreactors (Kuhn *et al* 2009) or removing the excess of solids from culture tanks or ponds by the use of settling devices (Ray *et al* 2010).

It is also important to note that bacteria protein-source plays an important role in the equilibrium and re-ingestion of particulate organic matter and faeces (coprophagia) left by shrimp results in a form of constant food supply. The colonization of shrimp gut by bacteria had been shown positive effects such as improvement of immune system (Kim *et al* 2014), digestive enzymes activity and increasing the availability of extracellular enzymes (Xu & Pan, 2012) acting as "natural probiotic" (De Schryver *et al*., 2012).

In biofloc culture system, there are several types of bacteria, the most important in the system stability are heterotrophic, nitrifying and cyanobacteria. Heterotrophic bacteria are those using organic compounds as a carbon source, under appropriate conditions (temperature and carbon source) have rapid growth. This group of bacteria consume organic matter and convert waste into bacterial biomass with high nutritional value. Huerta-Rábago (2013) found a positive correlation among total suspended solids and heterotrophic bacteria (Figure 1) in a tilapia culture, in the same study, the ammonia oxidizer bacteria

Emerenciano, M. *et al.* 2015. Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 51-61.

have a positive correlation with a heterotopic bacteria (Figure 2).

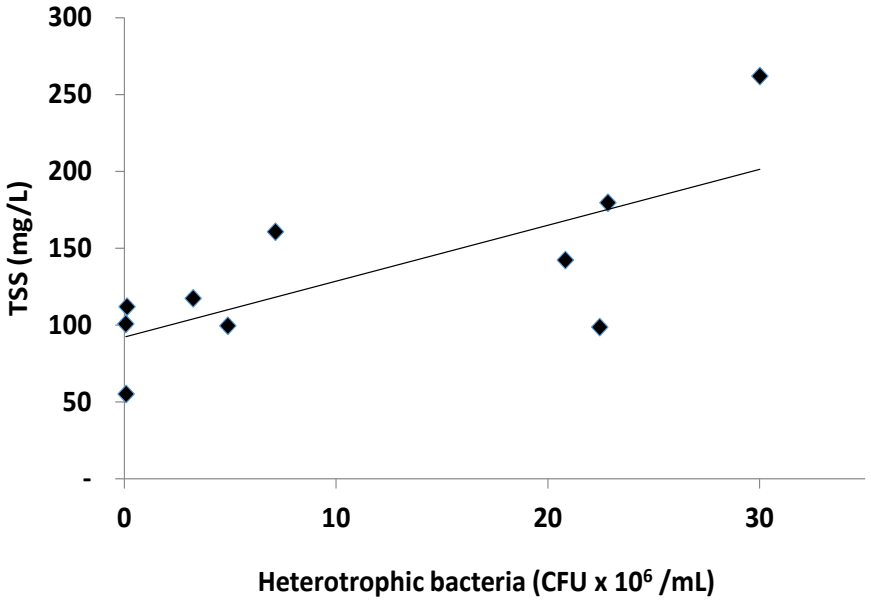

Figure 1. Relationship of total suspended solids and colony forming units of heterotrophic bacteria in a BFT Tilapia (*Oreochromis niloticus*) culture (r = 0.73), Huerta-Rábago (2014).

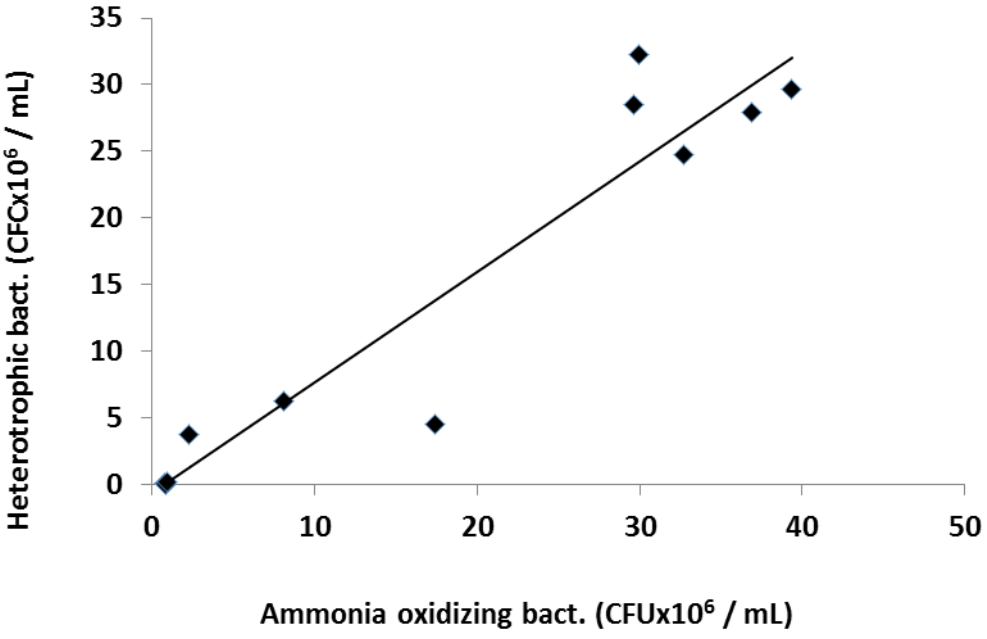

Figure 2. Relationship of colony forming units of heterotrophic and ammonia oxidizing bacteria in a BFT Tilapia (*Oreochromis niloticus*) culture (r = 0.90), Huerta-Rábago (2014).

Emerenciano, M. *et al.* 2015. Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 51-61.

Final considerations

The microorganisms in general play an important role in aquatic environments. There are wide ranges of opportunities to manage aquacultural systems in order to promote microbial growth that may have the major benefits for the farmed species. As the biosecurity is a priority in aquaculture industry, biofloc technology (BFT) are up today one of the most biosecure system used around the world with multiple and successful experiences. Moreover, microorganisms in BFT might partially replace commercial feed, protein content in diets or decrease its dependence of fishmeal. Biofloc technology will enable aquaculture grow towards an environmental friendly approach.

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Uso de Microalgas como Complemento Alimenticio de Especies Acuícolas: Efectos en la Condición Fisiológica, Sanitaria e Inmune

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Resumen

Las microalgas son un componente esencial de diversos ecosistemas acuáticos y la base de la cadena trófica en muchos de ellos. Se han utilizado desde hace mucho tiempo en acuacultura principalmente en el cultivo larvario de moluscos, crustáceos y peces. Recientemente se han estado utilizando en la nutrición de organismos acuícolas en las etapas posteriores al cultivo larvario, no solamente como una importante aportación nutricional sino además porque en ellas se encuentran constituyentes que pueden mejorar la condición fisiológica, sanitaria e inmune de los organismos que las consumen. El presente documento es una compilación de información reciente sobre el uso de microalgas en el cultivo de organismos acuícolas más allá de sus etapas larvales, ya sea desarrolladas directamente en las unidades de cultivo o suministradas exógenamente, tanto en fresco como preservadas o incluidas como ingredientes dietarios. . Se incluyen desde luego experiencias y datos originales de los autores

Palabras clave**:** Microalgas; Nutrición acuícola; Biopelículas; Compuestos bioactivos.

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Introducción

Las microalgas han sido ampliamente utilizadas en acuacultura, especialmente para la producción larvaria de moluscos, crustáceos y peces, para muchos de los cuáles se consideran insustituibles. En este sentido hay una vasta literatura científica que documenta el valor nutricional de muy diversas especies microalgales. Las técnicas de cultivo son también muy variadas y en la mayoría de los casos bastante bien dominadas, de tal manera que se pueden producir a nivel masivo, volúmenes enormes que son utilizadas de muy diversas maneras y para muy variados propósitos además de la nutrición de larvas. Entre ellas se pueden mencionar sus usos cosméticos y como complemento alimenticio humano, ya que se les consideran propiedades antioxidantes, antiinflamatorias e inclusive anticancerígenas (Sarakoon *et al*. 2013).

Recientes investigaciones han mostrado que las microalgas pueden ser utilizadas también como biomasa alimenticia en las etapas posteriores al cultivo larvario de crustáceos y peces, ya que ellas contribuyen con el aporte de proteínas de alta calidad, aminoácidos esenciales, lípidos (especialmente ácidos grasos poli y altamente insaturados) y carbohidratos.

Adicionalmente se ha encontrado que la inclusión de microalgas en la dieta de organismos cultivados, tiene un efecto positivo en su condición fisiológica y en su estado inmune debido al aporte de compuestos fenólicos, antioxidantes e inmunoestimulantes.

Después de sus etapas larvales, la mayoría de los peces y crustáceos son poco eficientes en la captura de microorganismos (incluyendo microalgas), cuando éstos se encuentran suspendidos en la columna de agua, sin embargo son capaces de aprovecharlos adecuadamente, cuando están asociados a sustratos ya sea fijos (biopelículas o perifiton) o suspendidos (bioflóculos). En este contexto, las investigaciones se han enfocado más al uso de bacterias autótrofas y heterótrofas y en mucha menor medida al uso de microalgas. Sin

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embargo en los últimos años se ha comenzado a dar importancia al uso de estas últimas y ya se pueden encontrar publicados algunos de los resultados obtenidos.
Por otra parte, la biomasa bacteriana o microalgal, puede utilizarse eficientemente como ingrediente en dietas para organismos acuícolas, proporcionando beneficios no solo nutricionales directos (crecimiento, reproducción, etc.) sino además en la mejora del estado fisiológico e inmune de los consumidores.

El presente documento compila información reciente de diferentes partes del mundo y de diferentes autores e instituciones, sobre el uso de microalgas en el cultivo de organismos acuícolas después de sus etapas larvales, ya sea consumidas directamente en las unidades de cultivo o proporcionadas exógenamente, tanto en fresco como preservadas o incluidas como ingredientes dietarios. Se incluyen desde luego experiencias y datos originales de los autores.

El Valor Nutricional de las Microalgas

Las microalgas son organismos eucariontes fotoautotróficos, generalmente microscópicos pertenecientes a las divisiones: Cryptophyceae, Dinophyceae, Prymnesiophyceae, Chrysophyceae, Bacillariophyceae, Dictyochophyceae, Euglenophyceae y Chlorophyceae. Son altamente eficientes en la fijación de CO_2 así como en la utilización de la energía solar para producir biomasa. Están presentes en todos los cuerpos de agua, como lagos, mares y ríos, pero se encuentran también presentes en el suelo y en la mayoría de los ambientes terrestres. Dada la gran diversidad taxonómica las microalgas presentan diferencias notables en cuanto a su forma, tamaño, hábitat, y composición bioquímica. Sin embargo estas diferencias están no solamente asociadas a la especie, sino además a la etapa de desarrollo o edad del cultivo, a las condiciones ambientales, a la intensidad luminosa y a la disponibilidad de nutrientes entre otros factores (Roy y Pal, 2014).

Emerenciano, M. *et al.* 2015. Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 62-81.

Composición químico-proximal

En la tabla 1, se presentan algunos reportes sobre la composición químico-proximal de algunas de las principales microalgas utilizadas en la acuacultura. En este caso se incluye solamente el contenido de proteínas, lípidos, carbohidratos y cenizas. En general las microalgas marinas contienen en mayor proporción proteínas con valores entre 17 y 70 % (Gatenby y col., 2003, Becker, 2004). En menor proporción se encuentran los carbohidratos, con porcentajes desde un 5 hasta un 12 % (Brown, 2003) y los lípidos fluctúan entre un 7 y un 23 % (Becker, 2004).

Componentes funcionales (compuestos bioactivos)

Las microalgas han despertado fuertemente la atención por contener diferentes componentes funcionales o compuestos bioactivos que pueden ser aprovechados tanto en la nutrición y salud humana, como en la de animales cultivados, incluyendo desde luego los acuacultivos. Estos componentes son principalmente: antioxidantes como carotenos y astaxantinas; compuestos fenólicos de muy diversa composición química y eventualmente se ha considerado también la presencia de inmunoestimulantes o inmunomoduladores. En la Tabla 2, se enlistan algunas microalgas comunes y su contenido de compuestos bioactivos.

Emerenciano, M. *et al.* 2015. Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 62-81.

Tabla 1. Composición químico-proximal de microalgas cultivadas para acuacultura

Especie/condición cultivo	PC (%)	L (%)	CH (%)	C (%)	Fuente
Chaetoceros muellerii/ Lab. Comercial Sonora	17.8	9.6	6.2		López Elías *et al* . (2004)
Chaetoceros muellerii/ Lab. Comercial Sinaloa	18.5	5.3	4.9		López Elías *et al* . (2004)
Spirulina (*Arthrospira platensis)*/ Biorreactor	**55.8** **18-73**	14.2 7-23	22.2	7.8	Tibbetts *et al* . (2015) Sassano *et al* (2010)
Chlorella sp./ Biorreactor	**53.3**	15.7	25.2	5.8	Tibbetts *et al* . (2015)
Nannochloropsis granulata/Fotobiorreactor	33.5	**23.6**	**36.2**	6.7	Tibbetts *et al* . (2015)
Tetraselmis chuii/ Fotobiorreactor	**46.5**	12.3	25.0	16.2	Tibbetts *et al* . (2015)
Chlorella pyrenoidosa/ cultivo cerrado, 3 y 10 días	19.2 44.1	14.1 **28.3**	2.1 6.2	-	Yadavalli *et al* . (2014)
Chlorella pyrenoidosa/ cultivo abierto, 3 y 10 días	18.0 43.2	15.3 **27.8**	2.0 7.3	-	Yadavalli *et al* . (2014)
Chlorella ovalis/ cultivo en laboratorio	32.1	0.9	**27.4**	34.0	Samarakoon *et al* . (2013)
Nannochloropsis oculata/ cultivo en laboratorio	30.9	1.3	17.8	32.9	Samarakoon *et al* . (2013)
Amphidinium carterae/ cultivo laboratorio	21.5	6.3	25.0	41.5	Samarakoon *et al* . (2013)
Phaeoductylum tricornutum/ C. laboratorio	34.7	2.5	15.7	35.7	Samarakoon *et al* . (2013)
Galdieria sulphuraria/ cultivo heterotrófico	26.5	0.1	6.9	-	Graziani *et al* . (2013)
Galdieria sulphuraria/ cultivo autotrófico	32.0	0.2	6.3	-	Graziani *et al* . (2013)
Thalasiossira weisflogii/ fase estacionaria 25 UPS	32.5	21.9	**25.6**	-	Lopez-Elías *et al* (2012)
Thalasiossira weisflogii/ fase estacionaria 50 UPS	21.2	16.2	20.9	-	Lopez-Elías *et al* (2012)
Chaetoceros muellerii / fertilizante agrícola	22.7	26.3	22.7	35.5	Martinez-Cordova *et a* . (2012)
Chaetoceros muellerii / Medio F	18.1	27.3	18.4	34.9	Martinez-Cordova *et al*. (2012)

Emerenciano, M. *et al.* 2015. Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 62-81.

PC (proteína cruda); L (lípidos); CH (carbohidratos); C (cenizas)

Tabla 2. Componentes funcionales de microalgas comunes en acuacultura

Especie/condición cultivo	PUFA/HUFA (% d total)	Aminoácidos (% d total)	Antioxidantes (mg/kg)	Fenoles (mg GAE/g)	Fuente
Galdieria sulphuraria/ cultivo heterotrófico	C18:2 19.5 C18:3 2.7		Vit E: 9.0 ☐ carot.: ND Astax.: ND Luteina: ND		Graziani *et al* . (2013)
Galdieria sulphuraria/ cultivo autotrófico	C18:2 45.2 C18:3 1.1		Vit. E: 15.0 Astax: 575 Luteina: 387 ☐ carot. : ND		Graziani *et al* . (2013)
Chaetoceros muellerii/ Lab. medio F2	C18:2 0.25 C18:3 4.1 C20:4 2.1 C22:6 6.9	Leu: 9.2 Arg.. 5.5 Lys.L 4.8 Met.: 4.2			Pacheco-Vega *et al* . (2009)
Chaetoceros muellerii/ Lab. Fertilizante agrícola	C18:2 0.16 C18:2 4.9 C20:4 1.4 C22:6 3.8	Leu: 10.2 Arg.: 5.5 Lys.: 4.3 Met.: 4.3			Pacheco-Vega *et al* . (2009)
Dunaliella salina/ 7 días Medio F2			☐ caroteno: 0.52 pg/cel.		Gireesh (2009)
Dunaliella salina/ 7 días Fert. liquido			☐ caroteno: 0.64 pg/cel.		Gireesh (2009)
Chlorella spp/ biorreactor	C18:2 17-25 C18:3 7-20 C20:4 ND C20:5 ND		Trans luteína 2600-7400 Caroteno tot. 3088-15600		Guil-Guerrero *et al* . (2008)
Modolus subterraneus/ biorreactor	C18:2 2.5-3.3 C18:3 0.2-1.3 C29:4 4.7-5.1 C20:5 24-27		Trans luteína 15-64 Caroteno tot. 680-4200		Guil-Guerrero *et al* . (2008)
Chlorella pyrenoidosa/ Lab.				Fenoles tot. 17.2	Li *et al* (2007)
Nitzschia laevis/ Lab				Fenoles tot.	Li *et al*

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				8.62	(2007)
Nostoc ellipsosporum/ Lab				Fenoles tot. 60.3	Li *et al* (2007)
*Nannochloris sp./*Cultivo masivo biodisel			□ caroteno: 1080-1190 Luteina: 190-2290 Caroteno Tot 1620-3060	Ác.Galico: ND-60 Ac. Cumárico 60-70 Ac. Salicilico ND-640 Fenoles Tot. 70-1100	Pereira *et al* (2015)

Uso de Microalgas en Acuacultura

Como alimento vivo en forma de células libres

Como es ampliamente sabido las microalgas se han utilizado durante mucho tiempo para la alimentación larvaria de diversos organismos en acuacultura; en este caso, lo más usual es utilizarlas como alimento vivo en forma de células libres en la columna de agua de donde las larvas de camarones, peces y otros organismos son capaces de capturarlas y utilizarlas. Adicionalmente, en tiempos más recientes, se han estado utilizando también para complementar la alimentación de peces y camarones, más allá de sus etapas larvales, reportándose beneficios muy importantes tanto en el aspecto nutricional en sí, como en la condición fisiológica y el estado inmune de los organismos que las consumen.

Godoy *et al*. (2012) evaluaron la maternización de *Litopenaeus vannamei* en un sistema con solo diatomeas (*Thalassiosira weissflogii* y *Chaetoceros muelleri*), con solo bioflóculos y en una combinación de ambos. Los resultados indicaron que las diatomeas fueron un elemento de gran importancia, ya sean solas o en combinación con bioflóculos, teniendo un efecto muy positivo en la respuesta productiva de los camarones, tal como se observa en la Figuras 1, 2 y 3 (datos tomados del documento mencionado).

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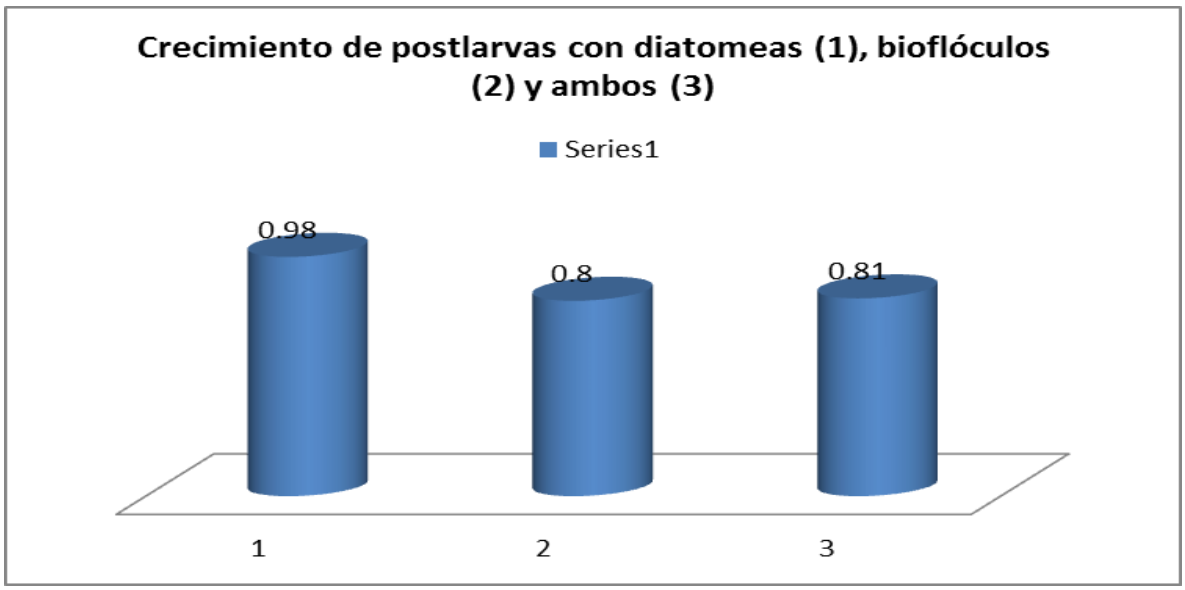

Figura 1. Crecimiento (g) de *L. vannamei* durante la maternización, utilizando diatomeas, bioflóculos y ambos.

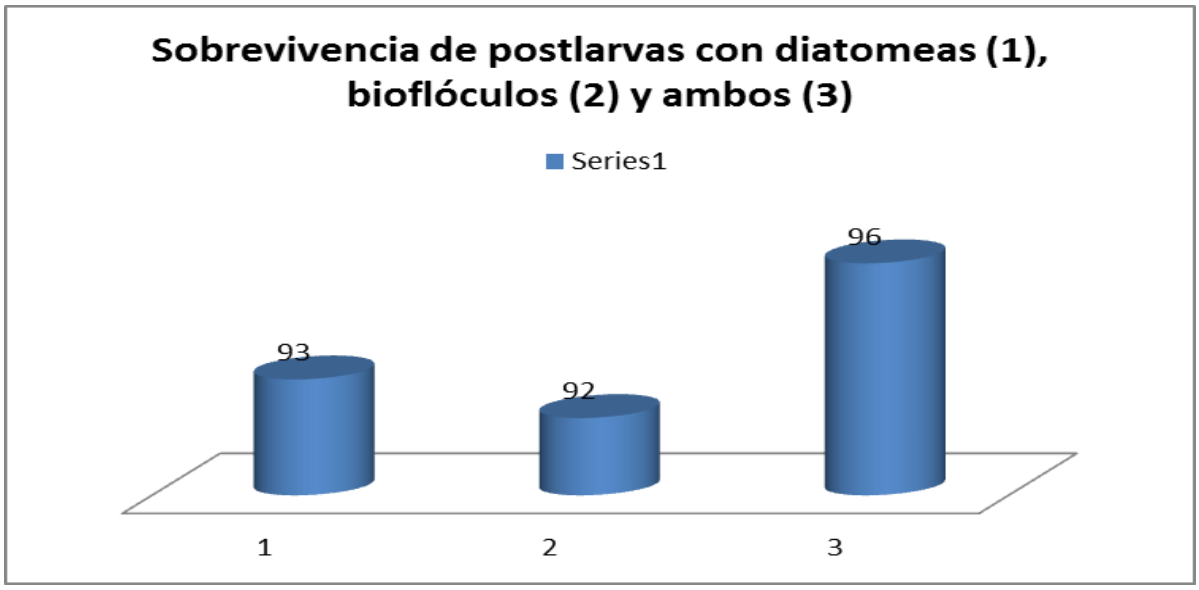

Figura 2. Sobrevivencia (%) de *L. vannamei* durante la maternización, utilizando diatomeas, bioflóculos y ambos.

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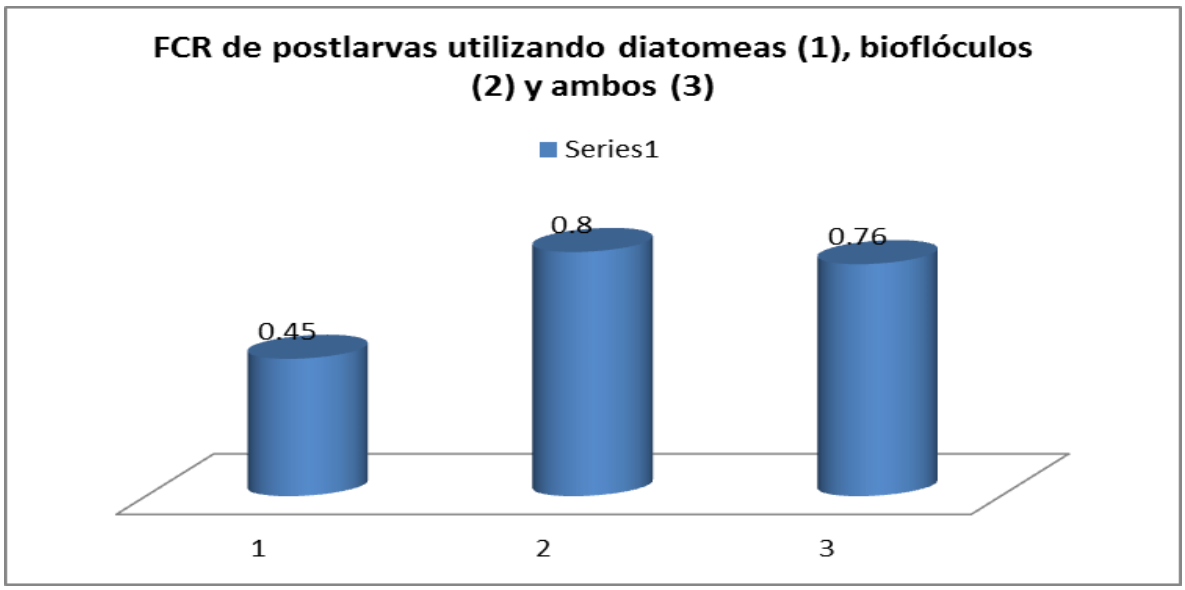

Figura 3. Factor de conversión alimenticia (FCR) de *L. vannamei* utilizando diatomeas, bioflóculos y ambos.

En una investigación similar, Ferreira-Marinho *et al.* (2015) evaluaron la adición de *Navícula* sp. sola o en combinación con alimento formulado, en el desempeño de postlarvas de camarón blanco, *L. vannamei*, encontrando que el crecimiento, la sobrevivencia, la biomasa final y el FCA fueron mejores en el tratamiento que combinó alimento formulado y microalgas, tal como se aprecia en las Figuras 4, 5 y 6 (datos tomados del documento mencionado).

Emerenciano, M. *et al.* 2015. Recent Advances in Aquaculture Systems Based on Microorganisms: The Biofloc Technology (Bft) Case. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 62-81.

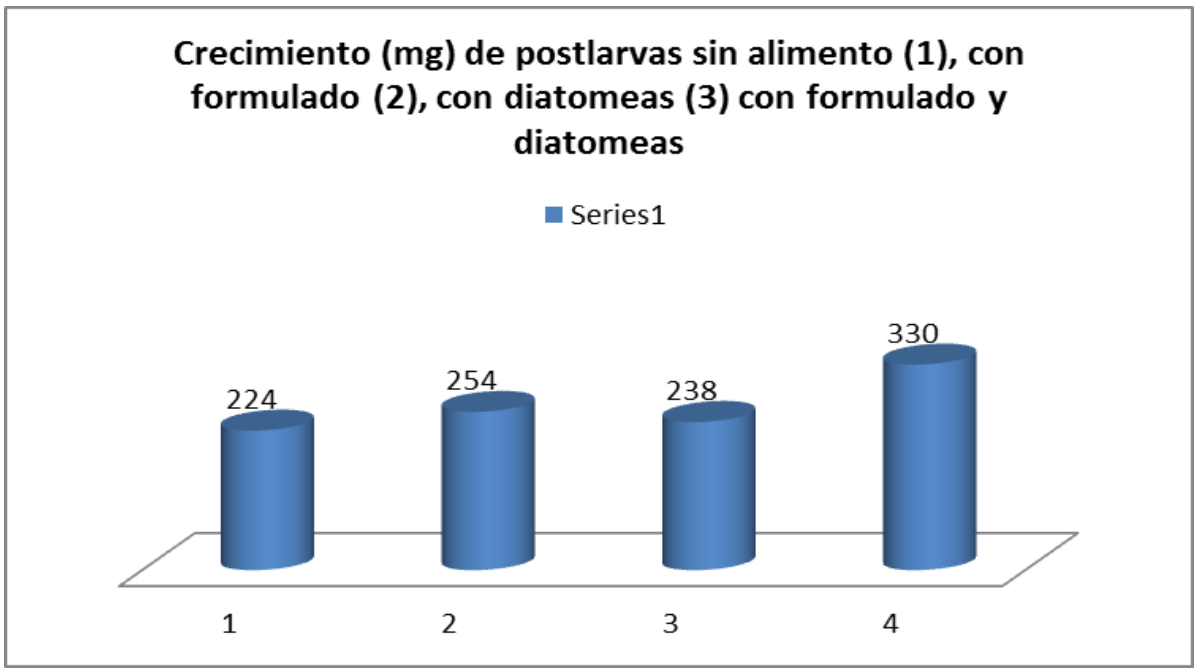

Figura 4 Crecimiento (mg) de postlarvas de *L. vannamei* con y sin alimento y/o diatomeas.

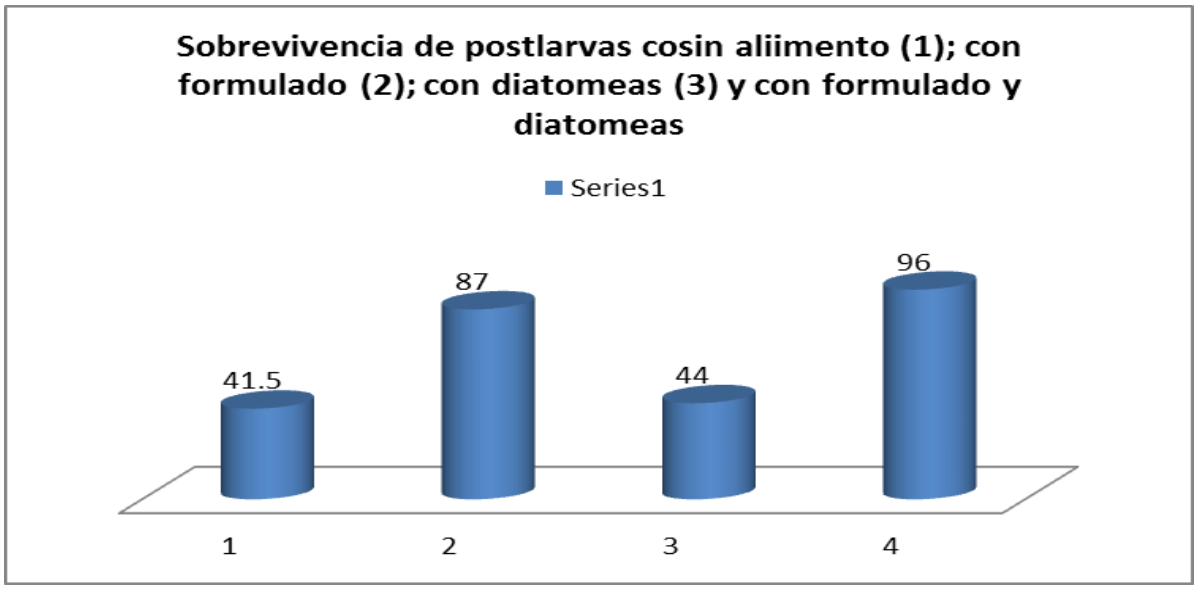

Figura 5. Sobrevivencia (%) de postlarvas de *L. vannamei* con y sin alimento y/o diatomeas.

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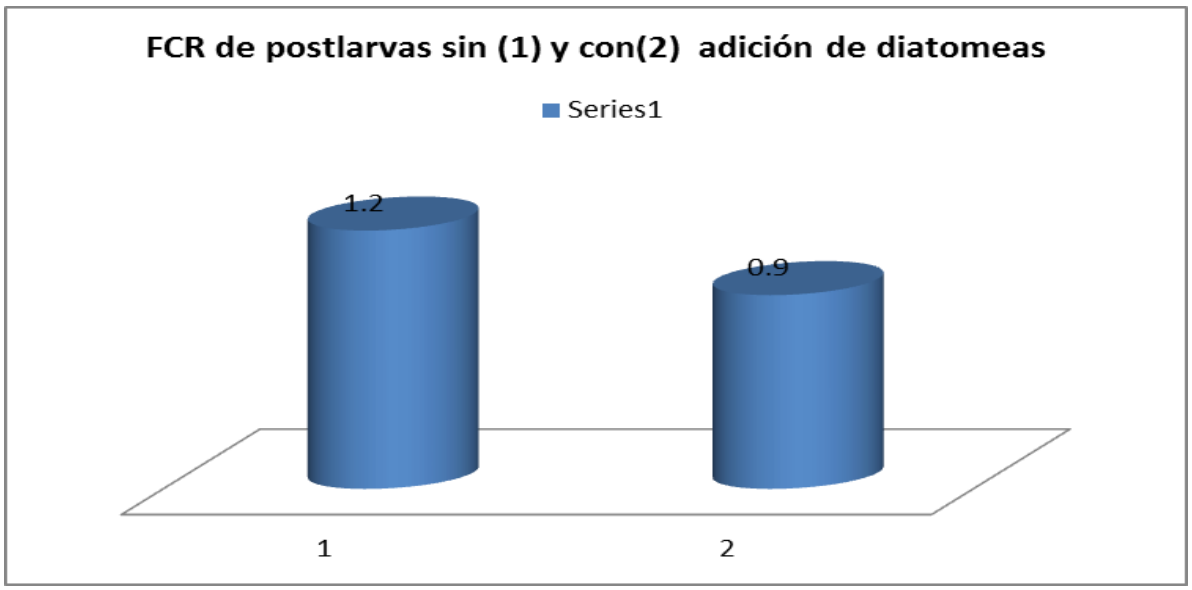

Figura 6. Factor de conversión alimenticia (FCR) de postlarvas de *L. vannamei* con y sin inclusión de diatomeas.

Como alimento vivo en forma de células inmovilizadas.

Las microalgas bentónicas tienen la capacidad de adherirse a sustratos fijos o flotantes, formando en el primer caso lo que se conoce como biopelículas o perifiton y en el segundo, lo que se denominan bioflóculos. También algunas microalgas que no son bentónicas, así como otra clase de microorganismos son capaces de asociarse a estos consorcios, una vez que los organismos iniciadores han formado una matriz a base de exopolímeros y otros componentes aglutinantes (Joyce and Utting, 2015).

La composición biológica de la comunidad perifítica puede ser muy variable y depende de muy diversos factores como: tipo de ambiente acuático, tipo de sustrato, condiciones ambientales, disponibilidad de nutrientes y de luz, entre muchos otros. A continuación (Figura 7) se presenta la composición por grupos de una comunidad de perifiton desarrollada en estanques de cultivo de camarón en Colombia (tomada de Caballero Ávila, 2013).

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El valor nutricional y el uso de perifiton y biopelículas en la alimentación de organismos acuáticos, ha sido reportado desde hace tiempo en diversos estudios, tal como se muestra en la Tabla 3.

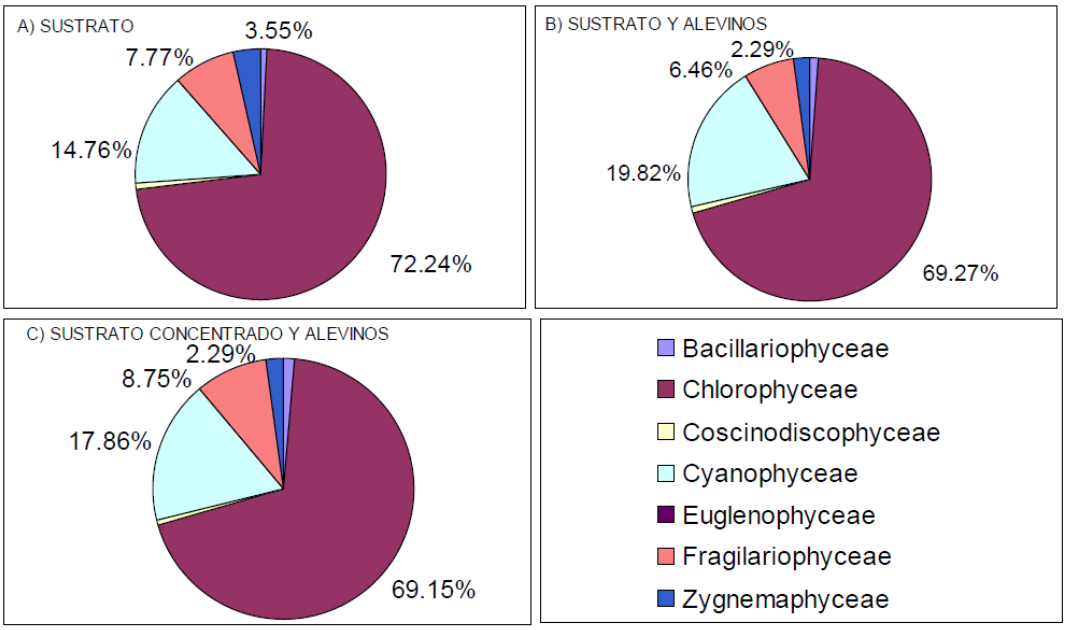

Figura 7. Composición porcentual de grupos de microalgas en el perifiton desarrollado en estanques de cultivo de camarón

Tabla 3. Composición químico proximal de perifiton en diversos ambientes y condiciones

		Proteína (%) base seca	Lípidos (%) Base seca	Fuente
Perifiton	Cultivo tilapia en tanques	14 a 17	1.77 a 4.25.	Azim *et al*. 2003
Perifiton	Policultivo tilapia y un crómido.	35 a 41	13.5 a 15.5	Garg *et al*. 2007
Perifiton	Tanques de peces con sustratos	3.66 a 3.77 (base húmeda)		Gangadhara & Keshavanath (2008)
Perifiton	En varas de bambú	25.96±0.51	2.65±0.21	Anand *et al*. (2013)

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Con respecto a la utilización de perifiton y biopelículas en la nutrición de organismos acuícolas, se tienen documentada varias experiencias exitosas.

Sakr *et al*. (2015) introdujeron perifiton desarrollado en palos de madera, a jaulas flotantes de cultivo de tilapia alimentadas con dietas bajas en proteína (25, 20 y 15 % de PC). Sus resultados mostraron que la dieta con menor nivel proteico, pero complementada con perifiton tuvo la mejor respuesta productiva y fue la mejor en cuanto a costo/beneficio. La composición bioquímica de los camarones no presentó diferencias significativas.

Gangadhar *et al*. (2012) utilizaron bagazo de caña de azúcar para promover el desarrollo del perifiton en el cultivo de carpa de la india, *Labeo rohita* y aunque no encontraron diferencias significativas en la densidad de perifiton, la respuesta productiva de los peces, en términos de biomasa final fue mejor en un 60, 129, 123 y 119 % en los tratamientos en que se usaron 1.5, 2.0, 2.5 y 3.0 toneladas por hectárea de bagazo, respectivamente.

Asaduzzaman *et al*. (2009) investigaron el efecto de la adición de tilapia y sustratos para la formación de perifiton, en la ecología de estanques y en la respuesta productiva de *Macrobranchium rosenbergii*. Los resultados indicaron que la inclusión de tilapia disminuyó la biomasa de perifiton, mientras que los sustratos la aumentaron. La presencia de tilapia disminuyo el FCA en un 14 %, mientras que los sustratos artificiales, lo hicieron en un 16 %, al mismo tiempo que aumentaron la sobrevivencia de los langostinos de 54 a 77 %.

Viau *et al.* (2012) evaluaron la contribución de biopelículas (formadas mayormente por microalgas) en la calidad del agua y la respuesta productiva de la langosta de agua dulce, *Cherax quadricarinatus*, encontrado que en el tratamiento que combinó biopelículas con alimento formulado se obtuvieron mejores crecimientos y sobrevivencias que en aquellos que solo usaron alimento o solo biopelículas (Figuras 8, 9 y 10; datos tomados de

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los mencionados autores). Se observó además un efecto muy positivo en la calidad del agua.

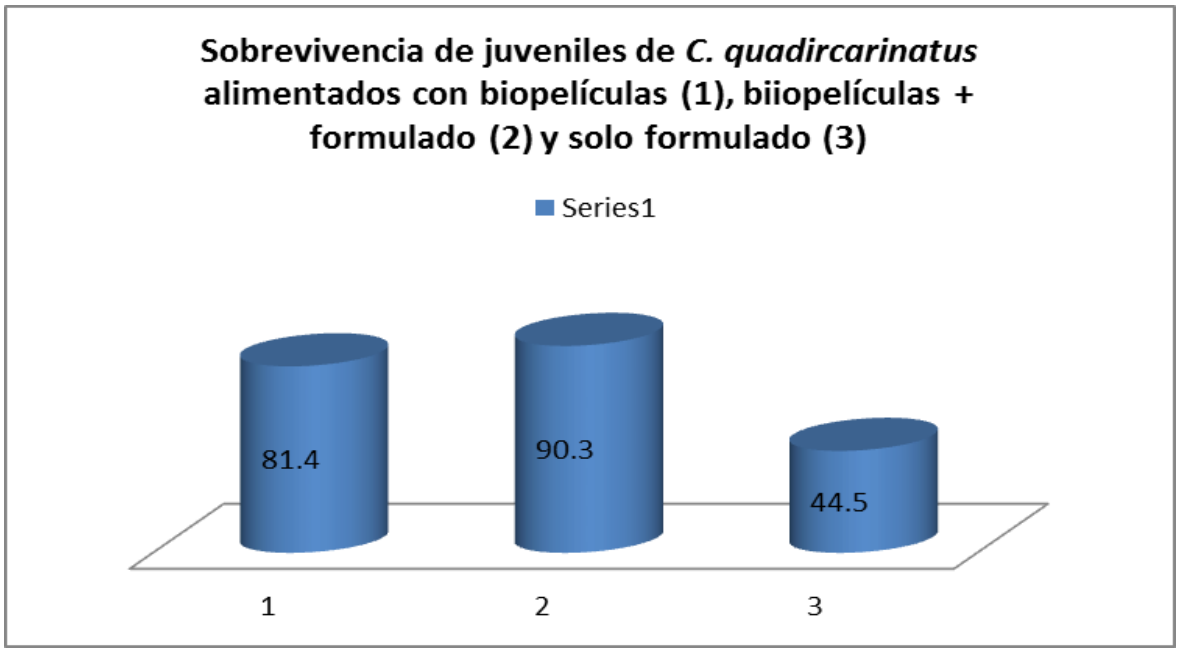

Figura 8. Sobrevivencia (%) de juveniles de *Cherax quadricarinatus* alimentados con biopelículas, alimento formulado y ambos.

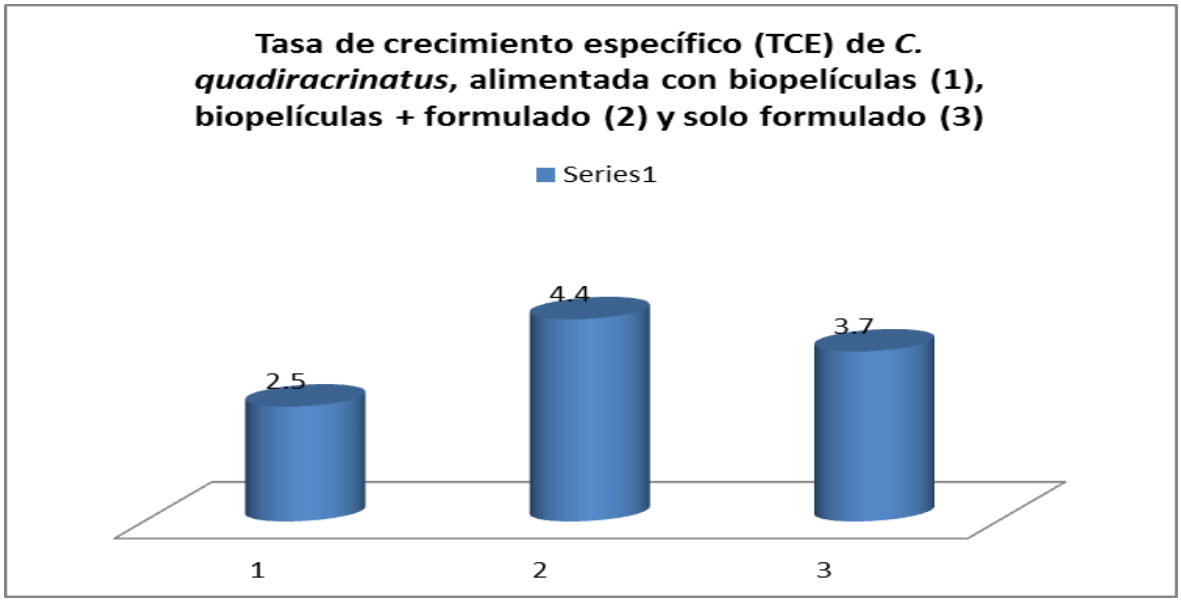

Figura 9. Tasa de crecimiento específico (%/día) de juveniles de *Cherax quadricarinatus* alimentados con biopelículas, alimento formulado y ambos.

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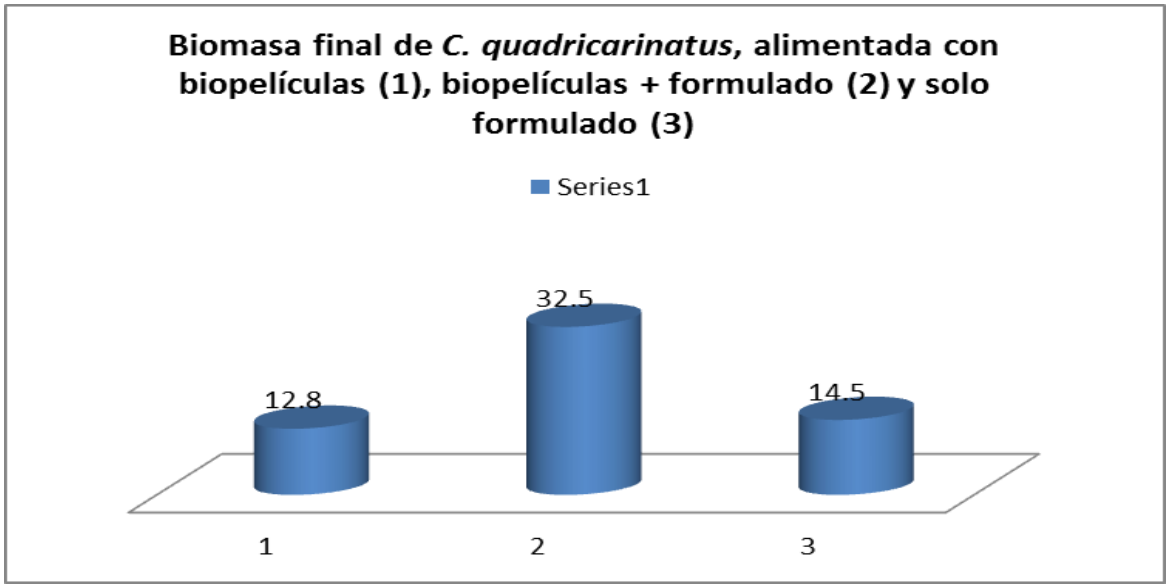

Figura 10. Biomasa final (g/m^2) de juveniles de *Cherax quadricarinatus*, alimentados con biopelículas, alimento formulado y ambos.

Como alimento inerte en dietas

El uso de las microalgas como ingrediente dietario de humanos data ya de algún tiempo atrás. La Spirulina por ejemplo se ha usado para consumo en muy diversas formas; en principio como complemento nutricional debido a su alto contenido de proteínas, lípidos, ácidos grasos y aminoácidos esenciales. Posteriormente ha despertado el interés por sus posibles propiedades terapéuticas y beneficios potenciales a la salud humana (Belay *et al*. 1993). Spirulina ha sido también exitosamente utilizada como ingrediente en dietas de organismos acuáticos. Kim *et al.* (2015) probaron la efectividad de la inclusión dietaria de *Spirulina pacifica* en la respuesta inmune y la resistencia al patógeno *Edwardsiella tarda*, por parte del lenguado *Paralichthyis olivaceous*.

Anand *et al.* (2013; 2015) suministraron perifiton desarrollado en varas de bambú como ingrediente (0, 3, 6 y 9 %) en dietas para el camarón tigre *Penaeus monodon* cultivado y encontraron un efecto muy positivo en el crecimiento, FCA, tasa de eficiencia

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proteica y actividad enzimática digestiva. Adicionalmente en un control positivo en el que no utilizaron el perifiton como ingrediente pero utilizaron las varas de bambú para su desarrollo en las unidades de cultivo, encontraron una mejora significativa en la calidad del agua, especialmente una disminución en la concentración de nitritos y fosfatos.

Sprague *et al*. (2015) sustituyeron parcialmente el aceite de pescado por una harina de la microalga *Schizochytrium* sp, rica en DHA que fue incluida en un 5.5 y un 11 % en la dieta de salmones cultivados. Aunque los parámetros de producción fueron ligeramente menores en las dietas experimentales, ellos concluyen que los resultados son importantes por la posibilidad de sustituir el aceite de pescado y sus consecuencias negativas como la cantidad de toxinas presentes en el mismo.

Kupchinsky *et al*. (2015) evaluaron dietas con diferentes niveles de inclusión (0, 10. 20 y 40 %) de harina de *Chlorella* sp., para alimentar bagres (*Ictalurus punctatus*) en cultivo, sin que se encontraran diferencias significativas en cuanto a la sobrevivencia y composición proximal de los peces; sin embargo un mayor consumo de alimento, una mayor ganancia en peso y un mejor FCA fueron observados en los peces alimentados con las dietas conteniendo 10 y 40 % de harina de la microalga.

Das *et al*. (2015) incluyeron harina destoxificada de las microalgas *Nannochloropsis* sp y *Tetrasemis* sp. en la dieta para cultivar carpa dorada (*Carassius auratus*) en un sistema abierto, encontrando una mejor tasa de crecimiento con la inclusión de la segunda al compararla con la primera y con la dieta comercial.

Medina-Felix *et al.* (2014) probaron la efectividad de la inclusión dietaria de harina de *Dunaliella* sp. la cual tenía altos contenidos de carotenos (propiciados por deficiencia de nitrógeno en el medio), en la alimentación del camarón blanco, *L. vannamei* infectado con WSSV, pudiéndose comprobar que dicha inclusión mejoró la respuesta fisiológica de los camarones, lo que se tradujo en una mejor sobrevivencia y mayor crecimiento en comparación de los camarones alimentados con una dieta comercial.

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Conclusiones

1. El valor nutricional y la contribución de las microalgas en la nutrición de organismos acuícolas está suficientemente bien documentada.
2. Aunque hay avances en el manejo de biopelículas basados mayormente en microalgas (perifiton), todavía queda mucho por investigar.
3. El uso de microalgas particulares o perifiton inespecífico como ingrediente dietario para organismos bajo condiciones de cultivo, es un asunto muy promisorio.
4. El efecto de las microalgas en el mejoramiento de la condición fisiológica y el estado inmune de los organismos acuícolas es en general muy positivo.

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Avances en Requerimientos Nutricionales de Langostinos Nativos

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Resumen

El cultivo de langostinos nativos del género *Macrobrachium* se ha propuesto como una alternativa para su conservación y la creación de fuentes de empleo para los pescadores que se dedican a la captura de estas especies. En el presente trabajo se presentan los resultados obtenidos de pruebas de alimentación realizadas en el Laboratorio de Producción Acuícola con los estadios larvarios de *M. acanthurus* y *M. carcinus*, organismos que se distribuyen a lo largo del Golfo de México. Así mismo, se presenta una revisión sobre el conocimiento actual de los requerimientos en estas especies en los estadios de juvenil y adulto.

Palabras clave: adultos, langostinos, larvas, juveniles, nutrientes, requerimientos

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Introducción

Los langostinos o camarones de agua dulce pertenecen a la familia Palaemonidae y son los crustáceos más diversos dentro del orden Decápoda, tienen una amplia distribución geográfica, batimétrica y están representados por numerosas especies en los sistemas marinos, estuarinos y dulceacuícolas (Hernández-Sandoval 2008). México cuenta con 104 especies de langostinos que se distribuyen en ambos litorales: en el Atlántico bordeando todo el Golfo de México y el mar Caribe; y en el Pacífico, desde Baja California hasta el estado de Chiapas (de los Santos-Romero, Silva-Rivera & Ruiz-Vega 2006). En los últimos años, muchas poblaciones de estos organismos han disminuido o desaparecido debido a dos factores principales: la sobrepesca y la contaminación de los ambientes acuáticos. Este fenómeno es particularmente grave en las cuencas de los ríos Papaloapan y Coatzacoalcos (Estado de Veracruz), en las que dos especies, el camarón prieto *Macrobrachium acanthurus* (Figura 1a) y la acamaya *M. carcinus* (Figura 1b), son sujetas a pesquerías artesanales durante mayo a agosto, meses en los que los organismos migran hacia las lagunas costeras y se reproducen. Usualmente se capturan organismos de talla adulta incluyendo hembras ovígeras y en algunos casos se utilizan sustancias toxicas para facilitar la pesca (Hernández-Guzmán, Cruz-Hernández, Mejía-Ortíz, Ortega & Viccon-Pale 1999). Así mismo, las industrias (papelera y cervecera, principalmente) y poblaciones asentadas en la zona litoral, descargan aguas residuales y desechos municipales directamente en los ríos, aumentando la concentración de materia orgánica y afectando directamente la calidad del agua y por ende, a las especies (Hernández-Guzmán *et al.,* 1999). Desde el punto de vista social, estos organismos representan un importante recurso para las comunidades, tanto desde un punto de vista comercial, como nutricional y de subsistencia, por lo que la sobrepesca obliga a los pescadores a tener un mayor esfuerzo de captura o buscar otras especies acuáticas, afectando la diversidad de los sistemas.

Por ello y como una opción para la conservación de estas especies se ha propuesto desarrollar su cultivo, lo que disminuiría la presión de pesca sobre las poblaciones aún existentes, crearía fuentes de empleo para los pescadores de la zona y en el futuro, aplicarse programas de repoblamiento. Considerando que el éxito de un cultivo esta directamente

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ligado a una alimentación adecuada (Casas-Sánchez, Vaillard-Nava & Re-Araujo 1995), la investigación sobre los requerimientos nutricionales de cada especie es básico para el desarrollo de dietas balanceadas y que a la larga permitirá que el cultivo de los langostinos sea más eficiente y económico. Los requerimientos nutricionales para los paleomonidos se han reportado principalmente en el langostino malayo *Macrobrachium rosenbergii*, (D´Abramo & New, 2010) y la información para las especies mexicanas empieza a generarse y este trabajo presenta avances realizados en el Laboratorio de Producción Acuícola y una revisión de la información hasta ahora publicada sobre los requerimientos nutricionales de los langostinos nativos, particularmente de las especies *Macrobrachium acanthurus* y *M. carcinus*.

(a) (b)

Figura 1. Langostinos macho de *Macrobrachium acanthurus* (a) y de *M. carcinus* (b).

Estadios larvarios

La determinación de requerimientos cuantitativos de nutrientes en los estadios larvarios de paleomonidos representa un reto. Aún cuando previamente se ha reportado que larvas de *M. carcinus* presentan una mejor supervivencia cuando se alimentan con una dieta húmeda con calamar (dos Santos, Gonçalves, Moraes & de Souza 2007) en el Laboratorio de Producción Acuícola se han utilizado microdietas aglutinadas con zeina y κ-carragenan como primer alimento en larvas *M. acanthurus* y *M. carcinus* sin éxito, pues causan una mortalidad alta durante los primeros días de iniciar las pruebas. Por ello, hemos utilizado

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nauplio de *Artemia* como alimento durante los primeros 20 días después de la eclosión. Para determinar el efecto de diferentes nutrientes en la supervivencia de las larvas de langostinos, los nauplios se someten a un proceso de enriquecimiento de corto plazo con microencapsulas de fosfatidilcolina y colesterol (Monroig, Navarro, Amar, Hontoria 2007). Hasta ahora, se ha utilizado DL-metionina (Evonik México, S.A. de C.V.), un aminoácido sulfurado indispensable y precursor en la síntesis de proteínas y de compuestos como el S-adenosilmetinonia (SAM), L-cisteína, glutatión, taurina, fosfatidilcolina y otros fosfolípidos (NRC 2011). Así mismo, se ha utilizado un derivado del ácido ascórbico, el 2-fosfo-L ácido ascórbico trisodio (Sigma Aldrich Comp.). La vitamina C está involucrada en el metabolismo antioxidante (Halver 2002). En una primera prueba, larvas de tres días después de la eclosión de *M. acanthurus* se alimentaron con nauplio de *Artemia* enriquecida con dos concentraciones del aminoácido DL-metionina (40 y 60 mg/ml). Como grupo control se utilizó nauplio sin ningún tipo de enriquecimiento. Grupos por triplicado de 100 larvas se alimentaron con sus respectivos tratamientos y diariamente se determinó la supervivencia. Las curvas de supervivencia se analizaron con una prueba de Gehan-Breslow-Wilcoxon con el programa Prism 6 for Mac OS X (GraphPad Software, Inc.). Después de 18 días de alimentación, se observó una mejor supervivencia de las larvas alimentadas con los nauplios enriquecidos con 40 mg/ml de DL-metionina (Figura 2).

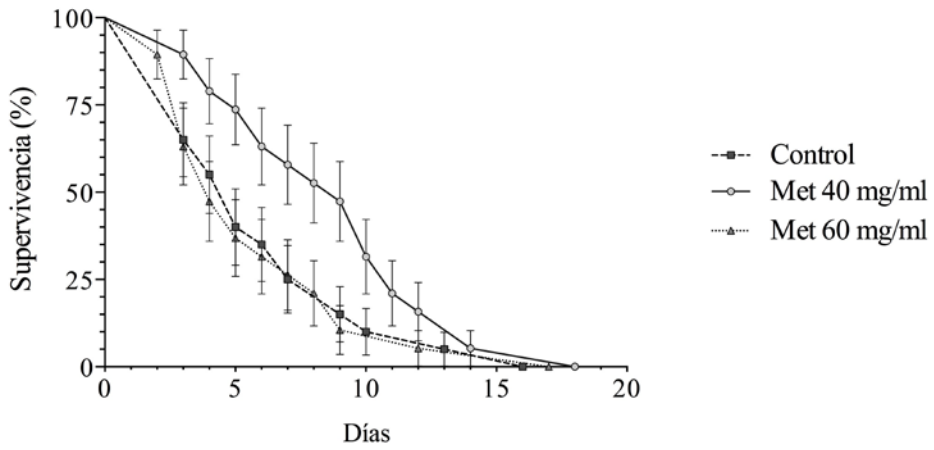

Figura 2. Supervivencia de larvas del langostino *Macrobrachium acanthurus* alimentadas con nauplio de *Artemia* enriquecidos con dos concentraciones de DL-metionina.

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Con esta base, se desarrollo una nueva prueba de alimentación con larvas de *M. acanthurus*, considerando un grupo experimental con la concentración de 40 mg/ml de DL-metionina y la adición de 40 mg/ml de vitamina C, así como un grupo con 40 mg/ml de vitamina C y un grupo control alimentado con nauplio sin enriquecer. Se siguió el procedimiento ya mencionado previamente y el grupo alimentado con la mezcla de vitamina C y DL-metionina, mostró una mortalidad de 100% a los 9 días de iniciada la prueba. Los otros dos grupos mostraron una supervivencia de 10% (grupo control) y de 20% (grupo alimentado con vitamina C) a los 33 días en que los organismos alcanzaron el estadio de postlarva (Figura 3).

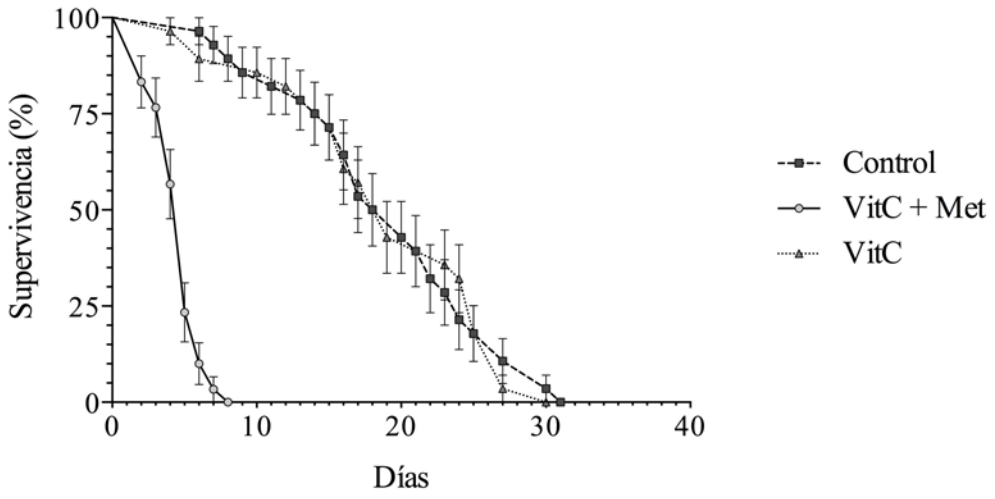

Figura 3. Supervivencia de larvas del langostino *Macrobrachium acanthurus* alimentadas con nauplios de *Artemia* enriquecidos con vitamina C más metionina y solo vitamina C.

Finalmente se realizó una prueba con larvas de *M. carcinus*, en la que grupos por triplicado de 50 larvas se alimentaron con nauplios enriquecidos con 40 mg/ml de DL-metionina, con 40 mg/ml de vitamina C y un grupo control sin enriquecimiento. La supervivencia de las larvas fue significativamente mejor con el tratamiento de vitamina C después de 35 días de alimentación (Figura 4). Estos resultados indican que durante el desarrollo larvario de *M. acanthurus* y *M. carcinus*, es necesario mejorar la calidad

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nutricional del nauplio de *Artemia*. Previamente, se ha reportado que la inclusión de vitamina C en el alimento vivo de larvas de *Litopenaeus vannamei* y *M . rosenbergii*, mejora el crecimiento y la supervivencia (Moe, Koshio, Teshima, Ishikawa, Matsunaga & Panganiban 2004), efecto similar al observado en las larvas de *M. carcinus* y *M. acanthurus* alimentadas con 40 mg/ml del derivado 2-fosfo-L ácido ascórbico trisodio en el nauplio de *Artemia*. Es necesario determinar el efecto de diferentes concentraciones en la supervivencia de las especies nativas de langostino, además de probar otros derivados del ácido ascórbico. El enriquecimiento del nauplio de *Artemia* con DL-metionina no mostró un efecto definitivo sobre la supervivencia de las larvas de *M. acanthurus* y *M. carcinus*. Sin embargo y debido a la importancia de este aminoácido indispensable, también es necesario determinar el efecto de diferentes concentraciones en la supervivencia y desarrollo de ambas especies.

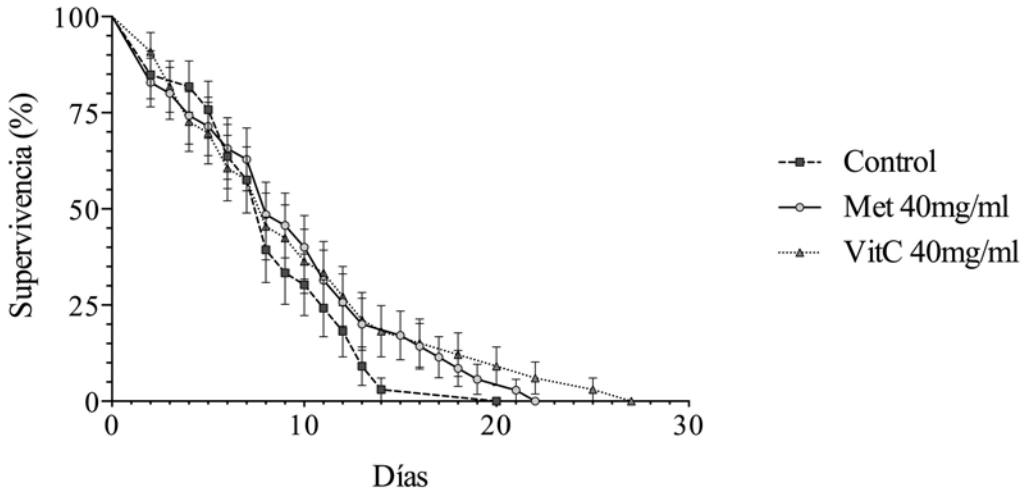

Figura 4. Supervivencia de larvas del langostino *Macrobrachium carcinus* alimentadas con nauplio de *Artemia* enriquecidos con DL-metionina y vitamina C.

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Juveniles

Probablemente es en el estadio juvenil donde mayor información se ha generado respecto a los requerimientos nutricionales del genero *Macrobrachium* (D´Abramo & New 2000). La determinación del requerimiento de inclusión de proteína es un primer paso para el desarrollo de dietas balanceadas, ya que estas moléculas son la principal fuente de energía para los crustáceos, además forman tejido nuevo y permiten la reparación de tejido dañado. Los requerimientos de proteína para los paleomonidos han sido reportados para juveniles de langostino malayo (*M. rosenbergii*) por diversos autores (D'Abramo & Sheen 1994; D´Abramo 1998; D´Abramo & New 2000) y Teshima, Koshio, Ishikawa, Alam & Hernandez (2006) reportan un requerimiento de 0.242 g de proteína por kg de peso por día, evaluado con el método factorial, lo que representa una inclusión aproximada de 40% de proteína en la dieta. Respecto a las especies de langostinos nativos, Casas-Sánchez *et al*. (1995) reportaron que juveniles de la acamaya *M. carcinus* no mostraron diferencias significativas en el crecimiento de organismos alimentados con una dieta con un contenido de 22% y otra de 42% de proteína proveniente de residuos vegetales y marinos. Recientemente Villafuerte, Hernández, Fernández & Angeles (en prensa) reportan un requerimiento de 37.8% para juveniles de *M. acanthurus* alimentados con dietas basadas en caseína. Estos datos indican que la inclusión de proteína en una dieta balanceada para estas especies debe de estar alrededor en un intervalo de 35 a 40%, lo que confirma también sus hábitos omnívoros de alimentación Albertoni, Palma-Silva & Esteves (2003).

Hasta ahora, no hay datos disponibles sobre los requerimientos cuantitativos de los 10 amino ácidos considerado como indispensables en la dieta, sin embargo se cree que al igual que en *M. rosenbergii*, otras especies requieren de metionina, lisina, treonina, arginina, leucina, isoleucina, histidina, fenilalanina, valina y triptófano (D´Abramo & New, 2010). Indudablemente, es necesario continuar con el trabajo de determinación de los requerimientos de aminoácidos.

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Respecto a lípidos, carbohidratos, vitaminas y minerales no hay reportes en especies nativas y solo existe información relacionada con *M. rosenbergii* (D´Abramo 1998; D´Abramo & New, 2000), que podría servir como base para el desarrollo de nuevas líneas de investigación en *M. acanthurus* y *M. carcinus*.

Adultos

Los requerimientos nutricionales en los adultos es, probablemente, el estadio con menos información disponible. La maduración sexual y reproducción son periodos demandantes en el ciclo de vida de los langostinos, particularmente de los nutrientes que serán utilizados en la producción de gónadas (NCR 2011). Los reportes sobre requerimientos en adultos se reducen al de Espinosa-Chaurand, Vargas-Ceballos, Guzmán-Arroyo, Nolasco-Soria, Carrillo-Fárnes, Chong-Carrillo & Vega-Villasante (2011) en ellos indican que adultos de *M. tenellum* requieren de 29% de inclusión de proteína y recientemente, Benítez-Mandujano & Ponce-Palafox (2014) reportaron que adultos de *M. carcinus* mostraron un mejor crecimiento y retención de proteína cuando se alimentaron con inclusiones de proteína de 40 y 45% de proteína proveniente de harina de pescado y de soya, principalmente. Así mismo, estos autores reportan que la utilización de 13% de inclusión de lípidos (aceite de pescado, lecitina y ácidos grasos poliinsaturados) mejoró significativamente el crecimiento cuando se combinaron con niveles altos de proteína (40 y 45%). Villafuerte *et al.* (En prensa) reportaron que hembras de *M. acanthurus* alimentadas con una dieta con 44% de proteína provisto de harina de pescado, harina de krill y harina de soya y 13% de lípidos (aceite de krill, aceite de pescado y lecitina de soya) produjeron huevos con un contenido significativamente mayor de proteína y más alto de lípidos que aquellas alimentadas con una dieta con la inclusión exclusiva de harina de pescado y soya.

Conclusiones

El conocimiento sobre los requerimientos nutricionales de los langostinos nativos de México es uno de los aspectos importantes para el desarrollo del cultivo de estas especies.

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A pesar de que la información generada hasta el momento es incipiente (Tabla 1), sirve de base para el desarrollo de nuevas líneas de investigación: en juveniles, más información debe obtenerse sobre los requerimientos de aminoácidos indispensables, de lípidos, carbohidratos, vitaminas y minerales. Así mismo, se deben realizar trabajos relacionados con el efecto de dietas balanceadas en el crecimiento y desarrollo normales, así como en la expresión de genes marcadores de crecimiento. La identificación y uso de ingredientes locales para substituir a los productos marinos (harina y aceite de pescado, principalmente) en las dietas balanceadas, es también un campo amplio de trabajo. En adultos y reproductores es importante entender el efecto de los lípidos y ácidos grasos sobre la producción y calidad de los huevos producidos, así como el efecto de vitaminas y carotenoides sobre el desarrollo temprano de las larvas. Este último aspecto no se ha trabajo en crustáceos. Finalmente y de particular importancia para el desarrollo del cultivo, la producción de larvas de buena calidad es la base para la engorda de las postlarvas. Por ello es necesario la optimización del alimento durante el desarrollo larvario de estas especies, desde el enriquecimiento (tanto de corto y largo plazo) del nauplio de *Artemia*, el uso de otras presas (como rotíferos o cladóceros) como primer alimento y hasta el desarrollo de microdietas que permitan el desarrollo y crecimiento normales de los organismos.

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Tabla 1. Requerimientos nutricionales reportados hasta ahora para las especies nativas de langostino del genero *Macrobrachium* y susceptibles de ser cultivadas.

Especie	Estadio	Requerimiento	Referencia
Macrobrachium acanthurus	Juvenil	Proteína, 37.8%	Villafuerte *et al*. (en prensa)
Macrobrachium carcinus	Juvenil	Proteína, entre 22 y42%	Casas-Sánchez *et al*. (1995)
	Adulto	Proteína, entre 40 y 45%	Benítez-Mandujano & Ponce-Palafox (2014)
	Adulto	Lípidos, 13%	Benítez-Mandujano & Ponce-Palafox (2014)
Macrobrachium tenellum	Adulto	Proteína, 29%	Espinosa-Chaurand *et al.* (2011)

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Recent Developments in Shrimp Feeds & Feeding

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Abstract

Farmed shrimp currently represent the most valuable segment of the global aquaculture production business at about US $ 22.7 billion in 2013 (farm gate value); total global farmed shrimp production estimated at 4.45 million tonnes (major country producers being China 38.1%, Indonesia 14.0%, Vietnam 12.1%, Thailand 7.4%, Ecuador 6.8%, India 6.5% and Mexico 2.7% in 2013), with production increasing at an average rate of 11.07% per year since 2000 (FAO, 2015). It is estimated that about 84% of total global farm shrimp production was based on the use commercially produced shrimp feeds in 2013, with total global shrimp feed production estimated at about 6.36 million tonnes or about 15.1% of the total estimated global compound aquafeed production in 2013.

With feeds and feeding representing the highest operating cost item of most shrimp farming operations (typically between 35-65 % of total farm operating costs), there is increasing pressure for shrimp feed producers and farmers alike to reduce feed costs per unit of shrimp production. However, despite its relative small size in global terms (97.2 million tonnes), the aquaculture sector is still the largest consumer of fishmeal and fish oil with the sector consuming 68% of the total global fishmeal production in 2012 and 74% of the total global fish oil production in 2012 (Mallison, 2013). The above is perhaps not surprising since fishmeal and fish oil represent ideal feed ingredients for the aquaculture sector by possessing a nutritional profile approximating to the nutritional requirements of most farmed aquatic species, including shrimp; fishmeal not only being an excellent source of dietary protein and essential amino acids but also being a good source of nucleotides, essential fatty acids, phospholipids, minerals, and trace elements (including calcium, phosphorus, magnesium, zinc, manganese, selenium, iodine, molybdenum, and chromium), and fat soluble and water soluble vitamins (including vitamin A, D, E, choline, inositol, and B-vitamins).

It follows from the above discussion therefore that efforts to replace fishmeal with alternative and more sustainable protein-rich feed ingredient sources should focus not only on making good any amino acid

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imbalances through dietary supplementation with aquaculture-grade free amino acids and dipeptides, but must also consider the dietary supplementation of the numerous other essential nutrients usually by fishmeal, including nucleotides, taurine, cholesterol, HUFA, minerals and trace elements. The current paper discusses how the aquaculture feed sector has been able to address the above issues to ensure the continued growth and development of the sector, including through improvements in feed ingredient selection and feed formulation (including the use of amino acids and feed enzymes), improvements in feed manufacturing technology, improvements in on-farm feed storage and management, and improvements in water management and shrimp health. Finally the paper also discusses the need for the improved labeling and reporting of dietary nutrient levels within compound shrimp feeds.

Keywords: shrimp, feeding, management

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Control of Pathogenic Vibrios in Shrimp Aquaculture

Global shrimp production & aquafeed production

Farmed shrimp currently represent the most valuable segment of the global aquaculture production business at about US $ 22.7 billion in 2013 (farm gate value); total global farmed shrimp production estimated at 4.45 million tonnes (major country producers being China 38.1%, Indonesia 14.0%, Vietnam 12.1%, Thailand 7.4%, Ecuador 6.8%, India 6.5% and Mexico 2.7% in 2013), with production increasing at an average rate of 11.07% per year since 2000 (Table 1). It is estimated that about 84% of total global farm shrimp production was based on the use commercially produced shrimp feeds in 2013, with total global shrimp feed production estimated at about 6.36 million tonnes or about 15.1% of the total estimated global compound aquafeed production (Figure 1), and the shrimp industry estimated to have a global economic FCR of about 1.7 in 2013 (Tacon & Metian, 2015).

Table 1. Top fed aquaculture species production in 2013 and estimated compound aquafeed use

Top fed species	Tonnes	APR 20-13	$ billion	Feed Tones
1.Chinese fed carp	13,158,580	5.2%	17.7	11,855,881!
2.Tilapia	4,823,160	11.3%	8.2	7,215,447!
3.Shrimp	4,454,602	11.1%	22.7	6,361,172!
4.Catfishes	4,274,110	10.1%	6.8	4,727,166!
5.Marine fish	2,283,456	8.1%	9.5	3,164,870!
6.Salmon	2,283,093	12.5%	13.8	2,968,021!
7.Misc FW/D fish**	2,206,437	10.5%	4.9	1,390,055!
8.FW crustaceans	1,953,773	4.9%	11.1	1,967,449!
9.Milkfish	1,043,936	8.9%	1.8	1,002,178!
10.Trout	836,569	2.7%	3.6	1,087,540!
11.Eel	231,682	-5.1%	1.3	355,863!
Total	37,549,398	7.3%	101.4	42,095,642!

*!Calculated!from!FAO!(2015); !**!Miscellaneous!freshwater!&!diadromous!fish!

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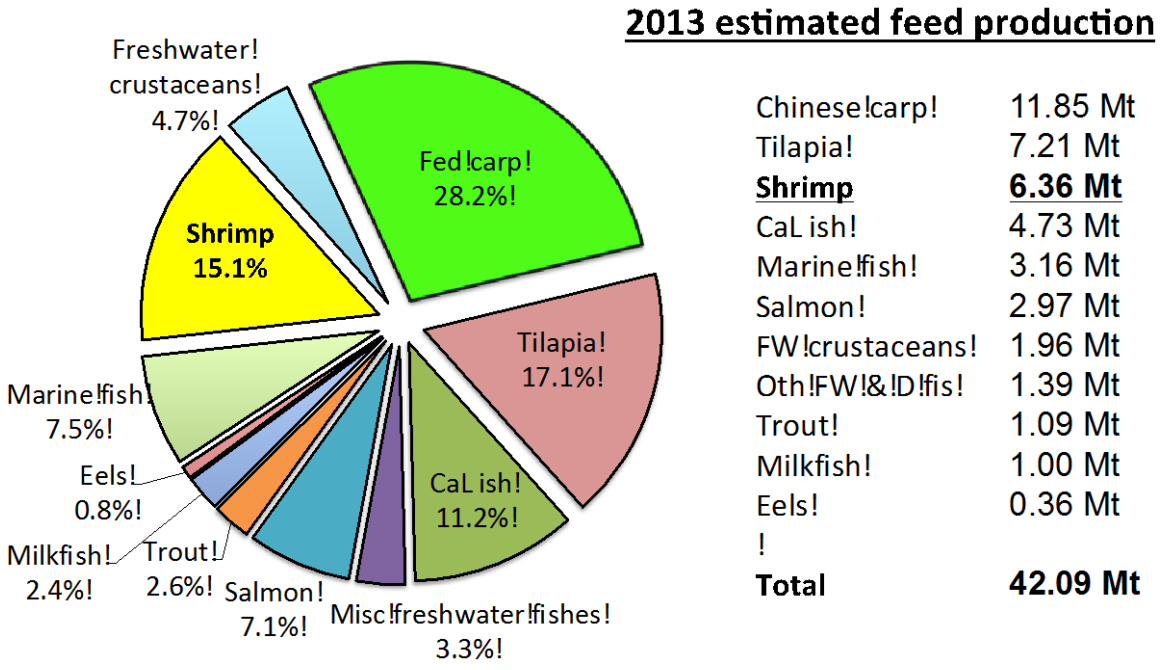

Figure 1. Total estimated industrially compounded aqua feed production in 2013

Shrimp still reliant on fishmeal as a major source of protein & other essential nutrients

According to the latest statistical information from the Marine Ingredients Organization (IFFO) marine shrimp and other crustaceans were the largest consumer of fishmeal in 2013 (estimated at 28% of the total fishmeal consumed by the aquaculture sector in that year (the aquaculture sector consuming 72% of total estimated global fishmeal production in 2013 (Dr Andrew Jackson, Technical Director of IFFO – personal communication). This dependence upon fishmeal is perhaps not surprising since fishmeal has a nutritional profile approximating to the nutritional requirements of most farmed aquatic species, including shrimp; fishmeal not only being an excellent source of dietary protein and essential amino acids but also being a good source of nucleotides, essential fatty acids, phospholipids, minerals, and trace elements (including calcium, phosphorus, magnesium, zinc, manganese, selenium, iodine, molybdenum, and chromium), and fat soluble and water soluble vitamins (including vitamin A, D, E, choline, inositol, and B-

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vitamins (Tacon & Metian, 2015).

It follows from the above therefore that efforts to replace fishmeal with alternative and perhaps more sustainable protein-rich feed ingredient sources should focus not only on making good any amino acid imbalances through dietary supplementation with aquaculture-grade free amino acids and dipeptides, but must also consider the dietary supplementation of the numerous other essential nutrients usually provided by fishmeal, including nucleotides, taurine, cholesterol, HUFA, minerals and trace elements. For a review of the major studies conducted to date concerning the replacement and/or reduction of dietary fishmeal levels within compound shrimp feeds see Tacon et al. (2014).

In general there has been a significant increased use of specific feed additives for shrimp to assist with dietary fishmeal replacement (ie. such as the use of specific limiting amino acids, trace minerals, proteolytic enzymes, fatty acids, attractants, and emulsifiers), to improve shrimp health and wellbeing (ie. such as the use of prebiotics, probiotics, antioxidants, and organic acids) and to reduced the environmental impact impacts arising from feed use (ie. such as the use of phytases, special binders etc.). For a review of the major studies conducted to date see the review of Tacon et al. (2014).

In addition to the above efforts there have also been considerable improvements through the use and blending of different plant and animal feed ingredient sources with complementary dietary essential amino acid (EAA) profiles, the determination of the EAA bioavailability within the feed ingredients used (using a combination of in-vitro and in-vivo digestibility techniques), and the consequent move away from the formulation of shrimp feeds on a total nutrient basis to a digestible or available nutrient basis.

Need for improvements in feed labeling and nutrient declaration

There is an urgent need for the improved labeling of shrimp feeds for the benefit of the farmer and consumer: including the need to move away from the current use of proximate chemical analysis (legal compliance for the declared proximate chemical composition of a feed ingredient or a formulated feed for the purposes of ingredient or feed registration, trade and sales) to the mandatory declaration of specific dietary nutrient levels.

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These specific nutrients could include specific EAA, vitamins, minerals, feed additives), the declaration of the use of genetically modified or banned feed ingredient sources or not (depending upon the country), use of specific feed antioxidants or not, and the possible estimated bioavailability/digestibility of key nutrients present (ie. digestible protein, digestible energy, digestible phosphorus etc).

For example, the current use of proximate chemical analysis (for moisture, crude protein, lipid, crude fiber, ash) to describe the composition of a shrimp feed (or feed ingredient for that matter) is of little or no value to the nutritionist or farmer as it gives no indication of the essential nutrients present or their potential bioavailability or not. Moreover, the current use of proximate analysis, and in particular the calculation of dietary protein level based on total nitrogen levels allows for the possible adulteration of feeds with non-protein nitrogen adulterants such as melamine, ammonium nitrate or urea (Moore et al. 2010).

Need for improved responsible on-farm feed management practices & training

Last, but not least, there is an urgent need for the development of improved responsible on-farm feed management practices, including the use of improved feed transportation and storage techniques, the use of improved record keeping and financial control, and the use of improved on-farm feed and water management, including natural food production and control.

Feeds and feeding represent the largest operating cost item for most semi-intensive and intensive shrimp farming operations, typically between 35 to 65 percent of total farm operating costs. However, in marked contrast to farmed fish species where feeding is usually very rapid and determined visually, shrimp usually feed on the pond bottom (primarily through olfaction) and consequently are not directly visible to the farmer or feeding technician, with pelleted shrimp feeds remaining immersed in water sometimes for several hours before being consumed. In view of the rapid deterioration of shrimp feeds on prolonged immersion in water due to nutrient leaching and the difficulty of shrimp farmers to accurately determine the optimum feeding level and feeding regime for shrimp under

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semi-intensive and intensive shrimp farming conditions, the success or not of the farming operation is still highly dependent upon the on-farm feed management skills of the farmer. As a direct consequence of the above difficulties, wide variations currently exist between individual shrimp farmers concerning shrimp growth (ie. weight gain) and feed performance (ie. food conversion ratio) with animals being fed the same feed, with the variability being greatest for small-scale shrimp farmers.

It is estimated that over 80% of Asia's aquaculture farms are currently small-scale operations, with poor production practices and disease outbreaks threatening the livelihoods of many smallholder shrimp farmers. Small-scale shrimp farmers currently represents the weakest link within most ASEAN shrimp producing countries, and the sector most vulnerable to the possible use of unsustainable farming practices and potential disease risks. Despite the above, feed companies and national government extension services rarely focus or target smallholder shrimp farmers in terms of training opportunities to improve knowledge and methodologies to improve or enhance their production efficiencies. Moreover, since most ASEAN shrimp is produced for export, it is increasingly subjected to strict controls by importing countries (such as the U.S., Japan and E.U.), including increasing market needs for compliance to BMPs and as well as strict import quality control restrictions regarding food safety issues, including spot checking for antibiotic residues and other unwanted contaminants.

Since the most frequent route of antibiotics use within shrimp feeds is through on-farm feed application by small-scale farmers it is essential that these farmers are made aware of these risks and other important on-farm feed management issues. For the purposes of this paper, on-farm feed management covers all those activities conducted by the farmer and his or her staff concerning the handling, storage and use of shrimp feed on the farm.

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Control of Pathogenic Vibrios in Shrimp Aquaculture using Antiinfectives from Marine Natural Products

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Abstract

Mid-culture outbreaks due to pathogenic *Vibrio* spp. are most common and frequent disease problems encountered in shrimp aquaculture. Literature showed that the decades old shell disease to recent early mortality syndrome are caused by vibrios as primary and/or secondary etiological agents. Poor water quality, deteriorated pond bottom due to over feeding, inadequate farm management, rapid intensification of stocking approaches etc. are major predisposing factors of disease outbreaks. Both reactive and proactive treatment methods are being progressed through R&D efforts, but tangible solution is remain to prevent/control mid-culture outbreaks in shrimp aquaculture. Alternate approaches to replace the use of antibiotics in aquaculture is a highly prioritized research area. Alternate approaches are being widened to explore marine natural products, smart biomolecules such as biosurfactants and poly-hydroxy butyrates (PHB), nanoparticles, quorum quenching molecules, small bioactive peptides and probiotics. The oceans are the single principal bio-resource of halo-metabolites produced by a range of marine organisms such as seaweeds, corals, sponges, molluscs, coelenterates, marine worms, tunicates, bacteria etc. but their utilization as aquaculture drugs are not being exploited. Application of seaweed secondary metabolites in treating shrimp bacterial diseases represents an easy, cost effective and environmentally benign venture for equitable and sustainable shrimp farming. Recently we reported antiadhesive activity of PHB biopolymer against *Vibrio alginolyticus* and *V. harveyi*, which were considered as the most significant pathogenic vibrios in the grow-out ponds of giant black tiger shrimp *Penaeus monodon*. In order to reduce the use of antibiotics, pesticides and other chemicals and to improve the ecological environment of shrimp farms, research is being focused on the potential use of marine probiotic bacteria in shrimp farms to improve water quality by balancing bacterial population in water and reducing pathogenic bacterial load.

Keywords: Shrimp, marine natural products, anti infectives

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Introduction

Shrimp Aquaculture is a profitable industry in several countries of Asia particularly Indonesia, Taiwan, China and India. However, diseases are recognized as a major constraint as well as a limiting factor for sustainable shrimp farming. Estimates of economic bases indicate that developing countries in Asia lost at least US$ 1.4 thousand million due to diseases in 1990 alone. Since then, losses due to diseases have been increasing. According to the 1996 World Bank report, global losses due to shrimp disease are around US$3 thousands million and the Bank recommended investment to the tune of US$275 million in shrimp disease research in the ensuing 15 years (Lundin, 1996). It is well known that aquatic organisms come in direct contact with the ambient microbes continuously, which may act as opportunistic pathogens (Raa *et al.,* 1992). Therefore, it is very difficult to prevent diseases caused by opportunistic or secondary pathogens during the entire culture period. Medication of aquatic organisms cannot be restricted to the diseased individuals and as a result, resistant microbial strains may develop, which change the normal microbial composition leading to massive outbreaks of the disease.

Environmental factors and poor water quality, resulting from increased effluent discharge, movement of aquatic animals, inadequate farm management, rapid proliferation of farms etc., have been implicated in major disease outbreaks occurring in epizootic proportion. However, the underlying courses of such epizootics are highly complex and difficult to pinpoint. Viral outbreaks have been damaging the shrimp culture in Southeast Asia and South and Central America. At present, over 20 viruses have been identified as important to shrimp, the most threatening being White Spot Syndrome Virus (WSSV) (Wang *et al.,* 1995), which was previously known as Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) (Wongteerasupaya *et al.,* 1995) in Asia and Taura Syndrome Virus (TSV) in USA. Diseases caused by bacteria are also considered as equally important in causing mass mortalities wherever shrimp are cultured. Among bacteria, vibrio has been implicated as the causative organism, which may trigger mortalities up to 100% (Nash, 1990). Such bacterial epizootics concomitantly emerge as facultative to shrimp due to

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primary viral infections or environmental stress (Lightner, 1988; Karunasagar *et al.,* 1996). The short generation time of bacteria ensures massive population, which develops rapidly in the infected host as well as in the host's environment.

Diseases of Shrimps

Although a wide variety of bacterial causative agents were reported in cultured penaeid shrimps, from the beginning, the most common group was being non-filamentaous, motile, Gram-negative, oxidase positive and fermentative rods (Bell and Lightner, 1987). Based on the bacterial aetiology and external clinical symptoms, the major bacterial epizootics reported could be grouped under the following seven categories:

1. Bacterial Septicemia

'White pleura' disease, diagnosed as bacterial septicemia, cause mass mortality in the postlarvae and grow-out phase of *Penaeus indicus* and *Penaeus merguiensis*. The causative bacteria were initially identified as Gram-positive cocci arranged in tetrads (*Micrococcus* sp.). 'Red Vien' disease in *Penaeus monodon* was reported as bacterial septicemic condition in hatcheries (Chong and Chao 1986). Penaeid bacterial septicemia in juvenile *Penaeus monodon* in Malaysia was reported to be caused by *Vibrio alginolyticus*, *V. parahaemolyticus* and *Pseudomonas* sp. (Anderson *et al.,* 1988). Earlier the same authors (Anderson *et al.,* 1987) reported that Gram-negative, rod-shaped bacteria were responsible for tissue level changes in *Penaeus monodon*. A new Vibrio pathogen, *V. gazogenes* was isolated from the blood of *Penaeus chinensis* affected by epizootic septicemia (Zhan *et al.,* 1997). In addition, the epizootic bacterial septicemia in *Penaeus chinensis* was reported to be caused by yet another new pathogen identified as *Providencia rettegeri.* This was considered as the first report of *Providencia rettegeri* infection in shrimp (Zhan *et al.,* 1997).

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2. Red Leg Disease

The acute bacterial infectious disease, 'red leg disease' was first described for an epizootic occurred in shrimp farm of Fujian province, China during 1987. The heart and nearby origin became light orange and the pleopods became red with reduced swimming activity. The causative bacterium, *V. alginolyticus* was isolated and reported as the causative pathogen for the first time (Zheng *et al.,* 1990). Subsequently, it was reported to be the most frequently isolated bacterium of diseased penaeid shrimp in Italy (Giorgetti, 1990). Xu *et al.,* (1992) characterized 'red leg disease' as expansion of chromatophores on the pereiopods and pleopods, giving these appendages reddish colouration, yellow pigmentation on the branchial region of cephalothorax and reduced swimming. Two strains of bacteria isolated from the haemolymph of moribund shrimps were identified as *Proteus vulgaris. Vibrio parahaemolytics* and *V. alginolytius* isolated from *Penaeus japonicus* and *Penaeus monodon* were also described as causative agents of 'red leg disease' and 'yellow gill disease' respectively (Su *et al.,* 1994). The 'Red disease syndrome' was characterized by the reddening of the shrimp body. However, Alapide-Tedenzia and Dureza (1997) first isolated four *Vibrio* phenotypes, namely, *V. harveyi*, *V. parahaemolytics, V. fluvialis* and *Vibrio* sp. from shrimp with red disease. Both *V. parahaemolyticus* and *V. harveyi* produced the characteristic red colouration in healthy shrimp, when administered experimentally.

3. Necrotizing Hepatopancreatitis (NHP)

Texas necrotizing hepatopancreatitis (TNHP) was an economically significant disease of the marine shrimp *Penaeus vannamei* cultured in Texas, USA. It was first recognized since 1985, and was reported as seasonal (Lightner *et al.,* 1992). Frelier *et al.* (1992) noted the symptom as granulomatous hepatopancreatitis' and the aetiological agent was characterized as Gram-negative, double-enveloped, intra-cytoplasmic bacteria. The NHP required specific environmental conditions for transmission (Frelier *et al.,* 1993) and it causes serious economic impact in the western hemisphere (Jory, 1997). The NHP

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bacterium infected only the epithelial cell lining of the hepatopancreatic tubules, and to date no other cell type have been shown to become infected. The hepatopancreas in shrimp was a critical organ involved in food digestion, nutrient absorption and storage and any infection leads to serious consequences from reduced growth to death. In penaeid shrimp, the hepatopancreas was also known to be affected by various viruses in addition to vibrio bacteria. During 1993, the epizootic NHP affected a number of commercial shrimp farms in the northwestern Peru and adjacent area of Ecuador (Lightner and Redman, 1994). TEM examination revealed that the causative was a pleomorphic intracellular Gram-negative bacterium. The agent was named Peru NHP (PNHP) for its geographical occurrence and it was very similar to the TNHP bacterium.

4. Shell Disease

Shell disease, the degradation of exokeleton is a common disease. The diseases were reported to be seasonal and size-related. Prevalence of 'cuticular lesion' (black spot disease) in brown shrimp *Crangon crangon* was reported to be most common in larger female specimen during late summer and autumn (Knust, 1990; Dyrynda, 1998). SEM observations of diseased shell showed the shell was destroyed to various extents and the epicuticle structure composed of phenolic compounds was damaged totally. Exocuticle and calcified endocuticle were destroyed seriously in most of the affected area (Yang *et al.*, 1992).

Vibriosis and shell disease were reported to be the major bacterial diseases in India. Black lesions (brown spot disease) were observed on abdominal appendages and telson of larvae and adult *Penaeus indicus* and *Panulirus homarus* (Hameed, 1994). Bacterial isolates belonging to the genus *Vibrio*, especially *V. alginolyticus* was isolated. The *V. alginolyticus* bacterium caused black lesion on abdominal segment of larvae in experimental transmission (Hameed, 1994). Luminous and non-luminous *V. harveyi* were associated with 'shell disease' in cultured *P. indicus* (Abraham and Manley, 1995). Several species of bacterial genera *Vibrio*, *Aeromonas* and *Pseudomonas* were commonly cited as causative agents of 'shell disease' of lobsters and shrimp (Aguado and Bashirsullah, 1996).

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Shell disease also prevailed among the wild caught *C. franciscor* and *C. nigricauda* (Arnold and Hendrickson, 1997). The causative bacteria associated with lesions were identified as *Vibrio* sp. and *Pseudomonas* sp. Song *et al.*, (1997) reported that the exoskeleton adherent and fouling bacteria degrade the chitin-based surface cuticle by chitinase, resulting in development of vibrio disease or generalized bacterial septicemia. Due to the destruction of surface cuticle, the opportunistic bacteria provided a route of entry for secondary pathogens such as *Leucotrix mucor* (Yang and Wu, 1992).

5. Black-gill Disease/other backening diseases

Although black-gill disease was well established as a fungal disease caused by *Fusarium* sp., Yang *et al.* (1992a) reported it as an epizootic syndrome with multiple aetiology. Bacteriological isolation made from the penaeid shrimps infected with epizootic black-gill and brown-spot of shell disease syndrome showed *V. pelagicus* and *V. alginolyticus* the major isolates. Subsequent experimental transmission in healthy host revealed that among these, *V. pelagicus* was the major pathogen among *P. chinensis*. Infections of *V. alginolyticus* together with *V. pelagicus* resulted in septicemia and high mortality.

Histological investigations revealed that bacterial gill-rot disease in *P. monodon* was caused by *Bacilli* infection. Moreover, the mortality occurred due to the infection of bacteria and bacteriotoxemia that caused gill and hepatopancreal functional blockages (Chen *et al.*, 1993). Alfaro *et al.* (1993) reported blackening disease in the reproductive system of *Penaeus setiferus* maintained for controlled maturation and reproduction. A progressive, melanised condition of the male reproductive tract was shown to be associated with bacterial infection. Three different species (*V. alginolyticus*, *Pseudomonas putrefaciens* and an unclassified strain) were isolated from the damaged tissues, which later successfully developed similar disease signs in challenge experiments. The authors concluded that the condition could be a progressive syndrome with bacterial invasion or could be of more than one aetiology.

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6. Vibriosis

Members of the genus *Vibrio* are autochthonous bacterial flora in the aquatic ecosystem and quite few of them are associated with infections in humans and aquatic animals. They are the normal bacterial flora of shrimp and the culture environment (Jiravanichpaisal *et al.,* 1994; Otta *et al.,* 1999), but often act as secondary or opportunistic pathogens that cause mortality ranging from few to 100% in affected populations under stress (Lightner, 1988). Vibriosis has been implicated as the cause of major mortality in juvenile penaeid shrimp (Lightner and Redman, 1994). Outbreaks of vibriosis occur only when fish/ shrimp are immunocompromised or under stress due to overcrowding. Reports on the epizootic luminescent bacterial diseases, especially in the shrimp farms of Asian countries have been connected to an increase in the shrimp production and intensive rearing systems (Karunasagar *et al.,* 1994). Luminescent vibriosis is mainly caused by *V. harveyi*, *V. campbellii*, and occasionally *V. splendidus* which can infect larval juveniles and adult stages of penaeid shrimp (Gomez-Gil *et al.,* 1998; Lavilla-pitogo *et al.,* 1998). In the Philippines, virulent *V. harveyi* strains have caused 100% loses in the larval production of *Penaeus monodon* with bacterial cell densities as low as 10^2 cells/ml (Lavilla-pitogo *et al.,* 1990). Bacterial infections related to *V. harveyi* luminescent strains have also been reported to cause major losses in the shrimp larviculture in Australia (Pizzutto and Hirst, 1995), South America (Alvarez *et al.,* 1998; Robertson *et al.,* 1998) and Mexico (Vandenberghe *et al.,* 1999). In India, luminous *V. harveyi* and *V. alginolyticus* were reported as prominent opportunistic and secondary shrimp pathogens of highly devastative mid culture outbreaks (Selvin and Lipton, 2003; Selvin *et al.,* 2005).

Species of *Vibrio* were among the most important bacterial agents known and formed typical normal microflora of the penaeids. They become opportunistic pathogen when culture conditions favour their growth at the expense of the shrimp host (Lightner *et al.,* 1992a). Infection with opportunistic bacteria of the genus *Vibrio* has been found to be a serious disease problem in the intensive brackish water culture of the giant tiger shrimp *P. monodon*. According to Nash *et al.,* (1992), in general infections are secondary and related

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to stress caused by high stocking density and inadequate management. *Vibrio parahaemolyticus, V. anguillarum, V. vulnificus, V. damsela* and *V. alginolyticus* were major pathogens. Lu *et al.*, (1992) reported the mass mortalities due to vibriosis caused by *V. alginolyticus*. *Vibrio* epidemics cause mortality over 90%. Guzman-Murillo *et al.* (1994) have developed a rapid membrane filter method to detect and quantify marine *Vibrio*s. The pathogenic *V. splendidus* I was reported to cause the epizootic disease, 'photobacteriosis' and consequent mass mortality in shrimp. Pathological studies indicated that, it was pathogenic to all stages of shrimp (Chen *et al.*, 1995). According to Ma *et al.*, 1995, an outbreak of explosive epidemic disease occurred in *Penaeus chinensis* due to the combined infection of *Vibrio* and *Micrococcus*. The challenge experiments demonstrated the virulence of *Vibrio* as very strong than that of *Micrococcus*. As seen above vibriosis affects all developmental stages, i.e., from larvae in hatchery tanks to juveniles and brood stock in grow out ponds. However, bacterial strain responsible for vibriosis in the successive stages may be different and virulence specificity was reported to change in the species and stage levels. Accordingly the 'syndrome 93' was considered as a seasonal juvenile vibriosis caused by *V. penaeicida* which affected *Penaeus stylirostris* in grow-out ponds and broodstock tanks. This pathogen did not cause any mortality in hatchery or nursery phases (Goarant *et al.,* 1998). However, some of the *V. penaeicida* strains demonstrated a very high virulence (Costa *et al.,* 1998). *V. cholerae* was found to be pathogenic to *Penaeus chinensis* larvae, which caused swellings in the intestine. The pathogenicity of the isolates was proved in the challenge experiments (Wang *et al.,* 1997).

In penaeid larviculture, frequent larval mortalities were reported to be caused by bacteria with external and internal necrosis (Palanisamy, 1993). Apart from *Vibrio* sp., the causative bacteria for external necrosis were *Myxobacterium* sp., *Aeromonas* sp. and *Psuedomonas* sp. The genus *Leucotrix*, though, non-pathogenic, caused surface fouling in gills or other external organs. Internal necrosis of larvae led to destruction of internal organs, especially the mid-gut gland, resulting in mortality of larvae. Main species of bacteria associated with internal necrosis were *Vibrio* sp., *Pseudomonas* sp. and *Aeromonas*

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sp. The pathogenic role of *Pseudomonas* sp. and *Aeromonas* sp. in shrimp was also reported by Yang *et al.* (1995).

Sung *et al.* (1999) studied changes in the composition of vibrio communities in pond water during *Penaeus monodon* cultivation and in the hepatopancreas of healthy and diseased shrimp. Results indicated that the diversity of vibrio decreased in the culture ponds prior to outbreak of vibriosis. During disease outbreak, *V. furnisii* was the major component of *Vibrio* community in pond water and hepatopancreas. However biotype studies indicated that 68.2% of the isolates were *V. harveyi* or *V. carchariae*. The characterization of extra-cellular products (ECP) from *V. harveyi* and *V. carchariae* was carried out by Montero *et al.* (1999). The ECP demonstrated a range of biological activities including the presence of caesinase, gelatinase and haemolysins.

Vibriosis generally manifests as a hemorrhagic septicaemia with extensive skin lesions, and focal necrosis (Hjeltner and Roberts, 1993). Many factors have been implicated in the pathogenesis of vibriosis. These include, the production of hemolysins (Munn, 1978), proteases (Norqvist *et al*., 1990), a capsule (Yoshida *et al*., 1985; Wright *et al*., 1990), iron binding proteins (Actis *et al*., 1985) and the presence of a 40 KDa hydrophobic surface Ag, VS-P1 (Espelid *et al*., 1987). Invasion of host cells by most pathogens requires penetration and damage of the cell membrane, which is mediated by either physical or enzymatic means, or a combination of two. Phospholipids and proteins represent the major chemical constituents of the host cell envelope. Therefore phospholipases are likely to be involved in the membrane disruption process that often occurs during host cell invasion (Waite, 1996).

7. Luminesent vibriosis

Luminescence in shrimp *Penaeus monodon* larvae was reported to cause mass mortalities in hatcheries in Indonesia (Sunaryanto and Mariam, 1987). Results from initial test had led to the authors to suspect the luminous vibrio as *V. albensis*. In India, luminous bacteria cause serious concern to the hatchery operations (Jayabalan *et al.,* 1996). Epibiotic

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infestation of luminous bacteria was observed in hatchery reared mysis larvae of *Penaeus indicus*. About 28.37% and 58.98% of total viable counts (TVC) in larvae and rearing water respectively. The luminous bacteria isolated from larvae and rearing water were identified as *V. harveyi* (Abraham *et al.,* 1997). The strain of *V. harveyi* isolated from diseased *Penaeus vannamei* was pathogenic in penaeid shrimp larvae, when given as a bath at 10^5 cfu/ml for 2 h. Koch's postulates were confirmed by reisolation and identification (Robertson *et al.,* 1998). These studies confirmed that the occurrence of luminous vibrios was mainly due to the high vibrio load in the rearing water.

The shift in bacterial profile of the rearing water, notably the dominance of luminous vibrio was observed preceding to the occurrence of mortalities (Lavilla-Pitogo *et al.,* 1998). The hepatopancreas (hp) load of luminescent vibrio was considerably increased ($9x10^4$ cfu/hp) during the outbreak in cultured *Penaeus monodon* when compared to the normal shrimp ($7.0x10^1$ cfu/hp) (Leano, *et al.,* 1998). High *V. harveyi* numbers (upto 10^5 cfu/larva) in the larvae were correlated with larval weakness (*Penaeus chinensis*) and mass mortalities (Vandenberghe *et al.,* 1998). According to Pillai and Jayabalan (1993), advanced postlarvae of *P. indicus* challenged with *V. harveyi* inoculum levels of 10^3, 10^4 and 10^5 cfu/l for 96 h did not induce luminescence or other clinical signs of luminous vibriosis. Though the exposed larvae were normal and accepted feed till the termination of experiments, the isolation of bacteria from haemolymph suggested their opportunistic pathogenicity.

One of the major problems in otherwise highly successful *P. monodon* hatchery in the Philippines was the occurrence of luminescent bacterial disease caused by *V. harveyi* (Lavilla-Pitogo *et al.,* 1992). Plate counts of the exoskeleton from all sampled female broodstocks revealed that *V. harveyi* was a minor component of the exoskeletal-associated microflora. Moreover, the authors stressed mid-gut content of mother was the massive source for luminescent vibrio. During 1996, in the southern Thailand, intensive luminescence was encountered in many shrimp ponds accompanied by massive mortality resulting in total crop loss within 3 or 4 days. The farmers named "tea-brown gill syndrome' (TBGS) due to the external signs. The causative bacteria were identified as *V.*

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harveyi (Ruangpan *et al.,* 1999). Recently, non-luminous *V. harveyi* biotypes were also reported to cause mass mortalities among *Penaeus indicus* (Abdel-Aziz and Dass, 2001).

Early Mortality Syndrome Outbreaks

Early Mortality Syndrome (EMS) also termed as Acute Hepatopancreatic Necrosis Disease or AHPND is considered as a new emerging shrimp disease that has attacked the shrimp farms in Southeast Asia (Zorriehzahra and Banaederakhshan, 2015). This disease had caused mass mortality in China (2009) as first time and then in Vietnam (2010), afterward in Malaysia (2011) and finally in Thailand (2012). It was named as EMS due to mass mortality during few days after shrimp post larvae stoking, especially within 20–30 days. The causative agent of EMS has been reported to be a bacterium—more specifically a pathogenic Vibrio belonging to the Harveyi clade, presumably *Vibrio parahaemolyticus* (De Schryver *et al*., 2014). There were some conversations about the possible presence of a bacterial phage or plasmid affecting the virulence of *V. parahaemolyticus*. Earlier, Lightner's team proposed that EMS pathogen has a unique strain of a relatively common bacterium, *V. parahaemolyticus*, that is infected by a virus known as a phage, which causes it to release a potent toxin, but later they denied it.

However, Ung Eng Huan *et al*. (2013) considered that the EMS-causing strains of *V. parahaemolyticus* were given virulence associated DNA from another species by lysogenic phage mediated lateral transfer. They even proposed a transmission mechanism of EMS pathogens. Ung Eng Huan (2014) stated that his group has successfully isolated a lytic phage that can help by killing 50% of all the Malaysian EMS-causing isolates, and the production of phage-probiotic combinations that change every month will not allow the pathogens to build up resistance easily.

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Marine natural products (MNPs)

Marine ecosystem is considered to harbor diverse communities of organisms and it serves as source of chemical diversity structures with promising biological activities. In addition to their uniqueness associated with those compounds, some of them possess unique mechanisms of action as well. So far, eight marine drugs have been approved by FDA or EMEA. It includes Cephalosporin C, Cytarabine (Cytosar-U ® ; Depocyt®), Vidrabine (Vira-A ®), Ziconotide (Prial®), omega-3-acid ethyl esters (Lovaza®), ET-743 (Yondelis®), E7389 (Halaven®), Brentuximab vendotin (SGN-35, Adcetris®), and other marine drugs such as Iota-carrageenan (Carragelose®), Plidítepsin (Aplidin®), PM00104 (Zalypsis®), DMXBA (GTS -21), Lurbinectedin (PM01183), CDX -011, SGN -75, PM060184, Marizomib, ASG -5ME are under clinical trials (Martins *et al*., 2014). Within the last four decades, over 20,000 marine natural products have been isolated from marine micro and macro organisms, many of which have demonstrated potent biological activities (www.grc.org; Marinlit, 2007). A number of these compounds, or synthetic analogues based on natural compounds, have entered clinical trials and some are currently administered as therapeutics. The "pseudopterosins" from the Caribbean sea whip *Pseudopterogorgia elisabethae* was the first clinically validated "cosmeceutical" derived from a marine source (Look *et al.*, 1986).

The exploration for novel bioactive compounds has been taking place in the terrestrial organisms long back, but, recently concern has cockeyed towards the marine biota and its products (Faulkner, 2000; Zhang *et al*., 2005). Marine organisms are the rich source of bioactive compounds, which are reported to have antibacterial, antifungal, cytotoxic, neurotoxic, immunosuppressive, antiviral, and anti-inflammatory activities (Blunt *et al*., 2007; Faulkner, 2002). There has been extensive study on medicinal properties of terrestrial plants as compared to their marine counterparts.

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MNPs in shrimp diseases

At present, application of bioactive natural products from marine source in mariculture industry appeared to be an alternate strategy for this knotty problem (Selvin and Lipton, 2003). Albeit, the bioactive potential of marine algae has been established long before, the application of algal-based products in shrimp disease management is a recently emerged approach (Selvin and Lipton, 2003; Huang *et al.,* 2006).

Among the diverse variety of marine natural products, the halogenated natural products have been the focus of attention in recent years (Gribble, 1998; Kladi *et al.,* 2004). The oceans are the single principal bio-resource of halo-metabolites produced by a range of marine organisms including algae, corals, sponges, molluscs, coelenterates, several marine worms, tunicates, bacteria and other marine life (Gribble, 1998). These compounds are rare in terrestrial plants. Halogenated compounds are biosynthesized mainly from marine red and brown algae and these compounds are dispersed in several different classes of primary and secondary metabolites, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Taskin *et al.,* 2010). These were reported as having biological activities including antibacterial and antitumoral (Cardozo *et al.,* 2007).

Oral administration of organic extracts from several species of marine algae and sponges has been reported to increase the propagation of haemocytes in *P. monodon* (Selvin, 2002; Huxley, 2002; Jose *et al.,* 2008). In addition, it has been known that treatment with hot water extract and polysaccharides from different marine algae progressively elevated the THCs in different species of shrimps (Huang *et al.,* 2006; Yeh *et al.,* 2006; Hou and Chen, 2005; Fu *et al.,* 2007).

1. Seaweeds

Among the diverse marine flora, marine algae (including microalgae) represents one of the most primitive photosynthesizing (contribute nearly 40 percent of global

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photosynthesis) autotrophic groups of ecologically and economically important vegetation of oceanic ecosystem with unique life-cycle and physiology. Nearly fifty thousand species of seaweeds have been discovered in the marine environment (Filho-Lima *et al*., 2002) and none is known to be poisonous (Zemke-White and Ohno, 1999). A relatively small percentage (1 to 5%) of seaweeds available is used as food by both humans and animal. About 221 seaweeds are utilized commercially world-wide of which 65% are consumed human (Zemke White and Ohno, 1999). Historically, seaweeds provide essential economic, environmental, aesthetic, and cultural benefits to humanity (Dhargalkar and Neelam, 2005). In contrast to terrestrial vegetation, the marine flora constitutes valuable source for drug development (De Vries and Beart, 1995). For centuries, many of the seaweed secondary metabolites (SSM) have been used for traditional medicines due to their therapeutic potentials (Fitton, 2006). Recent studies have shown that marine algae are a tremendous source of marine secondary metabolites (Williams *et al.*, 1989; Williams and Maplestone, 1992). Marine algae are continuously exposed to many biotic and abiotic pressures which influence the organism's physiology, which in turn leads to the production of multifunctional natural secondary metabolites (Schmitt *et al*., 1995). So far, more than 2,400 seaweed secondary metabolites (SSM) are described and many of the SSM are natural blueprints for the development of new drugs (Munro and Blunt, 1999; Faulkner, 2001 and previous authors). Several of these compounds exist in biologically active forms in healthy seaweeds. The major secondary metabolites produced by seaweeds are halogenated compounds (Blunt *et al*., 2007) displaying antibacterial, antifungal, antiviral, antifouling and antifeedent properties. The abundance and diversity of secondary metabolites in seaweeds elevated as the prime material for pharmaceutical Industry. Albeit thousands of bioactive compounds have been discovered, the need for novel therapeutic compounds is still urgent in concern of number of new diseases and resistant strains of microorganisms.

Moreover, the Indian red algal species belong to 136 genera, 36 families and 16 orders. The structural diversity of secondary metabolites from Indian red algal species were

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well reviewed by Sarma *et al.* (2006). Approximately 40 numbers of secondary metabolites have been reported from the Indian red algal species (Sarma *et al.,* 2006).

Application of seaweed secondary metabolites in treating shrimp bacterial diseases represents an easy, cost effective and environmentally benign venture for equitable and sustainable shrimp farming (Selvin *et al*., 2009). Currently, studies have been commenced to validate the efficacy of using algal metabolites in shrimp disease management (Selvin *et al*., 2009). Moreover, the range of bioactive compounds produced by the marine algae makes it an excellent source for the detection and characterization of bioactive compounds.

Dietary seaweed extracts "Vivanatural' prepared from an edible seaweed *Undoria pinnantifida*, demonstrated definite prophylactic activity against virus infection (Furusawa *et al*., 1991). The hot water extract of edible brown alga *Hijikia fusiforme* exhibited immunoenhancing activities and this property was associated with algal polysaccharides (Okai *et al*., 1997). Arsenosugar (AsSug) present in seaweed has induced different and interesting cellular responses in macrophages at high concentration (1-10 mM) (Sakurai *et al*., 1997).

The extracts of *Hijikia fusiforme* and *Meristotheca populosa* markedly stimulated human lymphophytes to proliferate whereas *Eucheuma musicatum* and *M. papulosa* gave weak stimulation of proliferation (Shan *et al*., 1999). The phosphate buffered extract of the red algae *Gracilaria verrucosa* and *Papenfum gigartinales* from Japan was known to contain lectin-based haemoglutinins (Kakita *et al*., 1999).

The efficacy of natural products from marine algae against various shrimp bacterial pathogens has been demonstrated in previous studies (Lipton *et al.,* 2009; Jose *et al.,* 2008; Kanjana *et al.,* 2011). Efficacy has also been demonstrated against other shrimp pathogens including virus such as WSSV in *P. monodon* (Witvrouw and De Clercqe, 1997; Chotigeat *et al.,* 2004; Manilal *et al.,* 2009), vibriosis in *Fenneropenaeus chinensis* (Huang *et al.,* 2006) *Litopenaeus vannamei* (Yeh *et al.,* 2006) and *P. indicus* (Immanuel *et al.,* 2004).

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Oral administration of natural antimicrobials is the preferred route of chemotherapy in shrimp aquaculture owing to the ease of use and lack of any additional stress to the shrimp during treatment. Furthermore, it is impossible to isolate infected shrimp for treatment purposes as done in mammals.The application of seaweed-based feed may be an effective means for increasing the immune-proficiency and disease resistance/control in shrimp. The disease resistance of shrimp has been found to be induced by feeding with an algal-based medicated feed that had been a successful strategy for disease management (Selvin, 2002). There has been only limited research effort in the development of therapeutics from natural products for shrimp disease management. This has included work on the marine sponges, mangroves, microalgae, terrestrial plants (Selvin, 2002). It is noteworthy that, apart from Selvin (2002) all work published to date, originated as a consequence of screening for diffusible inhibitory substances/extract *in vitro*. Algal-based medicated feed is a valuable vehicle for oral collective antibiotic treatments in shrimps provided that an adequate amount of the active ingredient is available for the animals. Marine red algae *Asparagopsis taxiformis* was found to be a highly active alga from the southwest coast of India. *A. taxiformis* showed 100% inhibition against pathogenic Vibrio strains isolated from moribund shrimps and MTCC culture of shrimp Vibrio pathogens. Considering the broad anti-vibrio potency, less *in vitro* shrimp toxicity and huge biomass availability, *A. taxiformis* is well suited for the development of potential therapeutic agent (Manilal *et al*., 2010).

There are no reports available on the role of Marine Secondary Metabolites (MSMs) in the shrimp disease management. However, some of the seaweed-based products were reported to act as immunomodulators in fishes. A commercially obtained spray-dried preparation of microalgae, *Tetraselmis sueccica* showed promise in controlling prawn pathogenic strains such as *Vibrio* sp., *V. alginolyticus, V. anguillarum, V. parahaemolyticus* and *V. vulnificus* (Austin and Day, 1990). The survival rate and growth of larvae of kuruma prawn *Penaeus japonicus* fed with *Ulva pertusa* was increased (Yamsaki *et al*., 1997). It was reported that epibiotic marine bacteria present on the larvae of some crustacean

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protected them from fungal infection by production of low molecular antibiotics (Boyd *et al*., 1998).

In a study conducted by Chen *et al*. (2015), the immune parameters of ammonia-stressed white shrimp *Litopenaeus vannamei* like hemocyte counts, phenoloxidase activity, respiratory bursts, and superoxide dismutase activity significantly increased, when they were immersed in seawater containing *Gracilaria tenuistipitata* extract. Several scientists including Smith *et al*. (1984), Kitikiew *et al*. (2013) and Chen *et al*. (2014) specified that shrimp hemocytes incubated with β-1,3-glucan, fucoidan, and carrageenan exhibit degranulation, alterations in cell size and viability, and better phenoloxidase activity and respiratory bursts. Chen *et al*. (2014) reported that white shrimp receiving carrageenan via immersion revealed increase in hemocyte count, and higher number of mitotic cells in hematopoietic tissue.

2. Sponges and its associated bacteria

During the 40 years of extensive investigations, more than 6000 compounds were discovered from the marine source (Davidson, 1995). A series of arabinosyl nucleosides, including spongothymidine and spongouridine, isolated from the Caribbean sponge *Cryptotethia* crypta were the first compounds discovered from marine source (Bergmann and Feeney, 1951). The bioactivities to some of these metabolites include ichthyotoxicity, phytotoxicity, cytoxicity, antibiosis, antiviral, insecticidal, antifeedant and pharmacological activities. Despite the vast bioactive potential found in the MSMs, only a few have reached preclinical/commercial level. The difficulties associated with the collection and isolation of marine samples in comparison to terrestrial samples has also hampered the progress (Naya *et al*., 1993). In addition, the synthesis of the active principles has only recently entered a similar growth phase (Albizati, 1991). According to Rinehart, (1991) only three marine-derived compounds have reached preclinical trails. One of these ‘didemin - B’ in phase II trials as an anticancer agent. ‘Ecteinasidins’- another group of compounds was proved to have potent solid-tumour activity. An analysis of phyletic distribution of MSMs revealed

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that the majority (93%) was confined to four groups (macroalgae, coelenterates, echinoderm and sponges), largely a reflection of the abundance and easy collection of these organisms (Attaway and Zaborsky, 1993). However, in the beginning of last decade, the contribution from macroalgae decreased significantly, whereas sponges become the dominant source for novel compounds. The increased studies on sponges could be attributed to their wider range of biosynthetic capabilities than any other group of marine invertebrates. Recently, marine microbes also received much attention as a renewable source of bioactive molecules for biomedical research (Bremer, 1998).

The marine bacterium *Pseudomonas* isolated from its host sponge *Suberea creba* from the Coral Sea of New Caledonia produced potent antibacterial quinones. However, the host sponge contained dibromo-verangiaquinol showing strong antibacterial activity. Moreover, it was promising for mariculture, as an antibacterial agent in cultures of *Pecten maximus* larvae, which was nontoxic in Artemia salina test (Debitus *et al.*, 1998).

Perusal of literature clearly indicated that the use of MSMs in shrimp disease management is an unexplored area, albeit it has a vast potential of developing potent safe antimicrobial/ immunostimulating drugs for shrimp disease management.

3. Marine microbe-associated products

As reported by Rattanachuay *et al*. (2010), marine microbes have the potential to produce many extracellular compounds against various shrimp pathogenic vibrios. The anti-vibrio compound produced by marine *Pseudomonas* is found to be pyocyanin (Priyaja *et al*., 2014). Likewise, many other products of marine microbes are under trial for application in shrimp farming. They are as follows:

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a. PHB

Poly-β-hydroxybutyric acid (PHB) is a natural, ecofriendly polymer accrued in the form of intracellular granules by a large variety of bacteria (Luzier, 1992). Kiran *et al.* (2014) reported the antiadhesive activity of PHB biopolymer from a marine bacterium *Brevibacterium casei* MSI04 isolated from a marine sponge *Dendrilla nigra*. PHB showed antibacterial activity against *Vibrio alginolyticus* and *V. harveyi*, which were considered as the most significant pathogenic vibrios in the grow-out ponds of giant black tiger shrimp *Penaeus monodon* in India. Also, Laranja *et al.* (2014) reported a PHB-accumulating *Bacillus* spp. isolated from the marine sediment improves the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae challenged with pathogenic *Vibrio campbellii*, which was in agreement with several reports (Halet *et al*., 2007; Defoirdt *et al.*, 2007).

b. Biosurfactants

Biosurfactants are surface active compounds having both hydrophilic and hydrophobic domain that allows them to exist preferentially at the interface between polar and non-polar media, thereby reducing surface and interface tension (Banat *et al*. 2010). Donio *et al*. (2013) reported a halophilic bacterium *Bacillus* sp. BS3 producing pharmacologically important biosurfactants that has antiviral property against the shrimp white spot syndrome virus by subduing the viral replication and considerably outstretched shrimp survival. In the review of Dinamarca *et al.* (2013), it has been mentioned that biosurfactants produced by marine bacteria can act as immunostimulating molecules that strengthen fish immune system and, thus, reduce the quantity of antibiotics required to control infectious outbreaks in aquaculture ponds. Biosurfactants produced by a hydrocarbon-degrading marine bacterium *Cobetia* sp. inhibits the quorum sensing ability of *Vibrio* species (Ibacache-Quiroga *et al*., 2013). This evidences that biosurfactants are quorum quenching molecules that may in turn neutralize the virulence of shrimp pathogenic vibrios. Hence,

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biosurfactants from marine biota is one of the potential MNPs in the control of aquaculture diseases.

c. Nanoparticles

Due to rapid disease outbreaks in the aquaculture industry, it is believed that many aquaculture experts are turning to an emerging technology termed as nanotechnology (Handy, 2012). Dominguez (2014) proposed that nanotechnology possibly will aid in aquaculture production by improving feeding formulation, controlling diseases and biofouling, etc. The synthesis of metal nanoparticles using the bioflocculant produced by a marine sponge associated bacteria could reveal improved safety and stability over existing methods (Sathiyanarayanan *et al*., 2013). Leaf extract of the coastal plant *Prosopis chilensis* was exploited by Kandasamy *et al*. (2013) for the synthesis of silver nanoparticles, which showed potential anti-vibrio activity in protecting the shrimp, *Penaeus monodon* from vibriosis. Rajeshkumar *et al*. (2009) attempted the synthesis of DNA-Nano vaccine made of VP28 gene of WSSV encapsulated within chitosan nanoparticle and succeeded in attaining the protective efficacy of oral delivery of that nanoparticle in black tiger shrimp (*Penaeus monodon*) challenged with WSSV. Perhaps their benefits, nanoparticles also reported to have considerable toxic effects on shrimps (Arulvasu *et al*., 2014).

Probiotics in shrimp aquaculture

In order to reduce the use of antibiotics, pesticides and other chemicals and to improve the ecological environment of shrimp farms, research is being focused on the potential use of probiotic bacteria in shrimp farms to improve water quality by balancing bacterial population in water and reducing pathogenic bacterial load. In addition, the use of probiotics can increase the population of food organisms, improve the nutrition level of aquacultural animals and improve immunity of cultured animals to pathogenic microorganisms. By applying these bacteria in shrimp farms, a biological equilibrium

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between competing beneficial and deleterious microorganisms could be produced. Reports indicated that addition of probiotic bacterial strains will repress the growth of *Vibrio* spp., fungi and other pathogenic microorganisms. It also noticed that probiotic bacteria could produce some digestive enzymes, which might have improve the digestion of shrimp, thus enhancing the ability of stress resistance and health of the shrimp (Selvin *et al*., 2009). According to some recent publications, the mechanism of action of the probiotic bacteria may have several aspects:

1. Probiotic bacteria may competitively exclude the pathogenic bacteria or produce substances that inhibit the growth of the pathogenic bacteria.
2. Provide essential nutrients to enhance the nutrition of the cultured animals.
3. Provide digestive enzymes to enhance the digestion of the cultured animals.
4. Probiotic bacteria directly uptake or decompose the organic matter or toxic material in the water improving the quality of the water.

According to the findings of Chinese researchers, when these bacteria were added into the water, they could decompose the excreta of shrimps, remaining food materials, remains of the plankton and other organic materials to CO_2, nitrate and phosphate. These inorganic salts provide the nutrition for the growth of micro algae, while the bacteria grow rapidly and become the dominant group in the water, inhibiting the growth of the pathogenic microorganisms. The photosynthesis of the micro algae provide dissolved oxygen for oxidation and decomposition of the organic materials and for the respiration of the microbes and cultured animals. This kind of cycle may improve the nutrient cycle, and it can create a balance between bacteria and micro algae, and maintaining a good water quality environment for the cultured shrimps (Selvin *et al*., 2009).

Microorganisms have a critical role in aquaculture systems because water quality and disease control are directly related and closely affected by microbial activity (Pillay, 1992). Intensive cultivation systems obviously led to a change in the composition of environment and indigenous protective flora of the cultured organisms. This leads to an

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increase in the susceptibility of the host animal to opportunistic / secondary pathogens as well as reduced feed conversion ratio due to the imbalanced microflora in the intestinal tract. However, it has been reported that both the health and survival of organisms in intensive rearing systems could be improved substantially by manipulating the gut / environmental microflora with "probiotic" microorganisms and/or prebiotics, which can be added to the diet and/or to the environment to promote the growth of beneficial bacteria in the gastrointestinal tract of the animal as well as in the detritivorous microbes in the pond bottom (Olafsen, 2001; Lin, 1995; Rengpipat *et al*. 1998a,b). The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular due to an increasing demand for environment-friendly aquaculture. Probiotics are, in general, defined as live microbial feed supplements, which beneficially affect the host (Fuller, 1989). Probiotics for aquatic organisms have been defined as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health" (Gatesoupe, 1999). There have been many studies involving probiotics for use in aquaculture (e.g. Moriarty, 1998; Gatesoupe, 1994; Gram *et al*., 1999; Nikoskelainen *et al*., 2001; Panigrahi *et al*., 2004; Salinas *et al*., 2005), but the mode of action is incompletely understood. However, it is widely accepted that the mechanism of probiotics include inhibitory interaction (antagonism), production of inhibitory compounds competition for chemicals and adhesion sites, improving the microbial balance, immune modulation and stimulation, and bioremediation of accumulated organic lead in the pond bottom (McCracken and Gaskins, 1999; Verschuere *et al*., 2000; Lin, 1995; Rengpipat *et al*. 1998a,b).

Albeit the potential of probiotics has been well established, the constraints in probiotic development need to be considered in the evaluation of novel as well as commercially available probiotics (Fig. 1).

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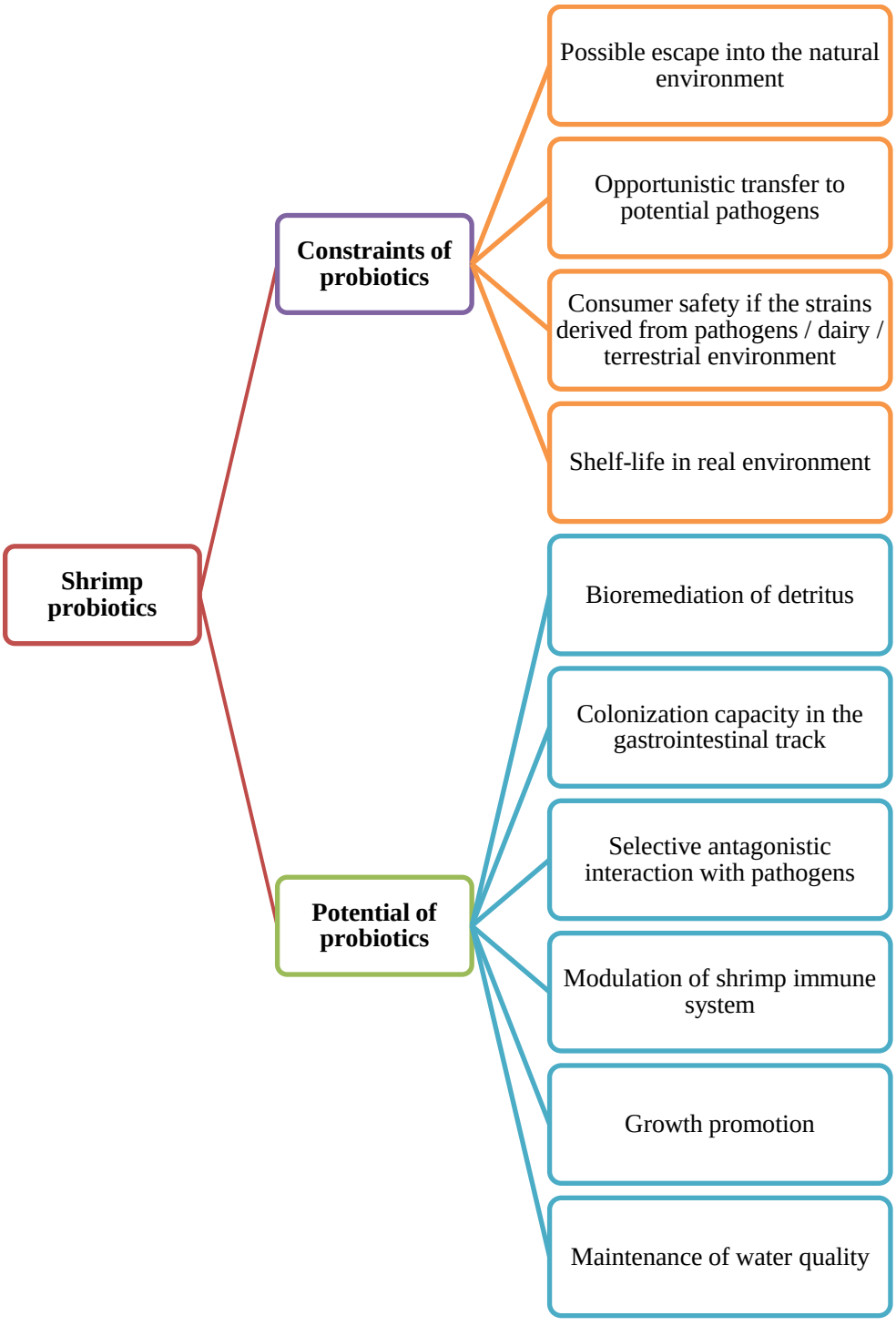

Fig. 1. Potentials and constraints of probiotics in shrimp aquaculture

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Potential sources of antagonistic probiotics

The marine environment has been mined for novel microorganisms used in drug development (Selvin *et al*., 2004) and is likely the largest contributor of bacteria to the aquaculture. Sfanos *et al*., (2005) made a survey of bacterial samples isolated from wild marine sources including macroalgae, sea water, and sea sediment to screen potential probionts. Numerous bacterial community have been explored from unique marine environments, such as hydrothermal vents (Jeanthon, 2000), marine sea sediments (Cifuentes *et al*., 2000; Llobet-Brossa *et al*., 1998), marine biofilms, microalgae blooms (Seibold *et al*., 2001), and marine sponges (Lafi *et al*., 2005). The representation of bacterial groups uncovered were mostly gamma proteobacteria tending to dominate in most communities (Eilers *et al*., 2000). Recently marine bacterial endosymbionts are emerging as potential source for the development of novel probiotics (Selvin *et al*., 2004: Selvin *et al*., 2007 unpublished data).

Bacteria in the aquatic environment and certainly those in the diet influence the composition of the fish intestinal tract where they can positively affect the health of the organism (Verschuere *et al*., 2000). In addition to gut microflora which can exclude the adhesion of other species to the intestinal wall, bacteria that can out-compete pathogens for carbon and energy sources in the aquatic environment may also be good candidates for probiotic mixtures (Verschuere *et al*., 2000). Furthermore, microorganisms displaying antibacterial properties have often been discovered associated with macroalgae and other sources in the marine environment (Sfanos *et al*., 2005), and these offer a third source of potential probionts. Literature evidenced that a wide variety of probiotics strains could be developed from different sources.

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Successful and commercial probiotics in aquaculture

In parallel to the growth of probiotic application ranging from food supplements to biotherapeutics, the biodiversity of strains exhibiting potentially probiotic functionalities has increased remarkably in recent years. The large majority of commercial probiotic products contain one or multiple strains of lactic acid bacteria primarily belonging to the genera *Lactobacillus* (Donkor *et al*. 2007; Geier *et al*. 2007), *Bifidobacterium*, *Lactococcus, Pediococcus, Enterococcus* and *Streptococcus*. In addition, other bacterial taxa such as *Propionibacterium spp., Bacillus spp*. and *Escherichia coli* and the yeast *Saccharomyces boulardii* have also been used in probiotic products (Holzapfel *et al*., 1998; Klein *et al*., 1998; Mercenier *et al*., 2003). Some *Bacillus* sp. (*B. megaterium, B. Polymyxa, B. subtilis, B. licheniformis*), lactic acid bacteria (*Lactobacillus sp., Carnobacterium sp., Streptococcus sp.,*), *Pseudomonas* sp. (*Pseudomonas fluorescens)* and *Vibrio* sp. (*V. alginolyticus, V. salmonicida*-like) have been proposed and tested as probiotics in aquaculture (Gatesoupe, 1991; Verschuere *et al*., 2000). Although studies have shown that lactic acid bacteria effective in inhibiting the growth of various vibrio species in Atlantic cod fry *Gadus morhua* (Gildberg *et al*., 1997) and turbot larvae (Gatesoupe, 1994), the probiotic effects lasted only for a brief time after feeding was discontinued. Lactic acid bacteria are known to produce growth inhibiting factors such as bacteriocins that are particularly useful against other gram positive bacteria (Stoffels *et al*., 1992), however, since most of the known pathogens in aquaculture are gram negative, and lactic acid bacteria account for only a small part of the gut microbiota of fish, their usefulness in aquaculture is debatable (Verschuere *et al*., 2000). *Pseudoalteromonas* have been found to synthesize biologically active compounds with antibacterial, algicidal, anti-algal and bacteriolytic properties (Holstrom and Kjelleberg, 1999). This relatively new genus has been exclusively isolated from marine environments throughout the world (Enger *et al*., 1987), and species within this genus are often found associated with eukaryotes (Holstrom and Kjelleberg, 1999). One isolate has even produced a bactericidal antibiotic against methicillin-resistant *Staphylococcus aureus* (Isnansetyo and Kamei, 2003). Maeda *et al*.

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(1997) showed that the addition of an anti-microbial strain of *Pseudoalteromonas undina* repressed the growth of pathogenic bacteria and viruses in fish and crustacean farming.

Four species including, *B. pumilus, Micrococcus luteus, Pseudomonas fluorescens* and *Pseudomonas putida,* are currently included in bacterial mixtures that are marketed as probiotics for aquaculture (Prowins Biotech Private Ltd., India). Additionally, *Bacillus* sp. have been successfully used as probiotics in the aquaculture of black tiger shrimp (*Penaeus monodon*) in Thailand, where there was an improvement in the growth rate (47%) and survival rate when challenged with *Vibrio harveyi* (Rengpipat *et al*., 1998a,b). *Aeromonas* media UTS strain A199 has been shown to be a potential probiotic for the management of bacterial (Gibson *et al*., 1998; Tan *et al*., 2003) and fungal pathogens (Lategan and Gibson, 2003; Lategan *et al*., 2004a,b) in the aquaculture industry (Lategan *et al*., 2006). *Pseudomonas fluorescens* (AH2) was shown to be strongly inhibitory against *Vibrio anguillarum* in model systems and it was found that this effect could be transferred to an in vivo situation where the mortality rate in rainbow trout infected with *V. anguillarum* was significantly reduced by the addition of the probiotic bacterium to the tank water (Gram *et al*., 1999). Rengpipat *et al*. (2000) showed that the survival and growth of the black tiger shrimp (*Penaeus monodon*), fed with probiont *Bacillus* S11 was increased when compared with non-treated shrimp. The addition of bacterium CA2 as a food supplement to auxenic cultures of *Crassostrea gigas* larvae was found to consistently enhance the growth of the oyster larvae regardless of the season of the year (Douillet and Langdon, 1994). Thus, probiotics have been shown to be effective in a wide range of species for the promotion of growth, enhanced nutrition, immunity and survival.

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Review of Amino Acid Nutrition and Digestibility in Shrimp: A Step Forward Toward the Formulation of Cost-Effective Feeds

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Abstract

Knowledge of nutrient digestibility coefficients for individual ingredients and the requirement of digestible nutrients for a defined production target, allows nutritionists to formulate diets that better match animals requirement. Significant information on the digestibility of nutrients, including amino acids from practical ingredients for pacific whiteleg shrimp (*Litopenaeus vannamei*), has been produced during the last years, allowing nutritionists to develop compound feeds that rely less and less on costly protein sources such as fish meal (FM). Fish meal has often been used as the protein source in commercial shrimp feed because of its excellent sources of nutrients, e.g., balanced amino acid profiles, essential fatty acids, and mineral content. However, in order to reduce feed cost, finding alternative protein sources to replace costly proteins such as FM is another major challenge facing nutritionists. Numerous research studies have demonstrated that substitution of FM with alternative protein sources in shrimp diets results in similar growth, survival, and feed conversion ratios, as long as nutrient composition, including amino acid profile, are balanced to cover animal requirements. We propose to review available data on amino acid recommendations and digestibility for whiteleg shrimp that will help making the process of least-cost formulation for shrimp more cost-effective.

Key words: Amino acids, methionine, amino acid digestibility, shrimp

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Review of available nutrient digestibility values in whiteleg shrimp

A major advantage of formulating diets in a digestible basis, is that it makes possible to ensure more predictable animal performance, when changing feed recipes due to changes in availability of feedstuffs and feedstuffs prices. Moreover, protein and amino acid digestibility coefficients of ingredients are needed for more accurate, environmentally friendly, and economical feed formulations. But for many years, the lack of information on amino acid digestibility has limited the switch from total into using digestible amino acid values in least-cost formulation. As a result, shrimp feed is still often formulated in terms of crude protein and amino acids content without considering the nutrient digestibility. The number of studies reporting nutrient digestibility, including amino acid digestibility has increased significantly during the last decade. Digestibility coefficients of dry matter (DM) and crude protein (CP), are by far the most commonly reported in shrimp. But information about amino acid digestibility in white leg shrimp is now available for several ingredients, including those commonly used as alternative protein sources to FM. Digestibility coefficients for individual essential amino acids, except tryptophan, have been reported for ingredients like blood meal (Villarreal-Cavazos *et al*., 2014, Liu *et al*., 2013), corn gluten meal (Yang *et al*., 2009; Liu *et al*., 2013), cottonseed meal (Liu *et al*., 2013), extruded soybean meal (Yang *et al*., 2009), feather meal (Villarreal-Cavazos *et al*. 2014), fermented soybean meal (Yang *et al*., 2009), fish meal of different origins and sources (Yang *et al*., 2009; Terrazas-Fierro *et al*., 2010; Liu *et al*., 2013), full fat soybean meal (Cruz-Suarez *et al*., 2009), meat and bone meal (Yang *et al*., 2009; Liu *et al*., 2013), peanut meal (Yang *et al*., 2009; Liu *et al*., 2013), plasma protein meal (Yang *et al*., 2009), pork by-product meal (Terrazas *et al.,* 2010; Villarreal-Cavazos *et al*., 2014), poultry by-product meal (Yang *et al*., 2009; Terrazas *et al*., 2010; Liu *et al*., 2013, Villarreal-Cavazos *et al*., 2014), rapeseed meal (Liu *et al*., 2013), different shrimp by-product meals (Yang *et al*., 2009, Terrazas-Fierro *et al*., 2010; Liu *et al*., 2003), soybean meal (Cruz-Suarez *et al*., 2009; Yang *et al*., 2009; Terrazas *et al*., 2010; Liu *et al*., 2013), soy protein concentrate (Cruz-Suarez *et al*., 2009), different squid meals (Terrazas-Fierro *et al*., 2010; Liu *et al*., 2013), and of different wheat products (Yang *et al*., 2009; Terrazas *et al*., 2010; Nieto-López *et al*., 2011). For

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many of these ingredients, also the digestibility of the non-essential amino acids is given. The digestible content of CP, lysine and methionine, of selected raw materials relatively to FM (taken as reference; 100%), is shown in Figure 1. Relatively to FM, digestible CP content is low in all of the ingredient analyzed with the exception of blood meal, feather meal and wheat gluten. Lysine and methionine contents are both limiting in feather meal, meat and bone meal, peanut meal, pork by-product meal, being in the case of feather meal and peanut meal severely limiting (<45%). Results found for feather meal illustrate well the existence of discrepancies between digestible CP and essential amino acid contents, and the need of moving away from using digestible or even available CP content as a criterion in the evaluation of ingredient quality. Digestible content of lysine is also limiting in corn gluten meal (<40%) and wheat gluten meal (< 45%), and that of methionine also limiting in soybean meal (<65%), blood meal (<80%), and slightly limiting in poultry by-product meal (90%). On the other hand, ingredients like blood meal and soybean meal have a relatively high digestible content of lysine, while corn gluten meal and wheat gluten meal have a relatively high digestible content of methionine. Regression of digestible CP content against digestible lysine or methionine showed no clear linear relationship, with a coefficient of determination of 0.63 and 0.36 found for CP vs lysine and CP vs methionine, respectively. Despite the limited number of measurements used to perform these analysis, the results highlight the limitations of using digestibility CP as a single criterion to define ingredient quality.

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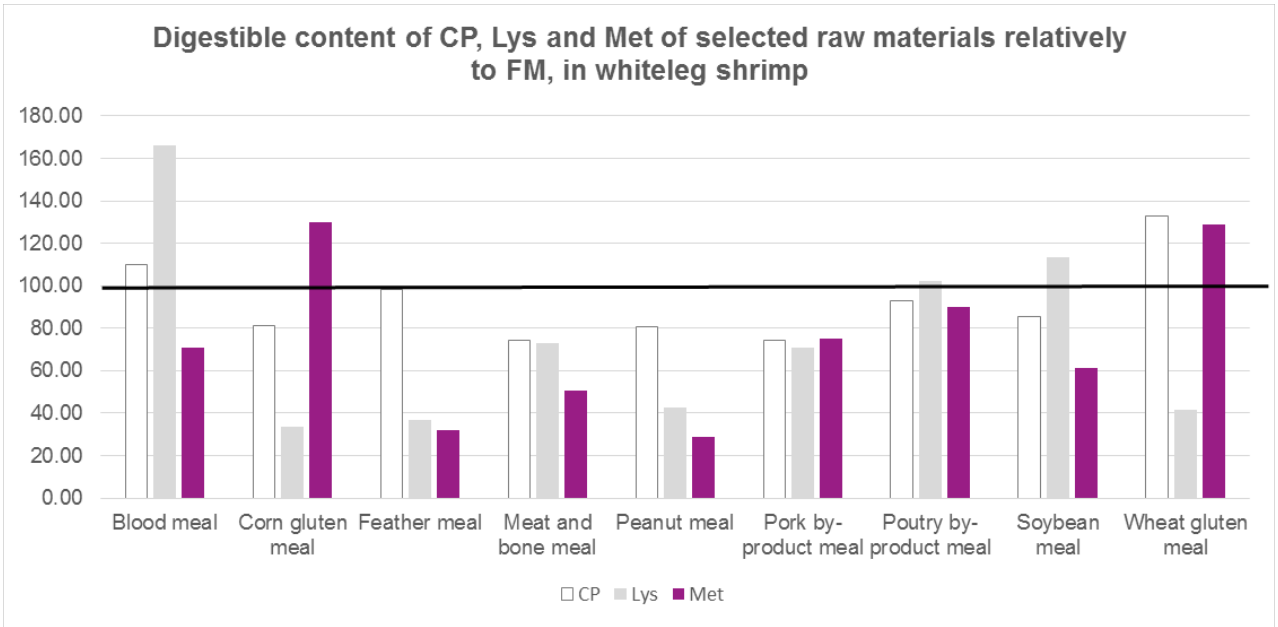

Figure 1. Digestible content of crude protein (CP), lysine (Lys) and methionine (Met) of selected raw materials relatively to fish meal (FM) in whiteleg shrimp.

Advances in the amino acid nutrition of shrimp

The slow feeding behaviour of crustaceans such as, for example, whiteleg shrimp, may result in a long residence time of the feed in water and thus of nutrients being dissolved or leached out. A major challenge that shrimp nutritionists are presented with is, thus, to find valid strategies to minimize leaching losses. Nutritionists, feed manufacturers and farmers have long recognized this issue and the need to improve feed stability in water so that wasting of nutrients due to physical deterioration is minimal. Besides the obvious economic losses, leaching of nutrients and in particular of amino acids and other nitrogen-compounds is long recognized to lead to eutrophication of the water and thus negatively impacting the environment. It is in this context that Evonik Industries started in 2008 an R&D project with the objective of developing a second generation methionine source for shrimp, prawn and other crustaceans. Such a product would remain sufficiently stable in water reducing leaching, and would be digested as slowly as protein-bound amino acids,

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improving protein synthesis. From several different molecules developed, the dipeptide DL-methionyl-DL-Methionine (or *DL*-Met-Met for short) was selected for its exceptional physical and chemical characteristics. The mixture of four different methionine stereoisomers (*DL*-Met-Met, *LD*-Met-Met, *DD*-Met-Met and *LL*-Met-Met) confers unique characteristics to this product –branded AQUAVI® Met-Met - due to its extremely low water solubility when compared to other methionine sources available in the market. Most importantly all four different stereoisomers were shown to be effectively cleaved by fish and crustacean digestive enzymes to free D- and L-Methionine, in several *in vitro* digestion experiments (Figure 2). This seems to agree with data obtained in Atlantic salmon (Sveier *et al*., 2001; Facts and Figures 1620), rainbow trout (Kim *et al*., 1992) and in hybrid striped bass (Keembiyehetty and Gatlin III, 1995), showing that D- and/or DL-Methionine is at least as effective as L-Methionine. The next step was to evaluate the efficacy of AQUAVI® Met-

Met by following a two-step systematic evaluation approach:

1) Studies under clear water/controlled conditions for an accurate evaluation of the AQUAVI® Met-Met efficacy,

2) Studies under green water/field conditions for a more practical oriented evaluation of Met-Met application in commercial feeds optimized to producer-specific conditions.

First studies were conducted to compare the effectiveness of AQUAVI® Met-Met and DL-Methionine in regard to shrimp overall performance (Facts and Figures 1623; Facts and Figures 1624; Fox *et al*., 2012; Lemme *et al*., 2012). Although DL-Methionine and AQUAVI® Met-Met are both efficiently utilized by whiteleg shrimp, significant differences in final body weight and specific growth rate (SGR) are seen when comparing both methionine sources. Altogether, data show that AQUAVI® Met-Met is about 2 times (200%) more efficient in promoting growth of shrimp than DL-Methionine.

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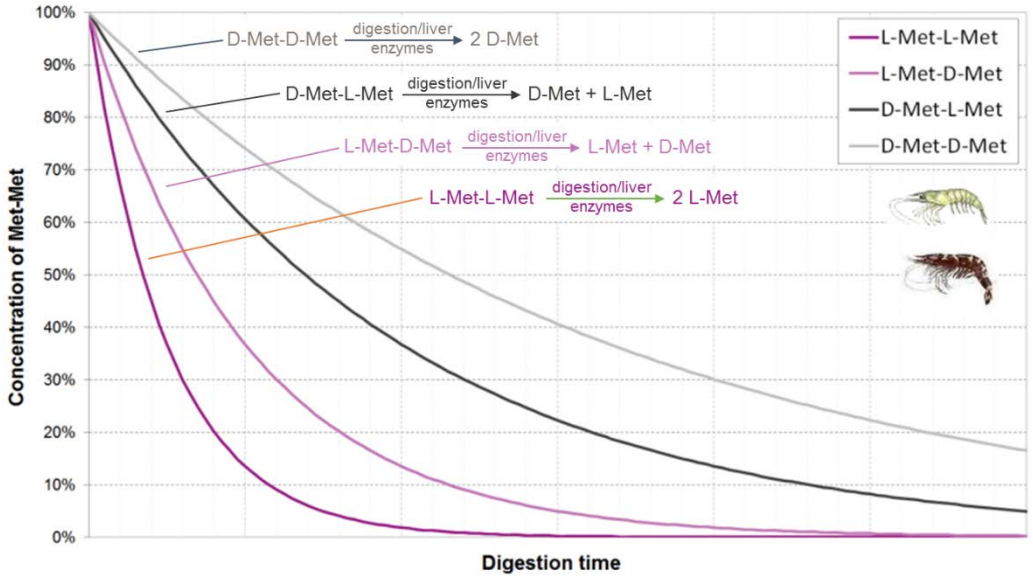

Figure 2. Cleavage kinetics for the four different *DL*-Met-Met stereoisomers (*in vitro* study; unpublished data).

Provided with evidence that AQUAVI® Met-Met is highly effective in covering methionine requirements of shrimp, the efficacy of this novel methionine source to reduce FM in whiteleg shrimp was evaluated (Facts and Figures 1625). AQUAVI® Met-Met proves to be a key element in formulating cost-effective reduced FM diets for whiteleg shrimp. Similar performance as a feed including 26% FM can be achieved with FM10% feeds supplemented with 0.19-0.28% DL-Methionine or 0.09% AQUAVI® Met-Met (Facts and Figures 1625).

The impact of natural food productivity when defining methionine recommendations and applying them to practical diet formulation in shrimp, was also evaluated. In fed-based aquaculture ponds, marine shrimp can be raised under various levels of intensification, from 10 to more than 120 shrimp/m^2. In these confined environments, phytoplankton productivity is the common dominant ecological factor. Microscopic algae typically can make up the base of the aquatic food chain, contributing to the abundance of a variety of shrimp food items, such as prey (polychaetes, amphipods, copepods, foraminifera, nematodes, molluscs), and other plant and organic matter in

various stages of decomposition, including microbial flocculated material. Although the precise relative contribution of pond food to shrimp growth is not completely understood, it is recognized as an important dietary component for farm-raised shrimp. This idea was corroborated in a recent study comparing responses of shrimp grown under two different rearing systems: flow-through (close to clear water conditions) and static green water systems (close to green water conditions) (Facts and Figures 1626). This study indicated that in systems where natural food productivity is scarcer or stocking densities are higher, an increase of about 20% of dietary methionine plus cysteine (Met+Cys) in a total basis, might be required to sustain maximum growth of shrimp. Furthermore, shrimp performance was shown to respond exponentially to graded levels of AQUAVI® Met-Met, with a dietary Met+Cys content of 1.05% (1.15 % dry matter) being required to reach 95% of maximum body weight. That AQUAVI® Met-Met significantly improves growth performances of shrimp grown under green water system was further corroborated by a study conducted under pond production conditions in India (Facts and Figures 1627). In this study, shrimp performance has also responded exponentially to increasing doses of AQUAVI® Met-Met, with a dietary methionine and Met+Cys content of, respectively, 0.72% (0.78% dry matter) and 1.16% diet (1.25% DM) being required to reach 95% of maximum weight gain. Overall, results demonstrate that AQUAVI® Met-Met significantly improves growth performances of shrimp grown under green water conditions, corroborating data produced under clear water conditions (Facts and Figures 1623, 1624, 1625). Although available data is not yet sufficient to determinate the impact of natural food on the Met+Cys specifications of shrimp feed, it supports the idea of adjusting the Met and Met+Cys dietary contents to the specific conditions of the farm.

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Facts and Figures 1620. Atlantic salmon respond equally well to both DL-Methionine and L-Methionine supplementation.

Facts & Figures 1623: AQUAVI® Met-Met is more effective than DL-Methionine in whiteleg shrimp: Study at Texas A&M University, US. 2015. http://feed-additives.evonik.com/product/feed-additives/en/services/technical-literature/Pages/default.aspx

Facts & Figures 1624: AQUAVI® Met-Met is more effective than DL-Methionine in whiteleg shrimp: Study at South China Sea Fisheries Research Institute, China. http://feed-additives.evonik.com/product/feed-additives/en/services/technical-literature/Pages/default.aspx

Facts & Figures 1625: AQUAVI® Met-Met is effective to reduce fish meal from 26% to 10% in shrimp feeds: Study at South China Sea Fisheries Research Institute, China. http://feed-additives.evonik.com/product/feed-additives/en/services/technical-literature/Pages/default.aspx

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Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California.

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Resumen

La integración de macroalgas en los sistemas de cultivo acuícolas, es una alternativa para expandir la industria de una manera sustentable y amigable con el medio ambiente. En 2012, la empresa Aonori Aquafarms inicio la operación de su granja piloto para el cocultivo de camarón café *Farfantepenaeus californiensis* (Holmes) y la macroalga verde *Ulva clathrata* (Roth). En 2012, la producción de camarón fue de 2.5 toneladas métricas por hectárea, utilizando larvas nacidas en el laboratorio a partir de camarones reproductores capturados del medio silvestre. Para 2013, la producción de camarón se incrementó a 3.8 toneladas métricas por hectárea, utilizando larvas de camarón a partir de reproductores nacidos en cautiverio. El aporte nutricional de la *Ulva* al camarón representa al menos el 50% del total de la dieta, obteniendo una tasa de conversión del alimento peletizado (TCAp) de entre 0.5 y 0.8 tanto a nivel experimental en laboratorio como en estanques de cocultivo, en contraste al monocultivo del mismo camarón café la TCAp es de 1.4 o más. En el presente trabajo se describen algunos de los avances más recientes en el cocultivo del camarón *F. californiensis* y la macroalga *U. clathrata*, demostrando con éxito la viabilidad de sistema.

Palabras clave: camarón café, macroalga, cocultivo

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Introducción

La acuicultura es uno de los sectores de producción de alimentos con mayor crecimiento en el mundo, lo que implica una mayor demanda de alimentos balanceados. La producción de camarón y de macroalgas marinas por acuicultura, representaron en 2013 un mercado de más 22,600 millones de dólares y 5,400 millones de dólares (USD) respectivamente (FAO 2013). El cultivo de camarón ha presentado una tasa de crecimiento de más del 18% anual en los últimos 20 años (Benzie 2009). El volumen actual de producción en el mundo sobrepasa los 4 millones de toneladas, siendo en su mayor parte de camarón blanco *Litopenaeus vannamei* (FAO 2013). Sin embargo, los intervalos ideales de temperatura para el crecimiento de esta especie están limitados a zonas tropicales y, en algunas épocas del año, a subtropicales por lo que existe un área de oportunidad para el cultivo de especies de camarón en regiones de aguas templadas, tales como el camarón café *Farfantepenaeus californiesis*. El camarón café se encuentra ampliamente distribuido en el Pacífico Oriental desde la bahía de San Francisco, en Estados Unidos, hasta Paita, Perú (Hendrickx 1996). El camarón *F. californiensis* ha demostrado crecer a una tasa razonable a temperaturas alrededor de 20ºC, por lo que esta especie es buen candidato para ser cultivado durante el invierno en el Golfo de California, y en aguas más frías en la costa occidental de la Península de Baja California (Martínez-Córdova, Porchas-Cornejo, Villarreal-Colmenares y Calderón-Pérez 1998; Ocampo, Villarreal, Vargas, Portillo y Magallón 2000; Portillo-Clark, Casillas-Hernández, Servín-Villegas y Magallón-Barajas 2013).

Por el otro lado, las macroalgas marinas son una fuente valiosa de nutrientes y se han utilizado como alimento desde hace más de 2000 años, especialmente en Asia. En la actualidad el uso de macroalgas se ha diversificado debido a que contienen compuestos con actividad biológica específica, por lo que son utilizados en productos con alto valor agregado, nutracéuticos, farmacéuticos, aditivos alimenticos entre otros (Holdt y Kraan 2011). Debido al incremento en la demanda generado por su amplia utilización en diferentes campos, en la actualidad sólo es posible cubrir la demanda utilizando métodos de

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cultivo en gran escala (acuicultura). Desde la década de los 1970´s se iniciaron los esfuerzos por desarrollar cultivos de especies de macroalgas de importancia económica, debido a que desde entonces se reconocía una disminución y limitación en las poblaciones naturales (Gellenbeck y Chapman 1983). Por ejemplo, en cultivos experimentales de las macroalgas rojas *Hypnea musciformis* y *Gracilaria sp.* en tanques de 350 y 600 L con mezclas de agua marina y agua de desecho acuícola, se obtuvieron cosechas tan altas como en los cultivos masivos de microalgas marinas, o como en cultivos comerciales de caña de azúcar o arroz (Lapointe, Williams, Goldman y Ryther 1976). Existen diversos métodos de cultivo de macroalgas en mar abierto, donde se utilizan balsas flotantes, líneas de monofilamento fijas entre postes anclados en el fondo, largas líneas flotantes entre otros (Pickering 2006). En el caso de la macroalga verde *Ulva clathrata,* Moll y Deikman (1995) demostraron el gran potencial para el desarrollo del cultivo de esta alga de acuerdo a su alta velocidad de crecimiento y tolerancia a un rango amplio de temperaturas y salinidad; posteriormente Moll (2004) desarrolló una tecnología patentada de cultivo de la macroalga en estanques similares a los de cultivo para camarón.

Con el incremento en el costo de ingredientes prácticos como la harina y aceite de pescado, es necesario desarrollar alimentos balanceados más eficientes además de nuevas tecnologías de cultivo que permitan continuar con el crecimiento de la industria acuícola sin incrementar significativamente los costos de producción. Dentro de las estrategias de desarrollo de nuevas tecnologías de cultivo, están los policultivos o cultivos integrados, donde desde hace décadas se discute las compatibilidades de los organismos para la convivencia espacial y/o temporal (Bardach 1986), donde se busca maximizar la utilización del alimento mediante la combinación de especies con hábitos y necesidades nutricionales diferentes, además de proponer tecnologías sustentables no solo en la parte ecológica sino también en la económica (Neori, Chopin, Troell, Buschmann, Kraemer, Halling, Shpigel y Yarish 2004). La integración de las macroalgas en granjas acuícolas se ha propuesto como una alternativa para expandir esta industria de una manera sustentable y amigable con el medio ambiente (Chopin, Buschmann, Halling, Troell, Kautsky, Neori, Kraemer, Zertuche-González, Yarish y Neefus 2001; Neori 2007).

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En la mayoría de los estudios realizados para determinar los beneficios de cocultivo de camarón y macroalgas (especialmente clorofitas), se ha encontrado una mejoría en el rendimiento productivo del camarón. En el caso del camarón café *F. californiensis*, se observó un incremento en el crecimiento en presencia de la macroalga verde *Caulerpa sertularioides* (Porchas-Cornejo, Martínez-Córdova, Magallón-Barajas, Naranjo-Páramo y Portillo-Clark 1999; Portillo-Clark *et al.* 2013). En camarón blanco *L. vannamei* cocultivado con la macroalga verde *U. clathrata* se mejoró la tasa de crecimiento así como una mayor pigmentación y disminución del contenido de lípidos en el músculo del camarón (Cruz-Suárez, León, Peña-Rodríguez, Rodríguez-Peña, Moll y Ricque-Marie 2010). En el camarón tigre *Penaeus monodon* se mejoró su crecimiento y redujo su tasa de conversión alimenticia en cocultivo con la macroalga *Chaetomorpha sp.* (Tsutsui, Songphatkaew, Meeanan, Aue-umneoy, Sukchai, Pinphoo, Klomkling, Ganmanee, Sud y Hamano 2015). En camarones *L. vannamei* y *Penaeus penicillatus* se ha visto mejores crecimientos en cocultivo con la macroalga roja *Gracilaria sp*. que en monocultivo (Liu, Jie y Zeng 1997; Xu, Fang y Wei 2008). Brito, Arantes, Magnotti, Derner, Pchara, Olivera y Vinatea (2014) encontraron que la inclusión de *Ulva lactuca* en biofloc promueve el crecimiento del camarón *L. vannamei*, además de la absorción de los nutrientes en el agua y en un sistema de cultivo intensivo.

El cocultivo de camarones y algas marinas se inició en México por la empresa Aonori Aquafarms Inc., con el uso de camarón blanco *L. vannamei* y la macroalga *U. clathrata* (Cruz-Suárez *et al.* 2010), sin embargo, *L. vannamei* tiene su temperatura óptima para el desarrollo alrededor de 30ºC, mientras que *U. clathrata* tiene un crecimiento limitado después de 30ºC, por lo que este sistema con ambas especies tenia limitaciones para ciclos de cocultivo prolongados. La empresa Aonori Aquafarms, está basada en San Diego, California y ha operado con instalaciones productivas en Los Mochis, Sinaloa y en San Quintín, Baja California, donde recientemente terminó su proyecto piloto comercial 2012-2014, para la producción de camarón café *F. californiensis* y la macroalga verde *U. clathrata* (ambos nativos de Baja California), mediante el desarrollo y puesta en marcha de

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

su granja piloto de 3 hectáreas de estanques y laboratorio de reproducción y cultivo larvario de camarón café, probando con éxito la viabilidad de la operación. En el presente trabajo se describen algunos de los avances más recientes en el cocultivo del camarón café *F. californiensis* y la macroalga verde *U. clathrata* como una forma de acuicultura innovadora y sustentable.

Desarrollo de reproductivo y cultivo larvario camarón café *F. californiensis*

A partir de 2012 se inició con el programa de reproducción y cultivo larvario de camarón café *F. californiensis* por la empresa Aonori Aquafarms en San Quintin Baja California. El laboratorio de reproducción y cultivo larvario consta de un tanque de recepción y observación de organismos, un tanque de reproducción y maduración con capacidad de 15 m^3 para mantener 150 organismos, cuatro tanques para desoves múltiples con capacidad de 1000 L, cuatro tanques para cultivo larvario de 10 m^3 y cuatro tanques para cultivo larvario de 30 m^3.

Debido a la inexistencia de un laboratorio comercial para la obtención de larvas de camarón café *F. californiensis*, se tuvo la necesidad de obtener estas larvas por medio de la captura de reproductores del medio silvestre con los permisos ante las autoridades correspondientes (CONAPESCA). Se capturaron en la bahía de San Quintin ejemplares de camarón café en etapa adulta con un estado de madurez de las gónadas avanzado. En total se capturaron 30 hembras y 60 machos con un peso entre 40 y 90 g y entre 25 y 40 g respectivamente (figura 1), y de los cuales se realizaron análisis de diagnóstico para patógenos específicos, resultando negativos para WSSV, TSV, YHV, IMNV y PVNV.

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

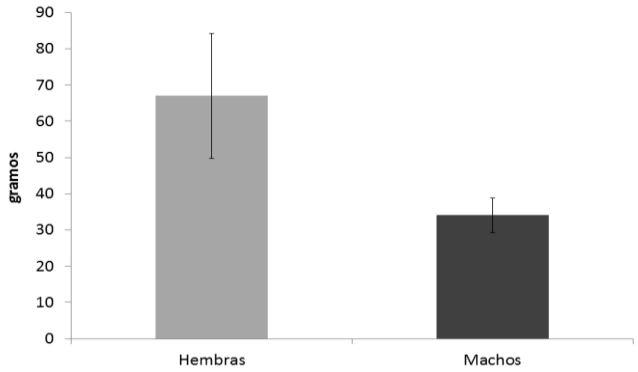

Figura 1. Peso promedio (±DE) de camarón café *F. californiensis* capturados en la bahía de San Quintin, BC, México.

A partir de este primer lote de reproductores (silvestres) se obtuvieron desoves bajo las siguientes condiciones de cultivo: fotoperiodo 12 horas de luz y 12 horas de oscuridad, a una temperatura de 24°C, recambio de agua de 50% diario, con una ración de alimento del 15% de la biomasa total. La dieta consistió en una mezcla de alimento fresco en partes iguales a base de poliquetos (Topsy Baits), ostión, calamar y artemia, que corresponden al 90% de la dieta y 10% de alimento balanceado (Royal oyster).

Se logró implantar la tecnología para la reproducción en cautiverio, desove, incubación, cultivo larvario y maternidad de postlarvas de camarón café. Las hembras en etapa 5 de madurez gonádica eran pasadas a un tanque de desove de 1000 L, colocando máximo 3 hembras por tanque, con aireación baja y con la introducción de agua dulce con baja conductividad a una tasa de 1 L min^{-1} para inducir el desove de las hembras. Posteriormente al desove, las hembras se retiraron de los tanques de desove y para retornar al tanque de reproducción. En los tanques de desove se mantuvieron las larvas hasta estadio de nauplio 5, para posteriormente ser trasladados a tanques de 10 ton, donde permanecieron hasta PL7 y después pasar a tanques de 30 ton para finalmente ser sembrados en los estanques de cocultivo con *U. clathrata* a una edad de PL 30 a PL 40. En promedio, el número de nauplios por hembra por desove fue de 400,000, con una mortalidad del 60% hasta la talla de siembra en los estanques de cocultivo. Se conformaron 8 lotes de larvas, de por lo menos 2 desoves de hembras diferentes cada uno, y con cada uno de estos lotes se sembraron 2 estanques en cocultivo con a macroalga *U. clathrata*.

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

Para 2013, se logró la reproducción en cautiverio de los camarones nacidos en laboratorio a partir de reproductores silvestres de 2011 y 2012 y cocultivados con *U. clathrata*. La selección de organismos para conformar el lote de reproductores, fue determinada en base a la talla de los organismos, seleccionando hembras y machos de todos los estanques (lotes diferentes). La talla de las hembras seleccionadas fue de entre 20 y 24 g, mientras que de los machos fue de entre 14 y 18 g. Se realizaron análisis de diagnóstico para patógenos específicos, resultando negativos para WSSV, TSV, YHV, IMNV y PVNV. Las condiciones y la dieta para inducir la maduración fueron similares que las utilizadas en el ciclo anterior de reproducción (ciclo 2012). El crecimiento de los camarones seleccionados para reproducción durante su acondicionamiento y maduración fue superior a 1 g semanal (figura 2).

En promedio, el número de nauplios por hembra a una talla de 32 g por desove fue de 200,000, sin embrago a una talla mayor de 42 g en promedio, el número de nauplios por desove se incrementó a 400,000 (figura 3). La mortalidad presentada en las larvas fue del 50% hasta PL40 cuando fueron sembradas en los estanques de cocultivo. Se generaron 8 lotes de postlarvas diferentes los cuales fueron sembrados en 10 estanques de cocultivo con la macroalga.

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

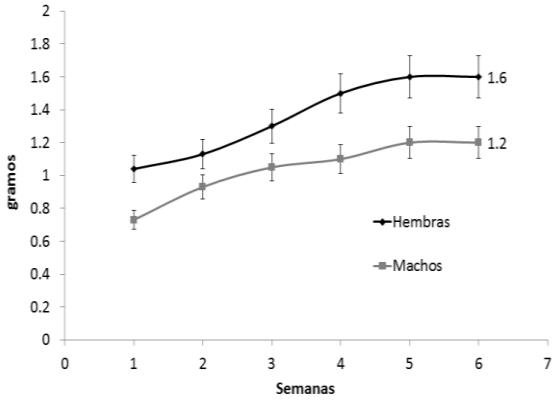

Figura 2. Ganancia en peso semanal de camarones seleccionados para reproducción, alimentados con alimento fresco y alimento balanceado.

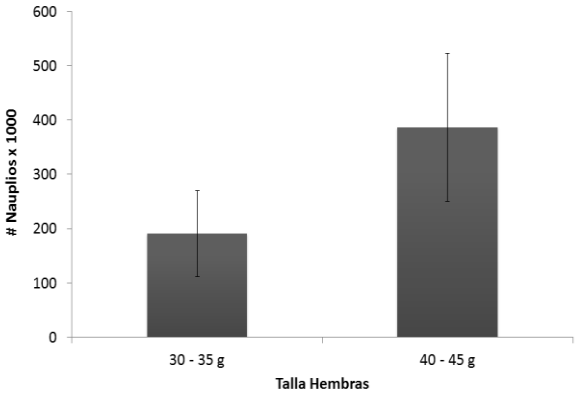

Figura 3. Número de nauplios por desove por hembra de acuerdo a su talla.

Preparación de estanques de cocultivo con *Ulva clathrata*

Los estanques de cocultivo tienen una dimensión de 25x75 m (1875 m^2) y están recubiertos por una membrana de polietileno de alta densidad. El agua marina se suministró vía una tubería de 8 pulgadas de diámetro alimentada por una galería filtrante enterrada 3 pies bajo el nivel de la arena. Una estación de descarga de agua se sitúa al final de la batería de estanques que están alineados. Esta estación de descarga bombea el agua de regreso al océano a un sistema de galería filtrante situada 200 yardas al sur de la toma de agua. Cada estanque se dividió en seis secciones donde se planta el alga superficialmente por medio de cuerdas, de acuerdo a lo descrito por Moll (2004). Cada semana se plantó una sección del estanque con *Ulva* para después de un ciclo de 6 semanas, el alga es cosechada y resembrada, de tal forma que la superficie del estanque esta entre un 40 y 60% cubierto por la macroalga durante todo el periodo de cultivo. Los requerimientos nutricionales para el crecimiento de *Ulva* fueron provistos principalmente por dos fuentes: 1) El alimento balanceado peletizado, el no consumido y el consumido y metabolizado por el camarón; 2) Fertilizantes inorgánicos, utilizando urea como fuente de nitrógeno y ácido fosfórico como fuente de fósforo. Los niveles de nitrógeno y fósforo total en el agua se mantuvieron a

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

niveles cercanos a 1 ppm y 0.25 ppm respectivamente. Los niveles de amonio y pH se monitorearon diariamente cuidando que los niveles de amonio no ionizado se mantuviera debajo de 0.25 ppm. Las fertilización de los estanques se suspendió un día previo a realizar recambio de agua (10% semanal, menos de 1.5% diario), esto con la finalidad de minimizar los niveles de nitrógeno y fosforo total en el agua de descarga. Según estimaciones de consumo así como estudios realizados en camarón blanco (Cruz-Suarez *et al.* 2010; Gamboa-Delgado, Peña-Rodríguez, Ricque-Marie y Cruz-Suárez 2011), el consumo de Ulva aporta al menos 50% de la dieta para el crecimiento del camarón bajo este sistema de cultivo.

Desarrollo de cocultivo

Para el año 2013, los estanques de cocultivo se sembraron a una densidad de 30 camarones por m^2, y se utilizó un alimento balanceado con 35% de proteína con 2 raciones diarias a las 19:00 y 00:00 horas debido a los hábitos nocturnos de la especie. La temperatura durante el ciclo de cultivo osciló entre los 20°C por la mañana, hasta los 26°C por la tarde (figura 4). El oxígeno disuelto en el agua se mantuvo en promedio en 5.7 mg $L\text{-}^{1}$, con niveles mínimos de 2.8 mg L^{-1} durante las mañanas una vez que la biomasa de camarón alcanzo más del equivalente a 2.1 tonelada por hectárea (figura 5). Debido al bajo recambio de agua semanal en los estanques (10%), la salinidad promedio en los estanques se mantuvo en promedio a 42 ‰.

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

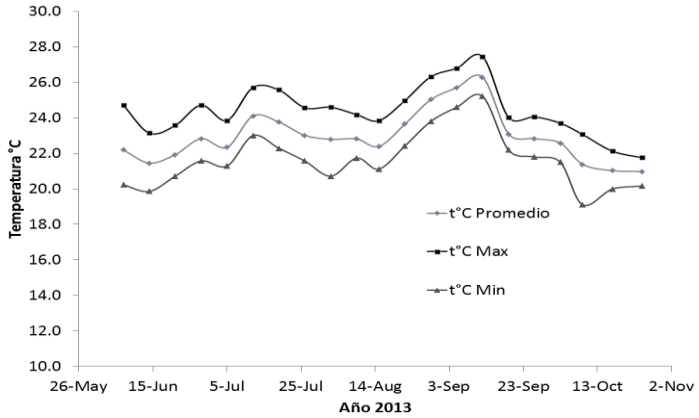

Figura 4. Temperatura (°C) del agua en los estanques durante el ciclo de cocultivo.

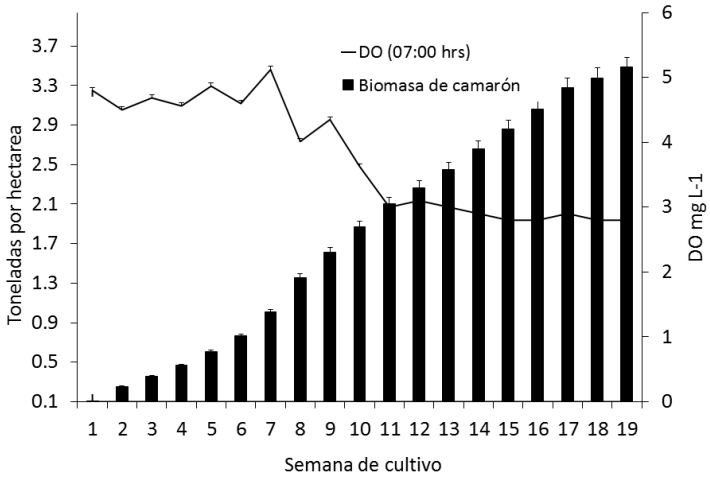

Figura 5. Biomasa de camarón *F. californiensis* generada en cocultivo con *U. clathrata* y promedio del pico mínimo de oxígeno disuelto (07:00 horas) en los estanques durante el ciclo de cocultivo

Durante el ciclo de cocultivo de camarón y macroalga de 22 semanas, se obtuvo una cosecha final promedio de 3.8 toneladas de camarón, 1.3 toneladas más que en 2012, con una sobrevivencia promedio de 75% y una tasa de conversión alimenticia promedio de 0.81. La talla final de los camarones a la cosecha varió entre 15 y 20 g, esto principalmente a la diferencia entre las tasas de crecimiento de los machos respecto a las hembras, siendo las hembras con una mayor tasa de crecimiento a partir de los 10 g de peso (figura 6). La

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tasa de crecimiento semanal promedio fue de 0.78 g, 15% superior a la tasa de crecimiento obtenida durante el ciclo de cocultivo de 2012 (0.68 g semana^{-1}).

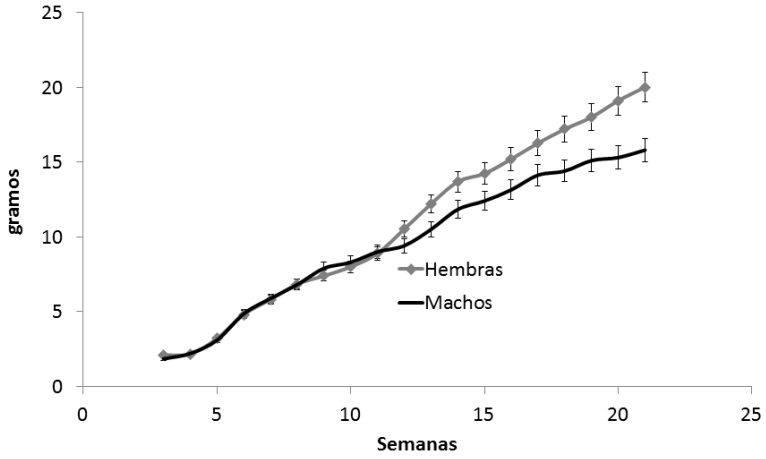

Figura 6. Peso promedio de camarones *F. californiensis* macho y hembras durante un ciclo de cocultivo con la macroalga *U. clathrata*.

Eficiencia de utilización del alimento balanceado en el cocultivo de camarón café *F. californiensis* y la macroalga *U. clathrata*

En un estudio anterior con camarón blanco *L. vannamei* cocultivado con *U. clathrata*, se observó que hasta con 45% menos de alimento peletizado (balanceado comercial) se mejoraba significativamente la tasa de crecimiento respecto a camarones en monocultivo con el 100% de la ración del alimento peletizado (Cruz-Suarez *et al.* 2010). Con la finalidad de conocer el mínimo de alimento balanceado peletizado necesario para obtener un crecimiento óptimo de juveniles de camarón café en cocultivo con la macroalga *U. clathrata*, se evaluaron diferentes proporciones del alimento peletizado tomando como referencia un tratamiento control de camarones en monocultivo alimentados a saciedad considerando esta ración como 100%. Los tratamientos fueron: 0% alimento peletizado más Ulva (0PU), 15% alimento peletizado más Ulva (15PU), 25% alimento peletizado más Ulva (25PU), 35% alimento peletizado más Ulva (35PU), 45% alimento peletizado más Ulva (45PU) y el control 100% alimento peletizado sin Ulva (100P). Cada tratamiento se realizó por triplicado distribuidos en 3 bloques al azar, cada triplicado fue representado por

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un acuario de 55 L de capacidad con 8 camarones *F. californiensis* de peso promedio inicial de 2.58±0.1 g. Diariamente se realizó un recambio del 100% de agua y la temperatura promedio durante el experimento fue de 22±2°C. En el caso de los tratamientos con la macroalga, esta se obtuvo de los estanques de cultivo, colocando inicialmente 20 g húmedos de Ulva en cada acuario y remplazando diariamente el alga consumida en cada acuario. Después de 4 semanas de experimento se evaluó el peso final, tasa de crecimiento (%), Tasa de crecimiento especifico (g día-1) y porcentaje de sobrevivencia de los camarones bajo los diferentes tratamientos. Los parámetros zootécnicos evaluados para cada tratamiento se sometieron a un análisis de varianza de una sola vía (ANOVA); y en los casos en que se presentaron diferencias significativas, se realizó una comparación múltiple de medias por el método de Tukey (α=0.05).

Tras 28 días de experimento de alimentación, se observó un mayor crecimiento en los camarones del tratamiento con 45% de alimento peletizado y Ulva (45PU) respecto al resto de los tratamientos, son embargo no fue significativamente mayor (P>0.05) que los tratamientos 35PU y el control de monocultivo (100P) (Tabla 1 y Figura 4).

Tabla 1. Valores promedio (±DE) del peso final (PF), Tasa de crecimiento específico (g día^{-1}), Tasa de conversión alimenticia del alimento peletizado (TCAp) y porcentaje de sobrevivencia (%S).

Tratamiento	PF(g)	TCE(g día^{-1})	TCAp	%S
0PU	2.8±0.1a	0.3±0.1a	0.0a	75±0
15PU	3.4±0.2b	1.0±0.2b	0.55±0.15b	88±13
25PU	4.2±0.2c	1.8±0.2c	0.46±0.06b	92±7
35PU	4.6±0.2cd	2.0±0.2cd	0.53±0.06b	96±7
45PU	4.9±0.3d	2.3±0.2d	0.58±0.06b	96±7
100P	4.6±0.3cd	2.1±0.2cd	1.48±0.18c	96±7

Los valores están dados como promedios ± desviación estándar de determinaciones por triplicado. Letras diferentes en la misma columna indican diferencias significativas determinadas por análisis de Tukey (α=0.05). TCE = 100(ln PF – ln Peso inicial) / número de días; TCAp = alimento peletizado administrado / ganancia en peso. %S = 100(número final de camarones / número inicial de camarones)

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

Los tratamientos con una porción parcial de alimento peletizado y Ulva resultaron en una disminución significativa ($P<0.05$) en la tasa de conversión alimenticia considerando solo el alimento peletizado (TCAp), donde para el caso del tratamiento 25PU solo represento una tercera parte del valor obtenido para el control 100P, sin resultar significativamente diferentes entre ellos respecto al crecimiento observado. No se observaron diferencias estadísticas en la sobrevivencia entre tratamientos.

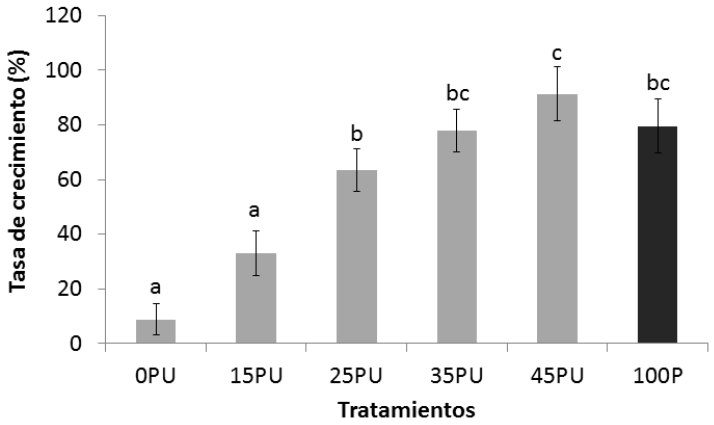

Figura 7. Tasa de crecimiento (%) de juveniles de camarón café *F. californiensis* alimentados con un porcentaje de alimento peletizado (P) y la macroalga *U. clathrata* (U)

Proyección del desarrollo del cocultivo de camarón café *F. californiensis* y la macroalga *U. clathrata*

La empresa Aonori Aquafarms (”la Compañía”) cuenta con los conocimientos y experiencia de los sistemas de producción totalmente probados y están listos para ser utilizados en una operación comercial a gran escala. Para el 2016 (año 1) se planea construir 25 hectáreas productivas en la región de San Quintin, para una operación comercial del cocultivo de camarón y alga a gran escala, con una inversión aproximada de 4.3 millones dólares USD. Para 2018, después de mejorar y ajustar los parámetros de la operación en estas 25 hectáreas, la Compañía requerirá capital adicional para invertir en 25 hectáreas productivas adicionales en el año 3. Esta escala permitirá a la Compañía continuar financiación sus planes de expansión con los flujos de caja operativo y la deuda

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

bancaria (hasta un 2,5x deuda / EBITDA de apalancamiento), que no requiere inyecciones adicionales de capital. Por parte del área de investigación y desarrollo, se tiene programado iniciar un programa de mejoramiento genético del camarón café con el Centro de Investigaciones Biológicas del Noroeste (CIBNOR), en La Paz, BCS, México.

Conclusiones y Discusiones

El aporte nutricional del alga al camarón se ha evaluado en anteriores estudios con camarón blanco *L. vannamei*, donde de acuerdo con su composición química, el alga es baja en lípidos y energía (Cruz-Suarez *et al.* 2010; Gamboa-Delgado *et al.* 2011; Peña-Rodríguez, Mawhinney, Ricque-Marie y Cruz-Suárez 2011), por lo que el uso parcial de alimento artificial (peletizado) es necesario para cubrir estos requerimientos, pero obteniendo tasas de conversión de alimento peletizado por debajo de 1, lo que significa un ahorro en este rubro que puede representar hasta un 60% del consto de producción del camarón. Adicionalmente, el tapete de Ulva provee de la protección a la luz así como refugio lo que puede contribuir a buenas sobrevivencias a altas densidades. Los niveles de oxígeno presentados en el sistema de cocultivo permiten mantener una densidad alta de camarón sin necesidad de aireación, debido principalmente a dos factores: la producción de oxígeno de *Ulva* en la columna de agua (Häder y Schäfer, 1994), y el bajo consumo de oxígeno del camarón café en las temperaturas a la que se lleva a cabo el cocultivo como se describe en el estudio realizado por Villarreal y Ocampo 1993. Por otro lado, también se ha descrito una mejor estabilidad del DO en presencia de macroalgas que en ausencia de ellas (Xu *et al.* 2008).

Se ha demostrado que las macroalgas son excelentes en la absorción de nitrógeno y de fósforo inorgánico proveniente de agua de descarga acuícola (Troell, Rönnbäck, Halling, Kautsky y Buschmann 1999; Copertino, Tormena y Seeliger 2008; Brito *et al.* 2014), lo que confiere una buena calidad de agua en los estanques de cocultivo, pudiendo ser un factor que mejora la salud de los camarones lo que se refleja en la sobrevivencia y en el crecimiento de los mismos. La biomasa de camarón producida bajos este sistema de

Peña, R. *et al*. 2015. Avances en el Cocultivo de Camarón café *Farfantepenaeus californiensis* y la Macroalga *Ulva clathrata* en la Costa Occidental de Baja California. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 151-168.

cocultivo (ton ha^{-1}) está dentro del rango de producción de granjas semi-intensivas de monocultivo de camarón *L. vannamei* a densidades entre 10 y 25 camarones por m^2 (Tacon, Jory y Nunes 2013). Considerando que las postlarvas de camarón café utilizadas en los ciclos de cultivo 2012 y 2013 fueron obtenidos a partir de reproductores silvestres y reproductores nacidos en laboratorio de padres silvestres, es de considerar un mejoramiento significativo en las tasas de crecimiento conforme se domestique a la especie y se adapte genéticamente al sistema de cocultivo con la Ulva (Gjedrem y Fimland, 1995).

Los resultados hasta el momento demuestran el potencial y viabilidad del cocultivo del camarón café *F. californiensis* y la macroalga *U. clathrata* en aguas templadas como las de la costa occidental de Baja California, México. Se planea la expansión de esta tecnología a una escala comercial en los próximos años, esperando detonar la economía en zonas con tierras costeras sin uso agrícola por la carencia de agua dulce para riego. Se contempla además continuar con el desarrollo del programa de domesticación, selección y mejoramiento genético del camarón café para mejorar las tasas de crecimiento y por tanto la rentabilidad del sistema de cocultivo.

Agradecimientos

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Seaweeds as Sustainable Feed Ingredients for Farmed Fish species: Effects on Growth, Immunological Response and Flesh Quality

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Abstract

The potentiality of seaweeds, produced in IMTA systems, as dietary feed ingredients for rainbow trout and Nile tilapia was evaluated. The dietary inclusion of the best performing seaweed in each fish species was further evaluated in juvenile fish, concerning its effects on growth and nutrient utilisation, immunological response and flesh quality. *Gracilaria vermiculophylla* was selected for rainbow trout, whereas in tilapia *Ulva* spp. was selected instead. The inclusion of *Gracilaria* meal in diets for rainbow trout was possible up to 5%, but a higher inclusion level impaired growth. Flesh iodine content doubled in fish fed 5% *Gracilaria* meal, confirming seaweed as a natural and effective tool to increase the nutritional value of rainbow trout. Moreover, in Nile tilapia, the inclusion of *Ulva* spp. meal seems to be possible up to 10% without major effects on growth performance or flesh organoleptic properties, but enhancing the innate immune response of the fish.

Keywords: seaweeds, immunological response, rainbow trout, Nile tilapia

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Introduction

Global population growth and increase in living standards will push up the demand for fish-derived protein in the future. Fish meal has traditionally been the major dietary protein source for fish, but its reduction in aquafeeds is now a priority goal for the further expansion and sustainability of the farmed fish production. On the other hand, the increasing consumer demand for products containing food ingredients from natural sources encourages the use of environmental friendly alternatives to fish meal (FM) able to produce a final product that retains adequate levels of n3 LC-PUFA (Gatlin, Barrows, Brown, Dabrowski, Gaylord, Hardy, Herman, Hu, Krogdahl, Nelson, Overturf, Rust, Sealey, Skonberg, J Souza, Stone, Wilson & Wurtele 2007).

Exogenous feeding in aquaculture unlocks the possibility to tailor fish composition towards increased health-promoting properties, without compromising its sensory attributes and consumer´s acceptance. Over recent years, there has been a renewed interest on the use of seaweeds in aquaculture feeds, not only as a nutrient supply (Valente, Gouveia, Rema, Matos, Gomes & Pinto 2006; Soler-Vila, Coughlan, Guiry & Kraan 2009), but also as a valuable source of bioactive compounds like pigments, vitamins and minerals (Holdt & Kraan 2011; Ribeiro, Gonçalves, Colen, Nunes, Dinis & Dias 2015; Valente, Rema, Ferraro, Pintado, Sousa-Pinto, Cunha, Oliveira & Araújo 2015a). Moreover, seaweeds have remarkably higher concentrations of halogens, rare earth elements and many transition metal elements than terrestrial plants (Hou & Yan 1998). Nevertheless, the protein content of most wild seaweeds is too low to fulfil the nutritional requirements of carnivorous fish. Brown seaweeds are poor protein sources (3–15 % of the dry weight), but red and green seaweeds can have higher protein contents (10–47% of the dry weight) (Fleurence 1999), depending on the season and production conditions.

In recent years, integrated multitrophic aquaculture (IMTA) has been gaining momentum as a way to diminish aquaculture environmental impacts using seaweeds as biofilters in the extractive component of the system (Neori, Chopin, Troell, Buschmann,

Valente, L. 2015. Seaweeds as Sustainable Feed Ingredients for Farmed Fish species: Effects on Growth, Immunological Response and Flesh Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 169-182.

Kraemer, Halling, Shpigel & Yarish 2004). In such ecologically-balanced systems, wastewater from aquaculture effluents is converted into potentially valuable biomass that could be used as an ingredient in fish feed, while reducing the cost of water treatment. Seaweeds produced in IMTA systems usually present higher productivity levels and less variability in protein contents than naturally harvested biomass (Lüning & Pang 2003; Schuenhoff, Shpigel, Lupatsch, Ashkenazi, Msuya & Neori 2003; Mata, Schuenhoff & Santos 2010; Abreu, Pereira, Yarish, Buschmann & Sousa-Pinto 2011).

Previous studies have demonstrated that seaweeds like *Ascophyllum nodosum* (Linnaeus) (Nakagawa, Umino & Tasaka 1997), *Ulva lactuca* (Linnaeus) (Wassef, El Masry & Mikhail 2001), *Gracilaria cornea* (Agardh) and *Gracilaria bursa-pastoris* (Gmelin) Silva (Valente *et al.* 2006), *Porphyra* sp. (Soler-Vila *et al.* 2009), *Macrocystis pyrifera* (Linnaeus) (Dantagnan, Hernández, Borquez & Mansilla 2009) and *Kappaphycus alvarezii* (Doty) Doty ex Silva (Shapawi, Safiin & Senoo 2014) can be used as partial substitutes of dietary fish meal (FM) in aquafeeds. However, the benefits of including seaweeds in fish diets are still in its infancy.

This manuscript summarises the potentiality of seaweeds, produced in IMTA systems, as sustainable feed ingredients in two important farmed fish species: rainbow trout (*Oncorhynchus mykiss*, Walbaum) and Nile tilapia (*Oreochromis niloticus, Linné).*

Material and Methods

A critical aspect when developing diets for fish is the evaluation of their capacity to digest different ingredients. Inclusion of highly digestible ingredients will improve the fish performance whilst reducing the production of wastes. This study evaluated seaweed species selected based on its availability on the Portuguese coast and its potential to be locally produced in integrated multi-trophic aquaculture (IMTA) systems (Pereira, Kraemer, Yarish & Sousa-Pinto 2008; Abreu *et al.* 2011). Three seaweeds, *Porphyra dioica* (J. Brodie & L.M. Irvine), *Ulva* spp. and *Gracilaria vermiculophylla* (Ohmi)

Valente, L. 2015. Seaweeds as Sustainable Feed Ingredients for Farmed Fish species: Effects on Growth, Immunological Response and Flesh Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 169-182.

Papenfuss were produced in tanks, in an IMTA system in the facilities of the aquaculture A. Coelho e Castro (Póvoa de Varzim, Portugal, N41°27′10″, W8°46′28″).

The apparent digestibility coefficient (ADC) of the three selected seaweeds was evaluated in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). Practical basal mixtures were formulated with the incorporation of 1% chromic oxide (Cr_2O_3) as inert marker. The reference diet consisted of 100% of the basal mixture. For each fish species additional test diets were subsequently produced by mixing 70% of the basal mixture with 30% of each seaweed. Feces were collected using the Choubert System (Choubert, De La Noüe & Luquet 1982).

The dietary inclusion of the best performing seaweed in each fish species was further evaluated in juvenile fish, concerning its effects on growth and nutrient utilisation, immunological response and flesh quality.

Cross sections from the anterior part of the intestine were fixed and embedded in paraffin for light microscopy evaluation of villi height, intestine diameter and muscle layer thickness. The immunological response of fish was evaluated by determining the activity of key components of fish defences: lysozyme, peroxidase and alternative complement pathway (ACH50) as previously described (Araújo, Rema, Sousa-Pinto, Cunha, Peixoto, Pires, Seixas, Brotas, Beltrán & Valente 2015).

Muscle chemical composition and organoleptic properties were evaluated in both rainbow trout and Nile tilapia. Flesh instrumental colour was also evaluated and L*, a* and b* values were recorded. Antioxidant activity of muscle carotenoids were determined by radical scavenging 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods according to Guimarães *et al.* (2007) and Brand-Williams *et al.* (1995).

Valente, L. 2015. Seaweeds as Sustainable Feed Ingredients for Farmed Fish species: Effects on Growth, Immunological Response and Flesh Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 169-182.

Results

Seaweeds impact on nutrient digestibility and fish growth

The ADC´s of seaweeds varied significantly according to both the seaweed and the fish species considered (Pereira, Valente, Sousa-Pinto & Rema 2012). In rainbow trout, protein ADC of *Gracilaria* (88%) was significantly higher than that of *Porphyra sp. (80%) or Ulva* (76%), but in tilapia, protein ADC was higher for *Ulva* (63%) than for the other two seaweeds (51-59%) (Figure 1). Hence, *Gracilaria* sp. was selected for further studies in rainbow trout, whereas in tilapia *Ulva* spp. was selected instead.

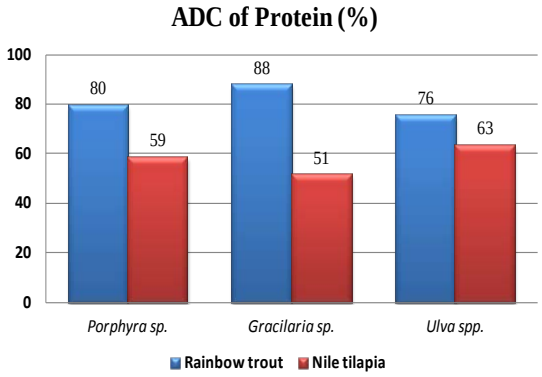

Figure 1. Protein apparent digestibility coefficients (ADC %) of seaweeds in rainbow trout and Nile tilapia (Adapted from Pereira *et al.*, 2012).

Increasing dietary inclusion levels (0, 5 and 10%) of IMTA-cultivated *Gracilaria vermiculophylla* were evaluated in 67 g rainbow trout. Growth and feed efficiency were determined after 91 days at 16 ^{o}C. Although protein intake was similar among groups, the inclusion of 10% *Gracilaria* (G10) induced the lowest protein retention and gain, resulting in the lowest final body weight (Araújo *et al.* 2015). However, the inclusion of 5% *Gracilaria* meal (G5) resulted in similar fish performance and nutrient retention efficiency.

In Nile tilapia juveniles (12g), increasing levels of a mixture of *Ulva* spp. meal (0, 10, 15 and 20%) produced in an IMTA system were evaluated as partial replacement of

Valente, L. 2015. Seaweeds as Sustainable Feed Ingredients for Farmed Fish species: Effects on Growth, Immunological Response and Flesh Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 169-182.

dietary fish meal. After 63 days at 26 ^{o}C, all groups of fish more than tripled their initial body weight. Fish fed 10% *Ulva* meal (U10) had the highest protein efficiency ratio and nitrogen retention efficiency, allowing this fish to growth and reach a final body weight similar to the control fed group (Marinho, Nunes, Sousa-Pinto, Pereira, Rema & Valente 2013). However, higher levels of dietary seaweed impaired growth.

Histological measurements of fish intestinal mucosa showed that seaweeds affected gut morphology and its components. In rainbow trout, G10 diet induced the smallest intestine diameter and lowest *villi* height (Araújo *et al.* 2015). In tilapia, the very same trend was reported with the dietary inclusion of 10% *Ulva*, but without statistical significance (Silva, Valente, Sousa-Pinto, Pereira, Pires, Seixas & Rema 2015).

Seaweed impact on fish immunological response

The immunological response of fish was evaluated by determining the activity of key components of fish defences: lysozyme, peroxidase and alternative complement pathway (ACH50) (Figure 2).

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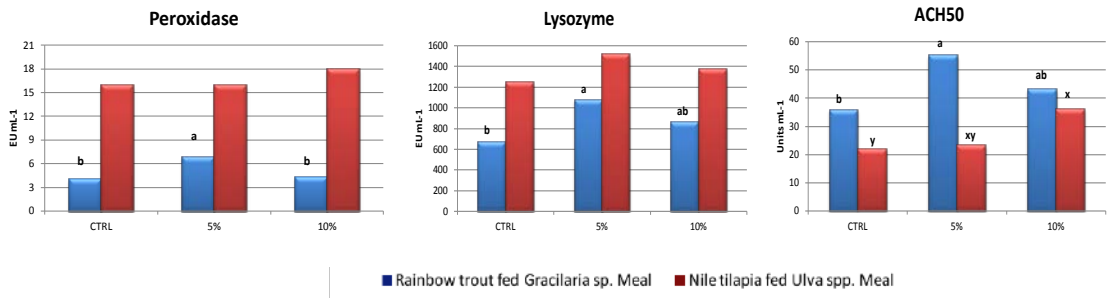

Figure 2. Innate humoral immune parameters of rainbow trout (blue) and Nile Tilapia (red) fed *Gracilaria* sp. meal and *Ulva* spp. meal, respectively (Adapted from Araújo *et al.*, 2015 and Valente *et al.*, 2015b)

In rainbow trout, humoral parameters analysed in plasma of fish fed 5% *Gracilaria* meal (G5) suggest a general and significant stimulation of the immunological system compared to those fed the control diet (Araújo *et al.* 2015).

In Nile tilapia, the peroxidase and lysozyme activities were similar among treatments. The alternative complement pathway increased proportionally to the dietary seaweed inclusion, and tilapia fed U10 showed a significantly higher activity than the control fed fish (Valente, Araújo, Batista, Peixoto, Sousa-Pinto, Brotas, Cunha & Rema 2015b).

Seaweed impact on fish flesh quality

The inclusion of *Gracilaria* meal increased rainbow trout flesh moisture and decreased lipid content, with significant differences between G5 and the control fed fish. Instrumental colour showed that raw and cooked fillets from *Gracilaria*-rich fish were more luminous (L*), less yellowish (b*) and more redish (a*). G10 samples yield the lowest colour intensity (C*), confirming the lowest carotenoid content in these fish (Araújo *et al.* 2015). Moreover, muscle carotenoid extracts presented no significant antioxidant activity through the ABTS and DPPH assays (<20%) (Valente *et al.* 2015a). The sensory evaluation showed that fish fed with seaweed had juicier fillets than the CRT with G5

presenting the most intense (pinkish) colour. Iodine levels in the flesh increased with the seaweed inclusion, with fish fed G5 doubling its iodine content (214.5 μg/kg) in relation to the CTRL (111.7 μg/kg) (Valente *et al.* 2015a).

In Nile tilapia flesh, colour evaluation of the tristimulus L*, a* and b* indicated significant differences between dietary treatments. The inclusion of 10% *Ulva* spp. meal resulted in fillets with the highest lightness (higher L* value) and yellowness (less negative b*), but the lowest redness (lower a* value). No differences were observed between U5% and the control fed fish. No carotenoids could be found in tilapia muscle. Moreover, sensory attributes showed no significant effects of dietary treatments on visual, olfactory, texture and flavour parameters, with the exception of sour parameter that was lowest in U10-fed tilapia (Valente *et al.* 2015b).

Discussion

The response of fish to dietary seaweed inclusion seems to be dose and species-dependent since nutritional composition and digestibility differ among seaweed species (Pereira *et al.* 2012; Silva *et al.* 2015). In European sea bass (*Dicentrarchus labrax* Linnaeus) juveniles *Gracilaria cornea* could replace dietary fish meal (FM) up to 5%, whereas *G. bursa-pastoris* or *Ulva rigida* could replace 10% FM without compromising growth performance and feed efficiency (Valente *et al.* 2006). The inclusion of *G. vermicullophyla* in rainbow trout diets was shown to be feasible up to 5% with higher levels impairing growth. Moreover, reduced growth observed in G10 could not be associated with poor dietary palatability as food intake was highest in this group, but could be due to the presence of anti-nutritional factors described in plants and algae that can affect the digestion processes.

Modifications in feed ingredients can modulate gut morphology, which is highly responsible for a good digestion and absorption of nutrients. The smallest intestine diameter associated with the lowest *villi* height observed in fish fed 10% *Gracilaria* sp may have

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reduced absorption surface, contributing to the lowest growth and nutrient retention observed in those fish (Araújo *et al.* 2015). In Nile tilapia, the dietary incorporation of 10 % *Ulva* spp. meal, either produced in IMTA systems (Marinho *et al.* 2013) or wild caught (Güroy, Cirik, Güroy, Sanver & Tekinay 2007; Azaza, Mensi, Ksouri, Dhraief, Brini, Abdelmouleh & Kraïem 2008) did not compromise dry feed intake, growth performance or protein utilisation. This is consistent with recent results from Pereira *et al.* (2012) demonstrating that *Ulva* meal could be considered a practical partial replacement for fish meal in Nile tilapia diets, being better digested than *Gracilaria* or *Porphyra* (Figure 1).

Previous studies showed that algal compounds, mainly polysaccharides such as carrageenan, fucoidan, alginates and β-glucans, modulate the immunological response, and often induce and enhance resistance against infectious diseases, representing primary tools in modern fish farming (Vetvicka, Vannucci & Sima 2013). Lysozyme, peroxidase and complement system are key components of fish defenses since they act against pathogens by directly disrupting their cell walls or through the production of harmful chemicals, such as oxidative radicals (Nayak 2010). However, the immunological response towards dietary seaweed inclusion seems to be dose and species dependent. In rainbow trout the dietary inclusion of 5 % *G. vermiculophylla* meal enhanced fish innate immune response, inducing the highest peroxidase, lysozyme and complement activities (Araújo *et al.* 2015), but in Nile tilapia, the dietary inclusion of *Ulva* spp.meal had no beneficial effect on lysozyme or peroxidase activities (Valente *et al.* 2015b). The complement activity (ACH50) in Nile tilapia increased concomitantly to the dietary inclusion level of *Ulva* spp. meal, reaching maximal activity with the highest seaweed inclusion level (U10). Similarly, supplementation of 5 % *Ulva* spp. meal in the diet for red sea bream (*Pagrus major*, Temminck & Schlegel) enhanced complement activity and disease resistance without impairment of growth (Satoh, Nakagawa & Kasahara 1987). This suggests that dietary inclusion of seaweeds initiates activation of fish innate defence mechanisms, which may be due to its high content of carbohydrates. El-Boshy *et al.* (2010) have previously reported immunostimulant properties of both β-glucan and laminaran in farmed Nile tilapia, suggesting its use under immune depressive stressful condition to increase their resistance

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to diseases. The main polysaccharide in *Gracilaria* spp. is agar, with similar structural and functional proprieties to carrageenan, but other compounds such as β-carotene, may also modulate fish humoral immune response. Studies evaluating the effects of such compounds in fish immune response are scarce. Although both *Gracilaria*- and *Ulva*-rich diets seems to influence innate immune system in rainbow trout and tilapia, respectively, only further experiments exposing fish to stress conditions could confirm their capacity to enhance fish immunity and disease resistance.

The nutritional modulation of flesh quality traits has been considered in several fish species as an effective way of enhancing the fillet quality improving its nutritional value for human consumption. Seaweeds present a valuable content of micronutrients, such as carotenoids, vitamin E and minerals (Holdt & Kraan 2011). One of the most important minerals in seaweeds is iodine, a halogenated trace element that is essential for growth and metabolism since it is involved in thyroid hormone synthesis in humans and animals. The dietary inclusion of 5% *G. vermiculophylla* in diets for rainbow trout resulted in a two-fold increase of fillet iodine content, confirming this seaweed species as a natural and effective tool to increase the nutritional value of rainbow trout (Valente *et al.* 2015a). Moreover the dietary inclusion of *Laminaria* sp. was shown to be a valuable way of increasing fillet iodine content in both freshwater (Schmid, Ranz, He, Burkard, Lukowicz, Reiter, Arnold, Le Deit, David & Rambeck 2003) and marine fish species (Ribeiro *et al.* 2015). In gilthead seabream *L. digitata*, an iodine-rich macroalgae, was an effective and natural strategy to fortify muscle with iodine, showing that a 160 g portion of steam-cooked fillets could cover approximately 80% of the Daily Recommended Intake for iodine and 370% of the Daily Adequate Intake of EPA+DHA for enhanced cardiovascular health in adults (Ribeiro *et al.* 2015).

In rainbow trout, the sensory panel perceived fillets from fish fed with 5% *Gracilaria* meal (G5) as the sample with higher colour intensity and juicer than those fed the control. Nevertheless, G5 could not improve flesh carotenoid deposition nor improve fillet preservation (Valente *et al.* 2015a). Previous studies demonstrated that the flesh

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dominant carotenoids in Nile tilapia are astaxanthin and canthaxanthin (Czeczuga, Czeczuga-Semeniuk, Káyszejko & Szumiec 2005). However, the absence of these two pigments in the *Ulva* spp. meal (Sefc, Brown & Clotfelter 2014) may explain the lack of detectable carotenoids in muscle of tilpia either fed 5 or 10% *Ulva* meal (Valente *et al.* 2015b), as cichlid like other fish cannot synthesise them. Moreover, the incorporation of 30 % *Sargassum siliquastrum* in diets for Silver seabream (*Sparus sarba*) significantly increased flesh total bromophenol content providing the desirable sea-like flavour (Ma, Chung, Ang & Kim 2005). But, the dietary inclusion of *Ulva* meal resulted in no major visual, olfactory, texture or flavour parameters in tilapia fillets (Valente *et al.* 2015b).

In conclusion, the inclusion of *Gracilaria* meal in diets for rainbow trout is possible up to 5%, but a higher inclusion level impairs growth. However, in Nile tilapia, the inclusion of *Ulva* spp. meal seems to be possible up to 10 % without major effects on growth performance or flesh organoleptic properties, but enhancing the innate immune response of the fish.

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Las Algas Marinas de los Géneros *Byothamnion* y *Halimeda* como Fuentes de Antioxidantes Naturales

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Resumen

El objetivo de este trabajo es analizar y comparar las propiedades antioxidantes mediante ensayos *in vitro* de extractos acuosos de las algas de los géneros *Bryothamnion* y *Halimeda* así como esclarecer sus posibles mecanismos de acción.

Se obtuvieron los siguientes resultados: *Bryothamnion*: DPPH; CI_{50}=1,15 ± 0,06, capacidad reductora; 128 mg/mL, DO=2,798, inhibición de la peroxidación lipídica; CI_{50}=5,09± 0,25, inhibición de la hemólisis con 5 mg/mL; 100 %, ensayo del β-caroteno-ácido linoléico; 1mg de extracto 15% de inhibición y Capacidad antioxidante mediante el ensayo xantina/xantina oxidasa como generador de radicales O_2; no presenta actividad. *Halimeda:* DPPH; CI_{50}=12,34 ± 0,30 mg/mL, capacidad reductora; DO=0,800, inhibición de la peroxidación lipídica; CI_{50}=1,25± 0,31 mg/mL, inhibición de la hemólisis; 24%, ensayo del β-caroteno-ácido linoléico; 1mg de extracto 68% de inhibición y Capacidad antioxidante mediante el ensayo xantina/xantina oxidasa como generador de radicales O_2; muy efectivo. Las algas de los dos géneros resultaron muy efectivas en los estudios de cultivos de células así como en el modelo de estrés oxidativo inducido por CCl_4 en ratas Wistar.

Al comparar los resultados de las propiedades antioxidantes entre las algas se comprobó que *Bryothamnion* resultó mucho más eficiente en algunas metodologías mientras *Halimeda* resulta más eficiente en otros ensayos.

Palabras clave: Algas marinas, antioxidantes, *Halimeda*, *Bryothamnion*, polifenoles

Vidal, A., *et al*. 2015. Las Algas Marinas de los Géneros *Byothamnion* y *Halimeda* como Fuentes de Antioxidantes Naturales. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 183-219.

Introducción

Las algas marinas forman parte de la dieta tradicional en algunas regiones del mundo, sobre todo asiática, con un alto valor nutricional como fuentes de proteínas, minerales, vitaminas y fibras dietéticas y desde épocas ancestrales han sido utilizadas como fitofármacos contra diferentes patologías (Mac Artain *et al*., 2007). En los últimos años, las investigaciones acerca de posibles propiedades terapéuticas de las algas marinas han cobrado una marcada importancia, motivado en cierta medida por su contenido de metabolitos secundarios bioactivos (Proksch *et al.*, 2003). Diferentes estudios *in vitro* y en modelos animales así como investigaciones epidemiológicas, han evidenciado una relación directa e inversa entre el consumo de algas y la incidencia de algunas patologías (Gómez-Gutierrez *et al.*, 2011).

La síntesis de determinados metabolitos secundarios en las algas marinas puede ser explicada como un mecanismo de defensa contra circunstancias adversas del medio ambiente entre los que se pueden citar la temperatura, luz solar, pH, estrés oxidativo y presencia de peces herbívoros. En los mares tropicales las algas están expuestas a una alta incidencia de luz solar lo que puede conducir a la formación de radicales libres, de manera que la ausencia de daños oxidativos de sus componentes estructurales y fisiológicos evidencian que estos organismos presentan un eficiente sistema de defensas antioxidantes (Sampth-Wiley *et al*., 2008).

Las propiedades antioxidantes de las algas marinas pueden ser explicadas por la presencia de moléculas como carotenoides, aminoácidos tipo micosporinas, terpenoides y polisacáridos sulfatados, aunque la mayoría de los investigadores consideran a los compuestos polifenólicos como los ácidos fenólicos y cinámicos, florotaninos y bromofenoles, entre los principales responsables de esta propiedad(Dutra Rocha *et al.*, 2007).

En trabajos previos de nuestro Laboratorio, con especies de los géneros *Bryothamnion* y *Halimeda* se demostraron propiedades antioxidantes en sistemas libres de células, en modelos experimentales *in vitro* y en modelos animales (Rivero *et al.*, 2003; Fallarero *et al*., 2006; Vidal *et al*., 2009; Batista-Gonzalez *et al*., 2012; Silva *et al*., 2012).

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Considerando estos antecedentes, el objetivo de este trabajo fue evaluar y comparar las propiedades antioxidantes de extractos acuosos de alga marinas de los géneros *Bryothamnion* y *Halimeda* mediante ensayos *in vitro*, cultivos de células y modelos animales de estrés oxidativo y su relación con el contenido de polifenoles así como esclarecer sus posibles mecanismos de acción.

Resultados y Discusión

En la actualidad se ha incrementado el interés por los extractos vegetales como fuentes de compuestos antioxidantes, al prevenir el daño celular por inactivación de los radicales libres y en este contexto las algas constituyen excelentes candidatos para la obtención de compuestos bioactivos (Dutra Rocha *et al*., 2007).

Las propiedades antioxidantes de los extractos acuosos de las algas de los géneros *Bryothamnion* y *Halimeda* se evaluaron a través de algunos de los ensayos más utilizados, con el objetivo de comparar sus propiedades antioxidantes y su relación con el contenido en polifenoles y adicionalmente esclarecer sus posibles mecanismos de acción.

I.-Contenido de polifenoles

Los polifenoles constituyen uno de los más numerosos y representativos grupos de metabolitos secundarios de las plantas, con propiedades beneficiosas para la salud humana. Estos metabolitos tienen la capacidad de neutralizar radicales libres, debido a la habilidad de donar los átomos de hidrógeno de los grupos hidroxilos presente en su estructura de anillos aromáticos, la capacidad de atrapar radicales libres, funciones como agentes quelantes y la inducción de enzimas antioxidantes (Vauzour *et al*., 2010).

Diferentes investigadores han demostrado la presencia de compuestos fenólicos en las algas marinas y su relación con las propiedades antioxidantes (Dutra Rocha *et al*., 2007). De acuerdo a Kuda e Ikemori (2009) el contenido de polifenoles de las algas marinas varía

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considerablemente aun en especies dentro del mismo género, desde muy bajos contenidos hasta valores altos.

El contenido de polifenoles para estas algas se presenta en la **Tabla I.** El contenido de polifenoles del extracto acuoso de *Bryothamnion triquetrum* fue superior a los valores obtenidos en trabajos previos por Vidal *et al*., (2001) (8,05 mg de EAG/ g de extracto seco), aunque estos investigadores emplearon un extracto acuoso con diferente esquema de extracción. Este valor de polifenoles totales para *B triquetrum* fue aproximadamente 2,6 veces superior al obtenido para el alga verde *H. opuntia.* Zubia *et al*., (2007) encontraron una relación similar (3,3 veces) del contenido de polifenoles de las algas *B. triquetrum* y *H. monile*.

Tabla I. Contenido total de compuestos polifenólicos de los extractos acuosos de *Bryothamnion triquetrum* y *Halimeda opuntia.* Se realizaron las determinaciones por triplicado y cada punto representa media ±DE. El método empleado fue la técnica de Folin-Ciocalteau según Vidal *et al.*, (2009). Tomado de Díaz *et al*., ***Ars Pharmaceutica***. 56(2): 89-99, 2015

Alga	Contenido de polifenoles (µg EAG/ mg de extracto)
Bryothamnion triquetrum	51,21± 2,25
Halimeda opuntia	19,99 ± 1,12

El contenido de polifenoles de *H. opuntia* presentado en este trabajo fue superior a los obtenidos por Silva *et al*., (2012) con un extracto acuoso de esta especie de alga (97.2± 7.3 µg EAG/ g) y Batista-González *et al*., (2012*)* con un extracto acuoso del alga *H. monile* (179,45 ± 18,54 µg/ g de extracto seco). Vidal *et al*., (2009) reportaron un valor similar de polifenoles totales (74.3 mg of polifenoles/g alga seca) en un estudio sobre la actividad antioxidante de *Halimeda* spp con fracciones de ácidos fenolicos. Costa-Mugica *et al*., (2012) encontraron valores de polifenoles totales para el alga *Halimeda incrassata* de 131 µg GAE/g alga seca.

Se ha identificados como los principales componentes de la fracción polifenolica del alga *B.triquetrum* a los ácidos *p*-cumárico, *t*-cinámico y ferúlico y se ha relacionado con sus propiedades antioxidantes (Vidal *et al*., 2001; Fallarero *et al*., 2006) mientras que para *H. opuntia* se identificaron y cuantificaron 8 ácidos fenólicos y cinámicos, resultando el componente mayoritario el ácido salicílico (Vidal *et al*., 2009). Resulta interesante señalar que del total de compuestos polifenólicos presentes en las algas *H. opuntia* y *H.monile* fueron identificados como ácidos fenólicos el 34.3 y 33.3%, respectivamente (Vidal *et al*., 2009). Otros investigadores encontraron cantidades apreciables de compuestos polifenolicos en especies del genero *Halimeda*. Yoshie *et al.,* (2002) han identificado ácido caféico en *H. macroloba.* Vidal *et al.,* (2006) encontraron cantidades apreciables de acido salícilico y en menor proporción otros ácidos fenólicos y cinámicos en *H incrassata*.

Nakai *et al*., (2008) evaluaron la actividad antioxidante de diferentes especies de algas y en *H. opuntia* encontraron actividad atrapadora de radicales hidroxilos ($^{\bullet}$OH), explicando las propiedades antioxidantes por la presencia de florotaninos y terpenoides. Otros metabolitos con función antioxidante, que en alguna medida pudieran influir en esta propiedad son los carotenos y el ácido ascórbico, también presentes en *B. triquetrum* (Vidal *et al.,* 2006). Zubia *et al*., (2009) señalan que los bromofenoles presentes en las algas rojas *B. byssoides* y *E lanosa* con similar ubicación taxonómica que *B. triquetrum* (Familia Rhodomelaceae, orden CERAMIALES), pudieran ser los responsables de su alta actividad antioxidante. Vidal *et al.,* (2001) detectaron cantidades apreciables de compuestos bromados en el alga *B. triquetrum*.

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II.-Actividad antioxidante con métodos *in vitro*

a.-*Capacidad reductora de los extractos de Halimeda y Bryothamnion*

Diferentes investigadores señalan que los compuestos reductores tienen la capacidad de romper las reacciones en cadenas de los radicales libres por la donación de un átomo de hidrógeno y de esta manera exhiben propiedades antioxidantes (Moon y Shibamoto, 2009).

En el intervalo de concentraciones evaluadas para los extractos de ambas algas se observa una alta capacidad de reducir el estado de transición del Fe^{3+} y consecuentemente la generación de radicales libres de manera dosis-dependiente.

Los resultados de la Capacidad reductora de los extractos de *Halimeda* y *Bryothamnion* se muestran en la Figura 1. Los valores de capacidad reductora obtenidos con el extracto de *Halimeda opuntia* resultaron similares a los valores encontrados en la literatura y en trabajos previos de nuestro Grupo para el género *Halimeda*. Silva *et al.*, (2012), obtuvieron un valor de absorbancia igual a 0,1 nm con una concentración de 10 mg/mL (λ = 700 nm) con un extracto acuoso de *H. opuntia,* valor similar al obtenido en este trabajo a esa concentración. Batista-González *et al*., (2012) trabajando con fracciones polares de ácidos fenolicos de *H monile* obtuvieron una DO de 0.13 nm con 20 µg de polifenoles totales, valores que convertidos en mg/mL también son comparables a los resultados de este trabajo.

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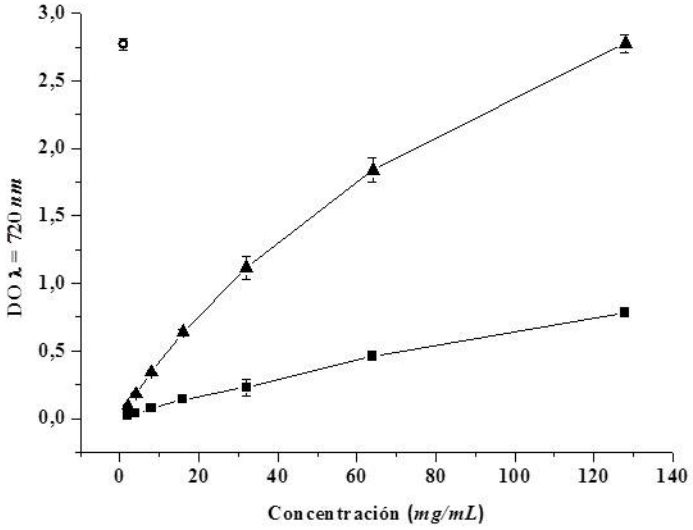

Figura 1. Capacidad reductora expresada en función de mg de extracto acuoso liofilizado de *Bryothamnion triquetrum* (▲) y *Halimeda opuntia* (■). Como control positivo se utilizó ácido ascórbico a 1mg/mL (○). El ensayo fue realizado de acuerdo a Oyaizu *et al.*, (1986). Los resultados están expresados como x± DE, n=3. Tomado de Díaz *et al*., ***Ars Pharmaceutica***. 56(2): 89-99, 2015

Al comparar los valores de absorbancia a una concentración de 128 mg/mL, en el extracto de *B. triquetrum* se obtuvo una DO de 2.798 nm mientras que con el extracto de *H.opuntia* el valor fue de 0.800 nm, de manera que *B.triquetrum* resultó 3,5 veces más eficiente en la reducción de los iones Fe^{3+} a Fe^{2+} que *H. opuntia.* Este resultado puede ser explicado por un mayor contenido de polifenoles en este extracto (aproximadamente 2,5 veces mayor con respecto a *H. opuntia)* y la estrecha relación que existe entre este parámetro y la capacidad reductora de extractos naturales. Kuda e Ikemori (2009) observaron una buena correlación entre contenido de polifenoles y la capacidad reductora en 12 especies de algas.

Se empleó como control de actividad antioxidante el ácido ascórbico en una concentración de 1 mg/mL, con una DO de 2,824 nm, valor de absorbancia igual al observado con el extracto de *B. triquetrum* y aproximadamente 3,5 veces superior al extracto de *H. opuntia*

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pero a una concentración 128 veces inferior. Sin embargo si consideramos que comparamos un compuesto químicamente puro (ácido ascórbico) con un extracto crudo podremos concluir que los extractos presentan una actividad antioxidante relativamente alta.

b.- ***Ensayo de actividad atrapadora de radicales DPPH•***

Dentro de los mecanismos de acción antioxidante más importantes de las algas marinas se encuentra la capacidad atrapadora radicales libres y por este motivo, el ensayo de inactivación del radical DPPH• es una de las metodologías más utilizadas para estudiar sus propiedades antioxidantes (Moon y Shibamoto, 2009). El DPPH• es un radical estable coloreado que puede aceptar protones donados por entidades antioxidantes presentes en estos extractos convirtiéndose en su forma no radicalaria incolora.

Los resultados del Ensayo de actividad atrapadora de radicales DPPH• de los extractos acuosos de *Halimeda* y *Bryothamnion* se pueden apreciar en la Figura 2. Los extractos acuosos de ambas algas provocaron la decoloración total, llegando a la máxima actividad de atrapamiento de radicales DPPH•. El extracto de *B. triquetrum* logró inhibir el 100 % del radical a la concentración de 5 mg/mL mientras que a esa concentración el extracto de *H. opuntia* produjo un 24% de inhibición. Al comparar estadísticamente los valores de CI_{50} obtenidos con esta metodología (1,15± 0,06 y 11,34± 0.30 mg/mL para *B. triquetrum* y *H. opuntia* respectivamente), observamos que el alga *B. triquetrum* resulta aproximadamente cuatro veces más potente que *H. opuntia,* resultado que concuerda con lo obtenido en el experimento de la capacidad reductora. También Boonchum *et al.,* (2011) encontraron un valor de CI_{50} de 0.837 mg/mL para un extracto acuoso de *Halimeda macroloba.*

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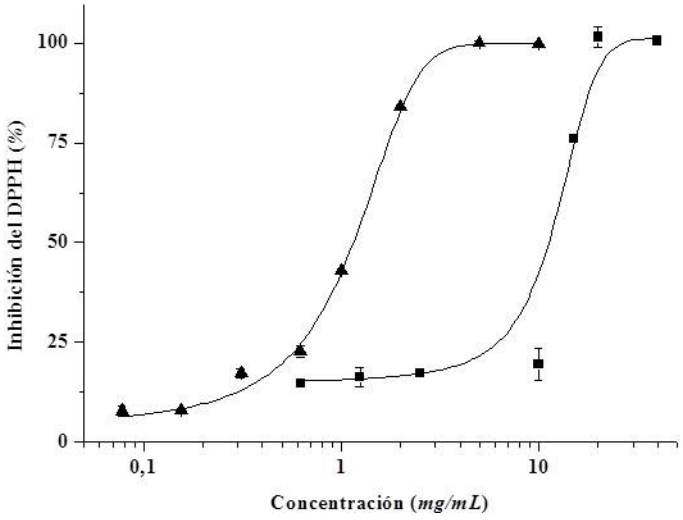

Figura 2. Actividad atrapadora de radicales DPPH• expresada en función de mg de extracto acuoso liofilizado de *Bryothamnion triquetrum* (▲) y *Halimeda opuntia* (■). El ensayo fue realizado de acuerdo a Goupy *et al.*, (1999). Los resultados están expresados como x± DE, n=3. Tomado de Díaz *et al*., ***Ars Pharmaceutica***. 56(2): 89-99, 2015

El valor obtenido con el extracto acuoso del alga *B. triquetrum* es superior al informado por Vidal *et al.*, (2006) que obtuvieron con 4 mg/mL un 38% de inhibición del DPPH· y también superior a lo reportado por Zubia *et al.*, (2007) con una CI_{50} de 12.9 mg/mL para esta especie de alga. Los resultados de *H. opuntia* se corresponden con valores informados por otros investigadores. Silva *et al.*, (2012) reportaron un 48% de inhibición con 7 mg/mL de extracto acuoso liofilizado de *H. opuntia*.

En esta investigación encontramos valores relativamente altos de polifenoles para el alga *B. triquetrum*, por lo que es de esperar que presente una capacidad atrapadora de radicales libres mayor que *H. opuntia*. Adicionalmente estos resultados evidencian que existe una relación directa entre el contenido de polifenoles de los extractos acuosos y las propiedades

antioxidantes en función de la capacidad reductora y el atrapamiento de radicales libres, siendo *B. triquetrum* más potente en estos sistemas que *H. opuntia*.

Zubia *et al*., (2009) estudiando las propiedades antioxidantes con el ensayo de DPPH y poder reductor de 24 algas rodófitas observaron una correlación significativa entre la actividad atrapadora del radical DPPH y el poder reductor en las algas rojas evidenciado en que ambos ensayos se explican porque se basan en la donación de electrones/hidrógenos.

Las propiedades antioxidantes de un extracto vegetal están en dependencia al radical libre al que se enfrenta la molécula antioxidante. Kaur *et al.,* (2006) demostraron que la actividad atrapadora de radiales libres de un extracto de flores de *Cassia siamea* difieren en dependencia del radical al que se enfrenta el extracto, para el radical $DPPH^{\cdot}$ se obtuvo un 100 % de inhibición a una concentración que no fue tan eficaz para otros radicales como $O_2^{\bullet-}$ y $OH^{\bullet}$, lo que pudiera ser explicado porque el mecanismo de atrapar $DPPH^{\cdot}$ está estrechamente relacionado al poder reductor, por la capacidad de donar un protón, mientras para los otros radicales se fundamenta en el atrapamiento. Este criterio podría corroborar los resultados de este trabajo, ya que la peroxidación lipídica espontánea está mediada principalmente por las EROs y pudiera resultar que *B. triquetrum*, más potente en cuanto a mecanismo de donar protones, no sea tan eficiente para atrapar radicales libres a diferencia de *H. opuntia*. Vidal *et al.,* (2006) demostraron que la actividad antioxidante de *B. triquetrum* puede ser explicada en parte por la capacidad atrapadora de radical $OH^{\bullet}$ y la dismutación de radicales $O_2^{\bullet-}$.

c.-*Inhibición de la lipoperoxidación espontánea en homogenado de cerebro de rata*

La peroxidación lipídica es un evento de daño celular, donde se afectan los lípidos poli-insaturados de las membranas, formándose productos citotóxicos como el *trans*-4-hidroxi-2-nonenal (HNE) y el malondialdehído (MDA), compuestos que a su vez desencadenan un estrés oxidativo y a nivel de las biomembranas lo que puede conducir a la despolarización y

permeabilización no selectiva de las mismas y cambios estructurales así como alteraciones a las proteínas embebidas en ella, de ahí que este constituya un evento de efectos altamente deletéreos para la célula (Moon y Shibamoto , 2009).

Los resultados del Ensayo de Inhibición de la lipoperoxidación espontánea en homogenado de cerebro de rata de los extractos acuosos de *Halimeda* y *Bryothamnion* se pueden apreciar en la Figura 3. Los resultados de este trabajo evidencian que ambos extractos de algas inhiben efectivamente la generación de TBARS con CI_{50} de 5,09 ± 0,25 y 1,25 ± 0,31 mg/mL para *B. triquetrum* y *H. opuntia* respectivamente. Al comparar estadísticamente los valores de CI_{50} se hace evidente que el alga *H. opuntia* es mucho más efectiva que *B. triquetrum*. En un trabajo previo, Batista-González *et al.,* (2012) estudiando un extracto acuoso de *H opuntia* encontraron un valor de CI_{50} similar a lo obtenido en este trabajo. Rivero *et al*., (2003) investigando las propiedades antioxidantes de un extracto acuoso de *Halimeda incrassata* reportaron valores de máxima efectividad en la inhibición de la peroxidación lipídica espontánea en homogenados de cerebro de rata a la concentración de 5 mg/mL. En este trabajo para el alga *Halimeda opuntia* se obtuvo a esa misma concentración aproximadamente un 75% de inhibición de la peroxidación lipídica. Nuestros resultados también concuerdan con Vidal *et al*., (2001) quienes investigando extractos de *B. triquetrum* observaron una CI_{50} de inhibición de la lipoperoxidación en cerebro de ratas de 6,46 mg/mL.

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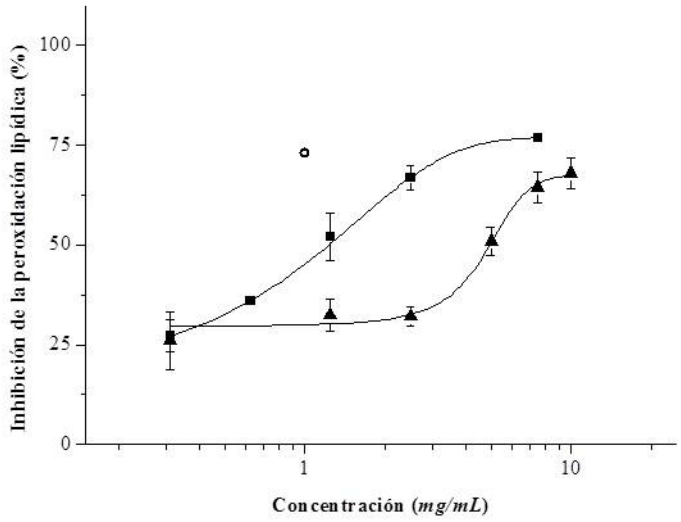

Figura 3. Inhibición de la lipoperoxidación espontánea en homogenados de rata expresado en función de mg de extracto acuoso liofilizado de *Bryothamnion triquetrum* (▲) y *Halimeda opuntia* (■).Como control positivo se utilizó ácido ascórbico 1mg/mL (○). El ensayo fue realizado de acuerdo a Ohkawa e*t al.*, (1999). Los resultados están expresados como x± DE, n=3. Tomado de Díaz *et al*., ***Ars Pharmaceutica***. 56(2): 89-99, 2015

Algunas moléculas pueden inhibir la lipoperoxidacion por mecanismos como la prevención de la iniciación de la oxidación de cadenas de ácidos grasos, enlazamiento de iones metálicos de transición catalíticos, y descomposición de peróxidos y además en adición el atrapamiento de radicales libres. En los ensayos de capacidad reductora y atrapamiento de radical libre $DPPH^{\cdot}$, el extracto acuoso del alga *B. triquetrum* había mostrado resultados superiores con respecto al extracto de *H. opuntia*, sin embargo en este ensayo se obtuvo mayor inhibición de la lipoperxidacion espontánea con el extracto acuoso de *H. opuntia.* Lim *et al*., (2006) reportaron mayor valor de CI_{50} para el ensayo de DPPH con respecto a valor obtenido en el ensayo de inhibición de la lipoperoxidación en homogenados de cerebro con un extracto de *N. aculeate*, lo que concuerdan con nuestros resultados. Matsukawa *et al.*, (1997) no encontraron correlación entre la inhibición de lipo-oxigenasa y la actividad atrapadora de DPPH, sugiriendo que estas actividades ocurren con mecanismos

no relacionados directamente. En el ensayo de atrapamiento del radical DPPH, los extractos de algas actúan como donadores de electrones y/o hidrogeno mientras que en la inhibición de la lipo-oxigenasa se pudiera bloquear la adición enzimática de oxígeno al ácido graso (sustrato de la enzima) y por tanto inhibir la formación de hidroperóxido. En estudios previos de estos autores, demostraron una correlación entre el consumo de oxígeno y la formación de hidroperóxidos durante el proceso de inhibición de la lipo-oxigenasa. Adicionalmente la diferencia en respuesta de acuerdo al método empleado también se pudiera explicar por las diferencias cuantitativitas en las composiciones químicas y/o en tipos de compuestos polifenólicos de estas algas por lo que resulta lógico que presenten diferentes propiedades antioxidantes como por ejemplo algunos mecanismos entre los que se comprenden la quelación de hierro (en la etapa de iniciación) o un incremento sinérgico de la actividad antioxidante de vitamina E en la etapa de propagación. Este criterio está en concordancia con resultados obtenidos por Yoshie *et al*., (2002) quienes demostraron que existían diferencias en cuanto a la composición de polifenoles y las cantidades de estos en extractos naturales de algas incluso dentro del mismo género. Otra explicación pudiera ser, la presencia de otros componentes en el extracto pudieran actuar de forma sinérgica con los estos y potenciar la actividad inhibitoria en la peroxidación lipídica espontánea (Fallarero *et al.*, 2006).

En este trabajo se encontró una relación directa entre la inhibición de la lipoperoxidación y el contenido de polifenoles para las dos algas. Otros autores (Rivero *et al*., 2003; Batista-González *et al*., 2012) también han encontrado una relación directa entre esta actividad antioxidante y el contenido de polifenoles. Chakraborty *et al.*, (2013) sugieren que la inhibición de la peroxidación lipidica de extractos de *Turbinaria* spp puede ser debida a la presencia de compuestos polifenólicos que disrumpen la reacción en cadena de los radicales libres por donación de un protón al radical acido graso y de esa manera inhibir la peroxidación lipídica.

Lim *et al.,* (2002) estudiando fracciones del alga *Sargassum siliquastrum* no encontraron una relación directa entre el contenido de polifenoles y la actividad antioxidante sin embargo encontraron una relación estrecha entre la inhibición de la peroxidación lipídica y la hemólisis de eritrocitos inducida por AAPH, concluyendo que una de las fracciones estudiada probablemente contenía un potente antioxidante bloqueador de la rupturas de cadenas no polares**.**

d.- Capacidad inhibitoria de la hemólisis inducida por el AAPH

AAPH es un radical peroxilo que inicia el proceso peroxidativo, generando a la vez otros radicales libres para inducir oxidación de los ácidos grasos y proteínas, ocasionando un daño sobre la organización de los eritrocitos y conduciendo eventualmente a lisis de la membrana (Pannangpetch *et al*., 2007). Adicionalmente la liberación del hierro desde los hematíes puede aumentar el efecto pro-oxidante de hidroperóxidos provenientes de la reacción del oxígeno y el AAPH, con un papel importante como agente catalítico redox de acuerdo a la reacción de Fenton y de Haber-Weiss.

Los resultados de este trabajo (Figura 4A) evidencian que ambos extractos protegen a los eritrocitos del efecto toxico del radical AAPH sobre las biomembranas de una manera dosis-dependiente. El extracto de *H. opuntia* resulta más potente ya que a bajas concentraciones (12.5 mg/mL) mostró un 82.2 % de inhibición frente a un 35.1% con el extracto de *B. triquetrum.* Estos resultados se encuentran en concordancia con los resultados del ensayo de inhibición de la peroxidación lipídica, lo que resulta lógico si consideramos que ambas metodologías estudian fundamentalmente las propiedades antioxidantes de una molécula para prevenir los efectos tóxicos producidos por un radical libre sobre los lípidos de las biomembranas. Estos resultados concuerdan con los obtenidos por Lim *et al.,* (2002), quienes trabajando con extractos del alga *Sargassum siliquastrum* encontraron una excelente correlación entre la inhibición de la peroxidación lipídica y la

protección contra hemólisis de eritrocitos, considerando como responsables de estas actividades a los compuestos fenólicos.

En la literatura existen pocos reportes de estudios de propiedades antioxidantes de extractos vegetales mediante este ensayo. Benites *et al*., (2011) demostraron que concentraciones superiores a 0,2% (p/v) de extractos de frutas inhibían la hemólisis en aproximadamente un 40%. Sin embargo para el extracto de *H. opuntia* a concentraciones superiores a 25 mg/mL (Figura 4B) se comienza a observar una disminución de la protección frente a la actividad hemolítica del AAPH, a diferencia de lo que ocurre con el aumento de las concentraciones de *B. triquetrum,* donde el incremento de las concentraciones no disminuye sus propiedades antioxidantes protectoras y/o incremento de efectos tóxicos. Similares resultados encontró Lim *et al*., (2002) trabajando con diferentes extractos del alga *Sargassum siliquastrum*, quienes encontraron una satisfactoria actividad antihemolitica de los eritrocitos de rata en las concentraciones de 0,2 a 10 μg/mL, sin embargo con la concentración de 50 μg/mL la protección disminuía aproximadamente 9 veces. En determinadas condiciones experimentales los compuestos polifenólicos pueden actuar como pro-oxidantes en presencia de Fe^{3+}, dada su habilidad para enlazarse y reducir el Fe^{3+} vía transferencia electrónica colateral, aspecto corroborado por Puppo (1992) quien observo un incremento en la producción de $^{\bullet}OH$ estudiando el efecto de los flavonoides sobre la formación de radicales hidroxílicos por la reacción de Fenton. De manera que un factor a considerar seria la cantidad de Fe, consecuentemente un incremento de la cantidad del extracto conlleva un incremento de polifenoles y a su vez del Fe presente en este extracto. Adicionalmente es conocido que el genero *Halimeda* contiene cantidades apreciables de este mineral. Anantharaman *et al.*, (2010) investigaron la composición de minerales y de metales trazas de 9 especies de algas (incluidas *H.macroloba* y *H.tuna*), resultando el Fe como el segundo mineral en contenido para todas las especies de algas, con las mayores concentraciones en *Halimeda macroloba.* Otra explicación acerca de la toxicidad observada en las dosis altas de *H. opuntia* puede ser el aumento en las concentraciones de determinados metabolitos secundarios presentes en esta especie de alga, lo que generaría una actividad pro-oxidante

tanto de los polifenoles, vitamina C y la posible presencia de metales de transición (Fe^{2+}, Cu^{+}, Zn^{2+}), que en presencia de radicales peroxilo inducido por el AAPH aumentarían el daño oxidativo por generación de radicales $OH^{\cdot}$.

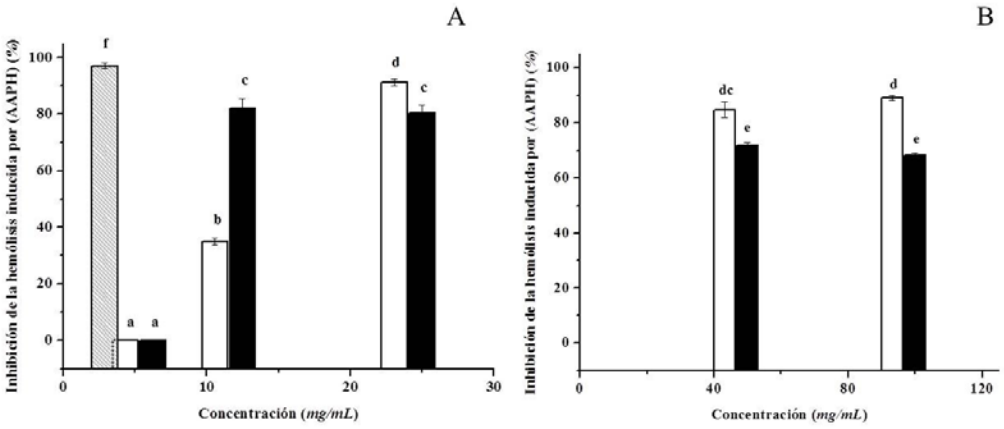

Figura 4. Capacidad inhibitoria de la hemólisis inducida por AAPH expresado en función de mg de extracto acuoso liofilizado de *Bryothamnion triquetrum* (□) y *Halimeda opuntia* (■).

A: Dosis 2-30 mg/mL

B: Dosis 35-128 mg/mL

El ensayo fue realizado de acuerdo a Aman *et al.*, (2013). Como antioxidante de referencia se empleó el ácido ascórbico en una concentración de 0.25-1 mg/mL (▒).Los resultados están expresados como x± DE, n=3. Tomado de Díaz *et al*., ***Ars Pharmaceutica***. 56(2): 89-99, 2015

En este sistema experimental (protección al eritrocito) actúan a la vez dos de los mecanismos de acción que explican la actividad antioxidante de extractos de algas: la capacidad de atrapar radicales libres y de inhibir la peroxidación de lipídica. Hipotéticamente se podría postular otro mecanismo de acción, la capacidad quelante de Fe,

entonces los resultados para cada extracto con este ensayo estarán en función de la composición química de moléculas que puedan contribuir más o menos a cada uno de estos mecanismos. Al parecer, la mayor actividad antihemolítica de *H. opuntia* con respecto a *B. triquetrum* se pudiera deber a mecanismos de atrapamientos de radicales libres más eficientes y a la inhibición de la peroxidación lipídica, independientemente del contenido de polifenoles que presenta y además la quelación de Fe^{3+}.

e.- ***Actividad antioxidante por el ensayo del β-caroteno-ácido linoléico***

La actividad antioxidante por el ensayo de β-Caroteno-Acido linoleico de los extractos acuosos de las algas de los géneros *Halimeda* y *Bryothamnion* se muestra en la Tabla II. Los resultados evidencian valores altos de actividad antioxidante, resultados en concordancia con informes anteriores para este género de algas pero con otras metodologías (Rivero *et al*., 2003; Linares *et al*., 2004).

Tabla II. Actividad antioxidante por el ensayo de β-caroteno-acido linoleico. El ensayo fue realizado de acuerdo a Miller (1971). Como antioxidante de referencia se empleó el BHA en una concentración de 0.1-0.2 mg. Los resultados están expresados como x± DE, n=3. Tomado de A. Vidal Novoa, conferencia magistral en la Facultad de Ciencias Farmacéuticas de la Universidad de Sao Paulo, Brasil, 24/abril/2015.

Vidal, A., *et al*. 2015. Las Algas Marinas de los Géneros *Byothamnion* y *Halimeda* como Fuentes de Antioxidantes Naturales. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 183-219.

Cantidad de extracto de alga	***H. incrassata***	***B. triquetrum***
1 mg	68%	15%
2 mg	75%	24%
	Control positivo BHA	
0.1 mg	88%	
0.2 mg	97%	

Tabla III. Valores de los parámetros y enzimas sericas y hepáticas relativas al estrés oxidativo en ratas Wistar tratadas con CCl_4, *Bryothamnion triquetrum* y Acido Ferulico. Los valores representan la media ± DE. Letras diferentes indican diferencias estadísticas significativas, *p < 0.05. Tomado de Vidal *et al*., en prensa en ***African Journal of Agriculture Research, 2015.***

	Suero		**Higado**	
Grupos	AST (U/mL)	ALAT (U/mL)	TBARS (nmol/mg protein)	GSH (µmol)
Control	84,36 ± 0,61^{a}	57,68 ± 2,70^{a}	0,19 ± 0.02^{a}	0,10 ± 0,02^{a}
CCl_4	116,30 ± 11,68^{bc}	90,87 ± 20,08^{bc}	0,87 ± 0,21^{b}	0,65 ± 0,110^{b}
FA 20	112,25 ± 10,22^{bd}	78,59 ± 13,11^{ac}	0,56 ± 0,29 ^{bc}	0,55 ± 0,12^{bc}
Bt 200	96,03 ± 8,68^{ad}	62,13 ± 8,41^{a}	0,45 ± 0,10^{ac}	0,41 ± 0,12^{c}

En resumen, las fracciones hidrófilicas obtenidas de *Halimeda* presentan una potente actividad antioxidante según la evaluación del ensayo de β-caroteno-acido linoleico, lo que podría ser al menos parcialmente explicado por la presencia de los ácidos salicílicos y

ferúlicos, aunque pueden contener otros compuestos bioactivos, los que pudieran también influir directamente en la actividad antioxidante, como los carotenoides y polisacáridos.

Cuando se compararon los resultados de actividad antioxidante en el sistema β-caroteno/linoléico con el ensayo del DPPH, para la concentración de 4 mg de extracto, se observo un valor de inhibición de la oxidación más bajo (12%).

El ensayo del Sistema β-Caroteno-linoléico esta basado en la capacidad de un antioxidante de proteger la lipoperoxidación mediante el atrapamiento de radicales libres tanto en la fase de iniciación como de propagación, resultados que evidencian una débil actividad atrapadora de radicales libres del extracto del alga *B.triquetrum*. Al analizar estadísticamente la correlación de estas dos metodologías (Sistema β-Caroteno-linoléico y DPPH) (Figura 5) se encontró un coeficiente de correlación satisfactoria (r2=0,912), y esto permite avalar el criterio de que el mecanismo de acción antioxidante de esta alga en cierta medida está relacionado con la capacidad atrapadora de radicales libres. Si consideramos trabajos previos (Vidal *et al.,* 2001; Fallarero *et al.,* 2006) donde se relacionan la actividad antioxidante de esta alga con la presencia de polifenoles, entonces además se debe considerar que los compuestos polifenolicos pueden actuar como antioxidantes no solo por un mecanismo de atrapamiento de radicales libres (Rice-Evans *et al.,* 1995; Yeh, Yen, 2006). Por otra parte, la actividad antioxidante pudiera ser explicada por un conjunto de mecanismos. Kang *et al*. (2005) encontraron que el alga roja *Callophyllis japonica* presentaba actividad atrapadora de radicales DPPH·, inhibía la peroxidación lipídica y además incrementaba la actividad de algunas enzimas antioxidantes.

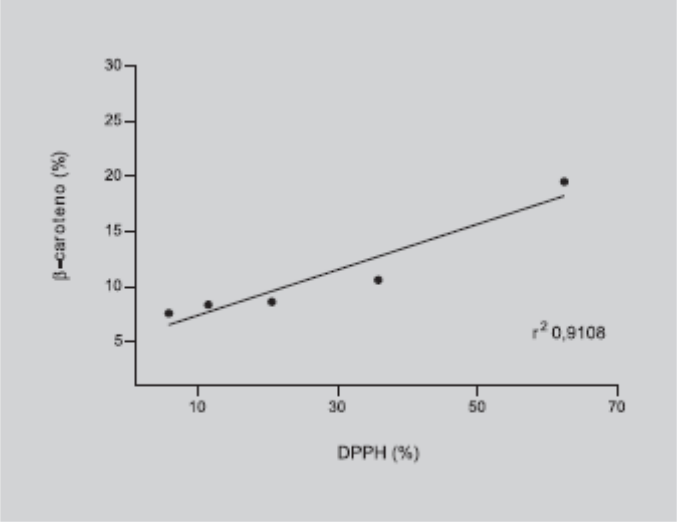

Figura 5. Estudio de correlación lineal de la actividad antioxidante del extracto acuoso de *B. triquetrum* mediante los ensayos de β-caroteno-acido linoleico y de atrapamiento de radicales DPPH. En cada punto se emplearon cantidades idénticas del extracto liofilizado (0,5; 1; 2; 4 y 8 mg). Tomado de Vidal *et al*., ***Braz J Pharm Sci***. 42(2): 589-599, 2006.

En un trabajo previo de nuestro Grupo, Mancini-Filho *et al*., (2009) investigaron las propiedades antioxidantes de un extracto acuoso del alga *Halimeda monile* mediante los ensayos de DPPH y β-Caroteno-Acido linoleico y demostraron que esta alga resultaba muy eficiente como fuente de antioxidantes, con resultados similares por ambas técnicas y adicionalmente encontraron una relación directa entre el contenido de polifenoles.y la actividad antioxidante.

f.- Capacidad antioxidante mediante el ensayo xantina/xantina oxidasa como generador de radicales $O_2^{\bullet -}$ (McCord y Fridovich , 1969)

La capacidad de extractos de *B.triquetrum* y *H.incrassata* para interaccionar con los radicales $O2^{\bullet -}$ fue medida a través de su efecto inhibidor sobre la reducción del NBT provocada por estos radicales y se presentan en la Figura 6. Se aprecia que ambos extractos presentan capacidad para reaccionar directamente con los radicales $O_2^{\bullet -}$ de un modo

dependiente de su concentración. El porcentaje de inhibición de la reducción del NBT mantiene una relación sigmoidal con el logaritmo de la concentración para las dos algas.

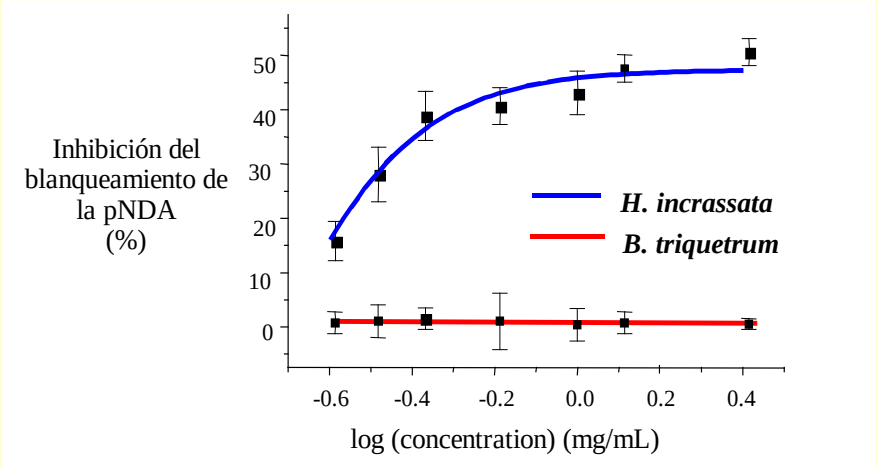

Figura 6. Actividad antioxidante atrapadora de radicales O2 •– en función de mg/mL de extractos acuosos de *Bryothamnion triquetrum* y *Halimeda incrassata.* El ensayo fue realizado de acuerdo a Aruoma, (1994). Los resultados están expresados como x ± DE, n=3. Tomado de A. Vidal Novoa, conferencia magistral en la Facultad de Ciencias Farmacéuticas de la Universidad de Sao Paulo, Brasil, 24/abril/2015.

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Los valores determinados de CI_{50} fueron 0,49 y 0.56 mg/mL para las algas *B.triquetrum* y *H.incrassata* respectivamente.

III-Actividad antioxidante en cultivos de células GT1-7

En la Figura 7 se puede observar los resultados de la viabilidad de la línea celular neuronal GT1-7 insultadas con H_2O_2 y con tratamiento con los extractos de las algas. En el control de H_2O_2 se puede apreciar una significativa afectación. Resultados similares fueron reportados por Heck *et al.*, (1999) en esta línea celular. Adicionalmente se puede observar una significativa cito-protección producida por los extractos de las algas. Ambos extractos, en concentraciones superiores a 0,20 mg/mL, pueden inhibir la muerte celular producida por H_2O_2, Del mismo modo, Yoon *et al*., (2000) han informado el efecto protector de un 0,50 mg/mL de metanol crudo extracto obtenido de la planta *Orostachys japonicus*, contra la apoptosis inducida por H_2O_2 en la línea celular GT1-1. El extracto puede aumentar la supervivencia celular 5,7 veces, que es inferior al valor similar para nuestros extractos.

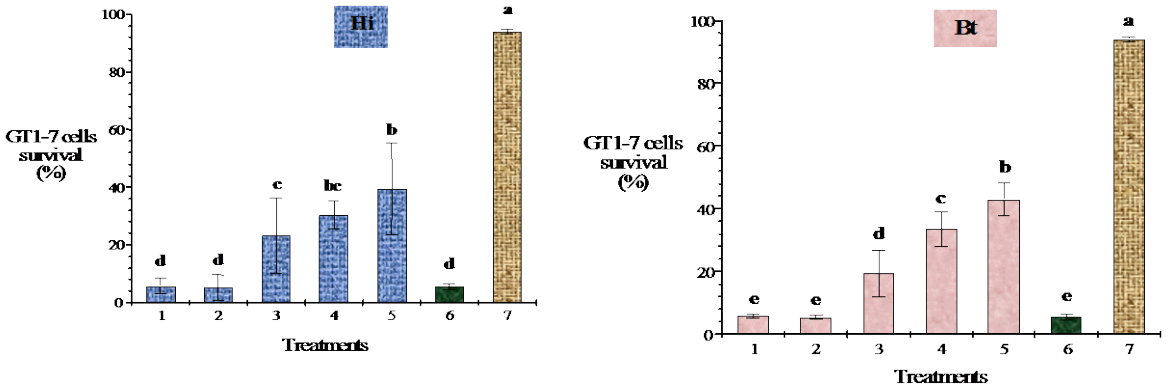


Figura 7. Efecto de diferentes concentraciones de las algas *B. triquetrum* y *H incrassata* sobre cultivos de células GT1-7 insultadas con H_2O_2. Las células fueron incubadas con diferentes concentraciones de las algas durante 3 h. y simultáneamente con 100 µM H_2O_2. Como control Positivo se empleo trolox 200 µM. Los valores se expresan como media ± DE. Tomado de Fallarero *et al*., ***Phytomedicine*** 10: 39-47, 2003

Vidal, A., *et al*. 2015. Las Algas Marinas de los Géneros *Byothamnion* y *Halimeda* como Fuentes de Antioxidantes Naturales. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 183-219.

Analizar el efecto de las algas sobre la producción de ROS podemos observar (Figura 8) que hay una reducción importante de estos metabolitos tóxicos, lo que evidencia en ambos casos que estos extractos tienen un efecto significativo directo sobre la producción y/o la inactivación de especies radicalarias del oxígeno.

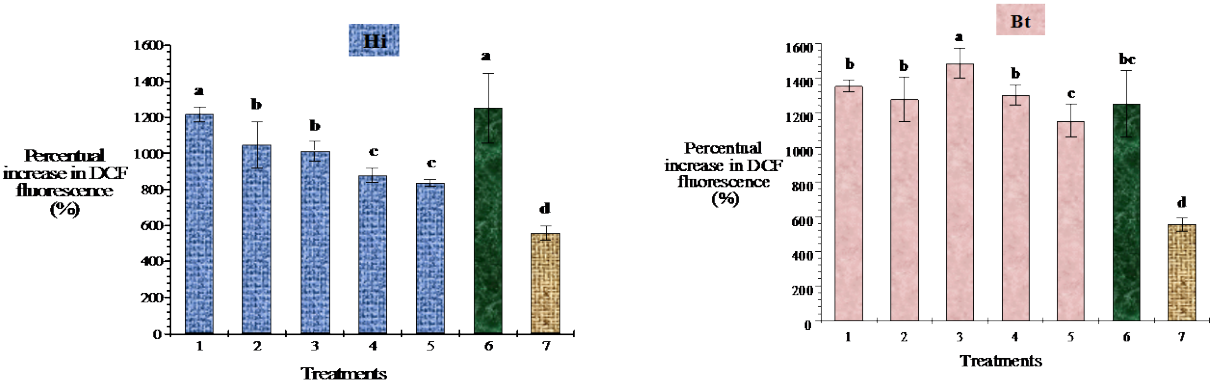


Figura 8. Efecto de diferentes concentraciones de las algas *B. triquetrum* y *H incrassata* sobre la producción de ROS en cultivos de células GT1-7 insultadas con H_2O_2. Las células fueron incubadas con diferentes concentraciones de las algas durante 3 h. y simultáneamente con 100 μM H_2O_2 Al final de la exposición la producción de ROS se determino de acuerdo a Loikkanen *et al*., (1998). Como control Positivo se empleo trolox 200 μM. Los valores se expresan como media ± DE. Tomado de Fallarero *et al*., ***Phytomedicine*** 10: 39-47, 2003

Los extractos de las dos algas son incapaces de mejorar el estado oxidativo de esta línea celular mediante el aumento de las reservas de GSH (Figura 9). Por lo tanto, un efecto protector de los extractos en la reducción de la fluorescencia basal DCF, no es al parecer, apoyada por el aumento en los niveles de GSH basales. Los altos niveles de GSH pueden actuar como reductores de la oxidación DCFH, no sólo porque GSH es una importante molécula antioxidante que controla la producción endógena de los radicales libres, sino

también debido a la capacidad directa de GSH para reducir químicamente radical intermedio DCF•, evitando su conversión a DCF (Zhu *et al.*, 1996).

En general, las propiedades antioxidantes de fenoles se encuentran en su habilidades para actuar como agentes reductores, los donadores de hidrógeno, desactivadores de oxígeno singlete y eficaces agentes quelantes de metales relaciones a reacciones redox (Rice-Evans *et al*., 1995). En el caso especifico de los ácidos trans-cinámico, p-cumárico y ferúlico, varios autores han reportado una actividad antioxidante, al menos similares al ácido ascórbico (Fukumoto y Mazza, 2000; Schroeter *et al*., 2000).

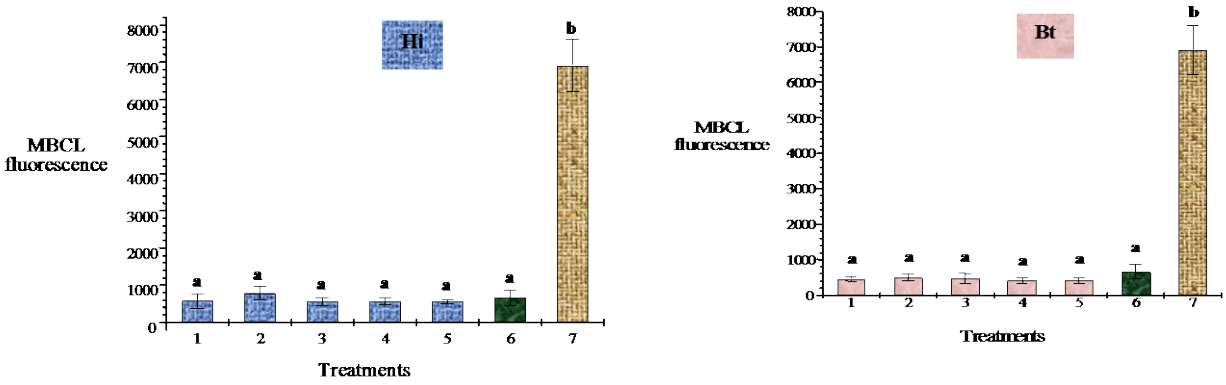


Figura 9. Efecto de diferentes concentraciones de las algas *B. triquetrum* y *H incrassata* sobre los niveles de GSH intracelulares en cultivos de células GT1-7 insultadas con H_2O_2. Las células fueron incubadas con diferentes concentraciones de las algas durante 3 h. y simultáneamente con 100 µM H_2O_2 Al final de la exposición los niveles de GSH se determino de acuerdo a *Loikkanen et al*., (1998). Como control Positivo se empleo trolox 200 µM. Los valores se expresan como media ± DE. Tomado de Fallarero *et al*., ***Phytomedicine*** 10: 39-47, 2003

En consecuencia, el efecto protector de extracto de *Bryothamniom triquetrum* en GT1-7 células neuronales pueden ser al menos parcialmente explicada por sus ácidos fenólicos.

En resumen, la presente investigación ha demostrado la capacidad de los extractos acuosos de algas de *Halimeda incrassata* y *Bryothamniom triquetrum* para proteger contra el estrés oxidativo producido por agentes químicos y prevenir así la toxicidad en células neuronales.

IV.-Modelos animales de estrés oxidativo

A.- Actividad antioxidante y hepato-protectora de un extracto acuoso de B. triquetrum en el modelo de estrés oxidativo inducido por CCl_4 de daño hepático en ratas Wistar

Las propiedades antioxidantes y hepato-protectoras de un extracto acuoso de *B.triquetrum* se investigaron en el modelo de daño hepático inducido por CCl_4 en ratas Wistar. En la Tabla III se pueden apreciar los resultados de las actividades de las enzimas ASAT y ALAT lo que evidencia el efecto hepato-protector ejercido por este extracto. Adicionalmente en el control positivo (ácido ferulico) se puede observar un efecto menor.

En la Tabla III, pueden ser apreciadas las concentraciones de TBARS en el tejido hepático, con niveles altos de este metabolito en los animales CCl_4-tratados mientras que en los animales tratados con el alga se observan valores bajos (36% de reducción de los niveles de TBARS hepático), lo que confirma las propiedades antioxidante a nivel hepático y por tanto una efecto hepato-protector.

En un estudio de ratas alimentadas con Saengshik, alimento típico coreano que contiene una mezcla de vegetales y algas, Kim *et al.,* (2008) observaron una reducción similar de los niveles de hidroperoxidos hepáticos en los animales tratados con CCl_4. Otros autores (Bupesh *et al*., 2012) observaran también una reducción de los niveles de TBARS en los hígados de animales tratados con CCl_4 y a su vez con las lagas marinas *H. muciformis* y *P. boergesenii*.

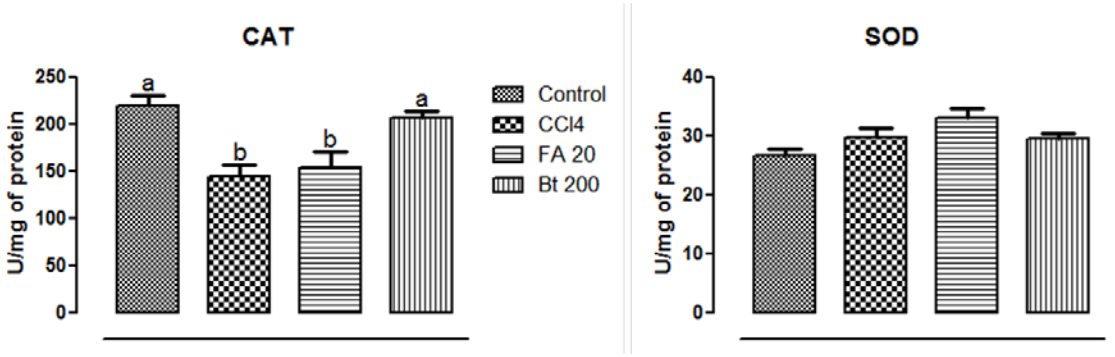
En este experimento, los animales con daño hepático producido por el CCl_4, tienen niveles de GSH incrementados estadísticamente con respecto a todos los grupos experimentales

(Tabla III). De acuerdo a Lu (1999), estos niveles incrementados pudieran ser explicados por una respuesta adaptativa contra el estrés oxidativo inducido por el CCl_4. Entonces en las ratas tratadas con el extracto de *B.triquetrum*, los valores menores observados con respecto a las ratas tratadas solo con el CCl_4 pudiera ser explicado por el efecto hepato-protector del extracto de alga. Bupesh *et al.*, (2012) también observaron niveles similares de GSH en ambos grupos.

Kim *et al.*, (2008) reportaron valores menores de GSH con respecto al grupo control en ratas tratadas con CCl_4 mientras que los niveles de GSH en las ratas tratadas con el extracto del alga fue intermedio entre ambos grupos. Aparentemente este resultado pudiera resultar contradictorio sin embargo es importante considerar que el tiempo de inducción del daño inducido por el CCl_4 y la recuperación del animal, incluida una respuesta adaptativa, así como la afectación de diferentes funciones y vías metabólicas relacionadas con este evento pudieran influir en estos valores.

Las enzimas antioxidantes CAT, SOD y GPx son consideradas como sistemas de defensas fundamentales en los mamíferos contra los radicales libres. Recknagel (1967) revisó el efecto toxico del CCl_4 y reportaron una significativa reducción en las actividades y síntesis de diferentes enzimas hepáticas.

Como se puede apreciar en la Figura 10, el tratamiento con el extracto de *B.triquetrum* produjo un incremento significativo en la actividad de la enzima CAT, lo que conlleva un incremento del sistema de defensa antioxidante. Adicionalmente fue observado un incremento en la actividad de la enzima SOD.



Vidal, A., *et al*. 2015. Las Algas Marinas de los Géneros *Byothamnion* y *Halimeda* como Fuentes de Antioxidantes Naturales. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 183-219.

Figura 10. Actividad de las enzimas CAT y SOD hepáticas de los animales: Controles (H_2O), CCl4-tratados, Acido Ferulico (FA) y *B. triquetrum* (Bt). Letras diferentes indican diferencias estadisticas significativas, *p < 0.05. En prensa en ***African Journal of Agriculture Research, 2015.***

Una alta actividad de enzimas antioxidante ha sido reportado por otros autores con la administración de dosis repetidas de extractos de *Sargassum* spp (Raghavendran, Sathivel, y Devaki, 2005). Adicionalmente otros autores observaron que ratas tratadas con dosis repetidas de extractos de *C. prolifera* y *L. obtusata* provocaban una elevación de la actividad de estas enzimas (Abdel-Wahhab, Ahmed y Hagazi, 2006).

La expresión de la enzima Catalasa por la técnica PCR-RT se muestra en la Figura 11. Como se pueden apreciar hay una sobre-expresión significativa (banda 4) en el tejido hepático de los animales tratados con el extracto de *B.triquetrum* mientras en los animales tratados solo con CCl_4 se observa una disminución (banda 3).. Estos resultados evidencian un efecto inductor del extracto a nivel de expresión génica.

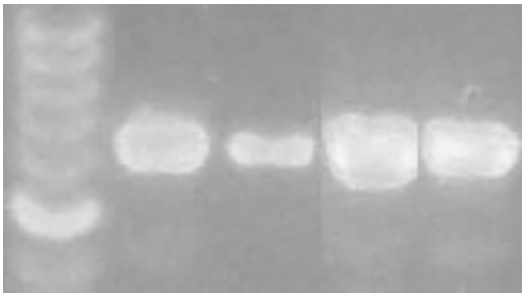


Figura 11. Electroforesis de la expresión génica de la enzima CAT hepática de rata de los diferentes grupos experimentales. 1: Ladder; 2: Control (H_2O); 3: CCl_4*;* 4: *B. triquetrum* 200; 5: FA 20. En prensa en ***African Journal of Agriculture Research, 2015.***

Stevenson y Hurst (2007), en una amplia revisión, discutieron algunas evidencias del efecto antioxidante indirecto de los polifenoles a través de la inducción de enzimas

endógenas protectoras, y estos efectos inductivos o de señalización pudieran ocurrir a concentraciones mayores que el efecto directo de atrapamiento de radicales libres.
En trabajos previos se determino que los ácidos fenólicos conformaban el 60% del contenido total de polifenoles de *B.triquetrum* y en esta fracción el 86.3% fue identificado como ácido p-coumarico mientras que una pequeña fracción correspondía a los ácidos ferulico y trans-cinámico (Vidal *et al*., 2001).

Adicionalmente, Yeh y Yen (2006) demostraron que los ácidos ferulico y coumarico modulan las enzimas antioxidantes CAT, SOD y GPx, y selectivamente inducen mecanismos de trascripción hepática de mRNA para la enzima CAT, probablemente a través de la regulación de genes de transcripción, como el factor de trascripción Nrf2. En resumen, el tratamiento con el extracto acuoso de *B.triquetrum* produce un significativo incremento de la actividad de la enzima Catalasa lo que provoca un incremento del sistema antioxidante. Esto pudiera atenuar el efecto tóxico inducido por el CCl_4, y por tanto el decremento de la actividad de las enzimas ASAT y ALAT así como los niveles de TABRS hepáticos. Estos resultados sugieren una actividad hepato-protectora altamente potente del extracto de alga *Bryothamnion triquetrum*.

B.- Actividad antioxidante y hepato-protectora de un extracto acuoso de Halimeda opuntia en el modelo de estrés oxidativo inducido por CCl_4 de daño hepático en ratas Wistar

Para investigar las propiedades antioxidantes y hepato-protectoras de un extracto acuoso de *H. opuntia* se empleo el modelo de estrés oxidativo inducido por CCl_4 en ratas Wistar se trataron con los animales con una fracción rica en polifenoles con un contenido de polifenoles totales de 5.92 ± 0.85 μg GAE/g alga seca. En un trabajo previo pero estudiando un extracto acuoso de *H monile* también se observo que las ratas tratadas con FPA de esta alga o acido galico (control positivo) fueron capaces de atenuar los cambios hepaticos inducidos por el CCl_4 (Mancini-Filho *et al*., 2009)

Los TBARS producidos como resultado de la peroxidación lipídica, son mostrados en la Figura 12. Como se puede apreciar los TBARS séricos y hepáticos de los animales tratados solo con CCl_4 se incrementaron significativamente lo que evidencia el daño hepático mientras que el pre-tratamiento con el extracto de *H.opuntia* (80 mg/kg) condujo a una reducción del 20 y 25% respectivamente de los TBARS séricos y hepáticos. Estos resultados concuerdan con los obtenidos por Kim *et al*., (2008) quienes observaron una reducción de hidroperóxidos en hígado y plasma de 30 y 15%, respectivamente en animales tratados con Saengshik respecto al grupo de animales tratados solo con CCl_4.

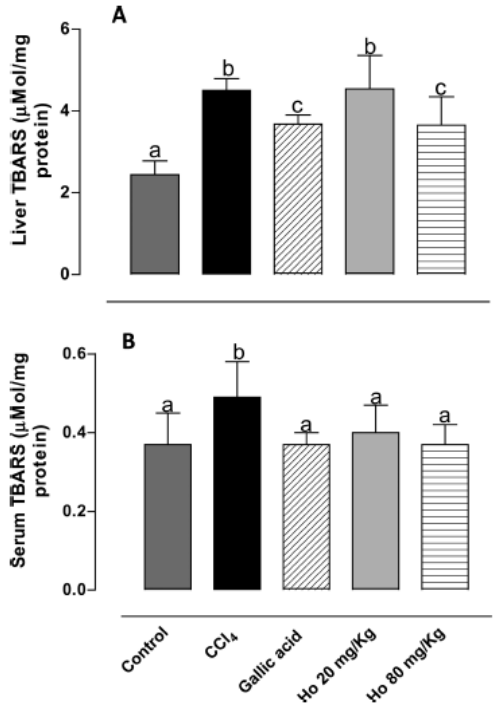


Figura 12. Valores de TBARS hepáticos relativas al estrés oxidativo en ratas Wistar tratadas con CCl_4, *Halimeda opuntia* y Acido Gálico. Los valores representan la media ± DE. Letras diferentes indican diferencias estadísticas significativas, *p < 0.05. Tomado de: Controles (H_2O), CCl4-tratados, Acido Galico y *Halimeda opuntia*. Letras diferentes

Vidal, A., *et al*. 2015. Las Algas Marinas de los Géneros *Byothamnion* y *Halimeda* como Fuentes de Antioxidantes Naturales. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 183-219.

indican diferencias estadisticas significativas, *p < 0.05. Tomado de Oliveira e Silva *et al*., ***Redox report*** 17 (2): 47-53, 2012.

En un trabajo previo de nuestro grupo se demostró que el extracto acuoso de *H. incrassata* redujo los niveles TBARS en un 55% in ratas con estrés oxidativo inducido por metil-mercurio (Linares *et al*., 2004).

En este estudio se investigo la habilidad del extracto de *H.opuntia* para inducir las enzimas antioxidantes. La actividad de estas enzimas puede ser apreciado en la Figura 13. Los valores de actividad de las enzimas CAT, SOD y GPx en los diferentes grupos de tratamientos experimentales evidencian un incremento en las actividades enzimáticas lo que conduce a un incremento de los sistemas de defensa antioxidantes, y a su vez evidencia las propiedades antioxidantes y hepato-protectoras de esta alga.

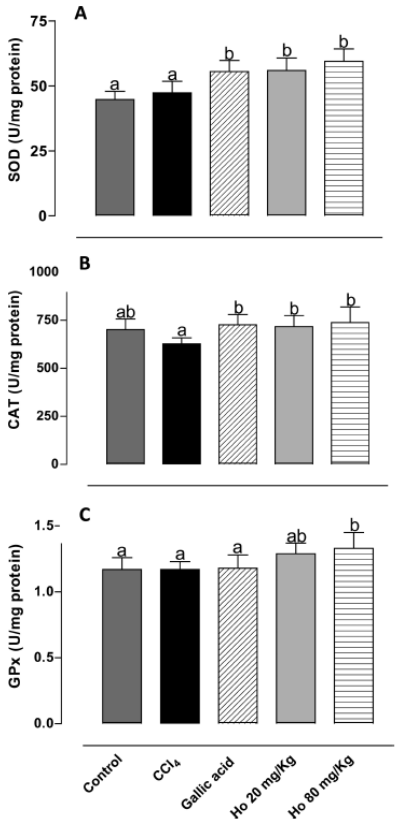


Figura 13. Actividad de las enzimas CAT, SOD y GPx hepáticas de los animales: Controles (H_2O), CCl4-tratados, Acido Galico y *H.opuntia* (Ho). Letras diferentes indican diferencias

estadísticas significativas, *p < 0.05. Tomado de Oliveira e Silva *et al*., ***Redox report*** 17 (2): 47-53, 2012.

Punitha y Rajasekaran (2001) demostraron que el tratamiento de ratas con CCl_4 reduce significativamente la actividad de las enzimas antioxidantes. Adicionalmente, Ozturk *et al.,* (2003) observaron que animales tratados con CCl_4 incrementaban significativamente la actividad de las enzimas CAT y SOD en los riñones.

También Kim *et al*., (2008) observaron un incremento similar de la enzima SOD en ratas tratadas con Saengshik por 4 semanas. En un trabajo previo de nuestro Grupo, Mancini-Filho *et al.,* (2009) reportaron un considerable incremento en las actividades de SOD y CAT en ratas tratadas con fracciones ricas en polifenoles de *H. monile* (80 mg/kg). Valores incrementados de actividad enzimática han sido observados en animales tratados con dosis repetidas de *Sargassum* (Raghavendran *et al*., 2005; Josephine *et al*., 2008) Diferentes autores han reportado valores incrementados de estas enzimas con otros modelos animales de estrés oxidativo, estudiando extractos de las algas *Caulerpa prolifera, Laurencia obtusata* y *Porphyra haitanensis* (Zhang *et al*., 2004; Abdel-Wahhab *et al*., 2006).

La expresión de las enzimas antioxidantes CAT y SOD hepáticas se investigó por la técnica de PCR-RT. Como se puede apreciar en la Figura 14 la administración de dosis repetidas del extracto de H opuntia y de acido gálico (control positivo) conlleva una sobre-expresión de la enzima Catalasa (banda 2) mientras se observa un decrecimiento en la actividad de las enzimas en los animales tratados solo con el CCl_4 (banda 4). Este extracto tiene alto contenido en polifenoles y de acuerdo con Stevenson y Hurst (2007) existen suficientes evidencias para indicar a los polifenoles como inductores de este tipo de enzimas. En un estudio previo, en el alga *H.opuntia* (Vidal *et al*., 2009) se identificaron por CGL a 9 ácidos fenólicos incluyendo acido ferulico, gálico y p-coumarico

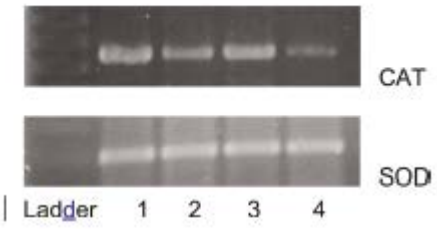


Figura 14. Electroforesis de la expresión génica de las enzimas CAT y SOD hepáticas de ratas de los diferentes grupos experimentales. 1: Control (H_2O); 2: *Halimeda opuntia* 80 mg/kg; 3: acido Gálico; 4: CCl_4 Tomado de Oliveira e Silva *et al*., ***Redox report*** 17 (2): 47-53, 2012**..**

Yeh y Yen (2006) sugirieron que estos 3 ácidos fenólicos modulan enzimas antioxidantes y de reacciones de la Fase II del metabolismo de xenobióticos (Conjugaciones) como las sulfo-transferasas hepáticas y así como selectivamente inducen procesos de trascripción de mRNA para Cu, Zn-SOD, GPx, y CAT, probablemente a través de la regulación de genes de transcripción mediante el factor de transcripción Nrf2.

Consideraciones generales

Las algas marinas constituyen organismos con perspectivas alentadoras como fuentes de bioactivos, con disímiles aplicaciones tanto en la prevención como en el tratamiento de diversas enfermedades. Diferentes autores han comprobado que los extractos de algas marinas presentan propiedades antioxidantes explicadas por una amplia variedad de moléculas, lo que a su vez determina que estos extractos posean diferentes mecanismos de acción. En este trabajo se comprobó que las algas de los géneros *Bryothamnion* y *Halimeda* poseen propiedades antioxidantes debidas al menos en parte a su contenido de polifenoles, con mayor cantidad de compuestos polifenólicos en el alga *B. triquetrum*.

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Al analizar detenidamente los resultados de los ensayos de actividad antioxidante, se observa que *Bryothamnion* sp. posee mayores potenciales como atrapadora de radicales libres aunque pueden estar presentes otras moléculas con otros mecanismos de acción, sin embargo *Halimeda* sp. Ofrece mayores perspectivas como inhibidor de los procesos lipoperoxidativos y en general en la protección de biomembranas.

En resumen, se puede concluir que ambos géneros de algas marinas resultan promisorias fuentes de antioxidantes naturales con aplicaciones como fitofármaco y/o nutracéutico.

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Chitin and Chitosan in Aquaculture

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Abstract

Aquaculture is an important economic activity on many countries. However face several challenges mainly associated to feed and diseases development. Among the strategies applied to avoid or prevent those problems are the use of chitin and its derivate. Chitin consists of β-1-4-linked N-acetylglucosamine residues and is estimated as the second most important polysaccharide found in the nature. The main sources exploited are crustaceans. Chitin and chitosan are considerably versatile and promising biomaterials. The effect of chitin on several cultivated organism it was reported. Detecting that the inclusion of chitin in feed farmed organism improves not only the growth and feed conversion also stimulated the immune system against virus and protozoea by increasing the serum lysozyme; moreover the costs of production decrease. However, oversupplies of chitin in some fish species induce excessive deposition of fat liver, heart and carcass. Whereas chitosan, the deacetylated chitin derivate, considering more useful and interesting bioactive polymer, it was evaluated as encapsulated bioactive compounds during the culture of some farmed organisms. Chitosan encapsulated vitamin C, improve its liberation without lost its bioavailability. Although, chitosan can encapsulate antigen against white spot syndrome virus and vaccines, its efficacy depends of the virus. Moreover, chitosan posses properties, that may be useful to improve aquaculture wastewater quality. There are still many questions about the chitin and chitosan application in aquaculture, as chitosan encapsulated stability, the right chitin concentration according with the cultured specie, among others.

Keywords: Chitin, chitosan, aquaculture

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Resumen

La acuacultura es una actividad económica muy importante en varios países. Sin embargo enfrenta varios retos, la gran mayoría asociados a las dietas y al desarrollo de enfermedades. De las diversas estrategias seguidas para solventar algunos de los problemas antes mencionados están el uso de la quitina y quitosano. Se sabe que la quitina es uno de los polisacáridos más abundantes en la naturaleza, que está constituido por moléculas de N-acetil-D-glucosamina, con enlaces β-1-4, estimándose que es el segundo polisacárido más importante en la naturaleza. Siendo la principal fuente de este polímero los crustáceos. Tanto la quitina como el quitosano son considerados biomatariales muy versátiles y con aplicaciones promisorias. El efecto de la quitina en organismos cultivados ha sido reportado en varias especies. Detectándose que la inclusión de quitina en alimentos de organismos cultivados mejora no solo el crecimiento y la conversión alimenticia, sino que también estimula el sistema inmune de los organismos contra el ataque de virus y protozoea, incrementando la actividad de una enzima sérica, la lisosima, más aún los costos de producción disminuyen. Sin embargo, el uso de concentraciones elevadas de quitina en algunos peces puede inducir la acumulación excesiva de grasa en el hígado, corazón y desechos. Por otro lado, el quitosano, que es un derivado de la quitina deacetilada, es considerado un polímero bioactivo más útil, y ha sido evaluado para encapsular compuestos bioactivos durante el cultivo de algunos organismos. Al encapsular a la vitamina C con quitosano, se mejoró la liberación de la misma, sin menoscabo de su bioabilidad y aunque el qutiosano es capaz de encapsular antígenos contra el síndrome de la mancha blanca y vacunas, su eficacia dependerá del virus a atacar. Por otro lado, el quitosano posee propiedades que pueden ser útiles en mejorar la calidad del agua de cultivo. Aún hay muchas preguntas por resolver respecto a la aplicación de la quitina y el quitosano en acuacultura, como la estabilidad de los encapsulados, las concentraciones correctas de quitina acorde a la especie de cultivo, entre otras.

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Introduction

The aquaculture industry has increasingly attracted much attention for the intensive farming of fish and shellfish. However, fish and shrimp farming are facing problems such as bacterial diseases, farming environment, and feed contamination (García-Morales *et a*l., 2015). To address the aforementioned problems, the use of chitin and chitosan as a protective material appears to be a potential alternative.

Chitin is a naturally abundant mucopolysaccharide, which is found in shells or walls of invertebrates, fungi and yeasts. It is the main component of crustacean exoskeletons and is made up of calcium oxide and protein units (Muzzarelli, 1977). Chitin is well known to consist of 2-acetamido-2-deoxy- β -d-glucose through a β $(1 \rightarrow 4)$ linkage (Kumar, 2000). Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas (Muzzarelli, 1973). Chitosan, an aminopolysaccharide, is prepared from shellfish chitin by treatment with alkali (Duta & Duta, 2004). Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is almost never complete (Duta & Duta, 2004).

Chitin and its derivatives exhibit a variety of physicochemical and biological properties resulting in numerous applications in many industries, including aquaculture. In addition to its lack of toxicity and allergenicity, its biocompatibility, biodegradability and bioactivity make it a very attractive substance for diverse applications as a biomaterial, especially its novel application in the form of nanocarriers for bioactive compounds for aquaculture industry (Alishahi & Aïder, 2012). In the present study, the applications of chitin and chitosan in aquaculture field will be reviewed.

Chitin and Chitosan Structure

Chitin is a linear, highly crystalline homo polymer of β-1,4 N-acetyl glucosamine that are arranged in antiparallel (α), parallel (β) or mixed (γ) strands, with the (α)

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configuration being the most abundant (Figure 1) (Cheba, 2011). Whereas, chitosan usually refers to a family of polymers obtained after chitin deacetylation to varying degrees. In fact, the acetylation degree, which reflects the balance between the two types of residues (Figure 1), differentiates chitin from chitosan. When the DA (expressed as molar percentage) is lower than 50 mol%, the product is named chitosan and becomes soluble in acidic aqueous solutions (Kafetzopoulos *et al.*, 1993). During deacetylation, acetyl groups are removed but also depolymerization reaction occurs, indicated by changes in MW of chitosan. Chitin can be converted to chitosan by enzymatic preparations (Tokuyasu *et al*., 2000, Ilyina *et al*., 1999, Aiba, 1994) or chemical (Kurita *et al.*, 1977). Chemical methods are used extensively for commercial purpose of chitosan preparation because of their low cost and suitability to mass production (No & Meyers, 1995).

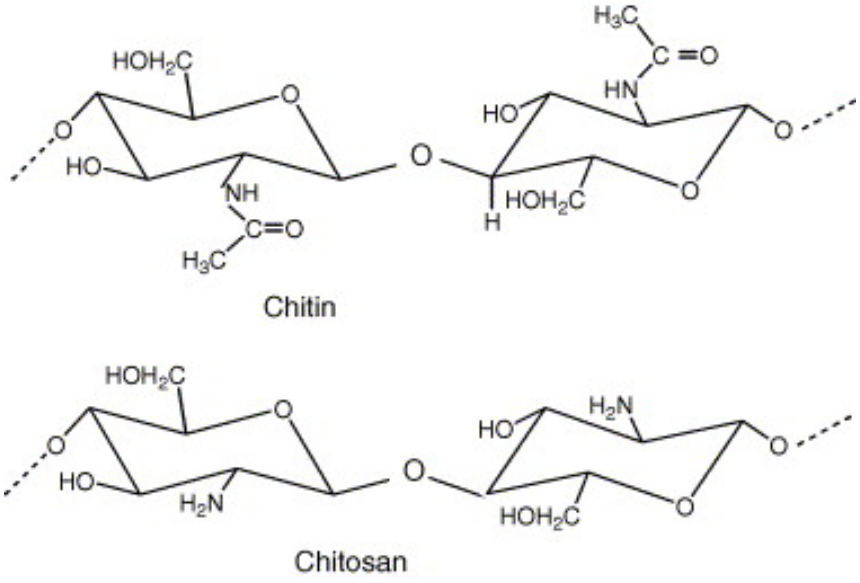

Figure 1. Chemical strucutres of chitin and chitosan. Source: Gamage & Shahidi, (2007)

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Chitin and Chitosan Biological Properties

A review on the biological activities of chitin and chitosan such as: antibacterial, antifungal, antitumor and antioxidant, was recently published (Younes & Rinaudo, 2015). It has been demonstrated that chitin, like other polysaccharides derived from cellulose, has good film-forming properties and good stability promoted by the establishment of a hydrogen bond network between extended chains. Therefore, chitin gives original properties to the new materials due to its biocompatibility, biodegradability and non-toxicity, with antimicrobial activity and low immunogenicity (Younes & Rinaudo, 2015).

Chitosan and derivatives possess many beneficially properties such as biocompatibility, biodegradability, safety and also interesting biological activities, much attention has been paid to their applications especially in biomedical, food, biotechnology and pharmaceutical fields (Younes & Rinaudo, 2015). These properties are specially recognized in the field of food preservation and packaging to avoid the use of chemical preservatives and to produce edible antimicrobial films due to the good film forming properties of chitosan. Chitosan, as a polymeric ingredient with a good antimicrobial and antioxidant properties, does not migrate easily out of the protecting film and has better barrier properties (Alishahi & Aider, 2012). The use of chitosan in the aquaculture industry was also described in the review of Alishahi & Aider (2012).

Chitin and Chitosan Applications in Aquaculture Industry

Chitin and chitosan compounds had potential and versatile uses in the aquaculture industry. These applications are summarized in tables 1. Among their attractive biological activities as feed supplementation, encapsulation and effluent treatment will be discussed in detail below.

Ezquerra, J. 2015. Chitin and Chitosan in Aquaculture. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 220-233.

Table 1. Effect of the chitin and chitosan application on cultivated organisms

Organism	Diet inclusion results	References
Cobia (*Rachycentron canadum*)	Shrimp waste: costs of production decrease	Lu & Ku, 2012.
Dover sole *(Solea solea)*	Chitosan encapsulated vaccines: improve immune system against some virus	Tian *et al.,* 2008
European seabass *(Dicentrarchus labrax)*	Chitosan encapsulated vaccines against *Vibrio anguillarum*: lower protection	Rajeskumar *et al.,* 2008.
Gilt-hear bream (*Sparus aurata*)	Chitin: improve immune system against virus and protozoea	Cuesta *et al*., 2003
Olive flounder (*Paralichthys olivaceus*)	Chitosan-coated diet: increased non-specific immune response and improved the water quality in the fish tank	Cha et al., 2008
Rainbow trout (*Oncorhynchus mykiss*)	Chitosan encapsulated vitamin C: improve vitamin C liberation without biodisponibility loss	Alishahi *et al*., 2011
Rainbow trout (*Oncorhynchus mykiss*)	Chitosan: enhance the haematological parameters and resistance against some environmental stress	Meshkini *et al.,* 2012.
Rainbow trout (*Oncorhynchus mykiss*)	Chitin: increase serum lysozyme and improved immune system	Vahedi & Ghodratizadeh, 2011.
Tilapia (*Orechromis aerus*)	Diet containing ChiB565, improved growth and feed conversion compared with a control diet.	Zhan *et al.,* 2014

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White shrimp (*Penaeus vannamei*)	Moderate chitosan concentration was beneficial to shrimp development	Niu *et al.,* 2011
White shrimp (*Penaeus vannamei*)	Chitosan encapsulated antigen against WSSV: improve protection	Rajeskumar *et al.,* 2009.

Feed supplementation

Chitin is an important food for fish, particularly during larval stages. Dietary chitin stimulates the innate immune response in gilthead sea bream (Esteban *et al.*, 2001) by increasing complement activity, cytotoxic activity, respiratory burst and phagocyte activity, but not lysozyme activity. Chitin in fish diets interferes with bacteriolytic activity of lysozyme in trout stomach (Lindsay, 1984). Thus, chitin may be of interest as an immunostimulant (Esteban *et al.*, 2001). The protective effects of chitin and chitosan by injection or immersion have been reported in rainbow trout, *Oncorhynchus mykiss*, against *Aeromonas salmonicida*and *Vibrio anguillarum* (Anderson *et al.*, 1995; Siwicki *et al.*, 1994; Sakai *et al.*, 1992), brook trout, *Salvelinus fontinalis*, against *A. salmonicida* (Anderson & Siwicky, 1994) and in yellowtail, *Seriola quinqueradiata*, against *Pasteurella piscicida* (Kawakami *et al.*, 1998). In contrast, Shiau & Yu (1999) reported that both chitin and chitosan supplementation depresses tilapia (*Oreoochromis niloticus*^{x}*O. aerus*) growth regardless of the supplementation level. Authors infer that the differences could be due to enzyme activity present in the organism studied.

There are several reports about the effects of dietary chitin and chitosan on crustacea. Deshimaru & Kuroki (1974) suggested that a dietary source of glucosamine is unnecessary for *Penaeus japonicus*. While Kitabayashi *et al.* (1971) demonstrated that the addition of glucosamine at 5.2 g/kg diet improved the growth of *P. japonicus*, but growth was retarded if chitin was added to the diet. Akiyama *et al.* (1992) recommended a minimum dietary level of chitin at 5 g/kg diet in shrimp feed. Wang & Chen (2005) established that fed *L. vannamei* with chitin at 6 μg/g or chitosan at 4 μg/g or less increased

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its immune ability and resistance to *V. alginolyticus* infection and Niu *et al.* (2011) reported that the level of chitosan supplemented in the postlarval *L. vannamei* diet should be between 2.13 and 2.67 g/kg to improve its growth and survival.

On the other hand, although the various enzymes and pathways for carbohydrates digestion have been detected in fish (Shimerno, 1974), they can develop signs of ill health if there is a high concentration of carbohydrate in their diet. The incorporation of inappropriate carbohydrate level in the diet has been identified to cause prolonged hyperglycemia (Hatlen *et al.*, 2005), fatty fish (Fernández *et al.*, 2007), liver dysfunction (Hilton & Atkinson, 1982), and impaired bone development (Tan *et al.*, 2007). Even though, if the carbohydrates are applied in the right concentration or presentation it can increase the development of the fish. Wang & Li (2010) detected that dietary chitosan nanoparticles supplementation improved, not only the growth performance of tilapia, also its meat quality.

Encapsulation

Vaccination plays a critical role in protecting commercially raised fish from bacterial, viral and parasitic diseases (Rivas-Aravena *et al.*, 2013). However, when incorporated into drug delivery systems, these bioactive components are often hydrolyzed by harsh conditions in the gastrointestinal tract (Alishahi *et al.*, 2011). It was stated that many oral delivery systems for bioactive aquaculture compounds meet three major barriers in passing through the gastrointestinal tract, namely enzymatic barriers from the host luminal and membrane bound enzymes, immunological cells present within both the enterocytes and underlying connective tissue and the physical barrier of the epithelial cells (Schep *et al.*, 1999). Then, the encapsulation of vaccines or any bioactive compounds could be a promising way to overcome these problems. Encapsulation is a process used to entrap active components and release them under controlled conditions (Deladino *et al.*, 2008).

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The beneficial effects on farmed organism of chitosan as an encapsulating agent have been demonstrated.

Alisha *et al.* (2011) showed that the shelf life of vitamin C was increased in rainbow trout feed for 20 days, whereas the control, which was fed vitamin C alone, lost significant vitamin C content in a few days. The authors also showed that vitamin C was released in the gastrointestinal tract of rainbow trout in a controlled manner and that chitosan nanoparticles protected vitamin C from the harsh conditions of acidic and enzymatic hydrolysis in the gastrointestinal tract of the rainbow trout.

Concerning the antiviral activity of chitosan, it has been demonstrated that chitosan nanoparticles could be used to encapsulate DNA, which was then beneficially incorporated into shrimp feed to protect them from white spot syndrome virus (Rajeshkumar *et al.*, 2009). Incorporated chitosan nanoparticles containing a DNA vaccine into Asian sea bass (*Lates calcarifer*) feed, moderate protection against experimental *Vibrio anguillarum* infection was detected (Rajeshkumar *et al.*, 2008). In addition, chitosan microspheres loaded with plasmid vaccine were used to orally immunize Japanese flounder (*Paralichthys olivaceus*) (Tian *et al.*, 2008).

Despite the apparent advantages offered by chitosan, there have not been sufficient studies on the effectiveness of encapsulating antigens with chitosan.

Effluent treatment

During the culture of any organism a lot of organic compounds and inorganic nutrients are produced, inducing deterioration of receiving water quality. Chitosan served as an effective coagulating agent in removing proteins from wastewater as well as for removal of metal ions for industrial wastewater (Renault *et al.*, 2009; Gamage & Shahidi, 2007) therefore it has been used to improve aquaculture wastewater quality. The chitosan could selectively remove pathogens like *Edwarsiella ictaluri* (Chung, 2010). Also, it was detected that chitosan is an effective bio-flocculant for phytoplankton removal in outdoor

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shrimp culture tanks (Lertsutthiwong *et al.*, 2009). Furthermore, chitosan bead immobilized algae system with *Senedesmus sp* was efficient in removing phosphate and nitrate from water (Fierro *et al.*, 2008). Cha *et al.* (2008) reported that fed olive flounder with chitosan-coated moist pellet diet besides to increase non-specific immune response of the organism, the water quality of the fish tank was improved. Finally, it is important to notice that the efficacy of the chitosan to remove suspended solids, organic material and pathogens will depend on its deacetylation degree and the pH of the wastewater (Chung, 2010).

Conclusions

Chitin and chitosan has attracted a great deal of attention in the aquaculture industry due to its properties. Both compounds can be used as feed, feed supplementation or as a new vehicle for the improvement of the delivery of active compounds. Chitosan could remove suspended solids, organic compounds, and pathogens, and then it can be used to improve aquaculture wastewater quality. Chitin and mainly chitosan could be successfully be incorporated into diet aquaculture organisms like fish and shrimp, to improve the growth, health and meat quality of those culture organism. However, there are still many questions about the chitin and chitosan application in aquaculture, as chitosan encapsulated stability, the right chitin concentration according with the cultured specie, among others.

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Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality

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Abstract

The aim of this study is to evaluate the tilapia silage as alternative protein source in diets for *Litopenaeus vannamei* reared in clear-water and biofloc conditions. The experiment was performed in a "macrocosm-microcosm" device consisting of two individual systems: biofloc and clear-water. The trial used forty 40L rectangular bins in a density of 63 shrimp/m^2. The juveniles were distributed in a bi-factorial completely randomized experimental design with four replicates to each treatment. The treatments were based on the percentage of silage inclusion (control, 1.5, 3.0, 4.5 and 6.0% of inclusion) in biofloc or clear-water based system, totalizing ten treatments. Survival were not affected by both system and diet and stayed above 80% in all treatments. Shrimp mean final weight and SGR was statistically influenced by system ($p<0.01$) but not by the diet, presenting high values in biofloc condition. Shrimp quality was not affected by diet. Replacement of fishmeal by fish silage in *L. vannamei* diets is a good option regarding to shrimp production and quality.

Keywords: silage, nutrition, waste, replacement, Pacific white shrimp

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1. Introduction

In recent years, studies approaching the production of Pacific white shrimp *Litopenaeus vannamei* in biofloc system desired great attention (Avnimelech, 2012). The recent diseases outbreaks and low productivity lead the scientists to search for an alternative system to improve efficiently the aquaculture growth.

Biofloc system, also called as biofloc technology (BFT) has the advantage to allow the production of a great amount of shrimp per area or volume with no water exchange. This provides better biosecurity for the production, especially if the farm is situated in areas with high concentration of aquaculturists using the same water source. BFT has gained popularity because it offers a practical solution to maintain water quality and recycle feed nutrients simultaneously (Xu and Pan, 2012). Other advantage of the biofloc system is the possibility to use alternatives low protein diets and consequently decrease the production costs (Ballester *et al*., 2010; Scopel *et al*., 2011), mainly due to the continuous availability of natural food source in a form of bacteria, protozoa, nematodes, microalgae, rotifers and copepods (Decamp *et al*., 2002; Azim and Little, 2008; Ray *et al*., 2010).

Fishmeal is one of the most expensive and unsustainable ingredient used in aquaculture diets (Naylor *et al*., 2009). Therefore, the replacement or reduction of fishmeal use is of great interest for the aquaculture industry. On the other hand, problems related to the fishmeal replacement by alternative ingredients have been identify including deficiency of some essential amino acids, presence of anti-nutritional factors, palatability and digestibility (Forster *et al*., 2003; Naylor *et al*., 2009). Although problems exist, many cases of success have been reported in *L. vannamei* diets (Davis and Arnold, 2000; Forster *et al*., 2003; Samocha *et al*., 2004; Amaya *et al*., 2007; Cruz-Suarez *et al*., 2007; Hernández *et al*., 2008; Suarez *et al*., 2009; Bauer *et al*., 2012)

Fish silage can be produced using by-products of the fisheries and aquaculture processing residues. Fish silage is an alternative protein source to the fishmeal (Vidotti *et al*., 2003) and possesses a simpler and cheaper production method (Gallardo *et al*., 2012). Furthermore, the use of fish silage as a substitute for protein ingredients, in feed for aquatic organisms, is an alternative to solve sanitary and environmental problems caused by the

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lack of adequate disposition for the waste from the fish industry. Besides, it is also a way of decreasing feeding costs, and, consequently, production costs, since feeding corresponds to about 60% of the overall expenses with production (Arruda *et al*., 2007).

On this context, tilapia, the main species in Brazilian aquaculture, has demonstrated positive results as fish silage incorporated into diets (Carvalho *et al*., 2006; Fernandes *et al*., 2007), possible due to its nutritional quality (Oliveira *et al*., 2006).

The aim of this study is to evaluate the tilapia silage as alternative protein source in diets for *L. vannamei* reared in clear-water and biofloc conditions.

2. Material and Methods

2.1 Experimental design and culture conditions

The study was conducted in the Aquaculture Sector, Department of Animal Sciences, Universidade Federal Rural do Semi-Árido (UFERSA) in Rio Grande do Norte State, Brazil. The Pacific white shrimp *L. vannamei* post-larvae were supplied by a local commercial hatchery.

Before the experiment, shrimps were stocked in a 15m^3 fiberglass circular tank (macrocosm) aiming to an acclimation and prior biofloc formation. Water was vigorously aerated using one air diffuser (composed by ¾" PVC pipe with several 1mm holes) located in the center of the macrocosm tank. In order to maintain biofloc culture medium, shrimp were stocked at a density of 200 shrimp m^{-2} and maintained until the end of the experiment. Shrimp were fed twice a day (08:00am and 6:00pm) with 35% crude protein commercial feed (Aquabalance 35 Presence™) in two feed tray in order to monitor the food consumption. Liquid sugar cane molasses as a carbon source was added daily after the feed addition to maintain a high C:N ratio (20:1) to ensure optimal heterotrophic bacteria growth (Avnimelech, 1999; Crab *et al*., 2009). Vertical substrates were added to provide an additional area of 30% of the tank. Limited water exchange (not exceeding 0.5% daily) was carried out by a central drain to prevent accumulation of sludge throughout the experimental period. Dechlorinated freshwater was added to compensate sludge removal and evaporation losses.

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

The experiment was performed in a “macrocosm-microcosm” device (Wasielesky *et al*., 2006; Emerenciano *et al*., 2012a) consisting of two individual systems: biofloc (BS) and clear-water (CWS). The trial was initiated stocking *L. vannamei* juveniles (1.43±0.33g) in forty (20+20) 40L rectangular bins (27x37x54cm) in a density of 63 shrimp m^{-2} (12 juveniles per bin). The juveniles were distributed in a bi-factorial completely randomized experimental design (water type and % of silage inclusion as the main factors) and reared for 45 days. Four replicate tanks were randomly assigned to each treatment. The treatments were based on the percentage of silage inclusion (control, 1.5, 3.0, 4.5 and 6.0% of inclusion) in biofloc or clear-water based system, totalizing ten treatments. The formulation of diets was described below. The juveniles were fed twice a day (08:00am and 06:00pm) using a feed tray to monitor feed consumption. The water was pumped from the macrocosm tank to the experimental units by a submerged pump (¾ HP pumps) and returned by gravity. Water flow in all experimental units was checked two times per day in order to maintain a minimum flow to recirculate the water. The experimental tanks were also siphoned to remove debris once a week.

For the clear-water treatments, the same scheme described above was performed, except by the macrocosmo tank that was not stocked with animals and receive no carbon source in order to maintain the water clear. Aeration were supplied by two 4 HP blowers connected to an emergency diesel electric generator to keep optimum dissolved oxygen levels in both systems.

Temperature, salinity, pH (YSI model ph100) and dissolved oxygen (YSI model 55) concentration were monitored 2 times per day after food time. Settling solids (Imhoff cones) was monitored daily (08:00am). Ammonia (NH_4-N) and nitrite (NO_2-N) were measured once a week (UNESCO, 1983). All shrimps were weighed to the nearest 0.1g at the beginning and the end of experiment. Specific growth rate (%), final weight (g), survival (%) and feed conversion rate (FCR) were measured.

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

2.3 Fish silage production

The Nile tilapia silage used in this study was produced in the Laboratory of Seafood Technology and Quality Control (LAPESC/UFERSA) using residues of Nile tilapia processing including head, bones, skin, fins and viscera. The acid silage was produced using the methodology described by Arruda *et al*. (2006) with some modifications: 2% formic acid and 3% phosphoric acid, and 1% ascorbic acid as antifungal. The fish silage was dried in the oven at 60^{o}C by 24 hours (to obtain moisture below 13%), ground in a Rotor Mill (Rotating Knives and Swing Hammer MA900, Marconi Equip. Lab. Ltda, Brazil) and homogenized (10 mesh). Before formulation of the experimental diets, fish silage was neutralized by adding 1.6% calcium hydroxide to raise the silage pH from 2.8 to 7.1. The Nile tilapia silage contained 83.8% dry matter, 33.7% crude protein, 37.4% crude lipid and 21.5% ash on a dry matter basis.

2.4 Diet formulation

Five experimental diets were formulated to be isocaloric and isoproteic and to attend the nutritional requirements of the species (Table 1). Tilapia silage ranged from zero to 6% of the diet in replacement of fishmeal. All diets were processed in the Laboratory of Aquatic Animal Nutrition of the Universidade Federal do Ceará using the method described by Nunes *et al*. (2011). All diets were kept frozen at − 20°C until use.

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

Table 1. Ingredient and chemical composition of diets used in the study.

Ingredients	Unit	Dietary Inclusion (%, as is basis)				
		CTL[1]	SIL[2] 1.5%	SIL 3.0%	SIL 4.5%	SIL 6.0%
Soybean meal	%	40.00	40.00	40.00	40.00	40.00
Wheat bran	%	20.00	20.00	20.00	20.00	20.00
Fishmeal 01	%	12.00	12.00	12.00	12.00	12.00
Salmon meal	%	8.00	7.54	6.82	6.10	5.38
Tilapia silage	%	0.00	1.50	3.00	4.50	6.00
Wheat midlings	%	5.24	3.90	3.62	3.33	3.04
Salmon oil	%	3.18	3.48	2.99	2.50	2.01
Powder molasses	%	3.00	3.00	3.00	3.00	3.00
Soy lectin	%	2.81	2.81	2.81	2.81	2.81
Calcium phosphate	%	2.00	2.00	2.00	2.00	2.00
Sardine hydrolisate	%	2.00	2.00	2.00	2.00	2.00
Vitamin and mineral premix DSM[3]	%	1.00	1.00	1.00	1.00	1.00
Aglutinant	%	0.50	0.50	0.50	0.50	0.50
Coline chloride 60%	%	0.22	0.22	0.22	0.22	0.22
Ascorbic acid (Stay C. DSM)	%	0.04	0.04	0.04	0.04	0.04
Etoxiquim 66% (Impextraco)	%	0.01	0.01	0.01	0.01	0.01
Basic Nutrients						
Ash	%	8.33	8.52	8.72	8.91	9.11
Crude Fat	%	9.24	10.00	10.00	10.00	10.00
Crude Fiber	%	3.14	3.02	2.99	2.96	2.93
Crude Protein	%	35.00	35.00	35.00	35.00	35.00
Digestible Energy	kcal/kg	3,036	3,015	2,942	2,868	2,794
Digestible Protein	%	30.83	30.41	29.98	29.56	29.13
Total Energy	kcal/kg	3,746	3,709	3,624	3,538	3,453
Water	%	10.42	10.46	10.59	10.73	10.86

[1] CLT= control; [2] SIL= fish silage; [3] Vitamin A 1.250.000 UI; Vitamin D3 350.000 UI; Vitamin E 25.000 UI; Vitamin K3 500 mg; Vitamin B1 5.000 mg; Vitamin B2 4.000 mg; Vitamin B6 10,0 mg; Nicotinic acid 15.000 mg; Pantothenic acid 10.000 mg; Biotin 150 mg; Folic acid 1.250 mg; Vitamin C 25.000 mg; Choline 50.000 mg; Inositol 20.000,0 mg; Iron 2.000 mg; Copper 3.500 mg; Chelated Cooper 1.500 mg; Zinc 10.500 mg; Chelated Zinc 4.500 mg; Manganese 4.000 mg; Selenium 15, mg; Chelated Selenium 15 mg; Iodine 150 mg; Cobalt 30 mg.

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

2.4 Effect of growing systems and diets on shrimp production

The shrimp were counted and weighed in the end of the experimental trial in order to verify the influence of growing systems and diets on shrimp performance. Specific growth rate (SGR), mean final weight, survival and food conversion ratio (FCR) were evaluated.

2.4 Effect of growing systems on shrimp quality

To verify the influence of growing systems and fish silage replacement on the shrimp quality and shelf life, the Quality Index Method (QIM) was used. The aspects analyzed were based on QIM schemes developed by Otwell and Marshall (1986), Oliveira *et al*. (2009) and Oliveira (2013). Three assessors were selected among the staff of the LAPESC, and trained according to international standards (ISO 8586, 1993), including detection and recognition of tastes and odors, use of measurement scales, and in the development and use of descriptors. Changes that were occurring during the storage of raw shrimp (15 days storage in flake ice at 1±0.2°C) were described day-by-day according to standardized methodology published by Martinsdóttir *et al*. (2001; 2002). The quality parameters observed in the samples were odor; presence of melanosis; texture; head adherence; shell adherence; and overall appearance. The amount to a total value called Quality Index (QI) ranged in this experiment from 0 to 36. Sensory analysis took place shortly after samples were taken for microbiological and physico-chemical analyses (each 72 hour). They were performed mostly on the same hours on the determined dates, in an adequate environment inside the laboratory, and panelists did not discuss samples amongst each other. All observations of the shrimp were conducted under standardized conditions following the general guidance for the design of test room and testing conditions described in ISO 8589 (2007).

Nitrogen of total volatile bases (TVB-N), trimethylamine (TMA-N) and pH analyses were performed in triplicate at each 72 hours and were made using official methodology (BRASIL, 1981). Microbiological analyses (total mesophilic and psychrotrophic count)

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

were performed according to the Manual of Brazilian Official Analytical Methods (BRAZIL, 2003).

2.5 Statistical Analysis

After check for homoscedasticity and normality, shrimp performance data were analyzed using a two-way ANOVA and Tukey's test to compare the means with α fixed in 0.05 using R (version 3.0.2). Survival data in percentage was transformed using the arcsine transformation in order to normalize the data before the analysis, however the original means and standard deviation are presented.

For the shrimp quality data, the linear equation (for QIM scheme), which was best fit and the correlation coefficient (r) between the QI and the storage time in ice, were calculated using the software SigmaPlot for Windows V. 10 (Systat Software, Inc.). Calibration models were calculated using the average QI of three samples evaluated per storage day. All regressions were calculated using XLSTAT Trial Version 2014.2.02 (Addinsoft 1995-2014).

3. Results

Water quality parameters stayed in the usual ranges for *L. vannamei* with temperature ranging from 24 to 32 ºC, pH 6.7 to 9.7, salinity 4 to 5 and dissolved oxygen always kept > 3.7 mg/l. Ammonia (<0.52mg/l) and nitrite (<0.25mg/l) stayed in safe concentration to the animals during all the experiment. Settling solids were maintained between 10 and 15 ml/l.

Regarding to growth performance (Table 2), survival were not affected by both system and diet and stayed above 80% in all treatments. Shrimp mean final weight and SGR was statistically influenced by system ($p<0.01$) but not by the diet, presenting high values in biofloc condition.

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Table 2. Performance of *L. vannamei* fed increasing percentages of tilapia waste silage in clear-water and biofloc systems during 45-d.

Diet (% of tilapia silage)	SGR (%/day)	Mean final weight (g)	Survival (%)	FCR
0	1.86 ±0.20	6.51 ±0.76	85.29 ±17.18	1.59 ±0.37
1.5	1.90 ±0.14	6.62 ±0.60	90.15 ±6.27	1.56 ±0.10
3.0	1.93 ±0.18	6.79 ±0.80	92.93 ±9.74	1.38 ±0.29
4.5	1.83 ±0.23	6.43 ±0.94	94.32 ±4.30	1.61 ±0.15
6.0	2.07 ±0.11	7.46 ±0.56	88.88 ±13.94	1.35 ±0.23
System				
Clear Water	1.82 ±0.20b	6.35 ±0.54b	87.50 ±12.12	1.65 ±0.23b
Biofloc	2.01 ±0.12a	7.17 ±0.79a	94.23 ±6.25	1.35 ±0.18a

Values are means (±standard error) of three tanks; Different letters in columns denote significant differences between experimental systems with $\alpha = 0.05$ level by Tukey's HSD multiple range test; SGR, specific growth rate; FCR, food conversion ratio.

During ice storage, the intensity of the sensory attributes changed in all shrimp farmed in biofloc systems (BS) and clear-water systems (CWS), showing gradual and consistent changes for all sensory parameters evaluated, reaching a score of 30 demerit points at 15th day of storage. The QIM scheme developed for shrimp farmed in BS and CWS stored in flake ice for 15 days is shown in Figure 1.

As can be seen in Figure 1, the sensorial attributes analyzed presented increased scores over time. These were added up to become the Quality Index (QI), a tool used to measure the shelf life of the group samples. The QI was calculated for each storage day of sampling and showed a linear relationship with storage time. High correlation (for all groups) between the total QI score (sum of all attributes) for each storage day and days in flake ice was found (Figure 1, Table 3) indicating loss of freshness during the days of storage. Its evolution could be expressed by the equations (linear regression model).

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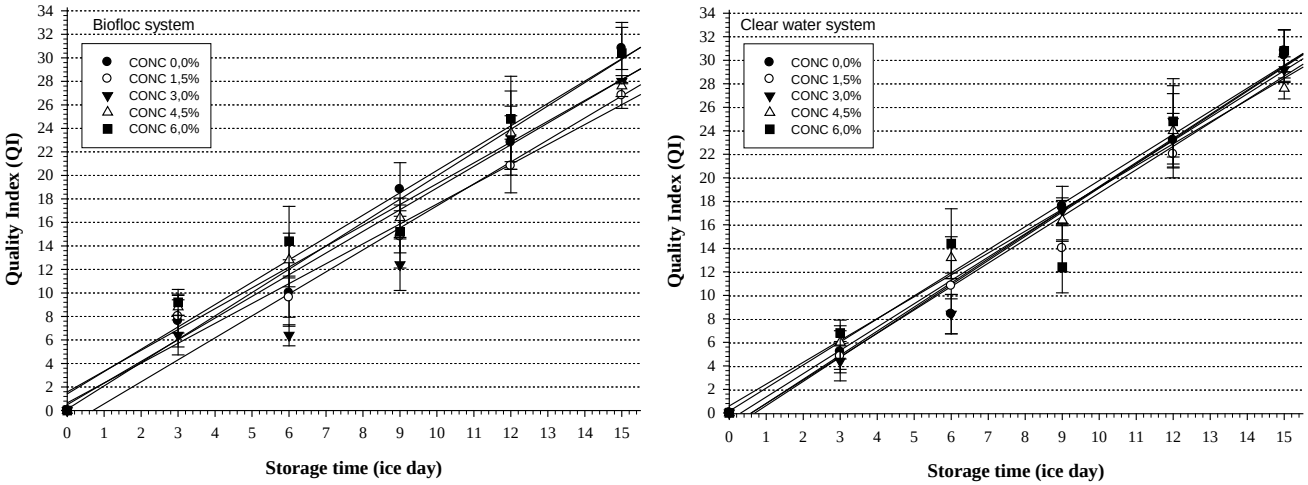

Figure 1. Results of the Quality Index Method (QIM) for shrimp grown in biofloc system, and in clear water system during storage on ice (0±1 °C).

Although mean QI scores of CWS group were lower throughout the experiment, there was no difference between BS group on the following days ($p<0.05$). Linear regressions were plotted to predict the shelf life of each sample group. Considering the maximum QI score for acceptable shrimp to be 65% of the total, i.e., 23.4 demerit points, it can be stated that the average shelf life of BS and CWS (Figure 1, Table 3) samples is, respectively, 12.18±0.16 and 13.19±0.86 days.

According to Figure 1 and Table 3, there are no significant differences among groups (i.e., percentage of fish silage replacement) on shrimp shelf lives in CWS and BS. These results showed that attributes gradually deteriorated with time as it is assumed in the Quality Index Method that the scores for all quality parameters increase with storage time. End of shelf life is usually determined when spoilage related sensory attributes become evident and most panelists detect them. The quality rejection of white shrimp (without additives, i.e. sodium metabisulphite) has reached the average limit of acceptability at 12 days of storage of shrimp farmed in CWS, and 13 days for BS (Table 3), according to the external attributes of sensory evaluation used and the maximum acceptable IQ (65% of total demerit points, i.e., 23.4).

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

Table 3. Results of linear regression and shelf life of the samples of white shrimp (*L. vannamei*) grown in clear water system and in biofloc system during storage (0 ± 1°C).

Clear Water System			
	Linear regression model	**r^2**	**Shelf life (days)**
Control	y = 2,0495 x - 1,2381	0,9856	12,02
1,5%	y = 1,9886 x - 1,1809	0,9782	12,36
3,0%	y = 2,0571 x - 1,4286	0,9839	12,06
4,5%	y = 1,8590 x + 0,5905	0,9903	12,27
6,0%	y = 1,9619 x + 0,1524	0,9399	11,85
Biofloc System			
	Linear regression model	**r^2**	**Shelf life (days)**
Control	y = 1,9848 x + 0,1143	0,9853	11,73
1,5%	y = 1,6914 x + 0,6476	0,9808	13,45
3,0%	y = 1,8705 x - 1,2952	0,9418	13,20
4,5%	y = 1,7714 x - 1,5810	0,9828	14,10
6,0%	y = 1,9010 x + 1,4095	0,9654	12,04

y = maximum acceptable IQ (65% of total demerit points, i.e., 23.4); x = ice days; r^2 = coefficient of regression.

The pH analysis during storage, all samples began with values of 6.8 in both BS and CWS groups. On the 9th day, there was a significant increase and values rose to 7.7 and 7.3, respectively; and from this moment forward pH stabilized in BS and increased and increased in small proportion, presented values of 7.9 and 7.8, respectively, until the end of the experiment (Figure 2).

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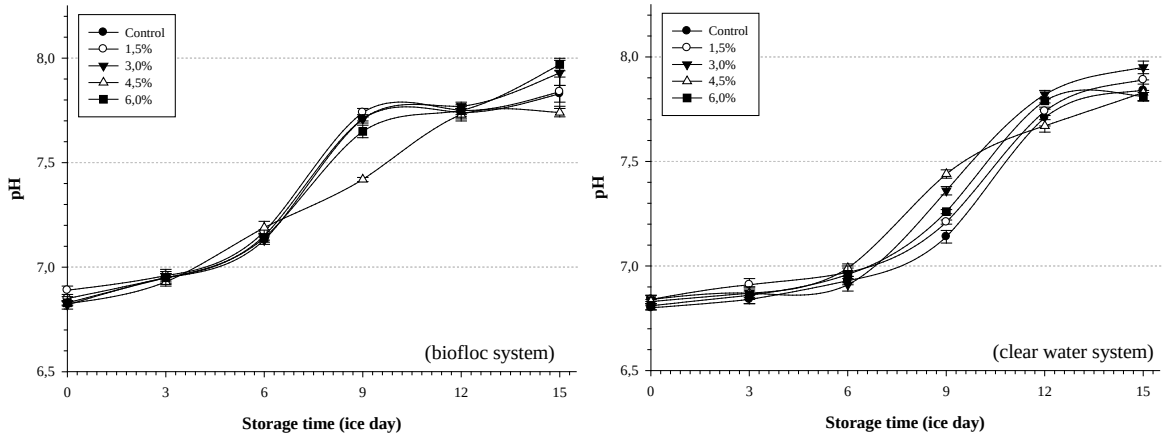

Figure 2. pH results in shrimp grown in biofloc system and clear water system during storage (0 ± 1 °C).

The microbiological results for total mesophilic and psychrotrophic count in shrimp stored on ice (0°C) for 15 days are shown in Figure 3. The results showed a decrease of mesophilic bacterial count from the 1st day (probably due to initial chilling) and the recovery of bacterial flora, remaining stable and remained above the limit of 4 log CFU/g until the end of the experiment. Instead, it was found that from the 1st day of storage, the psychrotrophic bacteria increased almost linearly during storage, reaching values above the limit of 5 log CFU/g, demonstrating its influence on the shelf life of shrimp. Considering both systems of farming (Biofloc and Clear water), the results were more stable in CWS (Figure 3). There was no difference among the groups (replacement of fish silage) in CWS along the storage time, but in BS the results showed more unstable.

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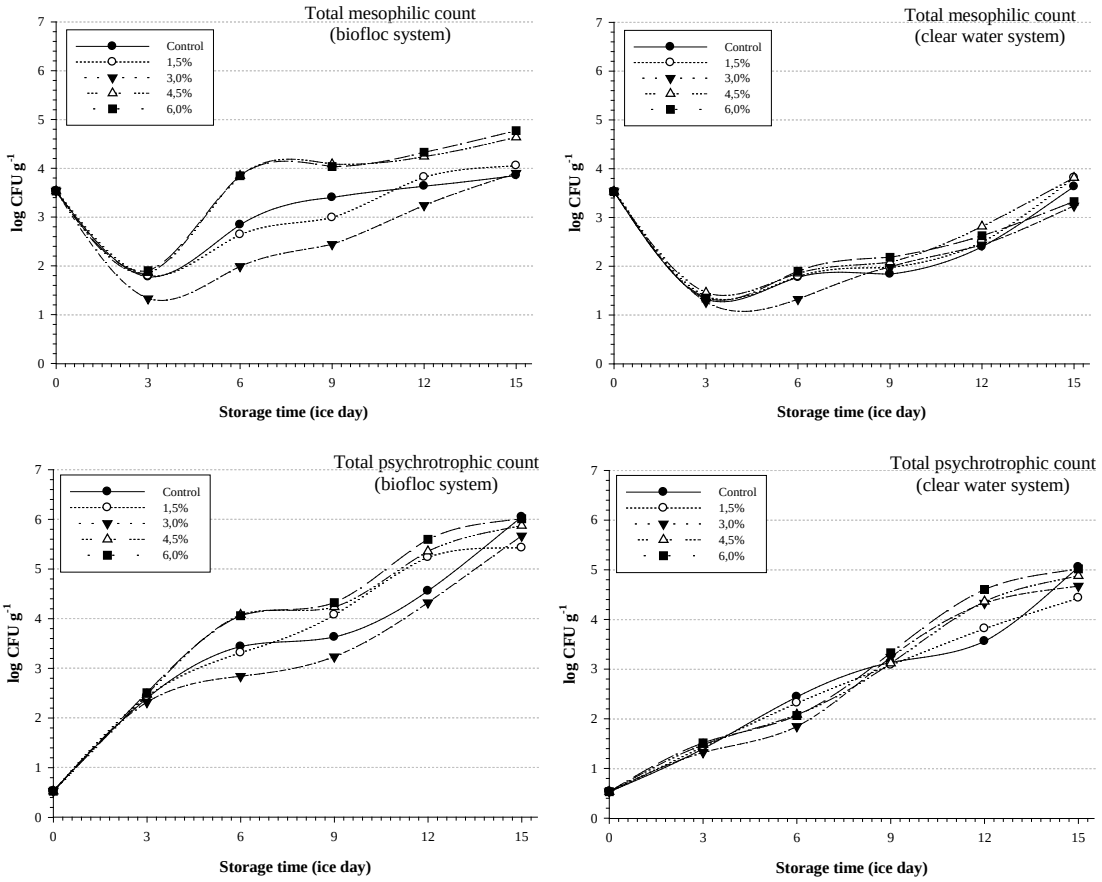

Figure 3. Mesophilic and Psychrotrophic bacteria counts in shrimp grown in biofloc system and clear water system during storage (0 ± 1 °C).

TVB-N values for BS showed an increasing trend for all samples (Figure 4) throughout the entire storage period (1.34 - 1st day), with higher values (18.56 mg N/100g on 15th day of storage), but all values were within the limit set by internationally (30 mg N/100g). TVB-N values for CWS showed the same tendency for all samples (Figure 4) throughout the entire storage period (1.34 - 1st day), but at the end of experiments (on 15th day of storage) showed higher values (20.16 mg N/100g), but all values were within the limit set by internationally (30 mg N/100g). These results could probably due to the log phase in bacterial growth (Figure 3).

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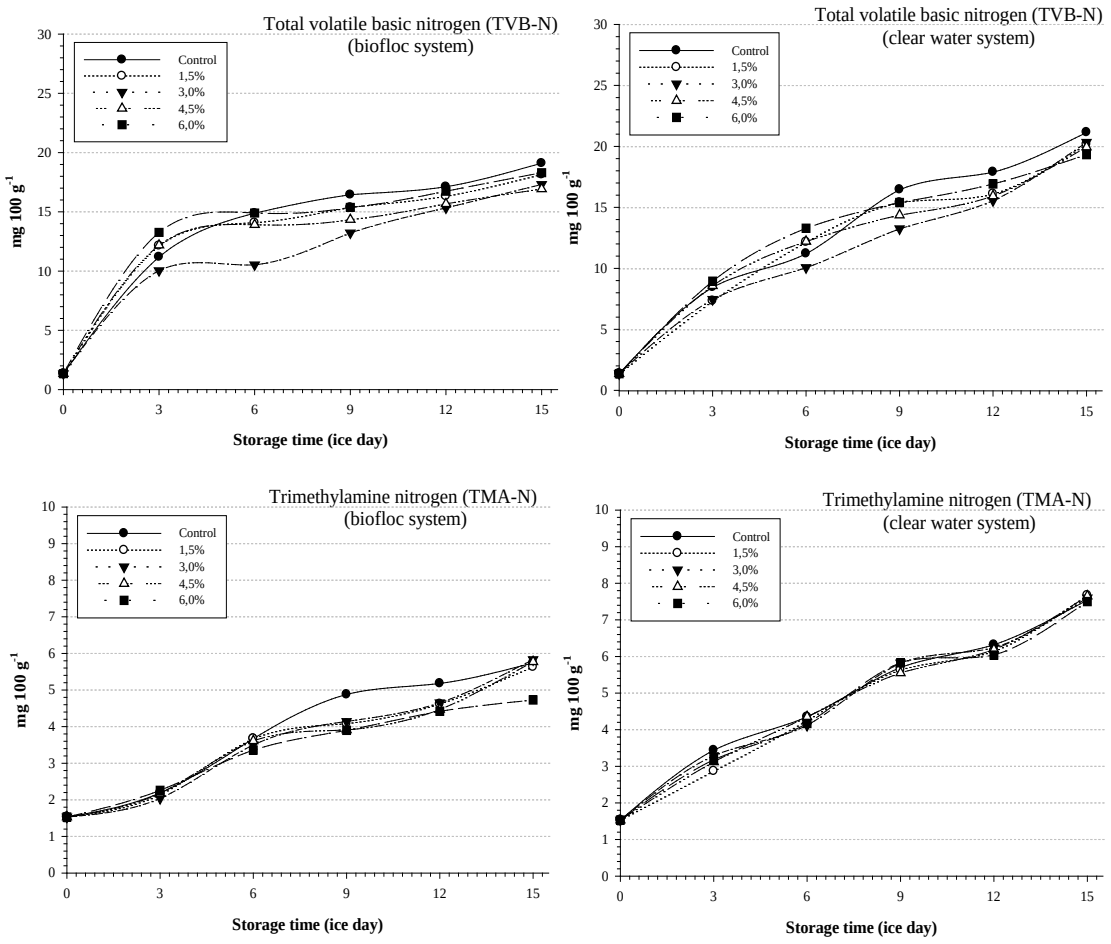

Figure 4. Total volatile bases nitrogen (TVB-N) and Trimethylamine nitrogen (TMA-N) in shrimp grown in biofloc system and clear water system during storage (0 ± 1 °C).

4. Discussion

The water quality parameters remained within the recommended range for *L. vannamei* culture (VanWyk and Scarpa, 1999), including settling solids maintained below to 15 ml/l (Taw, 2010).

In our experimental conditions, both biofloc and clear-water systems fish silage could be included at the highest level (6.0%) without losses in growth and survival. On the other hand, in biofloc condition shrimp got the best performance as compared to clear-water,

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probably due to the continuous availability of natural food source in a form of bacteria, microalgae, protozoa, nematodes, copepods and rotifers (Decamp *et al*., 2002; Azim & Little, 2008; Ballester *et al*., 2010; Ray *et al*., 2010). These microorganisms are a rich source of lipids (Maicá *et al*., 2012), vitamins and essential aminoacids (Ju *et al*., 2008), as well as highly diverse "native protein". The concept of "native protein" is related to protein source without previous treatment mainly including live food (Emerenciano *et al*., 2012b). Bacteria protein-source plays an important role in the equilibrium and re-ingestion of particulate organic matter and faeces (coprophagia) left by shrimp results in a form of constant food supply. The colonization of shrimp gut by bacteria had been shown positive effects such as improvement of shrimp digestive enzymes activity (Xu *et al*., 2012) and increasing the availability of extracellular enzymes (Xu & Pan, 2012) acting as "natural probiotic" (De Schryver *et al*., 2012).

Many studies have been done in the past decades with penaeid shrimp diets replacing fishmeal by alternative protein sources such as vegetable grains and terrestrial animal industry by-products. Problems related to the fishmeal replacement by alternative sources include palatability, digestibility, deficiency of essential amino acids, and presence of anti-nutritional factors (Forster *et al*., 2003; Naylor *et al*., 2009).

Although problems exist, many cases of success have been reported. Forster *et al*. (2003) and Suarez *et al*. (2009) recommended levels until 75% and 80% of fishmeal replacement using cattle by-product and a mixture of canola and soya, respectively. Amaya *et al*. (2007) and Hernández *et al*. (2008) concluded that is possible to replace 16% and 35% of fishmeal by poultry and swine by-product, respectively, without shrimp performance losses. Samocha *et al*. (2004) and Cruz-Suarez *et al*. (2007) achieved success replacing 100% and 80% of fishmeal by soya meal and poultry by-product in *L. vannamei* diets. Paripatananont *et al*. (2001) achieved 50% of replacement using soya protein concentrate in *Penaeus monodon* diets. Recently, Bauer *et al*. (2012) suggested that a mixture of soy protein concentrate and microbial floc meal can be utilized as a substitute for fishmeal in diets for *L. vannamei* juveniles. These studies have been carried out in clear-water condition and few efforts have been done to investigate alternative sources in biofloc conditions. Scopel *et al*. (2011) evaluated the replacement of fishmeal (0, 12.5 and 21.0%)

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by a combination of soya and animal terrestrial by-products. The authors found that 12.5% of replacement did not affect shrimp growth, resulting in growth rates of 0.7 g/week similar to those found in our study in clear-water condition, but less than 0.9 g/week observed in biofloc.

No literature was found related to the use of fish waste silage in *L. vannamei* diets under biofloc condition. In a recent study using clear-water, Gallardo *et al*. (2012) evaluated in *L. vannamei* juveniles feeds containing fish waste silage, fish waste silage with soybean meal or fish waste meal as protein source. The authors reported that shrimp fed with diets containing fish waste silage with soybean meal gained 0.7 g/week higher than those fed with fish waste silage or fish waste meal (0.3 g/week). These values are lower than observed in our study in biofloc conditions (0.9 g/week). Additionally, in our study values of FCR were 1.3 and 1.6 for biofloc and clear-water, respectively, higher than 2.8 and 2.5 observed by Ray *et al*. (2010) and Xu *et al*. (2012) using soy protein-based diets and low protein content diets, respectively, both in biofloc conditions for *L. vannamei.*

In contrast with our work, Costa *et al*. (2009) did an interesting study evaluating shrimp silage in juvenile tilapia (*Oreochromis niloticus*) diets. The authors concluded that it is possible to include 2.75% of shrimp silage, reducing the diets costs in 3.3%, without losses in fish performance. In a similar work, Cavalheiro *et al*. (2007) tested shrimp head silage, which contained approximately 40% protein, as a substitute for fishmeal in tilapia diets at 0%, 33.3%, 66.6% and 100% dietary levels. The results indicate that the shrimp silage could replace fishmeal at 100% level with economic advantages and without sacrificing the quality of the feed.

It is important to note that our experiment was carried out in euryhaline conditions, typically observed in Brazilian Semi-Arid region. The possibility of producing a marine shrimp in the continent is very interesting, as inland cultures demonstrate higher economic viability than the coastal cultures, mainly due to the high cost of land and the rigorous environmental protection legislation of these regions. As *L. vannamei* presents a salinity tolerance ranging from 0.5 to 40 g/l, together with good sensorial qualities and growth performance using alternatives protein source diets makes such species attractive for new investments in many regions around the world.

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The QIM is useful essentially because it evaluates sensory parameters and attributes that change most significantly in each species during degradation (Erkan & Özden 2006; Huidobro *et al*., 2000). According to quality experiments (Figure 1, Table 3) indicate that loss of freshness during the days of storage was verified. The loss of freshness evolution could be expressed by the equations (linear regression model) and these results are similar to studies of QIM for shrimp *Pandalus borealis* (Martinsdóttir *et al*., 2001) and for white shrimp *L. vannamei* (Oliveira *et al*., 2009; Oliveira, 2013).

Considering the maximum QI score for acceptable shrimp to be 65% of the total, i.e., 23.4 demerit points, it can be stated that the average shelf life of BS and CWS (Figure 1, Table 1) samples is, respectively, 12.18±0.16 and 13.19±0.86 days. Similarly, Oliveira *et al*. (2009) found 60% to be the maximum acceptable QI score for whole white shrimp (*L. vannamei*), and their samples reached this score in 12 days of iced storage. On the other hand, Norway lobsters stored in flake ice, even though treated with sodium metabisulphite, were only sensory acceptable for 5 days (Aubourg *et al*., 2007) while deep-water pink shrimp on ice was unacceptable after 7 days (Gonçalves *et al*., 2003).

In this study, the pH was above of the permitted pH by the Brazilian law (Brazil, 1997), which establishes pH values for seafood meat suitable for consumption ranging from 5.8 to 6.4. However, Ogawa *et al*. (1970) considered lobster tails good for consumption at pH 7.0 and Ogawa *et al*. (1975) observed that pH of frozen tail lobsters did not demonstrating a great variation, showing maximum values of 6.9 during one month of storage. Vieira *et al*. (1990) observed increasing trend of pH (6.4 to 7.6) for *P. argus* during the storage period.

The pH variation during storage (Figure 2) happens due to the basic compounds, such as TVB-N and TMA, formed from microbial activity, and to the fact that crustaceans have higher content of non-protein nitrogenous compounds, which facilitates the rise in pH values (Huss, 1995; López-Caballero *et al*., 2007). Similar results were reported in deep-water pink shrimp (*P. longirostris*) packaged in different modified atmospheres (Gonçalves *et al*., 2003), Norway lobster (*N. norvegicus*) stored in either flake or slurry ice and treated with sodium metabisulphite (Aubourg *et al*., 2007) peeled shrimp (P. serratus) treated with thymol essential oil (Mastromatteo *et al*., 2010) and especially in white shrimp (L.

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vannamei) treated with catechin (Nirmal and Benjakul, 2009). Kirschnik & Viegas (2004) had pH values ranging from 6.62 to 7.44 for *Macrobrachium rosenbergii* stored on ice for 10 days. Reilly *et al*. (1985) had a pH (7.1 to 8.1) for *P. monodon* stored on ice for 17 days and attributed the increase to the high levels of volatile nitrogen compounds produced by tissue and microbial enzymes, as well as Shamshad *et al*. (1990), who obtained initial pH values of 7.05 rising to 8.25 after 16 days of storage on ice, higher values than those obtained in the present study, and they found that at pH greater than 7.6, the prawns *Penaeus merguiensis* were classified as unacceptable or putrid.

Brazil's official analytical methods (Brazil, 2003) employed in microbiological analyses states plate counts must only be done when there are at least 20 and at most 200 CFU/plate, therefore the maximum countable value is 5.4 log CFU/g.

According to Cyprian *et al*. (2008) the low total counts reported at the beginning of storage time were due to the flesh of newly caught fish being sterile, since the immune system of the fish prevents the bacteria from growing. However, when the seafood dies, the immune system collapses, and consequently during storage, bacteria invade the flesh (Sveinsdóttir *et al*., 2002). At the end of shelf life estimated to be about 12 days the bacterial (psychrotrophic) count reach values near to log 4 CFU/g and remain increase up to the 15^{th} day of storage.

The present results of TVB-N and TMA-N is agree with results found by Vieira *et al*. (1990) after the first week of storage values tended to increase. TVB-N values vary according to the methodology used, the seafood species and the deterioration stage. This can be seen in Moura *et al*. (2003) experiments, which showed TVB-N values between 27.6 and 73.0 mg N/100g, much higher than recommended for freshness and consumption values. Cheuk *et al*. (1979) studying the pink shrimp (*Penaeus duorarum*) and brown shrimp (*Penaeus aztecus*) observed that the onset of deterioration coincided with the values of TVB-N reaching the limit of 30 mg N/100g, which occurred, respectively, 16 and 19 days of storage in ice. Angel *et al*. (1981) studying the Giant Malaysian Prawn (*M. rosembergii*) cooled on ice, detected slow increases on TVB-N, exceeding the value of 30 mg N/100g on 14 days of storage. Basavakumar *et al*. (1998), in experiments with tiger shrimp (*Penaeus monodon*), observed signs of deterioration at 11 days on ice (32.2 mg

de Azevedo, F. 2015. Replacement of Fishmeal By Fish Silage in *Litopenaeus Vannamei* Diets in Biofloc System: Growth Performance and Shrimp Quality. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 234-258.

N/100g). Kirschnik & Viegas (2004), studying with *M. rosembergii* stored on ice for 10 days, found, respectively, initial and final values of TVB-N 18.65 to 26.00 and from 10.23 to 27.10 mg N/100g.

Considering the species studied by the cited authors were different from that used in this study, it was not possible to draw a comparison among them. However, in all reports, there was an increase of TVB-N with storage time. It has been suggested that the TVB-N value is affected by species, season, harvesting area, age and sex of fish. According to Mitsubayashi *et al*. (2004) and Siripatrawan *et al*. (2009) TMA-N is produced by decomposition of trimethylamine N-oxide (TMAO) by microorganisms. Volatile compounds such as ammonia, dimethylamine (DMA) and TMA are products of microbial degradation and are collectively regarded as TVB-N. Although TVB-N values in all samples increased throughout the storage period, the TMA-N increased in small proportion, probably due to the fact that lobster has very low values of TMAO.

As mentioned by Barbosa & Vaz-Pires (2004), QIM is a method that implies the transformation of scientific knowledge of the products in a consumer friendly solution that can be used by the seafood retailer and the consumer in common, which is both rare and desirable.

5. Conclusion

Replacement of fishmeal by fish silage in *L. vannamei* diets is a good option regarding to shrimp production and quality.

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Broodstock Nutrition: Enhancement of Egg Quality in Channel Catfish

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Abstract

To facilitate our understanding of the interaction of nutrition and reproductive performance from female channel catfish, two experiments were performed in earthen ponds. The first experiment evaluated the interaction of feed quality (42 and 32% protein) and feed frequency (feed offered 3 or 6 times per week) in two strains (high and low spawning strains). The second experiment assessed the influence of different lipid sources and n3:n6 ratios using a commercial catfish feed containing 32% protein and 5% lipid as the basal diet. Reproductive performance in terms of spawning and egg production was not influenced by changing protein level of the diet from 32 to 42%. Increasing the feeding frequency from 3 to 6 times per week negatively affected spawning in one of the strains, but did not affect egg production. Age and period of spawning affected reproductive performance. In addition to having bigger eggs than their younger counterpart, older fish performed better than younger fish in terms of spawning success, and egg production. Biochemical composition of the eggs was affected significantly by dietary treatment in terms of lipid, fatty acids and free amino acid content.

Lipid supplementation on a 32% protein, 5% lipid commercial catfish diet using soybean and /or, linseed oil, or menhaden fish oil enriched with docosahexaenoic acid (DHA) and arachidonic acid (ARA) had no significant effects ($p<0.05$) on spawning success neither egg production. The quantity of fry produced per female body weight and fry survival from fish fed top-coated feed with menhaden fish oil enriched ARA and DHA were two to five fold greater than those obtained from fish fed with feed supplemented with vegetable oils.

Keywords: Nutrition, Catfish, *Ictalurus*, Broodstock

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Introduction

The catfish industry is one of the largest and best developed aquaculture segments in the United States. However, catfish production has declined due to decreasing catfish operations and reduction in water surface used for production; thus, during 2005 catfish sales represented 68.7% of total sales from aquaculture production with a total value of 461.9 million dollars, while in 2013 catfish sales added 375.9 million dollars in sales which represented 51.3% of total sales from US aquaculture production in that year (USDA 2014). In order to maintain a competitive level in the market, the US catfish industry started to work on the development of technological solutions to improve production, one of them has been implementing hybridization programs with the objective of produce a fish that combine some of the more desirable characteristics of two parent species (Kerby & Harrell 1990).

The interspecific hybrid obtained from crossing channel catfish, *Ictalurus punctatus*, & x blue catfish, *I. furcatus*, was identified as the most suitable for culture conditions, because attributes related to increased growth rates, improved yield and better disease resistance (Aphis, 2011; Arias *et al*., 2012). Hence, there is a trend to grow hybrids even though there are additional costs associated with their production, in fact it is estimated that during 2010 approximately 3400 ha were dedicated to culturing hybrids, while in 2014 it was estimated at 5,128 hectares (Aphis, 2011; USDA, 2014).

Implementation of the hybridization program had to overcome some reproductive limitations associated with the artificial spawning, because a chronically low hatch out rate, typically half that of pure channel catfish (Dunham & Argue, 2000). One of the first steps to overcome this challenge was to conduct basic research on brood stock nutrition to optimize egg quality, hatch and fry production. This article presents results from broodstock nutrition research conducted to improve egg quality in channel catfish.

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Broodstock management

One key factor in broodstock management is the nutritional status of the broodstock fish. Bromage (1995) indicate that both quality and quantity of the diet may influence gamete and fry quality. This is because the contents of an egg must be incorporated into the egg when it is an oocyte within the ovary in a process called vitellogenesis (Sargent, 1995; Wiegand, 1996; Brooks *et al*., 1997). Food restriction itself can affect spawning success, and thus has been reported to cause an inhibition of gonadal maturation in several species such as goldfish *(Carassius auratus* L), European sea bass (*Dicentrarchus labrax* L), and male Atlantic salmon (*Salmo salar* L) (Izquierdo *et al*., 2001). Dietary components as diverse as protein and fatty acids have all been shown to affect egg and embryo survival (Brooks *et al.*, 1997). Proteins act as a source of amino acids and as a reservoir of materials used during the many biosynthetic activities that are essential for the early stages of embryogenesis (Metcoff, 1986). Takeuchi *et al*. (1981) reported higher hatch rates for eggs from trout maintained on a low protein diet (36%), whereas Roley (1983) found that the eggs produced by trout brood fish fed on a 47% protein diet had higher survival than those produced by fish given either 27 or 37% protein diets.

Lipid and fatty acid composition of brood stock diets have been identified as major dietary factors that determine successful reproduction and survival of offspring. Highly unsaturated fatty acids (HUFA) with 20 or more carbon atoms affect directly or through their metabolites, fish maturation and steroidogenesis (Fernandez-Palacios *et al*., 1995; Izquierdo *et al.,* 2001). Altering the lipid composition of brood stock diet effected egg quality in the European sea bass. Eggs considered to be of better quality had a higher content of total n-3 fatty acids, which included enhanced levels of both docosahexaenoic acid and eicosapentaenoic acid (Brooks *et al.,* 1997). In contrast to these studies, an analysis of the lipid and fatty acid composition of Atlantic halibut eggs showed that batches of eggs with widely differing viabilities had very similar lipid compositions (Bruce *et al*., 1993). In the case of Nile tilapia, performance was much higher in fish fed a basal diet

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supplemented with soybean oil (high in n-6 fatty acids) and relatively low in fish fed a diet supplemented with 5% cod liver oil (high in n-3 fatty acids) (Santiago & Reyes, 1993).

Catfish broodstock management in US

Catfish broodstock management in commercial breeding operations in U.S. follows different practices in regards to feed management, and varies according to the season. Feeding in the spring through early summer (pre-spawning and spawning) is mostly either on a daily basis (35.2%) or every other day (35.1%), followed by every third day (18.9%), less often than every third day (5.4%), and no feed (5.4%) (USDA, 2010). The use of feed in breeding operations is identified based on the protein level content, thus 16.7% of the breeding operations use a 28% crude protein level feed, 69.4% of them use a 32% crude protein content, 5.6% of the operations use 35% crude protein level, and 8.3% of them use other protein levels. Finally, about 48.7% of the commercial operations used forage fish as a supplemental food source for broodfish (USDA, 2010)

Interaction nutrition and reproductive performance

To facilitate our understanding of the interaction of nutrition and reproductive performance from female channel catfish, two experiments were performed in earthen ponds. The first experiment evaluated the interaction of feed quality (42 and 32% protein), feed frequency (feed offered 3 or 6 times per week), channel catfish strains (high and low spawning strains) and brood fish age. The second experiment assessed the influence of different lipid sources and n3:n6 ratios using a commercial catfish feed containing 32% protein and 5% lipid as the basal diet.

Results of those experiments showed that reproductive performance in terms of spawning and egg production was not influenced by changing protein level of the diet from 32% to 42%, but affected egg size. Increasing the feeding frequency from three to six times

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per week negatively affected spawning in one of the strains, did not affect egg production and egg fertilization, but had a significant effect on egg size.

Brood fish age affected reproductive performance, with older fish performing better than younger fish in terms of spawning success and egg production. Similar findings were presented by Santiago (1979) who reported a very low spawning success in channel catfish 3-year-old females (12.7%). Davis *et al*. (2005) also suggested that age rather than size is a more important component of maturation. In the present study, there was not significant relation between egg size and either fish weight or fish length, but there was a significant difference in egg diameters due to fish age (older females had bigger eggs). Also, females given higher protein diets, and fed more frequently tended to have larger eggs. According to Shatunovskii (2006) this phenomenon of age affecting egg size, can be attributed to an increased reproductive function in ontogeny, which is realized as a more active synthesis of ovovitellin in the liver and its storage in oocytes as well as to an elongated period of trophoplasmatic growth of oocytes. This situation is also seen in walleye, where female age accounted for a greater amount of variation in egg mass than fork length or size (Johnston, 1997).

Biochemical composition of the eggs was affected significantly by dietary treatment in terms of fatty acids and free amino acid content, but not in the protein and lipid content (Table 1). The most abundant fatty acids were 16:0, 18:0, 18:1n9, 18:2n6, 20:3n3, and 22:6n3. There was a significant effect of protein level on the fatty acid composition of the eggs, except for 14:0,16:0, 18:2n6 and 20:1n9. Proportions and absolute values of linolenic acid and highly unsaturated fatty acids (ARA, EPA, and DHA), as well as the n3:n6 ratios were significantly higher in eggs from fish fed 42% protein diet (Table 1 and 2). The ratios DHA:EPA, ARA:EPA, and ARA:DHA were significant higher in eggs from fish fed 32% protein diet (Table 2). Egg viability at 48 hours of fertilization was not significantly different among treatments (feed protein x feed frequency) for each strain.

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The second experiment had as a goal to evaluate the effect of polyunsaturated fatty acids PUFAs (linolenic acid, 18:2n6 and linolenic acid, 18:3n3) in different ratios, as well as the effect of highly unsaturated fatty acids HUFAs (arachidonic acid - ARA, 20:4n6; eicosapentaenoic acid - EPA, 20:5n3; and docosahexaenoic acid - DHA, 22:6n3) on channel catfish, *Ictalurus punctatus* brood stock females to produce hybrid catfish fry. A commercial catfish feed (32% protein, 5% lipid) was top-coated with 2% lipids from different sources, using soybean and /or, linseed oil, or menhaden fish oil enriched with docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Quintero *et al*., 2011). Results of this study showed that reproductive parameters, such as spawning success, number of eggs either as per gram of egg mass or per female body weight did not exhibit significant effect from dietary treatments, but fatty acid composition of the eggs was affected by broodstock dietary treatment provide prior to the spawning season (Table 3 and 4). This has been observed in species that eat during sexual maturation and throughout the spawning season (Harel *et al*., 1994).

Results suggest that dietary essential fatty acids are readily incorporated into the eggs, and also mechanisms to elongate and desaturate fatty acids are very active in channel catfish. Freshwater fish are able to convert C18 PUFA to the biologically active C20 and C22 HUFA. Many freshwater fish posses both the fatty acid Δ6- and Δ5- desaturases required for the production of 20:5n3 and 22:6n-3 from 18:3n3, and of 20:4n-6 from 18:2n6 (Sargent 1995; Sargent *et al.*, 2002). Preferential accumulation of certain fatty acids was also observed especially with regards to saturated fatty acids such as C16:0 and C18:0, and monoenes as C:16:1 and C18:1n9, which represented from 62.2% to 63.7% of total fatty acids in eggs. This characteristic has been noted in other freshwater fish and could be related to the fact that these fatty acids are heavily catabolized to generate metabolic energy in fish (Kaitaranta & Linko, 1984; Tocher & Sargent, 1984; Henderson & Tocher, 1987; Anderson *et al*., 1990; Wiegand, 1996; Sargent *et al*., 2002). Linoleic acid (C18:2n6) and linolenic acid (C18:3n3) are considered primary precursors of highly unsaturated fatty acids, especially in freshwater fishes (Sargent *et al.*, 2002) and were found in lower proportions than those found in the feeds. These differences are more likely to be related to

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active processes in generation of HUFAs, such as ARA, EPA, and DHA, which tend to be deposited selectively into fish eggs (Henderson & Tocher, 1987; Wiegand, 1996; Sargent *et al.*, 2002). Thus, a relatively high presence of ARA (C20:4n6), EPA (C20:5n3), and DHA (C22:6n3) in eggs indicate either a selective mobilization of this fatty acid from other tissues or elongation and desaturation of C18:2n6 and C18:3n3. The elongation and desaturation of linolenic acid (C18:3n3) occurs in the absence of long-chain fatty acids of the n3 configuration in order to prevent essential fatty acid deficiencies (Farkas *et al*., 1977). There was not a defined trend in proportions of n3 HUFAs in the eggs, specifically EPA and DHA, when they were related to spawning success, egg production, and/or fry survival, in fact none of those parameters were affected significantly by dietary treatments.
Egg quality of channel catfish, it appears, is affected by more than the relative abundance of n3 HUFAs. The ratio between ARA (20:4n6) and EPA (20:5n3), or DHA (22:6n3) could be more determinant in that outcome, as was suggested by Bell & Sargent (2003). They consider that both the concentrations and ratios of the essential HUFAs (DHA, EPA and ARA) are likely to have important influences on both fertilization rates and survival of fish eggs, and make the generalization that a high ARA:EPA in fish eggs may be mandatory for survival. Moreover, embryogenesis could be influenced by essential fatty acids C20:3n6, C20:4n6, C20:5n3, and C20:6n3, since they are precursors for eicosanoid production, which in turn result in metabolites that include prostaglandins, leukotrienes and lipoxins (Leray *et al*., 1985; Mokoginta *et al*., 1998; Bell & Sargent, 2003).

Channel catfish females fed with feed top-coated with soybean oil (high n-6) produced eggs that were found to be significant smaller than the eggs produced by females under other treatments. Fatty acid composition of the eggs reflected the dietary lipid supplementation, making clear the incorporation of essential fatty acids during the pre-spawning season. Multivariate statistics using Principal Components Analysis (PCA) for the five fatty acids (linoleic acid, linolenic acid, ARA, EPA, and DHA) contained in the egg samples, lead us to differentiate groups of eggs based on scores assigned for each principal component which were reflection of the dietary treatments. Linseed oil (rich in linolenic acid C18:3n3) displayed the lowest hatch, which could be related to higher levels of ARA (C20:4n6),

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causing alteration in the immune cell composition. Similarly, higher proportions of C20:3n6 observed in diets 1 (SBO-LSO) and 2 (SBO) could be affecting embryo development due to immune response alterations. Supplying ARA, EPA, and DHA directly to brood stock females in diet 4 (MFO) increased fry production from two to five times when compared to females fed with diets top coated with soybean and/or linseed oil. Hence, commercial producers may consider using highly unsaturated fatty acids as lipid supplement on their brood stock diets to improve their fry production.

The quantity of fry produced per female body weight and fry survival from fish fed top-coated feed with menhaden fish oil enriched ARA and DHA were two to five fold greater than those obtained from fish fed with feed supplemented with vegetable oils. This difference was not significant ($p = 0.08$) and their impact on a commercial basis could be very important. Based on the results of these studies, it is recommended that the minimum dietary requirements for ARA, eicosapentaenoic acid (EPA) and DHA be evaluated for enhancement of egg quality in the channel catfish. Finally, further investigation is required to elucidate the mechanism that regulates fatty acid composition in eggs, in particular that related to proportions of linolenic acid and arachidonic acid, and the physiological implications of such relation reproductive performance parameters evaluated on channel catfish females, either spawning success, number of eggs per gram of egg mass, number of eggs per body weight, egg mass per body weight, fry production, or fry hatch.

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Table 1 - Proximate analysis from commercial channel catfish feed, and biochemical composition from eggs of channel catfish females, *Ictalurus punctatus*, including proteins, lipids, free amino acids as total ninhydrin positive substances (TNPS), and ratios between essential fatty acids from dietary treatments 42% and 32% protein level offered three times per week.

Parameter	42% -3 times/week	32% -3 times/week	p-values
Feed			
Moisture (%)	9.41 ± 0.09	7.12 ± 0.19	0.0048
Protein (%)	40.74 ± 0.83	33.99 ± 1.03	0.0187
Lipids (%)	6.22 ± 0.05	5.48 ± 0.11	0.0137
Energy (cal)	4,437 ± 6	4,231 ± 17	0.0036
Eggs			
Protein (%)	17.80 ± 1.93	17.21 ± 1.71	0.1590*
Protein (mg/individual egg)	3.20 ± 0.98	2.95 ± 0.94	0.1531
Lipids (%)	6.82 ± 1.05	6.49 ± 1.05	0.6159*
Lipids (mg/individual egg)	1.12 ± 0.42	1.02 ± 0.39	0.1415
Free amino acids as total ninhydrin positive substances (TNPS)			
TNPS (µmol/gr egg mass)	3.84 ± 1.89	2.43 ± 2.05	<0.0001
TNPS (µmol/individual egg)	6.91 ± 3.60	3.74 ± 3.09	<0.0001
Ratios			
DHA[1]:EPA[2]	6.46 ± 1.98	13.27 ± 3.09	0.0001
ARA[3]:DHA[1]	0.012 ± 0.005	0.015 ± 0.003	0.0004
ARA[3]:EPA[2]	0.08 ± 0.07	0.19 ± 0.07	<0.0001

*The smallest p-value ≥0.05 from Beta regression coefficients

1 – DHA: Docosahexaenoic acid

2 – EPA: Eicosapentaenoic acid

3 – ARA: Arachidonic acid

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Table 2 - Fatty acid analysis from eggs of channel catfish females, *Ictalurus punctatus*, by dietary treatments, commercial catfish diet 42% and 32% protein level offered three times per week

Fatty acid	42% - 3 times/week	32% - 3 times/week	p-value
14:0	0.99 ± 0.01	0.90 ± 0.04	0.0961
16:0	18.78 ± 0.02	18.37 ± 0.03	0.0702
16:1n7	3.08 ± 0.02	2.37 ± 0.04	<0.0001
18:0	12.17 ± 0.02	13.10 ± 0.02	<0.0001
18:1n9	28.67 ± 0.11	30.70 ± 0.05	0.0001
18:2n6	7.25 ± 0.07	6.97 ± 0.04	0.2471
19:0	5.92 ± 0.04	5.89 ± 0.04	0.8472
18:3n3	0.41 ± 0.01	0.26 ± 0.002	<0.0001
20:1n9	1.08 ± 0.01	1.13 ± 0.01	0.1607
20:2n6	1.16 ± 0.004	1.42 ± 0.01	<0.0001
20:3n6	2.65 ± 0.02	3.25 ± 0.01	<0.0001
20:3n3	3.88 ± 0.04	5.72 ± 0.01	<0.0001
20:4n6	0.08 ± 0.002	0.06 ± 0.001	<0.0001
20:5n3	1.20 ± 0.03	0.35 ± 0.01	<0.0001
22:4n6	0.24 ± 0.003	0.37 ± 0.002	<0.0001
22:5n6	1.11 ± 0.02	2.56 ± 0.02	<0.0001
22:5n3	0.74 ± 0.004	0.55 ± 0.01	<0.0001
22:6n3	7.34 ± 0.03	4.38 ± 0.08	<0.0001
Σ n-6	11.37 ± 0.08	13.10 ± 0.05	<0.0001
Σ n-3	13.66 ± 0.02	11.31 ± 0.07	<0.0001
n-3/n-6	1.22 ± 0.18	0.84 ± 0.12	<0.0001

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Table 3 - Proximate analysis from commercial channel catfish feed, and biochemical composition from eggs of channel catfish females, *Ictalurus punctatus*, including proteins, lipids, free amino acids as total ninhydrin positive substances (TNPS), and ratios between essential fatty acids from dietary treatments

Diet	1-SBO-LSO	SBO	LSO	MFO	p-values
Feed					
Moisture (%)	7.36 ± 0.25	7.15 ± 0.20	7.55 ± 0.10	7.18 ± 0.12	0.5512
Protein (%)	33.43 ± 0.40	33.58 ± 0.17	33.53 ± 0.31	33.39 ± 0.36	0.9726
Lipids (%)	7.12 ± 0.04	6.94 ± 0.20	7.12 ± 0.48	7.21 ± 0.26	0.7402
Energy (cal)	4,128 ± 15	4,207 ± 47	4,230 ± 66	4,220 ± 72	0.5312
Eggs					
Protein (%)	16.81 ± 0.18^{a}	16.65 ± 0.12^{a}	16.74 ± 0.24^{a}	16.33 ± 0.13^{b}	0.0360*
Protein (mg/ egg)	3.07 ± 0.30^{a}	2.73 ± 0.39^{b}	3.02 ± 0.46^{a}	3.16 ± 0.42^{a}	0.0001
Lipids					
Percentage (%)	7.27 ± 0.13	7.19 ± 0.09	7.40 ± 0.17	7.54 ± 0.09	0.2681**
mg/individual egg	1.12 ± 0.19^{ab}	0.97 ± 0.12^{b}	1.14 ± 0.28^{ab}	1.27 ± 0.40^{a}	0.0003
Free amino acids as total ninhydrin positive substances (TNPS)					
µmol/gr egg mass	3.29 ± 2.26^{c}	5.85 ± 1.83^{b}	3.72 ± 2.02^{c}	7.37 ± 1.63^{a}	<0.0001
µmol/egg	5.92 ± 4.15^{c}	9.62 ± 3.45^{b}	7.06 ± 4.74^{bc}	14.48 ± 4.45^{a}	<0.0001
Ratios					
DHA[1]:EPA[2]	7.54 ± 2.09^{b}	10.93 ± 3.62^{a}	8.14 ± 1.96^{b}	8.60 ± 1.97^{b}	<0.0001
ARA[3]:DHA[1]	0.021 ± 0.004^{b}	0.016 ± 0.003^{c}	0.026 ± 0.005^{a}	0.013 ± 0.002^{d}	<0.0001
ARA[3]:EPA[2]	0.15 ± 0.02^{c}	0.17 ± 0.05^{b}	0.20 ± 0.03^{a}	0.11 ± 0.03^{d}	<0.0001

* The largest p-value <0.05 from Beta regression coefficients

** The smallest p-value ≥0.05 from Beta regression coefficients

1 – DHA: Docosahexaenoic acid

2 – EPA: Eicosapentaenoic acid

3 – ARA: Arachidonic acid

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Table 4 - Fatty acid analysis from eggs of channel catfish females, *Ictalurus punctatus*, held under dietary treatments, commercial catfish diet 32% protein, 5% lipids, top-coated with 2% oil, and offered three times per week

Fatty acid	SBO-LSO	SBO	LSO	MFO	p-values
14:0	0.63 ± 0.12^{b}	0.67 ± 0.14^{b}	0.71 ± 0.11^{ab}	0.75 ± 0.10^{a}	0.0003
16:0	17.49 ± 0.75	17.25 ± 0.70	17.27 ± 0.26	17.62 ± 0.57	0.0485
16:1n7	2.06 ± 0.45^{b}	1.91 ± 0.29^{b}	2.35 ± 0.25^{a}	2.35 ± 0.38^{a}	<0.0001
18:0	13.09 ± 1.44^{b}	14.51 ± 1.34^{a}	13.04 ± 1.31^{b}	13.47 ± 1.39^{b}	0.0001
18:1n9	29.54 ± 1.15	29.48 ± 1.27	29.95 ± 1.22	30.21 ± 1.38	0.0537
18:2n6	7.46 ± 0.81^{a}	6.79 ± 0.90^{b}	7.08 ± 1.11^{ab}	6.53 ± 0.79^{b}	0.0001
18:3n6	0.45 ± 0.39^{b}	0.59 ± 0.26^{a}	0.58 ± 0.23^{a}	0.40 ± 0.11^{ab}	0.0075
19:0	6.61 ± 1.07^{ab}	7.09 ± 1.32^{a}	6.99 ± 0.78^{a}	6.25 ± 0.93^{b}	0.0040
18:3n3	0.85 ± 0.18^{b}	0.45 ± 0.20^{c}	1.11 ± 0.22^{a}	0.50 ± 0.11^{c}	<0.0001
20:1n9	0.97 ± 0.17^{b}	0.98 ± 0.15^{b}	0.92 ± 0.15^{b}	1.14 ± 0.19^{a}	<0.0001
20:2n6	1.18 ± 0.14^{ab}	1.25 ± 0.13^{a}	1.14 ± 0.13^{b}	1.24 ± 0.20^{ab}	0.0348
20:3n6	3.04 ± 0.26^{a}	3.05 ± 0.25^{a}	2.87 ± 0.31^{a}	2.63 ± 0.25^{b}	<0.0001
20:3n3	6.00 ± 0.42^{b}	6.38 ± 0.79^{a}	5.42 ± 0.49^{c}	5.38 ± 0.33^{c}	<0.0001
20:4n6	0.12 ± 0.03^{b}	0.08 ± 0.03^{c}	0.16 ± 0.03^{a}	0.09 ± 0.02^{c}	<0.0001
20:5n3	0.81 ± 0.23^{a}	0.54 ± 0.37^{b}	0.77 ± 0.14^{a}	0.87 ± 0.27^{a}	<0.0001
22:4n6	0.40 ± 0.04^{a}	0.41 ± 0.04^{a}	0.34 ± 0.05^{b}	0.36 ± 0.03^{b}	<0.0001
22:5n6	1.68 ± 0.35^{b}	2.42 ± 0.60^{a}	1.53 ± 0.22^{b}	1.56 ± 0.37^{b}	<0.0001
22:5n3	0.94 ± 0.16^{a}	0.72 ± 0.22^{b}	0.89 ± 0.17^{a}	0.76 ± 0.13^{b}	<0.0001
22:6n3	5.73 ± 0.69^{b}	4.77 ± 0.94^{c}	6.05 ± 0.58^{b}	6.98 ± 0.60^{a}	<0.0001
Σ n-6	13.14 ± 1.07^{ab}	13.33 ± 0.75^{a}	12.56 ± 1.35^{b}	11.56 ± 0.75^{c}	<0.0001
Σ n-3	14.33 ± 1.20^{a}	12.86 ± 1.70^{b}	14.25 ± 0.79^{a}	14.49 ± 1.08^{a}	<0.0001
n-3/n-6	1.09 ± 0.07^{b}	0.96 ± 0.10^{c}	1.14 ± 0.09^{b}	1.26 ± 0.10^{a}	<0.0001

Values followed by the same letter are not different (p>0.005, Tukey-Krammer test)

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Recent Advancements in Nutrition and Health Interactions Mediated by the Gastrointestinal Tract

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Abstract

Diet additives that influence immunity and disease resistance of aquatic species continue to receive considerable attention due their potential to positively influence intensive aquaculture. Prebiotics, probiotics and essential oils are some of the prominent types of feed additives that may influence the microbiota of the gastrointestinal tract (GIT). It has been documented that microbiota of the GIT play important roles in affecting the nutrition and health of the host organism. Thus, various means of altering the intestinal microbiota to achieve favorable effects such as enhancing growth, digestion, immunity and disease resistance of the host organism continue to be pursued.

The first line of defense within the GIT is the mucosa that separates the gut microbiota from direct contact with the epithelial cells of the GIT. It is because of this direct contact with the mucus that the immune system of the GIT, often referred to as gut-associated lymphoid tissue or GALT, has developed mechanisms to distinguish between potentially pathogenic bacteria and the normal, commensal bacteria. In the event that potentially pathogen bacteria are detected, the cellular and humoral mechanisms of the GALT activate the innate immune system and subsequently the adaptive immune system to prevent bacteria from causing and/or spreading infection. It is anticipated that continued advancements with diet additives that influence the GIT will allow nutritional modulation of immunity to be increasingly used as an effective and relatively inexpensive means of combating diseases in aquaculture.

Keywords: immunonutrition, essential oils, feed additives, fish health, immune responses, prebiotics, probiotics

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Introduction

It is well established that proper nutrition is critical not only to achieve optimal growth rates but also to maintain adequate health of cultured fish (NRC 2011). In recent years, the influence of nutrition not only on normal growth but also its role in health management through modulation of immune response and disease resistance has received heightened attention. The targeted goal of this research has been to lessen the dependence on chemotherapeutics and reduce disease-related losses (Kiron, 2012; Oliva-Teles, 2012). Some have referred to this research area as "immunonutrition", which may be defined as the study of enhancing immunological functions by means of using specific nutrients and/or other dietary compounds. It has become apparent in studying various nutrients and diet additives that the microbiota of the gastrointestinal tract (GIT) plays important roles in affecting the nutrition and health of the host organism. Thus, various means of altering the intestinal microbiota to achieve favorable effects such as enhancing growth, digestion, immunity and disease resistance of the host organism have been increasingly investigated in recent years. In particular, diet additives that affect the GIT such as probiotics, prebiotics, and essential oils have been evaluated with growing intensity, as have certain nutrients which are involved in specific functions in the GIT such as arginine and glutamine.

The aim of the present review is to highlight the importance of the GIT in maintaining fish health, and the influence of specific diet additives that can positively influence the GIT to enhance its functions for maintaining health and protecting against disease-causing organisms. Particular attention will be given to how the GIT mediates various immunological responses, and some of the prominent diet additives that influence the GIT either by influencing its microbiota or enhancing its immunocompetence.

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Discussion

The Gastrointestinal Tract Microbiota and Their Numerous Functions

It has become increasingly apparent in recent years that the microbiota of the fish's GIT may influence a wide variety of metabolic processes (Dubert-Ferradon, *et al*.,2008). This microbiota stimulates epithelial proliferation and expression of numerous genes. Prominent functions among these are various physiological, biochemical, and immunological responses that must be maintained or enhanced to improve health status, stress responses and disease resistance. In addition, there are various other responses mediated by the GIT that may synergistically enhance weight gain and feed utilization of the cultured organism. The important functions of the GIT microbiota in relation to the immune system are of particular relevance in terms of maintaining fish health.

The Gastrointestinal Tract and Immune Systems

The role of the GIT in maintaining fish health is an area that has continued to expand over the past decade. The first line of defense within the GIT is the mucosa that separates the gut microbiota from direct contact with the epithelial cells of the GIT (Dubert-Ferrandon *et al*., 2008; Pérez *et al*., 2010). Due to this direct contact with the mucus, the immune system of the GIT, also referred to as gut-associated lymphoid tissue or GALT, has developed mechanisms to distinguish between potentially pathogenic bacteria and the normal, commensal autochthonous bacteria. As a result, the GALT may determine whether to mount an attack or tolerate a specific bacteria's presence (Pérez *et al*., 2010). In the event that potentially pathogen bacteria are detected, various cellular and humoral mechanisms of the GALT activate the innate immune system and subsequently the adaptive immune system to prevent bacteria from causing and/or spreading infection (Gomez and Balcázar, 2008). There are various components of the innate or non-specific immune response which can be activated to kill invading pathogens including such factors as blood neutrophil oxidative radical production, serum lysozyme and superoxide anion production

in activated macrophages (Nayak, 2010). The purpose of these factors is to kill a wide variety of foreign or invading microorganisms, and their enhancement may lead to significant reductions in mortality of the host when exposed to various pathogenic organisms.

The adaptive immune system is more complex than the innate immune system but is activated and to some extent directed by the innate immune system. Components of the adaptive or specific immune system include lymphocytes such as B cells and T cells which allow the host to recognize and combat specific disease-causing organisms. The adaptive immune system allows vertebrates, including fish, to recognize and remember specific pathogens and generate immunity against future exposure to such pathogens. This complex part of the immune system has not been studied extensively in response to supplementation of various diet additives but some of its components appear to be enhanced. Thus, it is recommended that additional research in this area be conducted to more fully characterize the effects of additives such as probiotics, prebiotics and essential oils on adaptive immunity of the host organism.

Diet Additives that Influence Microbiota of the GIT

The intestinal microbial populations are composed of two primary groups: those that are permanent colonizers (autochthonous bacteria), and transients (allochthonous bacteria). The autochthonous bacteria are resident populations which colonize the epithelial surface of the host organism's GIT, including the microvilli. These health-promoting bacteria, such as lactobacilli, may provide a defensive barrier and protect against the invasion of bacterial pathogens via the GIT. It is this group of bacteria that is generally targeted for manipulation by prebiotics and probiotics (Ringo *et al*., 2010). Establishment of bacterial pathogens in the GIT also may be impeded by the mucus layer of the GIT, which provides physical as well as various types of biochemical protection. The GIT is among the most common sites of pathogen entrance in fish, because they are exposed to water which contains various types of potentially pathogenic bacteria. However, a healthy

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gut microbiota has the ability to prevent pathogenic bacteria from colonizing the intestine, thus preventing infection (Birkbeck and Ringø, 2005; Stecher and Hardt, 2008). The autochthonous bacteria of the GIT which are present under normal conditions act to competitively exclude pathogens simply by their presence. By taking up space and resources along the mucosal lining of the GIT, pathogenic bacteria are forced to continue in a transient state where the likelihood of damaging intestinal cells or causing infection is reduced. Autochthonous bacteria also have the capacity to produce antimicrobial substances, which enhance their ability to inhibit pathogens from attempting to colonize the GIT. However, when the natural equilibrium state of the microbiota is altered, conditions become more favorable for pathogenic organisms to flourish.

There is one broad group of diet additives that has been investigated with terrestrial and aquatic organisms which influence the microbiota of the GIT. These additives include prebiotics, probiotics and essential oils. Prebiotics are defined as non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the GIT, thus improving the host's intestinal balance (Gibson and Roberfroid, 1995). The health-promoting bacteria most commonly augmented by prebiotics include those of the genus *Lactobacillus* which tend to limit the presence of harmful bacteria. Many prebiotics are oligosaccharides composed of various sugars while other ingredients shown to possess prebiotic properties include GroBiotic®-A, a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products. The chemical nature and ways in which various prebiotic compounds may affect the host organism have been summarized by Gatlin and Peredo (2012). Prebiotics are not typically altered by diet processing and require limited regulatory approval such that their incorporation into diets is much simpler than required for chemical therapeutic agents.

Probiotics are live microbial additives that may alter microbiota of the GIT. The term probiotic was introduced by Parker (1974) as "organisms and substances which contribute to intestinal microbial balance". Fuller (1989) revised the definition as "live

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microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Subsequently, Moriarty (1998) proposed that the definition of probiotics be extended to microbial “water additives”. Administration of probiotics to the water has been associated with improving water quality such as reductions in the concentrations of nitrogen and phosphorus via microbial transformations (Wang *et al*., 2005). In addition to affecting water quality, probiotics administered to the water or diet also may inhibit the growth of pathogenic microorganisms, contribute digestive enzymes which may increase feed utilization, provide other growth-promoting factors as well as stimulate the immune response of the host organism.

Probiotics that have been shown to influence fish immunity, disease resistance and other performance indices include those of the genus *Bacillus* and various lactic acid bacteria (*Lactobacillus, Lactococcus, Carnobacterium, Pediococcus, Enterococcus* and *Streptococcus*). Bacteria of the genus *Bacillus* are Gram-positive rods that form spores and are resistant to environmental conditions and thus have extended shelf life. *Lactobacillus rhamnosus, L. delbrüeckii, Carnobacterium maltaromaticum, C. divergens, C. inhibens*, and *Enterococcus faecium* are other bacterial species which have been used as probiotics, as well as yeasts such as *Saccharomyces cerevisiae* and *Candida sake*. Viability of the bacteria during storage and processing is critical for probiotics to confer their beneficial effects to the host organism, however, the application of dead cells, lyophilized cells or cell-free supernatants or spores have all showed some degree of success (Merrifield *et al*., 2010). The logistical complication of culturing live microorganisms prior to application on the feed has constrained the use of probiotics at aquaculture facilities and thus administration of lyophilized cells or spores may be more practical. Potential applications of probiotics in fish, shrimp and molluscan aquaculture have been reviewed by Burr *et al*. (2005), Wang *et al*. (2008), Kesarcodi-Watson *et al*. (2008) and most recently by Ringø *et al*. (2010).

In order to help maintain the delicate balance between microbiota of the GIT, prebiotics or probiotics may be included in the diet to help reinforce the population of

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beneficial bacteria while decreasing the number of potentially pathogenic bacteria. Probiotics may directly accomplish this by providing an increased number of desirable bacteria when incorporated in the diet and ingested. A regular supply of beneficial bacteria added to the GIT will help to control or reduce the number of detrimental bacteria. In the case of prebiotics, they accomplish their goal more indirectly, by acting as a food source, preferentially, to the beneficial bacteria. It also has been shown that some pathogenic bacteria may become bound to certain prebiotics, as opposed to attaching to the mucosal lining of the GIT, and thereby may be passed from the GIT (Spring *et al*., 2000).

Another group of diet additives that may influence the microbiota of the GIT and is receiving a growing amount of attention in recent years is referred to essential oils. These essential oils are obtained from plants such as basil, cinnamon, and oregano, and may contain a rich mixture of highly functional molecules with a wide spectrum of biological activity. Some studies have demonstrated that these essential oils when added to diets can activate the fish antioxidant defense system, enhance various immune functions, change the intestinal morphology and microbiota, as well as increase digestibility and nutrient absorption (e.g., Harikrishnan *et al*., 2011; Giannenas *et al*., 2012; Saccol *et al*., 2013).

Practical application of prebiotics, probiotics and essential oils

As mentioned previously, maintaining the viability of probiotics during storage and processing is important for them to exert their beneficial effects to the host organism. Therefore, some logistical constraints may be encountered with the cultivation of live microorganisms in conjunction with manufacturing feeds. To ensure probiotic viability, its application to the feed typically must occur post extrusion so the probiotic organism is not subjected to excessive heat and pressure. As such, administration of probiotics in the form of lyophilized cells or spores may be less demanding compared to live organisms. In general, feed manufacturing constraints are of less concern when dealing with prebiotics because they are not living organisms. Although the efficacy of several prebiotics has been

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shown when incorporated into extrusion-processed feeds, the potential chemical alteration of prebiotic compounds during feed manufacturing has not be studied to any appreciable extent and deserves further consideration. Similar feed manufacturing issues also have not been thoroughly addressed with various essetial oil compounds but deserve further evaluation.

Administration regimes for specific prebiotics, probiotics and essential oils also have not been widely studied to date. Although these compounds have immunostimulating effects, it does not appear that long-term administration causes immunosuppression as noted with other potent immunostimulants such as beta glucans. Therefore, it is generally assumed that these diet additives may be administered for extended periods. However, developing more refined administration protocols for individual diet additives should be investigated to optimize their effectiveness. For example, administering probiotics, prebiotics or essential oils at prescribed times before the culture organism is exposed to a stressful event or at particular times of the year when pathogenic organisms are most prevalent could possibly be developed to most efficiently derive the benefits of these compounds under particular culture regimes.

Nutrients of the Immune System

Another type of immunonutrition which has been investigated to a limited extent is the dietary supplementation of essential nutrients at levels above which are required for normal physiological functions and which are intended to specifically nourish the immune system. In quiescent immune cells, the utilization of nutrients happens at basal levels, merely for cellular maintenance. However, during an immune challenge, the utilization of key nutrients dramatically increases. In particular, there is an important demand for amino acids (Kiron, 2012; Uribe *et al*., 2011). For example, *in vivo* reports suggest important usage of glutamine in ill fish, as reflected by the rapid decrease in plasma levels of this amino acid (Walker *et al*., 1996). Likewise, arginine is the unique precursor of nitric oxide

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in activated macrophages (Buentello and Gatlin, 1999, Neumann *et al*., 1995; Wu and Morris, 1998), and is a potent microbicidal compound and potent modulator of the eukaryotic cytoskeleton (Kasprowicz *et al*., 2009; Moffat *et al*., 1996). Although the profile of the amino acids used will vary depending on if there is phagocytic or lymphocytic responses, arginine and glutamine both have been demonstrated to play key roles in the overall performance of these immune cells of fish (e.g., Pohlenz *et al*., 2012a), and supplementation in the diet may achieve favorable effects. Supplementing diets with nutrients that could be metabolically used by phagocytes has been an area of interest for enhancing that stage of the immune response. For instance, glutamine has been shown to provide metabolic fuel to support reaction kinetics (Crawford and Cohen, 1985; Newsholme and Newsholme, 1989).

Exogenous sources of nutrients should supply minimum levels to meet requirements for normal immune system performance and to protect/restore tissues from collateral damage. However, in certain situations, providing additional nutrient at levels above those required for normal fish maintenance and growth may sustain and/or enhance one or more functions of the immune system, thereby increasing its efficacy and protection capacity against invading pathogens (Kiron 2012; Sealey and Gatlin 2001). Dietary supplementation of both arginine and glutamine was able to enhance vaccination efficiency of channel catfish *Ictalurus punctatus* (Pohlenz *et al*., 2012b).

Conclusions

It can be seen from this review that various nutrients and diet additives can positively affect the health and immune responses of fish through either modulation of the microbiota of the GIT or specifically nourishing immune cells. Nutritional modulation of the immune system continues to be a potentially powerful tool to improve the health and growth of cultured fish, and hence to improve production efficiency and yield. However, because of the great diversity of fish being cultured along with a lack of full understanding

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regarding the fish's immune system, immunonutrition is still not fully developed in aquaculture. This area does warrant additional research in order to reach its full potential and application. In particular, there is a considerable need to fine-tune dosing of various additives, optimize feeding regimes, and supplementation strategies so that immunonutrition can become more effective and used efficiently to benefit the production of various aquatic species.

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Uso de Isótopos Estables por Componente Especifico para Conocer los Requerimientos de Proteína en Peces Marinos

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Resumen

Uno de los grandes retos dentro de la fisiología del crecimiento en peces sigue siendo el conocer los requerimientos específicos de los nutrientes y en este caso de aminoácidos, para maximizar el crecimiento con un mínimo de ingestión de proteína y por ende, de pérdida de nitrógeno en el medio ambiente. La técnica de isótopos estables ha demostrado ser precisa dentro de la nutrición animal, para determinar el nivel de retención de la proteína de distintas fuentes en estudios previos dentro de nuestro laboratorio. No obstante que logramos determinar que fuente proteica es más retenida que la otra, aún nos queda conocer la relación proporcional de los aminoácidos retenidos y así conocer con mayor detalle los requerimientos pudiendo distinguir entre aquellos que se asimilan y retienen (enrutamiento). Existe una modificación de la técnica en donde las muestras son primero separadas por aminoácidos antes de pasar al analizador elemental acoplado al espectrómetro de masas (análisis de isótopos estables por compuestos específicos), y de esta manera se puede obtener la relación isotópica por aminoácido. El uso de los AA dentro del pez aún no se conocen con precisión para establecer los requerimientos exactos entre los que se retienen en los tejidos y los que son utilizados para las actividades metabólicas que es a lo que se refiere el enrutamiento o "*routing*" que nos lleva a la fisiología básica del metabolismo y crecimiento. De tal manera que cuando la proteína se ofrezca en exceso o restricción, el nivel de enriquecimiento será distinto. Es así que en el presente trabajo se presentan resultados obtenidos hasta ahora en donde se demuestra la capacidad de esta técnica para conocer con mayor detalle el uso y destino de los aminoácidos.

Palabras clave: Isótopos Estables, Enriquecimiento isotópico, proteína, componente específico

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Introducción

La substitución de harina de pescado (HP) es un problema prioritario dentro de la alimentación animal y particularmente en la nutrición acuícola, ya que se asocia como un ingrediente esencial para cubrir los requerimientos nutricionales de las especies de cultivo. Esto puede deberse al desconocimiento de los requerimientos puntuales por aminoácido para formular a proteína ideal y así obtener una eficiencia mayor de los nutrientes, con el fin de bajar el consumo de proteína cruda (Yamamoto *et al*., 2005 y Gaylord T. y Barrows F. 2009). El uso de la técnica de isótopos estables en masa o por aminoácidos (componente específico) permite obtener el nivel de retención proteica así como la retención y tasa de recambio para cada uno de los aminoácidos esenciales en los distintos tejidos (McMahon K. *et al*., 2010). Esta técnica, a diferencia de las evaluaciones productivas, permite el estudio de los requerimientos de manera puntual (Dalerum y Angerbjorn 2005; Martinez del Rio 2009). El uso de isótopos estables en estudios de retención representa una alternativa no invasiva, en donde la tasa de recambio de los isótopos en tejido se utiliza para determinar la contribución de las distintas dietas en individuos o poblaciones (Gamboa-Delgado y Le Vay, 2009). Esta técnica se basa en que todo elemento en la naturaleza está compuesto por una mezcla de isótopos que son átomos de un mismo elemento en los cuales varía la masa en el núcleo atómico. Los átomos ligeros son favorecidos energéticamente para ser utilizados antes que los pesados, lo que hace que a través de las cadenas tróficas los átomos pesados sean retenidos concentrándose en los organismos. Es así que utilizando ingredientes con distinta huella isotópica en experimentos de nutrición se puede conocer la proporción de lo que se retiene entre estos dos ingredientes. Si bien la retención del N es lo que más interesa al ser un elemento característico de la proteína, el contar con el valor isotópico del C nos permite hacer modelos de mezcla más sensibles, por lo que es importante contar con ambos en muestras desengrasadas si se quiere discriminar entre el C proveniente de la grasa y el de la proteína. En estudios anteriores utilizando los isótopos estables (Badillo *et al*., 2014a y b) se demostró que, tanto en trucha como en *Totoaba macdonaldi*, una mezcla de una parte de harina de pescado (HP) por tres de subproducto de ave (HA) en la porción proteica dio los

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mejores parámetros de crecimiento, en especial en totoaba, donde el crecimiento fue hasta tres veces mayor que los organismos alimentados exclusivamente con HP. Esta diferencia si bien no se pudo detectar mediante el uso de los isótopos estables en masa, se pudo observar que existe una tendencia a retener de manera más eficiente la HA sobre la HP.

Los isótopos estables han sido utilizados desde hace mucho tiempo como trazadores no invasivos para hacer estudios de ecología y, posteriormente, nutrición (Martínez del Río *et al*., 2009). Esta técnica permite estimar, dependiendo del nivel de enriquecimiento isotópico, el grado de retención o recambio para cada tipo de tejido estudiado, aplicando modelos matemáticos de mezcla y utilizando los valores isotópicos del tejido alimentado con distintas proporciones de las mezclas en relación a los valores esperados contenidos en el alimento, bajo el supuesto de que ambas fuentes son retenidas de manera similar (Martínez del Río y Wolf, 2005). Dichos estudios también sirven para estudiar la calidad de la proteína, efecto de la frecuencia alimenticia, entre otros.

Si bien el uso de los isótopos estables en masa muestra de manera integral el comportamiento de los ingredientes a partir de los datos obtenidos del enriquecimiento del total del ^{15}N en relación del ^{14}N, y entre el ^{13}C y ^{12}C, e incluso entre la relación isotópica del hidrógeno (^{1}H, ^{2}H y ^{3}H), existen hoy en día enfoques más finos. Uno es mediante la separación de la proteína y grasa en sus componentes, ya sea aminoácidos o ácidos grasos previo a la estimación del valor isotópico. De esta manera se estudia el enriquecimiento isotópico por componente, sea por aminoácido o ácido graso. Esta técnica se denomina por componente específico y constituye una manera más fina de estudiar cada uno de los aminoácidos y su enrutamiento metabólico dentro del organismo cuando es alimentado con distintas fuentes proteicas y sea por tipo o calidad. Esta técnica consiste en la separación de los aminoácidos antes de pasar al analizador elemental acoplado al espectrómetro de masas (análisis de isótopos estables por compuestos específicos), y de esta manera se puede obtener la relación isotópica por aminoácido. El destino y uso de los aminoácidos dentro del pez no se conocen aún con precisión para establecer los requerimientos exactos entre los que se retienen en los tejidos y los que son utilizados para las actividades metabólicas, que es a lo que se refiere el enrutamiento o "*routing*" que nos lleva a la fisiología básica del metabolismo y crecimiento. De tal manera que cuando la proteína se ofrezca en exceso o

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restricción, el nivel de enriquecimiento será distinto.

El presente trabajo tiene como objetivo el dar a conocer los avances de investigación de nuestro laboratorio en donde podremos evaluar la retención proteica en masa y por componente específico para lo cual se darán ejemplos de varios experimentos desarrollados en los ultimos dos años. El estudio se enfocó en totoaba (*Totoaba macdonaldi*) proveniente del CREMES y de ejemplares de jurel (*Seriola lalandi*) obtenidos del cultivo comercial de la empresa Baja Seas S.A de C.V ubicada en Baja California.

Metodología común

El diseño experimental en general se basó en una distribución al azar con un factor, cuatro tratamientos y tres repeticiones. Para esto se utilizaron lotes de juveniles de *Totoaba macdonalli* (3 ± 0.2 g) alimentados con una dieta comercial. Para cada experimento se colocaron cerca de 35 organismos en cada uno de los 12 estanques circulares con capacidad de 500 L (4 dietas con tres repeticiones en un diseño completamente al azar). Los organismos se alimentaron a saciedad aparente durante 60 días con cuatro raciones diarias o el tiempo necesario para obtener un 1000% de incremento en peso.

Para medir el nivel de optimización de los aminoácidos y establecer el nivel de enrutamiento de los mismos durante el periodo de inanición se utilizó al jurel (*Seriola lalandi*), por considerarlo un pez con una alta tasa metabólica (Booth *et al.*, 2010). Los organismos fueron proporcionados en primera instancia al Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), y posteriormente CICESE facilitó los organismos con los que se realizaron los experimentos descritos en el presente trabajo. Los peces se distribuyeron aleatoriamente en 4 estanques de 500 L. Uno de ellos fue alimentado 3 veces al día a saciedad aparente hasta los 35 días que duró el experimento, en tanto que los otros tres estanques se mantuvieron en inanición y bajo las mismas condiciones de calidad de agua y temperatura (26 °C). El muestreo se realizó de manera aleatoria en cada uno de los 4 estanques tomando 1 organismo cada 0, 2, 4, 6, 9, 12, 15, 19, 23, 27 y 35 días. Se tomaron muestras de hígado y músculo para medir el valor isotópico

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en masa y por componente específico. Este último se realizó sólo en las muestras de hígado. Después de 35 días los organismos en inanición llegaron a perder hasta 30% de su peso inicial. Los organismos fueron sacrificados por hipotermia según la norma de ética establecida. Los organismos fueron etiquetados y metidos a una tina con hielo para posteriormente ser disectados y extrer el hígado y una porción de músculo.

El contenido isotópico en masa del C y N ($\delta^{13}C$ y $\delta\ ^{15}N$) de ambos experimentos, además del análisis por componente específico de los aminoácidos de muestras de hígado y músculo se analizaron en la Universidad de Davis California (EEUU) de acuerdo a sus protocolos. Con los datos obtenidos se estimó el factor de discriminación trófica, el modelo Tiempo-Base (Hesslein *et al.* 1993), la tasa de recambio proteico (t_{50}) del N^{15} en el organismo así como la tasa de enriquecimiento por aminoácido.

Para estimar las diferencias significativas entre los índices biológicos de los tratamientos experimentales, se comprobó que los datos fueran normales y homocedásticos antes de proceder a un análisis de varianza de una vía y pruebas *a posteriori* de Tukey ($P < 0.05$) utilizando el programa Statistica 10.0 (Stat Soft, Inc. Tulsa, OK, USA).

En el experimento de *Totoaba macdoladi*, se demostró que los organismos que fueron alimentados con un contenido alto de proteína y lípidos (dieta "D") presentaron diferencias significativas en su peso final, en el porcentaje de peso ganado, eficiencia proteica, tasa de eficiencia alimenticia y la tasa de conversión alimenticia. Sin embargo, en el coeficiente térmico de crecimiento, no se presentaron diferencias con los organismos alimentados con la dieta "C" pero si con la dietas "A" y "B", mismas que contenían el nivel más bajo de proteína y lípidos. En lo que respecta a los isótopos de carbono y nitrógeno se demostró que los organismos que se alimentaron con un mayor contenido proteico, a los 12 días alcanzaron el equilibrio isotópico con su dieta en el hígado, realizando así un recambio isotópico más acelerado en este tejido. La diferencia en tiempo de recambio se da dependiendo del tejido, su actividad metabólica y etapa de crecimiento. Esto se corroboró en este mismo experimento, en donde se calculó el tiempo de recambio isotópico para

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músculo, mostrando un mismo comportamiento de acuerdo al incremento de proteína, sin embargo su equilibrio con la dieta fue alcanzado desde los 50 a los 61 días respectivamente.

En el enriquecimiento isotópico por aminoácido en hígado se muestra una distribución variante a lo antes reportado, sin dejar de mencionar que los pocos trabajos de isótopos por componente específico están realizados en músculo. Los aminoácidos esenciales deberían mostrar una huella isotópica idéntica a la de la dieta, teniendo en cuenta que estos no se pueden sintetizar *de novo* por los peces, por lo que adquieren el valor isotópico de la dieta proporcionada. Los aminoácidos que más se fraccionan son los no esenciales o mejor conocidos como aminoácidos tróficos, los cuales pueden ser sintetizados según su requerimiento. En el hígado de *totoaba* observamos que los aminoácidos esenciales son enriquecidos en isótopos pesados en las cuatro dietas proporcionadas. Sin embargo, con la dieta C y D se encontraron enriquecimientos mayores a 8‰ en isoleucina, valina y ácido glutámico. Este comportamiento inusual se pudiera estar dando por la actividad metabólica del tejido, por la proporción aminoacidica en la dieta y las necesidades nutricionales del pez. Los aminoácidos enriquecidos en los isótopos ligeros son mayormente utilizados antes que los isótopos pesados, por lo que pudimos observar que la huella isotópica fue enriquecienda en isótopos pesados.

En el experimento de inanición realizado con jurel, se demostró que en masa el nitrógeno en músculo no presentó cambios en la huella isotópica conforme avanzan los días en inanición. Sin embargo, en el hígado se nota claramente que a los dos días comienza a enriquecerse en isótopos pesados de 15‰ hasta 17.5‰ (35 días). En isótopo de carbono, se encontró un patrón similar para músculo, en el cual no cambia de manera significativa la huella isotópica del músculo inicial con la muestra de músculo a los 35 días en inanición. Sin embargo, para el hígado en este mismo isótopo, se muestra claramente que del día 4 al 12, el músculo se vuelve ligero y constante. Esto podría darse a medida que el hígado sintetizó *de novo* ácidos grasos o aminoácidos no esenciales. Sin embargo, posterior al día 12, el hígado comenzó a enriquecerse de isótopos pesados hasta alcanzar los -17‰. En lo que respecta a los isótopos estables por componente específico (aminoácido) en hígado, se

obtuvieron tres niveles de enriquecimiento respecto al hígado inicial e hígado final. Los aminoácidos que menos se enriquecieron fueron la metionina, lisina y fenilalanina mientras que en los no esenciales solo la glicina (0.5, 0.6, 1.2 y 1.4 respectivamente). Los aminoácidos mayormente enriquecidos fueron la isoleucina, leucina y valina como esenciales y prolina como no esencial (4.9, 4.4, 3.2 y 3.2). En los aminoácidos intermedios se obtuvo la alanina, glutamina y asparagina (2.4, 3.6 y 2.9).

Uno de los problemas que se enfrentan al formular las dietas con diferentes niveles de proteína, es que se cambian las proporciones de los diferentes ingredientes que la conforman para obtener un 100% de la dieta (por ejemplo al aumentar la proteína y disminuir el almidón para su formulación), dejando un contenido energético diferente o igual pero con diferentes porciones de proteína, lípido o carbohidrato. Por ello, en un experimento reciente con *Totoaba macdonaldi*, se formularon cuatro dietas con diferente contenido proteico (40, 43, 46 y 49) y mismo contenido de lípidos y carbohidratos, rellenado el déficit de las dietas con una mezcla de harina pollo/pescado (66 /33) tratada con formaldehido para fijar la proteína disponible y que no estuviera apta para la asimilación en el organismo. Se obtuvieron resultados en crecimiento con diferencias significativas, donde las dietas con mayor contenido proteico, C y D, presentaron un porcentaje de crecimiento del 1000% comparado con las dietas A y B, que solo obtuvieron el 600%. Mismo patrón se obtuvo en el la tasa especifica de crecimiento de 4.05, 3.89, 3.34 y 3.08 (D, C, B, y A respectivamente). Actualmente estamos en espera de los resultados de isótopos por componente, mismos que nos pudieran revelar los requerimientos nutricionales para dicha especie.

Después del análisis de los datos experimentales, se concluye que es posible utilizar la técnica de isótopos estables con mayor precisión a lo los resultados obtenidos de manera tradicional midiendo únicamente crecimiento y consumo de alimento.

La técnica de isótopos estables por componente (aminoácidos) tiene un gran potencial en la nutrición de peces, ya que nos revela un panorama más certero en el entendimiento de los procesos metabólicos de los aminoácidos esenciales y no esenciales

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en los organismos marinos y así poder establecer una dieta balanceada a proteína ideal.

El enriquecimiento isotópico de los aminoácidos en el tejido del pez, nos puede mostrar el grado de necesidad y biosíntesis *de novo* de los aminoácidos no esenciales. De igual forma, nos muestra el reciclamiento de estos aminoácidos, ayudándonos a entender las necesidades nutricionales de los organismos marinos de una manera específica.

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Optimizing Performance and Profit for Better Sustainability: A Review on Protease Application in Aqua Feed

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Abstract

Optimizing utilization of nutrients in aqua feeds by supplementing various dietary enzymes have recently been considered by nutritionists and formulators worldwide to combat fluctuating price, availability and quality of commonly used plant and animal proteins. Biological factors such as growing stage, age, species types, environmental factors such as temperature, pH and dissolved oxygen, and feed composition appeared to significantly affect endogenous enzyme production. A better understanding of these effects on digestive proteases will allow optimizing the use of supplementary enzymes, and in turn, will assist in better utilization of dietary nutrients.

The main objective of using dietary protease has been to compensate digestive enzymes to promote growth and efficiency of nutrient utilization and reduce nutrient excretion. Little focus has been paid to assessing their effects on improving digestibility of raw materials or feed quality or gut health and specific immune response. This review mainly digestive proteases, their interactions with various ingredients or diet composition in different fed species, use of dietary proteases and its implications on the industry in optimizing performance and profit.

Keywords: digestive enzyme; dietary protease; aquafeed; profit optimization;

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Introduction

The historical use of dietary enzymes started with the application of amylases and proteases to the diets of various farm animals for better productivity in 1950s. Since then, the use of enzymes has been expanded to other carbohydrases, phytates and recently, to lipase in terrestrial animals (1). The feed enzyme market is dominated by phytases and carbohydrases. However, as with the increasing cost of proteins, application of protease is on the rise specifically in poultry feed and expected to exceed those of phytase in the coming decade.

Application of protease in aqua feed is not new and the research endeavor can be traced back to 1977, when graded level of a commercially available bovine trypsin was used in common carp diets (2). Until recently, studies on application of dietary protease in aqua feed have been sporadic (3). Dietary protease can compensate the deficiency of endogenous enzymes especially for young animal, and assist in the breakdown of macromolecular proteins, which are difficult to digest. Until now, studies regarding exogenous protease supplementation in aquafeeds have been reported in rainbow trout (*Oncorhynchus mykiss*) (4, 5, 6, 7, 8,), tilapia (*Oreochromis aureus* × *O. niloticus*) (9, 10), common carp (*Cyprinus carpio*) (11), Atlantic salmon (*Salmo salar L.*) (12), and Pacific white shrimp (*Litopenaeus vannamei*) (9, 13, 14).

Digestion, a complex physiological process, depends on molecular activation, recognition and hydrolysis of food in specific conditions. During the process, the digestive enzymes break down polymeric macromolecules into smaller building blocks facilitating and enhancing nutrient absorption. Presence of proteases for protein, amylases for carbohydrate, and lipases for lipid digestion in various fish (15, 16) and shrimp (17, 18, 19) species has already been reported. Detail knowledge on digestive physiology of aquatic animals is a prerequisite when formulating feeds. There are great differences in specific activities of alkaline protease for various fish and crustacean species. And within a species, activity of specific enzymes showed great deal of variations during larval developments,

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food and feed composition and understanding these variations is of great importance for the development of artificial feeds (20).

This paper highlights dietary influence on digestive protease activity and effects of dietary inclusion of alkaline proteases on performance in various fed aquaculture species.

Digestive protease

Dietary protein level, protein sources, age or life-stage, all can affect digestive enzyme activity in aquatic animals. For example, significant changes in digestive enzymes of rainbow trout (*Oncorhynchus mykiss*) not in sea bream (*Sparus aurata*) were reported when dietary fish meal were gradually replaced by plan proteins (0% - PP0, 50% - PP50, 75% - PP75, and 100% - PP100) in their diets (21). Alkaline protease activity in trout fed the PP0 diet peaked at 3 h post-feeding (7.17 U protease/mg protein/min), showing a 3/36 h TPA (total protease activity) ratio of 2.07. Trout fed diets PP50 and PP75 showed only a slight post-feeding increase in this activity, with 3/36 TPA ratios of 1.43 and 1.40, respectively. PP100 fed trout did not register post-prandial peak, showing a 3/36 TPA ratio of 0.73. Similar to rainbow trout, a decreasing trend in total protease activity in intestine and hepatopancreas of hybrid tilapia (*Oreocromis niloticus* x *O.* aureaus) was reported when fed diets with increasing level of soybean meal, where 0%, 25%, 50%, 75% and 100% replacement of dietary fish meal (20%) were replaced in a recent study (27). The authors reported a significantly higher rate of decrease in activity in hepatopancreas (from 123 U/mg protein in those fed 0% SBM to 12 U/mg protein in 100% SBM fed fish) than in intestine (from 415 U/mg protein in those fed 0% SBM to 216 U/mg protein in 100% SBM fed fish). Dietary protein level might also influence enzyme secretion e.g., in tilapia fed 30% and 48% CP diets (23). However, under starving conditions, the differences are notable among various carp species reflecting their natural feeding habit and conditions (24, 25). But when fed a same diet, the differences in proteolytic activity among the same group of species could be minimal as reported in a study with silver carp and common carp (26).

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Among the various types of protease enzymes, trypsin, carboxy-peptidase A, carboxy-peptidase B, amino-peptidase, chymotrypsin, elastase like, collagenase, and esterase have been reported in major farmed crustacean species such as *Litopeneaus vannamei*, *Peneaus monodon*, and *Macrobrachium rosenbergii* (27). For all three species, trypsin activity tend to decrease significantly from early metamorphosis stages i.e., during zoea and mysis stages to the post larval stages and onward.

In crustaceans, molting stages also affect digestive protease activity. In a study with red shrimp *Pleoticus muelleri*, authors reported significantly higher trypsin and chymotrypsin activity during the post-molt stage (2.4±0.19 and 3.7±0.29 abs/min/ mg, respectively) than those during the inter-molt (0.9±0.0.6 and 1.2±0.10 abs/min/mg, respectively) or pre-molt (1.4±0.07 and 2.1±0.07 abs/min/mg, respectively) stages (28). In Pacific white shrimp, trypsin activity was also higher during post-molt stages. The higher trypsin and chymotrypsin activity during the post-molt stages observed in this study corresponds well with the higher nutritional requirement for growth during this period.

Dietary protease

In most cases, supplementation of dietary protease has been proved to improve production performance of various farmed species (4, 7, 8, 10). For example, supplementation of an exogenous protease (175 mg/kg) in pelleted diet containing 30 g/kg fish meal significantly increased weight gain and decreased FCR of tilapia (10). In another study, a mixture of proteolytic enzymes and carbohydrases in diet containing 339 g/kg soybean meal significantly improved the growth and feed efficiency of Atlantic salmon (12). In contrast, no effect on growth performance was observed in a study with rainbow trout (6) fed dehulled lupin-based diets supplemented with protease and carbohydrases alone or in combination.

In a recent study on tilapia, low fish meal diet (30 g/kg) supplemented with a protease complex (175 g/kg) showed significant improvement in ADC of DM and CP while

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no effects were observed in those fed the high fish meal (90 g/kg) diets (10). The more pronounced improvement in digestible nutrient concentration of a poorly digestible diet supplemented with a protease than the highly digestible diets are also evident in other species such as poultry (29). In another study (4), authors reported better feed efficiency of rainbow trout by the addition of protease in diet containing canola seed and pea, and was improved numerically in diets containing flax seed and pea. The authors also reported significant improvement in the ADC of CP, lipid, energy and dry matter for diets containing coextruded canola:pea when supplemented with protease but not for the coextruded flax:pea diets. The lack of positive effects on ADC of CP, crude lipid and gross energy was also reported in Nile tilapia fed diets supplemented with 1.5 g/kg enzyme complex containing neutral protease, β-glucanase and xylanase despite a higher ADC of DM in 1.0 g/kg group compared to those 0 g/kg group (9). These results implicate the variable affects of dietary protease based on their types, composition of various enzymes, as well as substrate types and their composition.

A challenge remains for the feed formulators whether to chose a single protease, a protease complex or multi-enzyme complex containing protease, phytase and carbohydrases. There are very few studies comparing the effects of various types of protease or protease complex or multi-enzyme complex on animals. In a recent digestibility study with rainbow trout comparing effects of a single protease and multi-enzyme complex on soybean meal, rapeseed meal and sunflower meal, authors reported significant improvement in ADC of CP in soybean meal when supplemented with the single protease (7). Interestingly, authors could not find any effect of single protease or multi-enzyme complex on the digestibility of the other two raw materials.

In shrimps, effects of dietary protease have been investigated on various species such as black tiger shrimp, *Peneaus monodon* (30) and Pacific white shrimp, *Peneaus vannamei* (13). In *P. monodon* fed graded levels of canola meal (20%, 54% and 64%), performance (weight gain and FCR) was improved only in those fed 20% canola meal diets supplemented with 0.25% protease compared to the corresponding non-supplemented diets.

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In *P. vannameii*, effects of graded level (0, 0.2 or 0.4%) of a commercial feed grade protease (ENZECO® Bromelain FG, EDC, NY) on ADC DM and ADC CP were tested. The ADC CP was signifcantly higher (74.3%) in shrimps fed 0.4% protease diets than those fed the control diets (65.3%). In the subsequent growth trial, authors reported a decrease in growth performance (weight gain, feed efficiency, and protein efficiency ratio) in shrimps fed the diets with protease. This negative response was more pronouced in high protease (0.4%) diets than those with a lower concentration (0.2%) of the enzyme.

In a recent study, growth performances of *P. vannamei* fed high fish meal diet (HFD) and low fish meal diet supplemented with a protease complex (LFD+P) were significantly better compared to those fed the low fish meal diets (10). Compared with the LFD group, the addition of protease (LFD+P) improved weight gain by 11.0% ($P<0.05$) and decreased FCR by 0.13 ($P<0.05$). In the same study, shrimps fed the HFD diet, or LFD+P diet had a higher activity of hepatopancreatic protease than those fed LFD diet ($P<0.05$). However, no significant difference was detected in intestinal protease activity among the groups ($P>0.05$).

Application of Protease

There are about 14 different types of alkaline proteases identified in animals that include but not limited to trypsin like, several chymotrypsin and elastase like, and carboxypeptidases. Each of these proteases has specific temperature and pH optima, and the ability to breakdown protein molecules vary from substrates to substrates. A list of preferred substrates for activity analysis of some of these enzymes are listed in table 1.

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Table 1. A list of preferred substrates for the activity assays of various alkaline proteases

Enzyme types	**Preferred substrate**
Chymotrypsin-like	Benzoyl-L-tyrosine ethyl ester (BTEE)
Carboxypeptidase A	Hippuryl-L-phenylalanine (HPA)
Carboxypeptidase B	Hippuryl-L-arginine (HA)
Leucino aminopeptidase like	L-leucine-p-nitroanilide (LAPNA)
Total protease	Azocasein
Serine protease	Benzoyl-DL-arginine *p*-nitroanilide (BAPNA)
Chymotrypsin	Succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAPNA) OR N-succinyl-L-phenylalanine-*p-nitroanilide* (Suc-Phe-p-Nan)
Amino peptidase	Aminoacyl β-naphthylamide (AA-NA) with Arg, Leu, Phe, Val, Lys as substrates.

The substrate specificity of each enzyme may well explain why a single protease was able to improve digestible nutrient content in soybean meal but not in sunflower meal or rapeseed meal (7). However, in a subsequent study (8), authors showed ability of the multi-enzyme to breakdown some non-starch polysaccharides. These types of information are very useful to improve our understanding on protease enzymes and variations in their effects on different substrates. For example, a single protease working very well on soy-based diets may well not be able to exert similar effects on diets predominantly containing other protein sources. On the other hand, a multi-enzyme complex containing a protease may not be able to exert the same effects as a single protease or a protease complex.

Conclusion

This review highlights the need for better understandings of digestive enzyme types and their activity as ingredients, diets, age, size, molting stage, culture conditions, and pH,

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temperature and dissolved oxygen of the culture environment can significantly influence their specific activity. The range of digestive enzymes specifically proteases is diverse. Composition of these enzymes and their interactions with environment and diets influence digestive capacity and immune response of the animals. Further studies are recommended to enhance our knowledge on digestive enzymes and their relationships with modern diets, culture systems and environment. Total protease activity or activity of various protease enzymes may also vary significantly based on analytical methods chosen for example, substrates or inhibitors used and incubation parameters such as pH, temperature and duration.

Dietary enzymes are being considered one of the available solutions to improve quality of feeds. Supplementation of enzyme can improve gut health, compensate digestive enzymes when needed, and may also improve immune responses. However, feed manufacturing industry remains skeptical because of concerns on heat-stability of the enzymes, interactions with other enzymes, safety of recommended dosages, and lack of knowledge of suitable substrates for each type of enzyme.

It is important to understand that each specific enzyme usually attack specific active sites in a complex molecule. With the omnipresence crisis for quality raw materials, an industry wide understanding of dietary enzyme application in aqua feed has become essential. Further research is needed to understand the effects of different types of dietary proteases on improving the quality of proteins in feed, their interactions with various ingredients, gut health, and nutrient utilization. Appropriate application of dietary proteases available in the market has also been an issue because of the concerns on heat stability and fear of their destruction during manufacturing. Some better heat stable enzymes are available in dry powder form, can be readily added to the mixer, and can keep majority of their activity intact. The rests are available in liquid form to be applied after pelleting requiring specific mechanical tools. We need to better understand advantages and disadvantages of the various application methods when selecting an enzyme. Ability to apply directly to the mixer may have several advantages. In addition to the ease of

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handling, the protease may also alter the quality of feed proteins during cooking or conditioning resulting in better quality feed. We are still at infancy in our understanding of the complexity of the protease world. A renewed and targeted focus in our research agenda is recommended to improve our understandings.

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Lipid Nutrition Research for the Development of Practical Commercial Diets for Corvina and Alternative Ingredients for Aquafeeds

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Abstract

In the state of Sonora, Mexico, several local sciaenids are promising options to diversify the local aquaculture and venture into the marine fish culture industry; however, cost-effective aquafeeds for the development of this farming business are required. Aquafeeds for carnivorous fish, like sciaenids, typically contain high levels of fish meal and fish oil, but the high prices of these dietary ingredients, well above $2000.00 US dollars per ton (aqua grades) in January of 2015, makes the use of high inclusion levels unprofitable and unacceptable. Research work with sciaenids such as the Gulf corvina at the Nutrition Laboratory of the Department of Scientific and Technological Research of the University of Sonora has focused on establishing the minimum dietary lipid requirements as well as the replacement of fish oil, as a starting point. In addition, alternative oil and protein sources, such as ray fish liver oil and tilapia by-products silage, are being explored and evaluated using a sustainable approach. Ray fish constitute an important fishery in the Mexican shoreline, but only their meat is consumed, the rest is discarded. Their liver however, is a rich source of lipids and essential fatty acids. Recent studies showed high lipid levels (30.67-46.41%) in 5 ray species distributed in Sinaloa, Mexico, with a high proportion of unsaturated fatty acids (58-69%), and considerably high levels of eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (EPA+DHA= 4.79-14.47 g/100 g of liver oil), thus, ray fish liver oil can be considered an interesting alternative to fish oil as a source of EPA and DHA. Furthermore, aquaculture and fisheries are activities that generate tonnes of by-products. For tilapia, about 30% of the weight is recovered as fillet; the remaining by-products can be processed for the production of

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silage through fermentation in the presence of *Lactobacillus* spp. and molasses as a carbohydrate source, which, in turn, can be incorporated as an ingredient in diets for other cultured aquatic organisms, a viable and environmentally friendly usage of discarded remnants of tilapia to generate a value added fish by-product for commercialization. These are some of the first steps leading towards the diversification of the aquaculture industry in Sonora.

Keywords: Marine fish culture, Sciaenids, lipid dietary requirements, fish oil replacement, by-products.

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I-Introduction

The culture of marine fish species on a large scale has been a very successful economic activity in Europe for several decades now (FAO, 2014) and include a number of species such as salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*) and turbot (*Psetta máxima*), among others. Currently, the prices per kilogram in Europe for each of these species are $25.31 €/Kg (salmon fillet), $14.03 €/Kg, $15.45 €/Kg, and $45.20 €/Kg, respectively (http://thecornishfishmonger.co.uk; August 10, 2015). According to FAO (2014), finfish mariculture reached a world production volume of 5,551,905 tonnes in 2012, which represent only 12.6 percent of the total farmed finfish production by volume, but their value (US$23.5 billion dollars) represents 26.9 percent of the total value of all farmed finfish species.

In Mexico the culture of marine fish is emerging and it has great potential for the diversification of the species currently cultured on a commercial scale, coupled with the fact that it is a profitable choice for aquaculture producers, who, particularly in the state of Sonora, are mainly focused on shrimp culture. The shrimp culture industry has suffered a major drawback due to the manifestation of the white spot syndrome virus (WSSV) and the early mortality syndrome (EMS), and from 81,422.8 tonnes produced in 2009, only 32,616.0 tonnes were produced in 2014 (COSAES, 2014). Therefore, the mariculture of marine fish species presents itself as a promising and lucrative alternative to diversify this economic activity.

Among the marine finfish available within the Gulf of California with aquaculture potential are some species from the family Scianidae, commonly called drums or croakers because of the repetitive drumming sound they produce with the air bladder (Nelson, 1994). There are at least 30 known species belonging to the family in this area (Van der Heiden, 1985), and 3 of them are endemic to this region, the bigeye croaker *Micropogonias megalops*, totoaba *Totoaba macdonaldi*, and the Gulf corvina *Cynoscion othonopterus* (Thompson y McKibbin, 1978).

The Gulf corvina is a heavily overfished sciaenid, the second most important fishery in the Northern Gulf of California near the Colorado River delta, where it is captured

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during spawning aggregations, thus, it is listed as a vulnerable species but its capture is overseen by the norm NOM-063-PESC-2005, which establishes a yearly closed fishing season from May 1st to August 31st, and 65 cm as the legal minimum capture length during the open season (Castro-González, 2011). A catch quota of 3,620 tonnes was established for the 2013-2014 season by SAGARPA-INAPESCA (2013), less than the 3,727 tonnes registered capture for 2010 (Carta Nacional Pesquera, 2012).

Corvinas are appreciated in the regional and international markets for their firm mild-flavor white meat. Although the price in the local market is low, close to MXN$16.00 pesos per Kg during the first trimester of 2014 (http://datamares.ucsd.edu/eng/projects/fisheries/kilos-and-pesos-interactions-between-fisheries-catch-and-market-value/; August 11th, 2015), the international market for other corvinas is much better, average price per kilo for fillet is US$10-12 (http://eatwineblog.com/2006/03/24/debunking-the-chilean-seabass-myth/; August 11th, 2015).

Unlike other marine finfish, the Gulf corvina reaches sexual maturity at a relatively early size and age, at approximately 2 years old or less. This is a fortunate advantage because individuals can become the broodstock in a short period of time at a lesser cost, and they may be kept within smaller tanks or less expensive infrastructure, also implying a lesser cost for the mass production of fry. There is regional awareness and interest in pursuing the culture of corvina in Sonora, hence, research work with the Gulf corvina at the Nutrition Laboratory of the Department of Scientific and Technological Research of the University of Sonora (DICTUS) has focused on establishing the minimum dietary lipid requirements as well as the replacement of fish oil, as a starting point (Minjarez-Osorio *et al.* 2014; González-Félix *et al.* 2015; Maldonado-Othón, 2015). This research is essential to limit the utilization of fish oil in aquafeeds developed for these species.

Up until now, fish oil is the most important source of the essential fatty acids for aquafeeds, particularly eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acid. However, the sustained increment in fish oil demand over the last years and the projected increment in the near future, together with the limited availability and high cost, have driven the search for alternative sources of these essential fatty acids (González-Félix

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et al. 2010). An alternative oil source explored is ray fish liver oil. Ray fish constitute an important fishery in the Mexican shoreline, but only their meat is consumed, the rest is discarded. Their liver however, is a rich source of lipids and essential fatty acids, such as EPA and DHA, thus, ray fish liver oil can be considered an important and interesting alternative to fish oil (Ould el Kebir *et al.* 2017; Navarro-García *et al.* 2004a,b, 2014).

On the other hand, alternative protein sources for aquafeeds also have one of the most important research topics for some time now. An interesting alternative for fish meal replacement is the utilization of by-products from the fish-processing industry (e.g. heads, tails, guts, fins, bones and/or skin). It is estimated that about 25% of the total fisheries and aquaculture production is discarded. Only a small share of by-products is used for human consumption, the rest is used for production of fish meal, silage, and other goods (Venugopal, 1995). Taking into consideration that the amino acid profile and digestibility of fish proteins are exceptional, it would be valuable to intensify the use of the protein fractions present in fish by-products, for example, for the production of silages, peptide and amino acid concentrates, surimi, collagen, gelatin, etc. (Rustad, 2007). The preparation of silages is one of the most popular uses for animal by-products (Gildberg, 2002); their manufacture from aquacultural by-products, as well as their use in aquafeeds for several cultured species is under study. This work presents current research in aquaculture nutrition developed at the University of Sonora for the Gulf curvina, as well as research on alternative ingredients produced from by-products for the replacement of fish oil and fish meal in aquafeeds.

II-Dietary lipid requirements of the Gulf corvine

The Gulf corvina, *Cynoscion othonopterus* (Figure 1) supports one of the most important seasonal commercial fisheries in northwest Mexico, and it is currently a potential aquaculture candidate in the state of Sonora. Early studies (Perez-Velazquez *et al.*, 2015) demonstrated that organisms near 100 g of individual weight and up to market size, could grow well on diets with 40% crude protein (CP) and high lipid levels, close to 16%, although the optimal dietary lipid level had not been accurately determined. Gonzalez-Félix *et al.*

González, M. *et al*. 2015. Lipid Nutrition Research for the Development of Practical Commercial Diets for Corvina and Alternative Ingredients for Aquafeeds. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 308-323.

(2015) carried out a 56-day study at the Wet Nutrition Laboratory of Kino Bay Experiment Station (KBES, DICTUS) located in Kino Bay, Sonora, Mexico, to evaluate the dietary lipid requirement of juvenile Gulf corvina raised in a recirculation aquaculture system.

Diets with an iso-proteic dietary content of 40% evaluated the effects of incremental levels of dietary crude fat at levels of 2, 5, 8, 11, 14, 17, 20, 23 and 26%, on the performance of *C. othonopterus* juveniles, with an initial mean body weight of 32.86 ± 0.48 g. Fish were reared in a clear-water recirculating culture system (Figure 2), composed of 48 circular tanks of 250 L (0.4 m^2 bottom area) filled with 200 L filtered seawater, at a density of 3 fish $tank^{-1}$. Each treatment was assigned to five replicate tanks. Fish were fed approximately 3% of their wet body weight daily and the daily ration divided into three equal portions. Overfeeding was minimized while maintaining the feeding rate close to apparent satiation.

Figure 1. Gulf curvina, *Cynoscion othonopterus*.

A clear dose-response effect of dietary crude fat was observed on growth of the Gulf corvina, with the best results corresponding to fish fed 11% crude fat, while growth performance was reduced as dietary crude fat departed from this level. These results were significant for specific growth rate and thermal growth coefficient data (P = 0.0283 and 0.0450, respectively), and although not statistically significant, the same numerical pattern was observed for additional growth response variables measured (Table 1).

González, M. *et al*. 2015. Lipid Nutrition Research for the Development of Practical Commercial Diets for Corvina and Alternative Ingredients for Aquafeeds. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 308-323.

Figure 2. Indoor clear-water recirculating culture system at the Wet Nutrition Laboratory of Kino Bay Experiment Station, DICTUS.

A quadratic broken line analysis of the thermal growth coefficient data estimated a requirement for dietary crude fat of 11.4% for this species when fed a diet containing 40% crude protein, with a 95% confidence interval of 9.8 to 13.0%. Significantly increased lipid deposition and reduced moisture content for muscle and whole body were observed in response to incremental levels of dietary crude fat. This study provided the first documented data on lipid nutrition of the Gulf corvina.

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Table 1. Growth performance of *C. othonopterus* fed graded levels of dietary lipid. After González-Félix *et al.* 2015.

Dietary Lipid (%)	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%/d)	TGC
2	31.3 ± 1.1	42.7 ± 3.9	11.7 ± 2.6	0.6^{ab} ± 0.1	0.025^{ab} ± 0.004
5	32.3 ± 1.1	49.0 ± 2.8	16.7 ± 2.5	0.7^{a} ± 0.1	0.034^{a} ± 0.004
8	33.9 ± 2.0	51.1 ± 2.7	17.2 ± 1.5	0.7^{a} ± 0.1	0.034^{a} ± 0.003
11	33.7 ± 1.6	55.4 ± 6.8	21.8 ± 6.2	0.9^{a} ± 0.2	0.041^{a} ± 0.009
14	33.3 ± 1.6	49.2 ± 0.5	15.9 ± 0.8	0.7^{a} ± 0.1	0.029^{ab} ± 0.003
17	30.7 ± 1.0	48.7 ± 0.7	18.0 ± 1.5	0.8^{a} ± 0.1	0.038^{a} ± 0.003
20	32.7 ± 1.1	45.8 ± 3.0	13.1 ± 2.5	0.6^{ab} ± 0.1	0.027^{ab} ± 0.005
23	32.8 ± 2.0	45.1 ± 4.8	12.2 ± 3.1	0.5^{ab} ± 0.1	0.025^{ab} ± 0.005
26	34.4 ± 2.0	42.4 ± 3.9	8.0 ± 2.4	0.4^{b} ± 0.1	0.016^{b} ± 0.004
External	31.9 ± 1.7	48.4 ± 2.5	16.5 ± 2.2	0.7 ± 0.1	0.034 ± 0.004
ANOVA $Pr > F$ [1]	0.7563	0.3270	0.1040	0.0283	0.0450

Values are means ± standard error of five replicate samples.

Means with different superscripts are significantly different ($P \leq 0.05$).

[1]Not included in the statistical analysis.

Abbreviations: SGR = specific growth rate; TGC = thermal growth coefficient.

An additional 56-day study was conducted to evaluate the effects of three levels of dietary CP (40, 45, and 50%) and three levels of crude fat (CF: 8, 12, and 16%) on the growth performance and body compositions of *C.* juveniles (initial mean body weight of 102.6 ± 14.1 g) using a 3 x 3 factorial experimental design (Perez-Velázquez *et al.*, 2015). The levels of dietary CP and CF tested, or their interaction, did not influence significantly the various growth responses evaluated (*e.g.*, final weight, weight gain, thermal growth coefficient-TGC, or survival; Table 2). However, in muscle tissue, increased crude fat deposition was observed in response to increasing levels of this nutrient, as described in the previous study. However, the content of CP and ash decreased significantly with both dietary CP and CF. In turn, moisture content increased significantly with dietary crude protein, from 71.2% ± 2.5 (at 40%

CP), to 75.9% ± 1.4 (at 50% CP), but it was not affected by dietary CF. However, no significant of the CP × CF interactions were observed on any of components of the proximate composition of fish muscle tissue.

It was suggested that, apparently, 40% dietary CP was sufficient to promote adequate growth of the Gulf corvina. This species tolerated the manipulation of dietary levels of CP (40-50%) and CF (8-16%), without compromising its growth or survival, while storing significantly more crude fat in muscle tissue with increasing dietary lipid. In addition, increased protein deposition in muscle tissue was observed in response to increasing dietary crude protein (Perez-Velázquez *et al.*, 2015). Additional research is necessary to evaluate if the dietary protein requirements of the Gulf corvina might be below 40%, and to further elucidate the extent of the protein-sparing effect of dietary CF.

III-Alternative ingredients for aquafeeds

The Gulf of California is an important ray fish fishing area in Mexico; particularly the state of Sinaloa has a high diversity of elasmobranchs (CONAPESCA, 2010). The ray fish species incidentally captured in shrimp trawlers include the families Urotrygonidae, Myliobatidae, Narcinidae, Rhinobatidae, and Rhinopteridae. Most of the ray fish species are underutilized; only the meat is used and its price depends on its color (Navarro *et al.*, 2004a, b). No information is available on the liver quality and oil yield of the captured species that would encourage the utilization and commercialization of this resource in this area. However, reports (Ould El Kebir *et al.*, 2007) confirm that ray fish liver oil of three species from the Republic of Mauritania have concentrations of EPA and DHA ranging from 1.88-5.01% and 10.00-13.04%, respectively. In Mexico Navarro *et al.* (2004a,b) reported that liver of some rays from the Gulf of California represents between 5 and 11% of the animals' wet weight, and the lipid content of the liver corresponds to 50% of that weight. The liver oil had 16-18% of EPA+DHA.

González, M. *et al*. 2015. Lipid Nutrition Research for the Development of Practical Commercial Diets for Corvina and Alternative Ingredients for Aquafeeds. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 308-323.

Table 2. Growth performance and survival of *C. othonopterus* fed different levels of dietary crude protein and crude fat. After Perez-Velazquez *et al.* 2015.

Main effects means	Final weight (g)	Weight gain (g)	TGC	Survival (%)
Crude protein (CP, %)				
40	183.3 ± 22.8	76.3 ± 17.3	0.060 ± 0.012	100 ± 0
45	169.7 ± 26.7	69.8 ± 14.7	0.057 ± 0.008	100 ± 0
50	173.5 ± 21.4	73.5 ± 19.3	0.060 ± 0.015	100 ± 0
Crude fat (CF, %)				
8	172.5 ± 29.1	71.3 ± 16.3	0.058 ± 0.010	100 ± 0
12	172.0 ± 18.3	67.5 ± 16.0	0.055 ± 0.012	100 ± 0
16	182.1 ± 23.3	80.9 ± 17.0	0.064 ± 0.012	100 ± 0
ANOVA *Pr* > *F*				
Crude protein	0.3210	0.5807	0.7485	[b]
Crude fat	0.4667	0.1030	0.0632	[b]
CP × CF	0.9972	0.8313	0.5250	[b]

[a]Values are means ± standard deviation of five replicate samples.

[b]100% survival was recorded for all experimental units. Hence, there was no variability of data.

Three more species from the state of Campeche, in the Gulf of Mexico, *Rhinoptera bonasus, Aetobatus narinari* and *Dasyatis americana*, showed an oil yield of 43.0, 41.2 and 38.2% of the liver wet weight, respectively. The highest sum of eicosapentaenoic, docosahexaenoic and docosapentaenoic n-3 LC- PUFA were found in *R. bonasus* (22.4%) and *D.* (21.6%) (Navarro-García *et al.*, 2004a, b). Thus, a study to analyze the lipid content and fatty acid composition of the liver oil from *Urotrygon chilensis, Urobatis halleri, Rhinobatos glaucostigma, R. steindachneri* and *D. dipterura* captured in Sinaloa, México, was carried out.

Samples of *U. chilensis* (20), *U. halleri* (20), *R. glaucostigma* (5), *R. steindachneri* (3) and *D. dipterura* (7) were obtained from experimental shrimp trawl surveys conducted in Teacapan, Sinaloa. The livers were dissected, placed in polyethylene bags and frozen at -20°C for their transportation in coolers to the DICTUS, where they were stored at -80°C until

lipid extraction (Navarro-García *et al.*, 2014). Total lipid content in their liver was relatively high, ranging between 30.67-46.41%, the highest amount observed in *D. dipterura* (Table 3). All of these species showed important concentrations of poly and highly unsaturated fatty acids, particularly EPA and DHA, around 4.79-14.47 g/100 g of liver oil of EPA+DHA (Table 4). EPA and DHA have a significant nutritional value in aquafeeds, being essential for many marine finfish and crustaceans cultured around the world. Therefore, ray fish liver oil can be considered an important and interesting alternative to fish oil as a source of EPA and DHA.

Table 3. Total lipid in liver of various ray fish. After Navarro-García *et al.* 2014.

Species	Lipid (%)
U. chilensis	$36.18^{ab} \pm 10.18$
U. halleri	$36.27^{ab} \pm 7.51$
R. glaucostigma	$31.83^{b} \pm 10.27$
R. steindachneri	$30.67^{b} \pm 4.84$
D. dipterura	$46.41^{a} \pm 4.98$

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Tabla 4. Fatty acid composition of liver oil from *U. chilensis*, *U. halleri*, *R. glaucostigma*, *R. steindachneri* and *D. dipterura* (g/100 g of liver oil). After Navarro-García *et al.* 2014.

	U. chilensis		***U. halleri***		***R. glaucostigma***		***R. steindachneri***		***D. dipterura***	
Fatty acid	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
16:0	15.28	4.17	16.70	4.94	15.20	1.76	4.74	1.11	23.44	10.85
18:0	10.71	2.93	7.21	1.95	6.48	0.66	3.10	0.87	8.41	3.24
18:1	6.24	1.78	12.37	3.38	8.15	1.30	2.86	1.13	17.61	6.96
18:2n-6	1.73	0.40	1.79	0.63	1.44	0.31	0.61	0.27	2.12	1.04
18:3n-3	0.39	0.11	0.39	0.32	0.32	0.09	0.09	0.06	0.31	0.18
20:1	2.27	0.53	2.05	0.58	0.96	0.17	1.02	0.24	2.50	1.02
20:4n-6	3.24	0.85	3.10	0.87	2.86	1.21	1.30	0.24	2.88	0.95
20:5n-3	5.41	1.54	5.09	1.75	3.28	1.20	0.99	0.18	4.33	1.42
22:5n-3	2.27	0.41	2.21	0.62	1.21	0.23	1.10	0.41	1.85	0.55
22:6n-3	8.55	1.66	8.37	3.22	11.19	1.82	5.70	2.84	4.73	1.44
24:1	3.02	0.58	2.29	0.59	0.73	0.09	1.52	0.40	2.44	0.73
24:2	2.02	0.38	1.69	0.66	1.43	0.32	2.29	0.41	1.07	0.35

An additional activity generating tonnes of by-products from aquaculture and fisheries results from gutting, heading, and filleting fish. The term by-products is used to indicate something that can be utilized, and is usually referred to all the raw material, edible or inedible, left during the production of the main product. When producing fish fillets, the left products or fractions are fillet cuts, backbone, head, liver, gonads and guts, they are all by-products (Gildberg, 2002). For tilapia, *Oreochromis niloticus*, about 30% of the weight is recovered as fillet; the composition of the remaining by-product fractions after filleting a sample of thirty organisms of an average size of 643.3 ± 4.8 g and 29.6 ± 3.5 cm is shown in Table 5. Proximate composition of these by-product fractions was analyzed. Skin (43.53 ± 6.28%) and backbone (42.56 ± 2.85%) showed the largest protein content, probably because of the leftover muscle adhered to these tissues. In contrast, the gut showed the lowest protein content (16.1 ± 2.01%) (Table 6).

Table 5. Composition (%) of the by-product fractions obtained after filleting thirty tilapias (*O. niloticus*).

By-product	Percentage (%)
Head-tail	31.0 ± 2.1
Backbone	21.3 ± 1.7
Skin	9.5 ± 1.6
Gut	8.5 ± 1.9

Tabla 6. Proximate composition of by-product fractions of tilapia (*O. niloticus*).

By-product fraction	*Moisture (%)*	*Ash* (%)*	*Crude* Protein (%)*	*Crude Fat* (%)*
Fillet	75 ± 1.0	0.35 ± 0.07	71.73 ± 8.40	12.73 ± 2.20
Gut	57.3 ± 2.3	0.21 ± 0.03	16.1 ± 2.01	68.26 ± 4.22
Backbone	61.6 ± 1.2	1.36 ± 0.25	42.56 ± 2.85	39.46 ± 9.05
Skin	63.3 ± 1.5	0.15 ± 0.05	43.53 ± 6.28	51.76 ± 6.81
Head-tail	60.0 ± 1.7	1.43 ± 0.31	33.73 ± 1.79	42.4 ± 2.36

Values are means ± standard deviation of three replicate samples.*Dry matter basis.

Additionally, the fatty acid profile of these by-product fractions was analyzed. Palmitic acid (16:0) was the most abundant saturated fatty acid in all fractions, oleic acid (18:1n-9) was the most abundant monounsaturated fatty acid, and linoleic acid (18:2 n-6) the most abundant among the polyunsaturated fatty acids. The gut fraction demonstrated to be a good source of fat, with an n-6/n-3 ratio equal to 4.67. A light brown silage with a doughy consistency of pH below 4.5 was obtained from a homogenate of all by-product fractions through fermentation in the presence of *Lactobacillus* spp. and molasses as a carbohydrate source. It contained approximately 41% crude protein and 10% crude fat. This silage can be incorporated as an ingredient in diets for other cultured aquatic organisms, such as catfish (*Ictalurus punctatus*) for instance, where an inclusion level of 5% proved to be best for

promoting optimal growth, coinciding with other reports for the inclusion of silages or hydrolysates in balanced feeds (Forster *et al.*, 2011; González-Félix *et al.*, 2014). Incorporating these processed by-products in balanced feeds as a source of very digestible small peptides and amino acids may have beneficial effect on growth of cultured organisms, but their inclusion level requires evaluation. All the same, the manufacture of silage from by-products has demonstrated to be a viable and environmentally friendly usage of discarded remnants of tilapia to generate a value added fish by-product for further commercialization.

Conclusions

The opportunities to diversify the aquaculture industry in the state of Sonora with the development of marine fish mariculture are promising. Sciaenid species such as the Gulf corvina are interesting options, since corvinas are already well accepted in the national and international market. Developing cost-effective aquafeeds, while searching for alternative sources of protein and oil for fish meal and fish oil replacement is a necessary task. By-products from fisheries and aquaculture, for example, ray fish liver oil, which can be considered an important and interesting alternative to fish oil as a source of the essential fatty acids EPA and DHA, or the use of the fractions obtained from the tilapia filleting, which can be processed into silages and incorporated into balanced feeds as a source of very digestible small peptides and amino acids, are sustainable approaches to do so, and may also generate a value added fish by-product for further commercialization.

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Advances in Understanding of Taurine Functions in Fishes Across Species and Life Stages

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Abstract

Taurine is now widely recognized as an essential nutrient in many teleost species, and during the past decade investigations have focused on determining quantitative requirement levels and physiological and metabolic responses to dietary taurine. Although the current state of knowledge is biased toward high-value marine carnivorous species, evidence points to functional differences among species (e.g., bile salt conjugation, osmoregulation, membrane stability). Prediction of the qualitative or quantitative requirement based on ecological boundaries is difficult, although trophic level seems to be a better predictor even if several exceptions exist. Thus caution must be exerted when assuming the qualitative or quantitative taurine requirement in a given species. Additionally, a number of studies highlight changes in the quantitative requirement between life stages, particularly in larval stages. If knowledge of taurine functions and potential technological uses in larval stages is limited compared to juvenile stages, it is even scarcer in reproducing broodstocks. Consequently, the first part of this paper reviews the current understanding of the species- and life stage-dependent differences in in taurine function and requirement levels. In a second part, initial experimental results obtained in California yellowtail *Seriola lalandi* and Florida pompano *Trachinotus carolinus* broodstocks are presented. While the crucial importance of essential fatty acid in egg quality and overall reproduction performances needs no additional proof, results highlight the importance of proteins as well. In this context, not only were the total amount of protein and amino acid levels correlated with hatching success, but results also suggest the relationship between urea cycle and survival to 1st feeding in the newly hatched larvae.

Keywords: Taurine function, *Seriola lalandi, Trachinotus carolin*

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1. Introduction

Taurine had largely been considered a dispensable amino acid in teleosts until evidence accumulated in the late 1990s and early 2000s that this β-amino acid was indeed an indispensable nutrient for many species. Initial studies investigated the reasons explaining the superior performances of fish larvae when fed wild copepods compared to those fed traditionally-enriched rotifers and *Artemia* (Conceicao *et al*., 1997; Shields *et al*., 1999), and identifying the taurine content as a major difference between the two types of prey (Aragao *et al*., 2004; Helland *et al*., 2003). In the following years, the essentiality of taurine was further demonstrated when taurine supplementation to otherwise taurine-poor feeds successfully restored growth and survival in larvae and juveniles (Chatzifotis *et al*., 2008; Kim *et al*., 2005; Lunger *et al*., 2007; Rossi Jr and Davis, 2012; Salze *et al*., 2011; Salze *et al*., 2012a).

Concerns in environmental and economic sustainability of aquaculture feeds have been driving the vast research effort toward the replacement of fishmeal with other sources of protein. However, taurine is found in relatively large quantities in fishmeal (provided adequate ingredient processing), while mostly-used alternatives such as soybean products are practically devoid of it. Consequently, taurine must be supplemented to the diet when low-taurine ingredients are used; possible source include krill meal, other animal proteins such as poultry by-product meal, or crystal taurine. Knowledge of the taurine requirement's existence allowed the further reduction of fishmeal and other animal proteins sources as dietary ingredients, thereby improving the sustainability of fish feeds.

Differences in taurine requirement among species is evident (Salze and Davis, 2015). Most of the taurine-related studies have been conducted in marine species living in warm waters, although some have focused on cold and/or freshwater species. Some

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evidence also points to changes in requirement according to life stages. This study will first review the available information regarding differences in taurine requirement among species and life stage. Information regarding the latter is currently limited, hence in a second part experimental data pertaining to taurine supplementation in broodstock diet will be presented.

1 Essentiality & ecology – Prediction of requirement in juveniles

The quantitative or qualitative requirement for taurine has been evaluated in a number of species (Salze and Davis, 2015). Many marine carnivores were found to require taurine in their diet, in contrast with most freshwater species. However, the essentiality of taurine does not reliably follow broad, ecological categories, such as salinity (e.g., common carp vs. channel catfish vs. white seabass) or temperature (e.g., red tilapia vs. Florida pompano) gradients. Figure 1 shows that the taurine requirement does not exactly follow trophic level either: while a cluster of species seems to follow a linear relationship between trophic level and quantitative taurine requirement, other species markedly depart from this paradigm, e.g., Nile tilapia and channel catfish.

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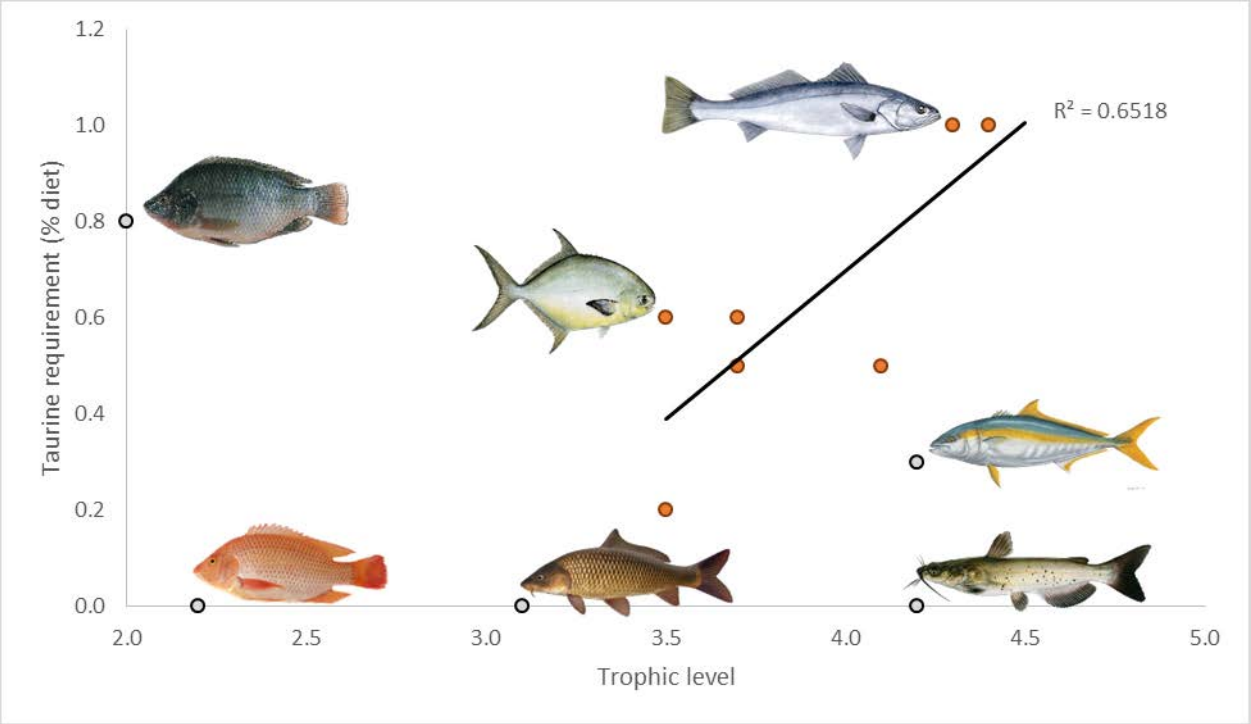

Figure 1: Correlation between taurine quantitative requirement and trophic level in teleosts

This shows that one should be cautious when predicting the essentiality of taurine based on ecological and environmental characteristics.

2 Taurine function among species

Many physiological functions have been attributed to taurine in mammals. However, knowledge of taurine physiology and metabolism in teleost is limited, and to which extent these functions are conserved between mammals and teleosts has not yet been studied in depth. Functional differences between teleost species are possible. Indeed, taurine would not be expected to be found in the diet of an animal to which taurine is not essential, and therefore may have different functions. For example, in contrast with most

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other teleosts, the bile of cyprinids such as carp and zebrafish is mostly made of bile alcohols, which conjugate with sulfate instead of taurine or taurine derivative. Therefore, taurine does not participate in the solubilization of lipids for digestion in these species. Other interspecific differences may be found as the functions of taurine in fish are further investigated.

Depending on their natural environment, different species are exposed to changes in salinity of various magnitude and frequency; hence their need to cope with changes in environment osmolality is different. In carp and tilapia expression of the taurine transporter is also significantly upregulated in response to increase is osmolality, but an increase in cellular taurine was observed (Takeuchi *et al*., 2000a; Takeuchi *et al*., 2000b) concomitantly with a decrease in plasma taurine (Assem and Hankf, 1979), thereby strongly suggesting that taurine is a major osmolytes in these species. Moreover, dietary taurine supplementation was shown beneficial for acute hypoxia tolerance in carp through a reduction in hemolysis rate (Yang *et al*., 2013). When subjected to hyperosmotic stress, salmon branchial cell also upregulated the expression of the taurine transporter, suggesting a role of taurine in osmoregulation in this species. However, taurine supplementation does not result in improved survival despite being cultured on taurine-free media. It is possible that other osmolytes present in the culture media (e.g., inositol) fulfilled the osmoregulation need in the absence of taurine (Zarate and Bradley, 2007), suggesting that taurine is not an osmolyte in salmon, or that other osmolytes may be preferred. Other examples are seen in marine species such as yellowtail *Seriola quinqueradiata* (Takagi *et al*., 2006), where hemolytic anemia was observed in taurine-deficient fish, along with reduced serum osmolality and osmotic resistance of erythrocytes. In contrast, red seabream *Pagrus major* does not exhibit such decrease in hematocrit in response to low dietary taurine intake (Takagi *et al*., 2011), nor does Florida pompano (Salze *et al*., Aquaculture, Submitted). Finally, tissue taurine levels did not change in banded killifish *Fundulus diaphanous*, despite exposing individuals to freshwater or full-strength sea water (Ahokas and Sorg,

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1977). Such differences suggest that taurine in not (or is less) involved in osmoregulation depending on the species. Similarly to the quantitative requirement, these differences are not aligned with broad ecological characteristics.

Another area of discrepancies in the roles of taurine among teleosts is seen in the changes in nutrient utilization and deposition with the dietary levels of taurine (Salze and Davis, 2015). Overall, there is a general positive trend of increased body lipid content with levels of dietary taurine in cobia (Lunger *et al*., 2007) Florida pompano (Salze *et al*., 2014), and turbot (Qi *et al*., 2012; Yun *et al*., 2012). However, the opposite trend have been observed in different species such as Atlantic salmon (Espe *et al*., 2012a; Espe *et al*., 2012b) and rodents (Tsuboyama-Kasaoka *et al*., 2006).

Taurine has long been reported to have hypoglycemic and properties (Das *et al*., 2012; Goldberg and Jefferies, 1946; Huxtable, 1992; Ito *et al*., 2012; Kim *et al*., 2007). In mammals the relationship between taurine and glucose and lipid metabolism is particularly complex and involves many interacting factors, all of which could explain interspecific differences observed. Although taurine has been found to directly bind with the insulin receptor (Maturo and Kulakowski, 1988), affinity is about 1% of typical extracellular concentrations, thus questioning such mechanism of action. Rather, the insulin-like action of taurine seem mediated by a pancreatic stimulation to liberate insulin it has been suggested that taurine exerts its hypoglycemic action through cAMP and protein kinase signaling pathways (Ribeiro *et al*., 2010). In fish, only a few studies investigated the effects of taurine on the glucose and lipid intermediary metabolism. In Atlantic salmon taurine supplementation of a plant-based diet decreased lipid deposition without significantly reducing final live weight compared to the same plant-based diet unsupplemented with taurine with results suggesting an increase in S-adenosyl methionine (SAM) and liver polyamine concentrations (Espe *et al*., 2012a), which may explain the decrease in lipid accumulation (Jell *et al*., 2007). In contrast, the hepatic lipid content totoaba does not

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decrease when fed taurine-supplemented, plant-based diets, not is there any influence on the activities of fatty acid synthetase or Malic enzyme (Bañuelos-Vargas *et al*., 2014). Rather, the activities of glycolytic hexokinase and gluconeogenesis fructose 1,6-bisphosphatase are significantly increased. This supports the existence of interspecific differences in the way that dietary taurine mediates metabolism in teleosts.

3 Function across life stages

Taurine is sometimes referred to as a conditionally required nutrient. This terminology should not be applied relative to ingredients used in feed formulations is incorrect: while using various ingredients may affect the bioavailability of a given nutrient, it is not likely to affect the requirement itself, i.e. the amount of nutrient needed to achieve a metabolic target. For instance, methionine is not conditionally required when feeding soybean-based diets; simply, soybean-based diets tend to be deficient in methionine, and must be supplemented using synthetic methionine or other, methionine-rich ingredients. The same applies to taurine, as it does for any other essential nutrients.

However, some studies have investigated the changes in taurine requirement with life stages / size, which would then constitute a conditional requirement. In turbot *P. maxima*, benefits of taurine dietary supplementation was 0.64% dietary taurine for 165.9g individuals, compared to 1.15% in 6.3g individuals, thus strongly indicating that the quantitative requirement in this species decreased as the fish grew in size (Qi *et al*., 2012). Moreover, feed intake increased in response to increasing dietary taurine in both sizes, but feed efficiency was improved only in the smaller fish. This suggests that taurine acted mostly as an attractant in the bigger turbot, but had a more complex effect in the smaller fishes.

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In Atlantic salmon parr (2g), dietary taurine caused an increase in polyamine synthesis, which implies an increase in S-adenosyl methionine (SAM) as methyl donor (Espe *et al*., 2012a). However, hepatic cells isolated from 1.4kg Atlantic salmon showed no changes in methylation capacity in response to taurine supplementation in the culture medium while also significantly reducing apoptosis (Espe and Holen, 2013). Though the essentiality of taurine in salmon remains to be clearly established, these data indicate that life stage is an important factor to consider when attempting to answer this question. The functional changes of taurine with life stages can be seen during major ontogenic milestones, such as during smoltification in Atlantic salmon. Indeed, expression of the taurine transporter gene *TauT* was decreased by dietary taurine in smolt but not parr held in sea water (Zarate and Bradley, 2007), thereby suggesting that taurine becomes more important as the fish adapts its physiology to cope with increasing salinity. Similarly, *TauT* expression was positively correlated with metamorphosis stage in Senegalese sole, suggesting an important role of taurine in this crucial developmental process (Pinto *et al*., 2011). In juvenile, *TauT* is particularly expressed in the hindgut (likely for enterohepatic recirculation) as well as in the stomach. It is not clear at which point during larval development *TauT* expression starts. It is quite possible however that it occurs prior to the completion of the acidic pepsin digestion capacity: dietary taurine supplementation significantly improved morphological and enzymatic development in larval cobia in the earliest phase (Salze *et al*., 2011; Salze *et al*., 2012a; Salze *et al*., 2012b), indicating that dietary taurine must be sensed and detected by the organism in order to produce such effect.

This overview of the current taurine literature clearly shows the limitations in our understanding of taurine function within and among teleost species, as well as among life stages; additional research is necessary to elucidate both the similarities and differences. Even among commercially-relevant aquaculture species, knowledge remains fragmented as the majority of studies were conducted on juvenile animals, and only a few performed with larvae. To the best of our knowledge there is to date only one study reporting the effects of

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taurine supplementation in sexually maturing individuals on reproductive performances (Matsunari *et al*., 2006). In this study, yellowtail *S. quinqueradiata* broodstock were fed 3 diets with graded levels of taurine: the group receiving taurine-unsupplemented feed did not spawn, and improved egg quality was observed in the group fed the highest taurine level. Acknowledging both the promising results and the dire need for additional information in this area, we conducted two studies concerned with the nutritional taurine status in broodstock and its effect onto reproductive output. The first study was an *a posteriori* analysis of historical egg samples from California yellowtail *Seriola lalandi*, matched with the performances of the resulting larvae. In the second study Florida pompano (*Trachinotus carolinus*) broodstock were fed diets that were supplemented or unsupplemented with taurine and the resulting egg quality was observed. These preliminary data provide initial information to better focus future studies on the subject.

4 California yellowtail

4.1 Introduction and methods

California yellowtail, along with other species in the *Seriola* genus, is a species of commercial importance: in 2013, aquaculture production of *Seriola* sp. was about 186k t (FAO, 2015). Hatchery production of California yellowtail is variable and typical survival rates of larvae at weaning range 1-2% to 30-40% (Ma *et al*., 2013; Roo *et al*., 2014; Stuart and Drawbridge, 2011). In an attempt to gain information on the role of taurine and other amino acids in broodstock maturation and gametogenesis, historical samples of fertilized eggs were analyzed for crude protein and amino acid profile. Results were then correlated with husbandry results such as spawn size, fertilization rate, hatching rate, and larval survival at weaning.

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4.1.1 Broodstock collection and holding

Broodstock were collected in 2003 and 2004 off San Diego and Santa Catalina Island, CA. The fish were captured with hook and line and transported by boat to a 555 m^3 net pen at Santa Catalina Island, CA, or directly to the Hubbs Sea World Research Institute (HSWRI) by live haul truck. The fish that were brought directly to HSWRI were introduced to the maturation pool in the winter of 2004, prior to the spring spawning season. All fish were weighed and individually PIT tagged (AVID, Norco, CA) after capture. The fish held at Santa Catalina Island were introduced to the maturation pool in 2008 (Stuart and Drawbridge 2013).

The broodstock were held in a 140 m^3 fiberglass pool (9.1 m diameter x 2.4 m deep) and exposed to shaded natural light and ambient seawater temperatures of 12 to 23°C. The seawater was recirculated at a rate of 1,135 L min^{-1} using an airlift-driven bead filter (0.7 m^3 PolyGeyser Bead Filter, Aquaculture System Technologies, New Orleans, LA). The bead filter performed the critical processes of solids capture and biofiltration. Water supplied to the pool by the airlift flowed by gravity from the top of the pool and a central bottom drain into an egg collector, so that all eggs were collected during the study period. The egg collector measured 1.27 m x 1.14 m x 0.64 m and contained a 500 µm mesh bag to trap the eggs before the water returned to the filter. Makeup water drawn from Mission Bay was sand-filtered and sterilized with ultraviolet light before being supplied to the pool at a rate of 5 – 20 L min^{-1}. Pure oxygen was supplemented as needed to maintain oxygen levels above 7 mg L^{-1} (90 – 100% saturation) during the warm summer months.

As described in Stuart and Drawbridge (2013), the broodstock diet consisted primarily of frozen sardines and squid that were thawed and injected with vitamins. Mackerel and anchovies were used occasionally to further supplement and vary the diet.

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The vitamin pack consisted of a custom premix (1.5% of the total diet), thiamin (0.02% of the total diet), vitamin C (0.5% of the total diet; ROVIMIX® Stay-C 35, DSM Nutritional Products, Basel, CH), lecithin (2.0% of the total diet), fish oil (3.3% of the total diet), and AlgaMac-3050 (10.0% of the total diet; used only during the spawning season; Aqua fauna, Hawthorne, CA). For all years the broodstock were provided a winter (non-spawning) and summer (spawning) ration. The winter ration was generally consistent at 4% body weight $week^{-1}$ based on near satiation feeding during the colder seasons. The summer ration was varied from 6 to 15% during the four year period and was subsequently evaluated relative to the performance of the broodstock.

4.1.2 Egg Collection and Incubation

The egg collector was checked daily at 0800 hours, and any eggs found were collected with a fine mesh aquarium net. Eggs were then placed into an 8.0 L container with aeration prior to volumetric estimation of spawn size using 1.0 L graduated cylinders. Eggs were poured into cylinders, and floating and sinking eggs were allowed to separate for 5 to 10 minutes; only the floating, fertilized eggs were considered viable and used for culture. Percent fertilization was calculated for each spawn as the ratio of the floating versus total eggs produced per spawn multiplied by 100. Following separation viable eggs were disinfected with 100 mg L^{-1} of formalin for one hour prior to stocking.

Eggs were stocked into 1600 L cone-bottom, fiberglass “incubator” tanks for culture. Flow rates were maintained at 3 – 6 turnovers day^{-1}, depending on the developmental stage of the larvae, and mild aeration was provided with air-stones and bubble-ring diffusers. Controlled lighting installed above the incubator tanks provided an illumination of 7,000 – 13,000 lux at the surface. For each spawn, five subsamples of 100 eggs were taken to estimate hatching rates. Subsequently, five subsamples of ten hatched larvae were taken to estimate survival to first feeding.

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4.1.3 Statistical analysis

Direct analysis of the results using multiple regression analysis was not possible due to high degree of collinearity between the levels of amino acids. Consequently a principal component analysis was performed to generate orthogonal variables. Initial variables included spawning year, all amino, crude protein and crude lipid contents of fertilized eggs. Resulting principal components were then regressed against fertilization rate, hatching rate and survival at first feeding. Regressions were significant at $P<0.05$. Quadratic regression was evaluated and deemed significant when the 2^{nd} degree parameter of the polynomial equation was significantly different from zero ($P<0.05$).

4.2 Results and discussion

The first three principal components, explaining 92.18% of the data variability were kept for subsequent analysis. Component patterns are illustrated by the vector plots in

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. Most of the amino acids and crude protein content of the egg heavily loaded on the 1st component, while hydroxylysine and ornithine loaded mostly on component 2. Spawning year loaded mostly on component 3, and egg crude lipid loaded almost equally on component 1 and 3.

Regression analysis on the components reveals that hatching rate is quadratically correlated with component 1 (Figure 3).This suggests that hatching rate is correlated with the egg protein content, and that hatching rates tend to increase quickly before stabilizing as egg protein content increases. Similar correlation has been found in angelfish *Pterophyllum scalare* (Shelar *et al*., 2014), where adults fed 52% dietary protein produced eggs with higher protein content and had improved reproductive performances, including relative fecundity rate, fertilization rate, and hatching rate. Conversely, no such relationship was found in turbot *Psetta maxima*: no correlations were found between the composition of the eggs and fertilization or hatching rates (Jia *et al*., 2014). Although best performances were observed in the middle of the reproductive season where content in some essential and non-essential amino acids was increased, the performance parameters were correlated with levels of specific fatty acids rather than amino acids. It remains unclear whether the discrepancy among these two species and California yellowtail may be explained by their different spawning strategy (pelagic vs. substrate spawners), environmental conditions (e.g., water temperature), or other factors.

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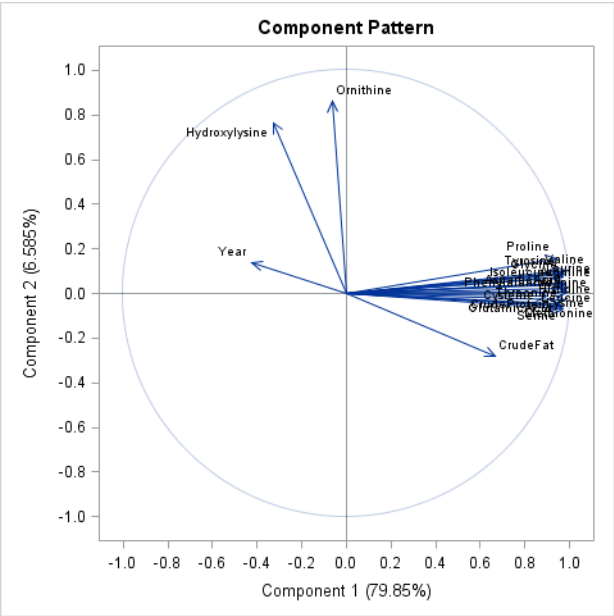

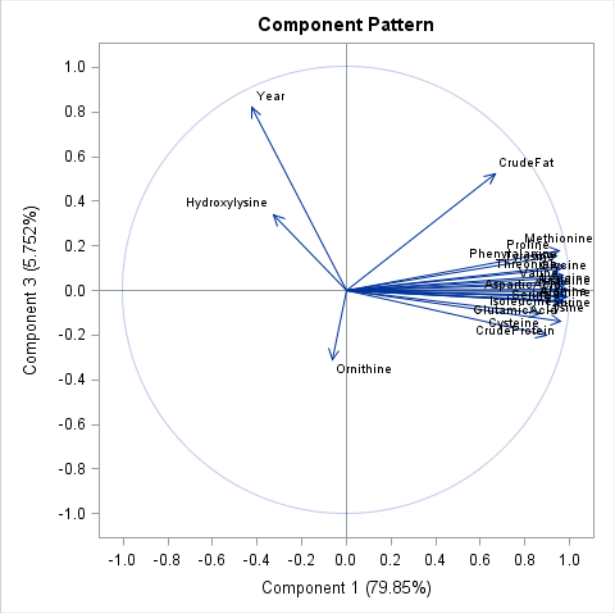

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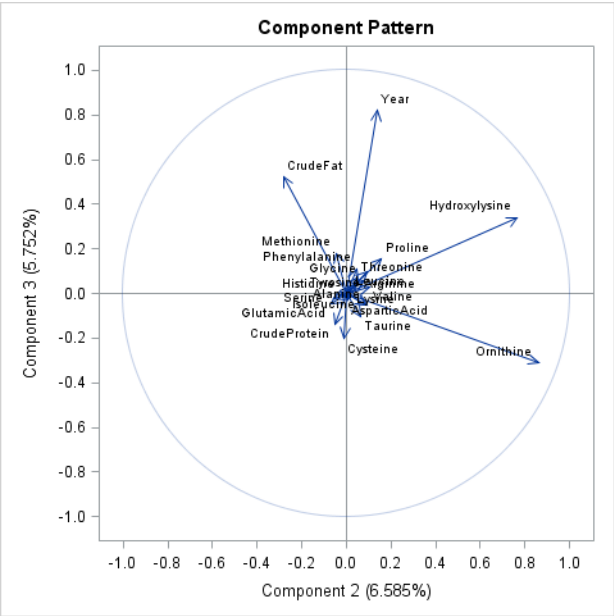

Figure 2: Vector plots illustrating the variable loadings onto the first three principal components

The fertilization rate was negatively correlated with component 3 (Figure 5), on which egg crude lipid content partly loaded together with the spawning year. Therefore this result may reflect genetic variability and/or aging of the broodstock as spawning pairs differed and aged over the years, negatively affecting egg fertilization.

Finally, survival at first feeding is negatively correlated with component 2 (Figure 4), where hydroxylysine and ornithine mostly loaded. The former is an important constituent of collagen, while the latter is used during urea production. It is known that amino acids are particularly used as metabolic fuel during embryogenesis and hatching (Cruzado *et al*., 2013; Moran *et al*., 2007), and this may point to suboptimal protein metabolism and nitrogen waste toxicity. Teleosts excrete nitrogen mostly in the form of ammonia (80 to 85%) while the remainder is excreted as urea. This is in contrast with terrestrial animals, which must convert ammonia to urea before it can be excreted; indeed

urea is far less toxic and can be temporarily stored in the urinary bladder. Because fish live in water, ammonia can be continuously excreted through the gills, thereby negating for the most part the need and associated energetic cost of conversion to urea. Nevertheless, the genes coding for the enzymes of the urea cycle are present in most but not all teleost species, though their activities remain barely detectable in juvenile and adult fish while being distinctively high during the first few days after hatching (Chadwick and Wright, 1999; Wright *et al*., 1995). The metabolism of yolk nutrient reserves leads to a significant production of cytotoxic ammonia, which must be disposed of. The egg chorion surrounding the embryo is characterized by a relatively low permeability; while ammonia slightly permeates, urea transport requires a specific carrier protein (Levine *et al*., 1973). Results in embryo and larvae of rainbow trout and Atlantic cod indicate that in spite of a detectable ammonia excretion, levels of both ammonia and urea still increase inside the developing egg. Trout eggs lack the urea specific transporter, and urea is excreted only after hatching (Wright *et al*., 1995). Although the urea concentration gradient between the animal and the water is highest at hatching, rates of excretion are low and sharply increase as the gills develop. Taken together, this suggests the urea cycle is a temporary but critical mechanism of ammonia detoxification during the early stages of life in teleosts. Therefore, the negative relationship between survival at 1st feeding and ornithine levels suggests a failure of this detoxification system, leading to increased mortality.

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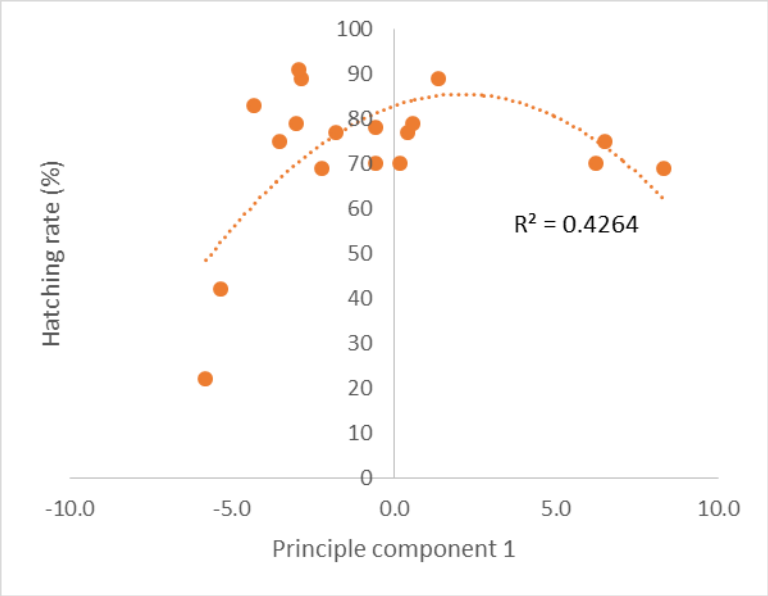

Figure 3: Regression of hatching rate in California yellowtail oocyte with principal component 1

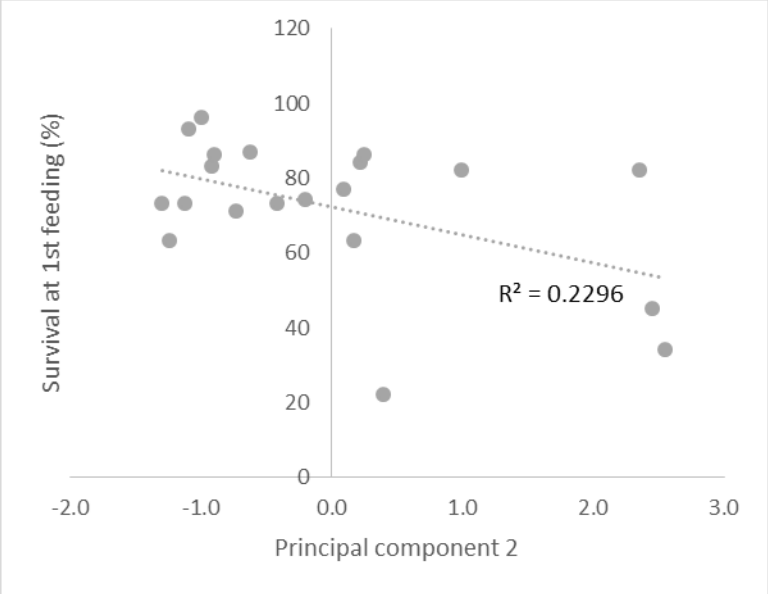

Figure 4: Regression of survival at 1st feeding in California yellowtail larvae with principal component 2

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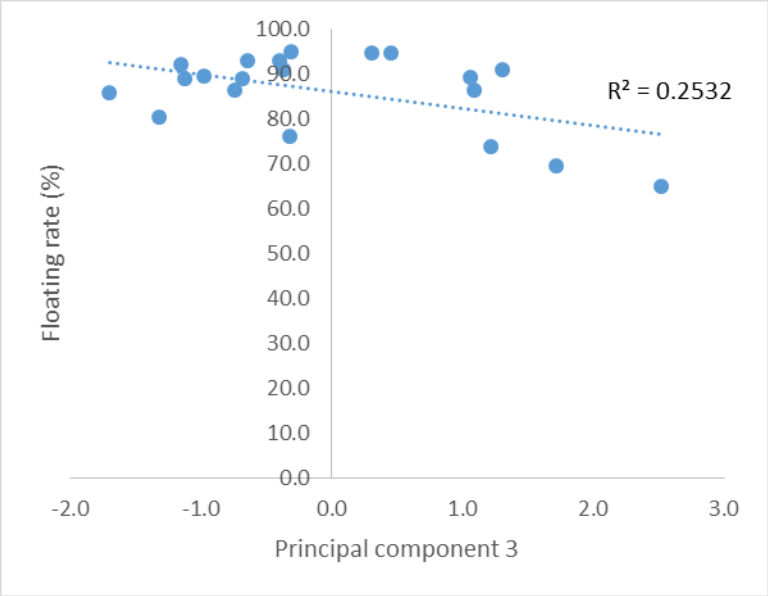

Figure 5: Regression of fertilization rate in California yellowtail oocytes with principal component 3

5 Florida pompano

5.1 Introduction and methods

Currently there is no commercial aquaculture production of Florida pompano. Some reports suggest that farms based in the Dominican Republic and Panama have now ceased their activities. Several research and pilot-scale operations are found in several countries including the United States; however, all commercial production is achieved through fisheries harvesting, which totaled 350 tonnes globally in 2013 (FAO, 2015). Nevertheless, Florida pompano combines highly desirable traits that makes it an excellent candidate for commercial operations, and has been widely recognized as such for years (Lazo *et al*., 1998; Main *et al*., 2007).

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5.1.1 System design and broodstock maintenance

Pompano broodstock were maintained in 25m^3 tanks (4.57 m diameter) equipped with an egg collector with a 500µm mesh bag, mechanical and biological filtration, a protein skimmer, and two 150 W UV units. Water quality was monitored daily, including dissolved oxygen (DO), salinity (ppt), pH, and temperature (°C). Optimal conditions for each of these variables were as follows: DO: 4.0 - 9.0 mg/l, Salinity: 35.0 ± 1 ppt, pH: 7.5 - 8.5, and Temperature: 27 ± 1 °C. Water chemistry was monitored weekly. Measurements taken were total ammonia nitrogen (TAN) maintained at <0.5ppm, nitrite nitrogen (NO_2-N) at < 1.0ppm, and nitrate nitrogen (NO_3-N) at <50ppm.

5.1.2 Maintenance and experimental feeds

The pompano broodstock population was composed of wild-caught and F1 fish. All were individually implanted with a PIT tag. Initially all fish were maintained on a diet of thread herring (40%), shrimp (30%), and squid (30%), and were fed close to satiation (4-5% of the tank biomass) daily, adjusting as necessary. Because of their high metabolisms and small guts, the fish were fed this amount over three daily feedings. Three weeks prior to spawning, experimental feeds were introduced and fed according to the same protocol as the maintenance regimen. Experimental diets were based on a blend of seafood (same proportions as maintenance diet) and gelatin, supplemented or not with taurine (

Table 1). The formulation was designed to approach the maintenance diet without compromising feed intake or physical characteristics of the pellets. Pellets were prepared by coarsely dicing the seafood and mixing with the rest of the ingredients. Gelatin was dissolved in warm water and added to the ingredient mix. The preparation was then poured into a large, shallow container and left to cool in the refrigerator before being cut into bite-

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size cubes. Diets were kept at 4°C until fed to the fish. These diets were well accepted by the fish, and fed for three weeks prior to spawning.

Table 1: Formulation and basic composition of the experimental diets

		Maintenance	Control	Taurine
Formulation (% as-is)				
Herring		40.00	19.20	19.16
Shrimp		30.00	14.40	14.37
Squid		30.00	14.40	14.37
Gelatin		-	18.00	17.96
water		-	33.12	33.05
fish oil		-	0.88	0.88
Taurine		-	0.00	0.20
Composition (calculated)				
Dry matter	(%)	19.72	27.3	27.2
Crude protein	(%, dry)	73.6	84.3	84.3
Crude lipid	(%, dry)	8.1	6.0	6.0
Taurine	(%, dry)	0.88	0.31	1.04

5.1.3 Maturation and spawning protocols

The maturation was controlled by manipulating photoperiod and water temperature in order to emulate Spring conditions in West coast Florida (13hr light:11hr dark, 27 ± 1.0°C). For resting we used winter conditions (11.5hr light: 12.5 dark, 22°C).

Spawning was stimulated by hormonal implants. To easily sample the fish and minimize stress, the tank volume was reduced by two thirds and the fish were corralled in a small area of the tank. The pompano were then removed one fish at a time, and placed in an anesthetic tank with 300ppm of buffered tricaine methanesulfonate (MS-222). When the

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fish were anesthetized, they were identified with their PIT tag, weighed, and measured for fork length. Males were sampled by applying light pressure to each side of the abdomen: assessments were made by recording if milt was expressed, and if it was, if the fish had a mild or heavy flow. Females were cannulated using an 8fr premature infant feeding tube mounted on a 10ml syringe. A few oocytes were removed and immediately placed on a slide with a small amount of salt water to assess their maturation stage using light microscopy. Females were considered mature when their oocytes were staged at or beyond the secondary growth final growth (SGfg) stage of oocyte maturation. Mature females were then implanted with ≥50µg/kg of Ovaplant® (sGnRHa). Since Ovaplant is only available in doses of 75 or 150µg, females weighing less than 1500, 3000, or more than 3000g were implanted with 75, 150, or 225µg, respectively. The fish were then revived and placed back in the main portion of the tank.

5.1.4 Egg collection

After receiving the hormonal implant, the fish were allowed to spawn volitionally. The egg collector was monitored by checking the bag and the tank for eggs every 2 hours, in order to minimize disturbance of the spawning behavior. Once eggs were found, water samples were taken every hour to ensure that the totality of the spawn has been collected. The 500µm bag was then emptied into a hatching cone filled to a known volume of aerated seawater. When the eggs reached the blastula stage (~6hr post-fertilization), three, 10ml aliquots were taken to determine the total number of eggs and fertilization rate. The aeration was then turned off to allow the settling and removal of unfertilized eggs, and floating viable eggs were cleaned on a 500µm sieve with clean salty water prior to being placed in a hatcher. Hatching occurred between 24-26hr post-fertilization, at which point the larvae were counted to determine the hatching rate. Samples of eggs and newly hatched

Salze, G. *et. al*. 2015. Advances in Understanding of Taurine Functions in Fishes Across Species and Life Stages. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 324-352.

larvae were taken, de-watered, and frozen at -80^{o}C pending dry matter, crude protein, and amino acid analyses.

5.1.5 Calculations and statistical analysis

The amount of sample was insufficient to measure lipid content of eggs and larvae. Consequently, the amount of lipid was estimated by assuming that both eggs and larvae contained negligible amounts of carbohydrates. Then lipids may be calculated by subtracting the moisture and protein content from 100%. Composition data of the egg and larvae were analyzed by 2-way ANOVA with developmental stage (egg or larvae) and treatment (control or taurine) as main effects. Results were considered significant when $P<0.05$.

5.2 Results and discussion

One spawn was obtained in each dietary treatment group: the absolute number of fertilized egg were close between the two treatments; however the taurine group spawned fewer eggs, leading to a much increased fertilization rate (Table 2).

Table 2: Spawn results from Florida pompano fed a control or taurine-supplemented diet for 3 weeks

	Taurine	**Control**
Total eggs collected	102,000	299,200
Total Fertilized Eggs	54,400	45,900
Percent Fertilization	53.5%	15.4%

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Table 3: Taurine and proximate composition (% as-is) of Florida pompano eggs and larvae from broodstock fed a control or taurine-supplemented diet for 3 weeks

	Taurine (% as-is)	**Crude Protein (% as-is)**	**Moisture (% as-is)**	**Lipid (calculated, % as-is)**	**Crude protein/Lipid**
Egg					
Control	0.05±0.01	5.02±0.71	92.33±1.15	2.65±0.45	1.91±0.12
Tau	0.07±0.01	6.85±1.18	89.95±1.82	3.20±0.66	2.15±0.12
Larvae					
Control	0.12±0.04	8.18±2.36	85.82±4.28	6.00±1.92	1.37±0.06
Tau	0.21±0.03	14.55±1.15	79.52±2.39	5.93±1.55	2.56±0.67
P-values (2-way ANOVA)					
Developmental stage	<0.0001	<0.0001	<0.0001	0.0008	0.7162
Treatment	0.0017	0.0005	0.0122	0.6637	0.0017
Interaction	0.0452	0.0132	0.1759	0.6392	0.0199

Value are averages ± SD with n=4 for egg samples, n=3 for larvae samples. Lipid content = 100-moisture-crude protein.

Proximate composition of the eggs and larvae are shown in

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Table 3. There was a significant increase in taurine content in both eggs and larvae, and the significant interaction indicates that the difference between treatments was greater in larvae. This shows that three weeks of feeding the broodstock with the experimental diets was sufficient to impact the composition of the egg and newly hatched larvae. Such rapid deposition of free amino acids in the eggs has been observed in red snapper *Lutjanus campechanus* broodstock injected with supplemental amino acids along with HCG injection to stimulate spawning (Hastey *et al*., 2015). Additionally, and although the pompano experimental diets were isonitrogenous and isolipidic, the taurine supplementation caused a significant increase in crude protein in both eggs and larvae at the expense of moisture. However, there was no change in total lipid content in response to taurine supplementation. This contrasts with the aforementioned red snapper study, where the oil globule diameter was significantly larger in amino acid-injected fish than in sham-injected fish (Hastey *et al*., 2015). The relative changes in crude protein and lipid contents result in a crude protein/lipid ratio to significantly decrease from egg to larvae in the control group whereas it remains relatively stable in the taurine-supplemented group. This suggests that in Florida pompano broodstock, dietary taurine signals for an increase in protein deposition and nutrient density in the oocytes, as well as different nutrient utilization as was also seen in red snapper (Hastey *et al*., 2015).

Unfortunately correlation with egg quality parameters was not possible due to the limited number of spawns and the very low fertilization rate in the control group. The trade-off between number of eggs produced and fertilization rates resulted in a similar number of fertilized eggs produced by each group. However, if the taurine-supplemented eggs and larvae are more nutrient-dense, it is reasonable to hypothesize that these stand better chances of surviving throughout the larval development stages than their control counterpart. This hypothesis is currently begin tested as the experiment is repeated with the same diets, and the resulted larvae will be cultured until completion of metamorphosis and weaning onto a dry larval diet.

Salze, G. *et. al*. 2015. Advances in Understanding of Taurine Functions in Fishes Across Species and Life Stages. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7,pp. 324-352.

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Importancia Nutricional de Taurina en Peces Carnívoros Como *Totoaba Macdonaldi*, Cuando es Alimentada con Dietas Ricas en Proteína Vegetal

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Resumen

La mayoría de los estudios en nutrición acuícola están enfocados en apoyar el desarrollo sostenible de los alimentos formulados para peces marinos debido a la reducción del suministro de harina de pescado de pesquerías silvestres, y a un aumento en su precio. Asimismo, para que la industria de la acuacultura y maricultura sean rentables, es necesario

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elaborar alimentos comerciales para peces carnívoros con ingredientes económicos y disponibles (Gatlin *et al*., 2007). En los últimos años, se han desarrollado investigaciones para sustituir la dependencia de harina de pescado con fuentes vegetales como harina de soya, harina de trigo y harina de maíz (Gaylord *et al*., 2007). La sustitución de harina de pescado por proteína de soya en la dieta presenta reducción de metionina y lisina, así como de taurina, por lo tanto, los peces que se alimentan con dietas a base de proteína vegetal requieren taurina exógena para el mantenimiento de sus funciones fisiológicas. Se han realizado estudios para mejorar los factores limitantes de proteína vegetal, como aminoácidos (Gaylord, 2007) y otros nutrientes que no están presentes en la proteína vegetal, de los cuales taurina ha sido de gran interés. Debido a que la taurina se encuentra en la harina de pescado, pero deficiente en las fuentes vegetales como la proteína de soya la suplementación de éste nutriente podría ser una práctica prometedora para mejorar el valor nutritivo de las dietas de peces carnívoros en la que harina de pescado se sustituye con fuentes de proteínas vegetales (Lunger *et al*., 2007; Takagi *et al*., 2011; Bañuelos-Vargas *et al*., 2014). La importancia de suplementar con taurina a los alimentos para peces en cultivo se ha realizado en diversos estudios (Yun *et al*., 2012; Lim *et al*., 2013; Watson *et al*., 2013; Bañuelos-Vargas *et al*., 2014; Khaoian *et al*., 2014; Kim *et al*., 2014; Salze *et al*., 2015;). Además de estos estudios, la deficiencia de taurina en jurel *Seriola quinqueradiata* (Takagi *et al*., 2005), pargo *Pagrus major* (Takagi *et al*., 2011) totoaba *T. macdonaldi* (Bañuelos-Vargas *et al*., 2014) se ha relacionado con el desarrollo de “síndrome de hígado verde”.

T. macdonaldi es una especie endémica del Golfo de California y se clasifica dentro de familia Sciaenidae alcanzando tallas cercanas a los dos metros de longitud y pesos superiores a los 135 kg (Cisneros-Mata *et al*., 1995). La sobreexplotación pesquera de ésta especie provocó un agotamiento de las reservas naturales y a partir de 1975 ha sido incluida en la lista de especies en peligro de extinción (CITES, 2005). Actualmente la Unidad de Biotecnología en Piscicultura de la Facultad de Ciencias Marinas de la Universidad Autónoma de Baja California, lleva a cabo programas de repoblamiento de juveniles

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obtenidos mediante el desove de reproductores mantenidos en cautiverio (True *et al*., 1997). Los avances logrados en la producción de juveniles han colocado a *T. macdonaldi* como especie de alto potencial para la acuacultura y maricultura comercial en México. Debido al gran interés que existe por apoyar a la industria de alimentos para acuacultura se ha creado vinculación con la U.S. Soybean Export Council para probar el uso del concentrado proteico de soya como sustituto de la harina de pescado. A partir de esta iniciativa se está probando el uso de la taurina como suplemento en alimentos ricos en proteína vegetal. Para ello, se están realizando estudios sobre el requerimiento de taurina al alimentar a totoaba con fórmulas a base de harina y aceite de pescado, así como también, con el uso del concentrado protéico y aceite de soya.

Uno de los estudios evaluó el efecto de diferentes niveles de taurina (0.05% (dieta control), 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 y 2.1%) con dietas isoprotéicas (50%) e isolipídicas (12%) sobre las respuestas productivas y de salud de totoaba. Después de 10 semanas de experimentación los peces mostraron una buena sobrevivencia y desempeño productivo. Los peces alimentados con 0.3% de taurina mostraron el crecimiento más alto y el mayor índice bilisomático, sin embargo, altos contenidos de colesterol y triglicéridos en sangre se presentaron en los organismos alimentados con la dieta con solo 0.05% de taurina (DC), además, el "síndrome del hígado verde" se presentó en los peces que consumieron ésta misma dieta, lo cual indica una condición fisiológica anormal debido a la deficiencia de taurina (Tabla 1). Así mismo, los resultados indican que la baja concentración de taurina en la dieta de totoaba afecta el metabolismo de lípidos.

En otro estudio se investigó sobre los efectos del concentrado de proteína de soya (CPS) y taurina en el crecimiento y salud de juveniles de totoaba. Fueron evaluadas dos dietas con 30 y 60% de CPS sin taurina y 30 y 60% de CPS más 1% de taurina, además de una dieta control (DC) sin CPS y sin taurina. El uso de taurina se reflejó en un aumento en el crecimiento (Figura 1), de la eficiencia alimenticia (EA) y eficiencia protéica (EP) comparado con la DC. Los niveles de actividad de las enzimas clave del catabolismo de aminoácidos (AA), de gluconeogénesis y la enzima glucosa 6-fosfato deshidrogenasa

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(G6PD) resultaron significativamente disminuidas por la falta del suplemento de taurina (independiente del nivel de CPS). Por su parte, los niveles de actividad de las enzimas claves de la glucólisis fueron disminuidas por el aumento de CPS en las dietas. La actividad de la enzima del sistema de defensa antioxidante catalasa (CAT) resultó significativamente aumentada con el suplemento de taurina. Además, la actividad de las enzimas CAT y G6PD fueron correlacionadas positivamente con la disminución de los niveles de lipoperoxidación (LPO) hepática. Asimismo, el suplemento de taurina, por sí mismo, indujo a la disminución significativa de los niveles de LPO y en el desarrollo del "síndrome de hígado verde" en totoaba (Tabla 2). Por otra parte, el aumento de CPS (60%) provocó un incremento significativo de agregados inflamatorios en el tejido hepático. En la respuesta hematológica y de bioquímica sanguínea, se observó que el aumento de CPS afectó la concentración de hemoglobina (HB), albúmina (AL), razón Al: globulinas (AL:Glb) y aumentó la glucosa plasmática (GLU). Por el contrario, la suplementación de taurina generó la recuperación de la mayoría de estas variables y además influenció significativamente la disminución de los eritrocitos circulantes (EC) y el hematocrito (HT). Se observó un efecto significativo de la interacción de factores (CPS*taurina) en el hematocrito (HT), la media de la concentración de hemoglobina corpuscular (MCHC) y la GLU. Así, mientras el nivel de CPS provocó una tendencia de aumento de HT, disminución de MCHC y aumento de GLU; la suplementación de taurina indujo al efecto contrario en cada una de estas variables.

Por lo que, nuestros resultados sugieren que la taurina puede ser un nutriente limitante para el desempeño productivo y mantenimiento de la salud de totoaba, sobre todo cuando se incluyen fuentes de proteína vegetal en la dieta, por lo que es recomendable suplementar taurina para prevenir problemas derivados de su deficiencia.

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Tabla 1. Hematología y parámetros de crecimiento de *T. macdonaldi* alimentada con diferentes raciones de taurina, durante 10 semanas.

Taurina (g/100g)	**0.05**	**0.3**	**0.6**	**0.9**	**1.2**	**1.5**	**1.8**	**2.1**
Peso ganado	74.8	82.1	78.4	77.1	77.0	77.2	75.9	76.9
Alimento ingerido	45.1	51.1	48.0	47.9	47.9	49.3	48.1	48.6
Hematocrito (%)	19.0	19.9	18.9	19.3	20.0	19.1	19.3	19.0
Hemoglobina (g/dl)	4.78	5.08	5.37	5.22	5.48	4.96	5.16	5.25
Colesterol (mg/dl)	82.3^{b}	84.9^{b}	93.4^{b}	100.3^{ab}	98.0^{ab}	106.3^{ab}	107.5^{ab}	111.3^{a}
Triglicéridos (mg/dl)	50.7^{c}	67.9^{bc}	73.7^{bc}	72.3^{bc}	84.2^{ab}	101.4^{a}	79.3^{ab}	75.0^{b}
Bilirrubina (mg/dl)	0.12^{c}	0.20^{b}	0.20^{b}	0.11^{c}	0.18^{b}	0.26^{a}	0.22^{ab}	0.24^{ab}
Índice hepatosomático	1.9^{ab}	1.5^{ab}	1.4^{ab}	1.6^{b}	1.4^{b}	1.8^{a}	1.6^{ab}	1.8^{ab}
Índice viscerosomático	2.3^{ab}	2.0^{b}	2.1^{ab}	2.0^{b}	2.2^{ab}	2.1^{ab}	2.1^{b}	2.5^{a}
Índice bilisomático	0.09^{b}	0.14^{a}	0.15^{a}	0.14^{a}	0.14^{a}	0.14^{a}	0.14^{a}	0.14^{a}
Lípidos en víscera	17.6^{b}	27.2^{a}	23.1^{ab}	20.5^{ab}	26.3^{a}	23.0^{a}	23.1^{ab}	24.7^{ab}
Lípidos en hígado	57.2	65.5	64.2	61.9	64.9	63.1	63.6	62.2
Glicógeno	4.7	5.3	5.2	5.1	5.0	5.2	4.9	4.6

Promedios con diferente letras son significativamente diferente ($P < 0.05$) (Budi Satriyo, en preparación).

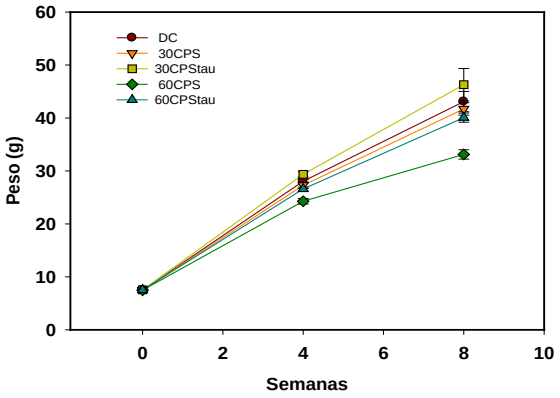

Figura 1. Crecimiento de *T. macdonaldi* alimentados con 30 y 60% de concentrado proteico de soya (CPS), y adicionados con y sin taurina (tau), así como una dieta control (DC) a base de harina de pescado sin adición de taurina (Bañuelos-Vargas *et al*., 2014).

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Tabla 2. Actividad específica de enzimas relacionadas con el metabolismo intermediario hepático de juveniles de *T. macdonaldi* alimentados con concentrado de proteína de soya sin y con el suplemento taurina. Los datos se expresan como mU mg^{-1} proteína.

Dietas	Control	CPS30	CPS30tau	CPS60	CPS60tau	SEM[1]
Catabolismo de aminoácidos						
Aspartato aminotransferasa (ALAT)	1747	984†	1991	1179†	1805	86
Alanina aminotransferasa (ASAT)	417	241†	441	238†	401	18
Glutamato deshidrogenasa (GDH)	414	277†	424	233†	370	16
Glucólisis						
Hexoquinasa (HK)	20.2	22.4	25.9‡	17.0	19.3	0.73
Glucoquinasa (GK)	3.50	2.74	2.34	4.06	5.01	0.43
Gluconeogénesis						
Fructosa-1,6-bisfosfatasa (FBPasa)	77.2	51.8†	73.7	49.3†	61.8†	3.2
Lipogénesis						
Glucosa-6-fosfato deshidrogenasa (G6PD)	72.3	56.8	82.1	37.5†	80.1	4.02
Enzimas málicas (EM)	17.2	18.3	21.7‡	17.3	18.0	0.64
Ácido graso sintetasa (FAS)	1.34	1.51	1.39	1.62	1.58	0.07

Palabras claves: taurina, concentrado proteico de soya, totoaba, nutrición

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Vitamin K, Much More than Blood Clotting: Roles, Metabolism and Specific Requirements. A Mini-Review.

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Abstract

Vitamin K (VK) is a fat soluble vitamin required in fish diets for normal growth and development since they are not able to synthesize it *de novo*. VK can be naturally found as phylloquinone (VK1; vegetable origin) or menaquinone (VK2; microbial or animal origin); and synthetically produced as menadione (VK3). Two major roles of VK metabolites have been described up-to-date. As a co-factor of the enzyme γ-glutamyl carboxylase which performs the conversion of Glu into Gla residues in VK-dependent proteins (VKDPs), through which VK is controlling blood clotting, representing the most widely recognized function of VK. VK also participates in transcriptional regulation, acting as ligand for the steroid xenobiotic receptor or pregnane X receptor (PXR), which is mostly known as a master regulator of xenobiotic metabolism. However, little is known about its metabolism and its specific requirements along fish development. Here, we will briefly review what is known, and identify what still remains to be unveiled about VK and fish physiology. In this sense, new findings regarding VK requirements on early fish development, metabolism and VK cycle regulation under different nutritional conditions will be enumerated. Finally, future perspectives on knowledge gaps, strategies and approaches to be applied will be discussed.

Key words: vitamin K, gamma-carboxylation, PXR, metabolism, proteomics, transcriptomics.

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Historical overview of the greatest milestones on VK physiology:

Vitamin K (VK) is a fat soluble vitamin discovered by Henrik Dam as the "*Koagulationsvitamin*" in 1935 (Dam 1935). However, its specific role in blood coagulation was only revealed in 1974, when its requirement for the conversion of glutamyl (Glu) to γ-carboxyglutamyl (Gla) residues, conferring calcium binding properties to the proteins nowadays known as VK-dependent proteins (VKDPs) was described (Magnusson *et al*. 1974; Nelsestuen *et al*. 1974; Stenflo *et al*. 1974). However, only 17 years later the enzyme catalyzing this conversion, the γ-glutamyl carboxylase (GGCX), was discovered and characterized (Wu *et al*. 1991). We still had to wait 10 years more to have a complete picture of the VK recycling process when the cloning of the enzyme vitamin K epoxide reductase complex 1 (VKORC1) was achieved (Li *et al*. 2004; Rost *et al*. 2004). Just one year before, and similarly to what has been found in the other fat soluble vitamins, the specific nuclear receptor to which VK is bind was discovered, the pregnane X receptor (PXR; Tabb *et al*. 2003). In this sense, PXR was largely known as as a master regulator of xenobiotic metabolism (Chen *et al*. 2012) and for cholesterol and bile acid metabolisms (Makishima 2005). More recently PXR was shown to have a key role in bone homeostasis, as demonstrated by the osteopenic phenotype induced in PXR knockout mouse (Azuma *et al*. 2010).

In contrast to invertebrates, vertebrate genomes include two paralogous enzymes VKORC1 and VKORC1-like 1 (VKORC1L1) likely resulting from a gene duplication of an early common VKOR ancestor. Until 2011, VKORC1 protein was considered as the only player supporting VK recycling activity. Westhofen *et al.* (2011) demonstrated that the VKORC1L1 is able to reduce VK epoxide (the VK by-product after γ-glutamyl carboxylation) to VK, although showing a low enzymatic efficiency. More recently, Hammed *et al*. (2013) reported a VK recycling activity of VKORC1L1 in extrahepatic tissues in the absence or inhibition of VKORC1 protein, completing the full picture of VK roles known up-to-date (Fig.1).

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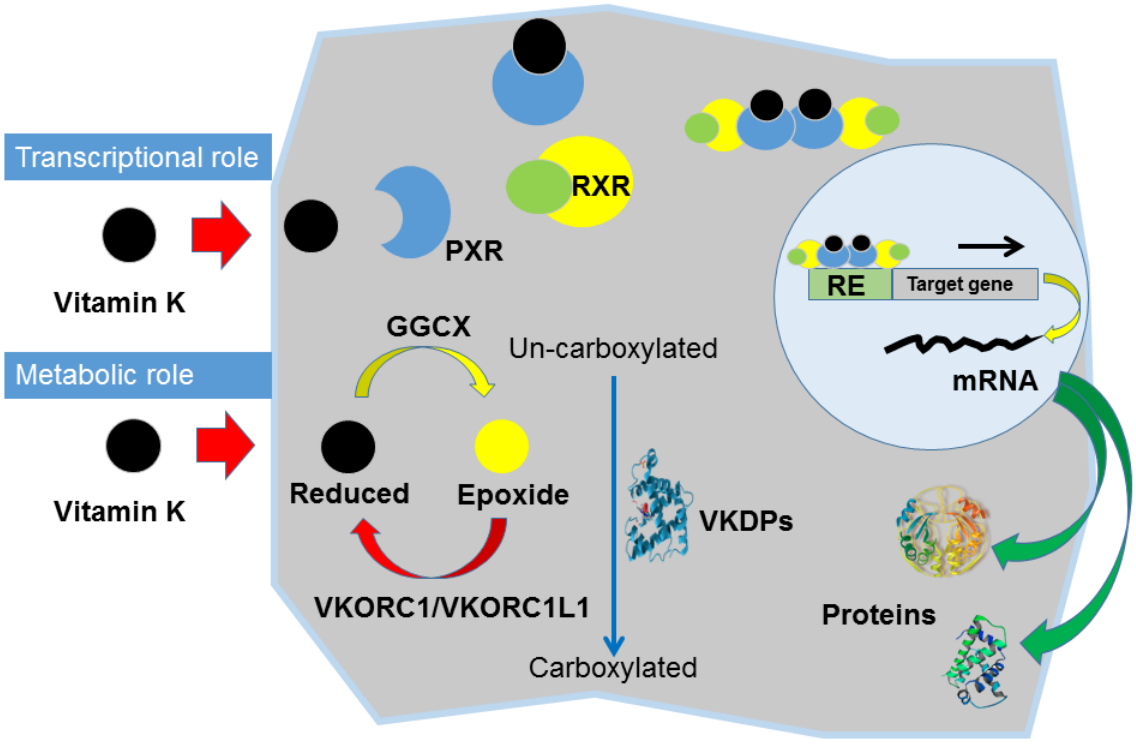

Figure 1. Vitamin K (VK) cycle showing the metabolic and transcriptional roles. VK, coming from dietary intake or glut microflora, is used by the γ-glutamyl carboxylase (GGCX) to convert glutamate into γ-carboxyl glutamate (Gla) residues in VK-dependent proteins (VKDPs), resulting in the production of a VK epoxide as a by-product. VK epoxide is then recycled to VK either by VK epoxide reductase complex 1 (VKORC1) or VKORC1-like 1 (VKORC1L1). In addition, VK is also a ligand of the pregnane X receptor (PXR). The activation of PXR by VK, promote the formation of a heterotetramer with retinoid X receptor (RXR), which finally bind to the PXR responsive elements (RE) on the promotor or enhancer regions of down-stream target genes, activating their transcription and thus the proteins synthesis.

During the last decades different members of the VKDPs have been also identified. The first ones were those involved in the blood coagulation cascade such as the different clotting factors (II, VII, IX, and X), being this the reason why VK has been largely associated with blood coagulation (reviewed in Brenner *et al*. 2009). Subsequently, other VKDPs involved different biological processes have been identified. Matrix Gla protein

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(MGP), has been identified by Price *et al*. (1983), and is known to act as an inhibitor of calcification in soft tissues such as arteries (Luo *et al*. 1997). Bone Gla protein (BGP), also known as osteocalcin, was already described in 1976 by Price and co-workers. In contrast to MGP, it is not only involved in the regulation of tissue mineralization, but it was recently shown to play a role also in glucose metabolism and spermatogenesis (Karsenty and Ferron 2012). Further, the product of growth arrest specific (GAS) gene 6, GAS6, was discovered by Manfioletti *et al*. (1993) and has been suggested to be an important regulator of vascular homeostasis and platelet signaling and being a ligand for the tyrosine protein kinase receptors AX1, TYRO3 and MER implicated in cell growth, survival, adhesion and migration (reviewed by Maree *et al*. 2007). The last member of the VKDP family to be discovered was Gla rich protein (GRP) that was found in the last decade (Viegas *et al*. 2008). It is the most densely γ-carboxylated protein and although it is expressed mainly in cartilaginous tissues (chondrocytes) and in bone cells (osteocytes) and thus, suggested to act as a modulator of calcium availability (reviewed in Cancela *et al*. 2012), however its molecular function is still to be unveiled.

The discovery of all that diverse set of VKDPs, in addition to the one of its nuclear receptor, evidenced a broader biological impact for VK than it was initially foreseen and it is expectable that it might be expanded in the nearest future.

What is known about VK in fish species?

Vitamin K metabolites

Vitamin K (VK) compile a family of compounds derived from quinone, all of them having a common 2-methyl-1,4-naphthoquinone ring, but differing in the side chain at the C3-position (Lambert and De Leenher 1992). Depending on their source, three different VK metabolites (or vitamers K) can be found. The first two can be found in nature as phylloquinone or the group of menaquinones. Phylloquinone or VK_1 is produced by photosynthetic plants serving as a cofactor for Photosystem I-mediated electron transport, with green leafy vegetables being the ones showing higher contents (Booth and Sutie

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1998). Menaquinones, VK2 or MK are a collection of isoprenologues with microbial origin that can be found in fermented products such as Nato or in foods of animal origin (Booth and Sutie 1998). Although more than 20 different menaquinoes have been described, named MK-n accordingly to the number (n) of prenyl groups in the unsaturated side chain, the most relevant from a nutritional point of view are MK-4 and MK-7 (Fodor *et al*. 2010).

Since the main sources of VK are the diet composition and the intestinal microbiota, an equilibrate intake of VK from diverse dietary sources and a healthy intestinal microbiota seems to be needed to the maintenance of proper VK levels in mammals; although contribution of colonic flora to vitamin K requirements remains controversial (Harshman *et al*. 2014). In this sense, some bacterial derived menaquinones have been found stored in the human liver (Suttie 1995). Although the use of antibiotics is known to affect intestinal microbiota (Mathers *et al*. 1990), the relevance and the impact of antibiotics on intestinal production of VK are still not known (Tan and Mai 2001). The third vitamer K is a synthetic water soluble salt, known as menadione or VK_3, majorly produced as menadione sodium bisulphite (MSB) and menadione nicotinamide bisulphite (MNB).

The complex metabolism and transformation from one vitamer K into another has been recently reviewed in detail and thus, for interested readers in this issue Shearer and Newman (2014) is recommended.

VK sources used, dietary levels recommended and biomarkers proposed in fish species

All natural vitamers K are insoluble in water, slightly soluble in alcohol and readily soluble in non-polar organic solvents. Although they are relatively thermostable compounds, vitamers K are highly sensitive to light and alkaline conditions (in Krossoy *et al*. 2011). In contrast, menadione is much more chemically unstable than the natural VK forms (Marchetti *et al*. 1999). Although it can be partly alkylated enzymatically to MK-4 in animal tissues when present in animal feeds (Graff *et al*. 2010; Krossoy *et al*. 2009), it is easily excreted and shows lower bioavailability than the naturally occurring K vitamers (reviewed in Krossoy *et al*. 2011). Furthermore, menadione cannot act directly as a co-factor for GGCX as demonstrated by Krossoy *et al*. (2010). When the different K vitamers

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

are compared metabolically, it seems that conversion of menadione to MK-4 is highly rate limited, as it is its retention compared to that of phylloquinone (Graff *et al*. 2010). Importantly, meal and oil from alternative vegetable sources for the replacement of fish meal and fish oil, like soybean oil or canola oil, may contain higher levels of natural vitamin K_1 compared to marine ingredients, although its incorporation might be hampered by anti-nutritional factors present in the same vegetable resources.

Regarding the safe levels of VK metabolites, while a 50 and 100 % mortality has been observed in zebrafish (*Danio rerio*) embryos exposed from 0-5 dpf to 0.25 and 0.5 mM VK_3, respectively, the same concentrations of VK_2 and VK_1 only induced a 15 % mortality and not significantly different from the control group (Fernandez *et al*. unpublished data). Similarly, when other aquacultured species have been fed with MSB (20-30 mg Kg^{-1}) a reduced growth has been verified (Grisdale-Helland *et al*. 1991; Grahl-Madsen and Lie 1997). However, upper tolerance of 100 mg Kg^{-1} of VK_1, 2500 mg Kg^{-1} for MSB and 2000 mg Kg^{-1} for MNB have been reported in different fish species (reviewed in Krossoy *et al*. 2011). Thus, the VK source and the amounts present in fish diets seems to depend on fish species and developmental stages.

The most common VK deficiency signs in fish are mortality, blood coagulation time, reduced growth, anemia, hemorrhages, loss of fin tissue and abnormal skeletogenesis or bone homeostasis. Based on those parameters, different studies suggested different optimal dietary VK content for a diverse set of fish species (Table 1). Although quite sensitive biomarkers for nutritional VK status in humans are commonly used at clinical level, such as the rate of circulating uncarboxylated BGP or the combined rate of uncarboxylated and dephosphorylated MGP in blood samples, no reliable, sensitive and easy to apply biomarker has been found for fish species. In this sense, GGCX activity has been previously proposed as a sensitive marker for evaluating VK status and intake (Krossoy *et al*. 2010). Atlantic salmon (*Salmo salar*) juveniles fed with increasing levels of MNB (from 0 to 50 mg Kg^{-1}) did not show differences in specific growth rate, condition factor, whole body proximate analysis, blood coagulation time, vertebra morphology or mechanical properties of vertebrae; although a positive dose-response relationship between dietary MNB and the level of MK-4 analysed in liver samples was reported (Krossoy *et al*.

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2009). However, when the enzyme activity and gene expression of GGCX was analyzed in the same samples, the authors reported a decreasing GGCX activity with the increase of VK dietary content and no differences on its gene expression (Krossoy *et al*. 2010). Taking into account those results, GGCX activity seems not to be an accurate VK biomarker since it showed differences in animals with the same biological performance. This could be due to the quantification of γ-glutamyl carboxylase by assaying the incorporation of $H^{14}CO3^-$ into synthetic peptides and subsequent quantification using liquid scintillation counting (Emson and Sutie, 1976). More sensitive and accurate quantification methods have been developed such as the described by Kaesler *et al*. (2012). Nevertheless, considering more recent results from *in vivo* and *in vitro* studies, the other two major players of the VK recycling, VKORs and PXR, have been proposed as more suitable biomarkers. Senegalese sole (*Solea senegalensis*) fed VK supplemented diets showed a better skeletal development and a lower expression of *pxr* and *vkorc1* genes (Richard *et al*., 2014). Conversely, in zebrafish larvae and osteoprogenitor cells under a VK-induced deficiency through the exposure to increased levels of warfarin (an inhibitor of VK recycling), *pxr* and *vkorc1* genes were up-regulated (Fernandez *et al*. 2014, 2015). These results, although in phylogenetically distant fish species, strengthen the value of these markers as a robust indicator of the VK status in fishes.

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

Table 1. Suggested optimal dietary levels of VK in fish feeds

Fish species	Developmental stage	Criteria	Vitamer K	Total VK content*	Reference
Lake trout	Juvenile	Haematology, coagulation time	-	**0.5-1**	Poston, 1976
Salmonids	Juvenile	Growth	VK1	**0.45**	Woodward, 1994
Atlantic cod	Juvenile	Mortality, haematology, coagulation time	VK3	**0.2**	Grahl-Madsen and Lie, 1997
Salmonids	Juvenile	Growth, mortality	VK3	**1.5**	Kaushik *et al.* 1998
European seabass	Juvenile	Growth, mortality	VK3	**1.5**	Kaushik *et al.* 1998
Salmonids	Juvenile	Growth	-	**10**	Halver, 2002
Haddock	Juvenile	Growth, bone health	VK3	**20**	Roy and Lall, 2007
Atlantic salmon	Juvenile	Growth, coagulation time, bone health	VK1	**0.1**	Krossøy *et al.* 2009
Senegalese sole	Larvae	Bone health	VK1	**4.5**	Richard *et al.* 2014.

* in mg kg^{-1}

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Insights on new roles and future research needs

The requirement of VK for the blood coagulation control has been largely known. More recent works also demonstrated its requirement in fish for the prevention of soft tissues calcification (Fernandez *et al*. 2014) and for a proper skeletogenesis (Richard *et al*. 2014) and bone homeostasis (Fernandez *et al*. 2014). Further, Richard *et al*. (2014) presented evidences that VK might also be critical for a broader set of biological functions such as muscular contraction, resistance to osmotic stress, intracellular Ca^{2+} homeostasis or energetic metabolism, by applying proteomic analysis. Undoubtedly, new approaches and technologies with a high throughput like Next-Generation Sequencing (NGS) will help us to fully unveil the biological processes where VK has a key role in fish species, and confirm those already suggested in mammalian systems. In this regard, VK have an important role in the synthesis of sphingolipids that are crucial for the nervous system, and some correlation with cognition has been found (Ferland 2012). The undercarboxylated form of BGP in which Glu 13 is not carboxylated was shown to be active on β-cells and Leydig cells. In this way it is proposed that undercarboxylated BGP acts as a bone-derived hormone stimulating insulin secretion and β-cell proliferation in the pancreas, energy spending by muscles, insulin sensitivity in adipose tissues, muscles and liver, as well as by stimulating testosterone synthesis in the testis, promoting male fertility (Karsenty and Ferron 2012). Furthermore, Gray and Squires (2012), found that the transactivation of PXR in Leydig cells increased the expression of several genes involved in steroidogenesis, including *cytochrome B5A* and *cytochrome B5 reductase 1*, as well as *hydroxysteroid* (17-beta) *dehydrogenase 4* and *retinol dehydrogenase 12*. Treatment with rifampicin, an agonist of mammalian PXR but not of fish PXR (Ekins *et al*. 2008), resulted in significantly decreased sex steroid production and significantly increased production of androstene steroids. It is well known that androstene steroids are androgens controlling the development and maintenance of male characteristics in vertebrates by binding to androgen receptors, but also functioning as paracrine hormones required by the Sertoli cells to support sperm production. Further, the expression of genes involved in the biosynthesis of cholesterol and steroid hormones was found to be decreased in rats fed with VK deficient

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

diets; being the mRNA levels of *cyp11a* – a rate-limiting enzyme in testosterone synthesis – positively correlated with the MK-4 concentration in testis (Shirakawa *et al*. 2006). The same authors compared testosterone concentrations among rats fed control, VK supplemented and VK deficient diets, demonstrating decreased concentrations of testosterone in plasma and testis from rats fed VK deficient diet. Similar results regarding the role of MK-4 in testis and testosterone were also reported by Ito *et al*. (2011). Thus, since VK and warfarin are able to regulate gene expression of *pxr* in fish (Richard *et al*., 2014; Fernández *et al*., 2014), and expression of PXR downstream target genes is also activated under warfarin exposure (which induces a VK-like deficiency), the VK nutritional status might affect fish gametogenesis. In this regard, although a previous report stated that VK deficiency in the parental fish did not affect the hatching rate of the eggs or the mortality of the larvae (Udagawa and Hirose 1998), little is known regarding the potential roles of VK in fish gametogenesis.

The last, but not the least, interaction of VK with other molecules should be revealed in order to find optimal nutritional levels in aquafeeds. In this sense, VK bound to its nuclear receptor can interact at the nuclear level with other fat soluble vitamins. Not only VK can activate PXR (Tabb *et al*. 2003), all forms of vitamin E are also able to activate gene expression via PXR (Landes *et al*. 2003). Since PXR form heterotetramers with retinoid X receptors (RXR; Wallace *et al*. 2013), it also might interact signaling pathways regulated by its ligands, the retinoids (vitamin A). Interestingly, vitamins A and D influence expression and synthesis of VKDPs (Darias *et al*. 2010; Fernandez *et al*. 2011), while VK is responsible for the posttranslational modification and activation of those VKDPs (Oldenburg *et al*. 2008). Another potential crosstalk between fat soluble vitamins which remains to be revealed might be the intestinal absorption mechanisms, since all fat soluble vitamins share some intestinal absorption proteins (Gonçalves and Reboul, 2011; Blomhoff and Blomhoff, 2006).

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

Conclusions

Studies from the 1990's and 2000's have revealed the basal requirements of VK in blood coagulation and evidenced its role in bone development and homeostasis (reviewed by Krossoy *et al*. 2011). More recently, research efforts on determination of VK dietary requirements on early developmental stages have also suggested new biological processes where VK might has an important role such as soft tissue pathological calcification, skeletogenesis, muscular contraction and energy metabolism among others. Certainly, new functions will be identified. Nevertheless, a reliable and accurate marker of VK nutritional condition in fish species is still lacking, hampering the determination of species and developmental stage specific VK requirements. Importantly, comparative studies on the suitability of the different K vitamers for aquafeed diets is an imperative for feed producers, which should be based on thermal and chemical stability, metabolic bioavailability and on toxicological parameters, for which the identification and characterization of enzymes and transport proteins are essential.

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Avances en Estudios Sobre Expresión y Actividad de Enzimas Digestivas en Juveniles de Totoaba (*Totoaba macdonaldi*, Gilbert 1890) Alimentados con Proteínas de Origen Vegetal.

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Resumen

En este documento se presentan los resultados de dos estudios donde se sustituyó harina de pescado (HP) por ingredientes de origen vegetal como el concentrado de proteína de soya (CPS) y harina de soya (HS), para el desarrollo de alimentos de calidad y económicamente viables para peces marinos carnívoros como totoaba (*Totoaba macdonaldi*). En ambos estudios se evaluó el efecto del CPS y HS como fuente alternativa en la sustitución parcial y total de la harina de pescado para conocer la actividad de enzimas digestivas y desarrollo productivo de los peces ante el cambio de fuente proteica en la dieta. En el primer experimento se formularon ocho dietas isoprotéicas, donde se incrementó el nivel de CPS a 0%, 15%, 30%, 45%, 60%, 75%, 90% y 100% en sustitución de la HP, mientras que el segundo estudio se formularon cuatro dietas experimentales isoprotéicas e isolipídicas con un incremento en la sustitución de harina de pescado por harina de soya: 33, 66 y 100% (HS-33, HS-66 y HS-100, respectivamente); y una dieta control de 100% harina de pescado (HS-0). Los resultados muestran que en el primer estudio con CPS la respuesta de la actividad de tripsina, quimiotripsina, fosfatasa alcalina y leucina aminopeptidasa mostraron diferencias significativas ($P<0.001$) entre los tratamientos, observándose mayor actividad en los peces alimentados con CD, SP15 y SP30. Así mismo con respecto a los niveles de expresión de tripsina y quimotripsina, se observó que los niveles de estas dos enzimas se ven afectados conforme se aumenta los niveles de CPS en la dieta en sustitución de la HP. En el segundo estudio con HS los niveles de actividad de enzimas digestivas no se ven afectados con dietas que contengan

Galaviz, M. 2015. Avances en Estudios Sobre Expresión y Actividad de Enzimas Digestivas en Juveniles de Totoaba (*Totoaba macdonaldi*, Gilbert 1890) Alimentados con Proteínas de Origen Vegetal. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Veja, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 377-401.

hasta el 33% de HS. En base los resultados encontrados en la presente investigación, el uso de la soya como CPS o HS puede ser utilizados como fuente de proteína en la dieta para juveniles de totoaba hasta un 33% sin afectar el crecimiento, desarrollo y salud de los organismos en cultivo.

Palabras claves. Expresión de genes, enzimas digestivas, soya, nutrición

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Introducción

Durante años se ha monitoreado la actividad pesquera y acuícola con el objetivo de encontrar un equilibrio adecuado entre las oportunidades y las amenazas a las que se enfrentan los ecosistemas naturales. La acuacultura durante las últimas décadas ha crecido de manera considerable, lo que ha ocasionado un incremento en la demanda de alimentos balanceado de alta calidad que son elaborados con proteínas y lípidos de origen marino, como harina y aceite de pescado, en especial de sardina y arenque, especies de pelágicos menores que son ricos de ácidos grasos (AG) y aminoácidos (AA) esenciales que requieren las especies en cultivo para un mejor desarrollo (García-Ortega 2010; Tacon, A.G.J, Hasan, M.R, Metian, M. 2011, FAO 2012). Sin embargo, los alimentos balanceados elaborados con estas fuentes ricas en AG y AA pueden representar arriba del 55% de los costos totales de producción, por lo tanto la industria dedicada a la elaboración de alimentos para acuacultura busca optimizar la manufactura, calidad y uso de los mismos con el fin de aumentar la eficiencia de producción, sin afectar el crecimiento, desarrollo y salud de los organismos en cultivo (Watanabe 2002). Tomando en cuenta que los principales ingredientes que se usan en la formulación de los alimentos provienen de las pesquerías, las cuales han llegado a su límite máximo de explotación y que año tras año existe una restricción en el uso actual y futuro de las harinas y aceites de pescado en alimentos acuícolas (Tacon, A.G.J. y Metian, M 2008), resultan relevantes las investigaciones enfocadas a encontrar fuentes alternativas de proteína y lípidos de origen vegetal para la elaboración de los alimentos acuícolas (Tacon & Metian 2008; Hardy R. W 2010, Ngandzali B. O., Zhou F., Xiong W., Shao Q.J. & Xu J.Z 2011). En este aspecto, los ingredientes provenientes de fuentes vegetales tienen un gran potencial en la industria acuícola debido a que algunos granos, como la soya, que contienen elevadas concentraciones de proteína, un buen perfil de aminoácidos, buena biodisponibilidad, y son económicamente accesibles en comparación con la harina y aceite de pescado. No obstante, existen ciertas restricciones en el uso de éstos productos vegetales debido a la palatabilidad, a la carencia de algunos aminoácidos esenciales (lisina, cisteína y metionina), así como a la presencia de factores antinutricionales tales como inhibidores de proteasas, glucosinolatos,

Galaviz, M. 2015. Avances en Estudios Sobre Expresión y Actividad de Enzimas Digestivas en Juveniles de Totoaba (*Totoaba macdonaldi*, Gilbert 1890) Alimentados con Proteínas de Origen Vegetal. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Veja, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 377-401.

ácido fítico, saponinas, anti vitaminas liposolubles, entre otros (Francis G, Makkar H.P.S. & Becker K 2001, Chou R.L, Hera B.Y, Sua M.S, Hwang G, Wu Y.H & Chen H.Y 2004). Los factores antinutricionales presentes en la harina y derivados de soya limitan la utilización de ciertos nutrientes esenciales (por ejemplo los aminoácidos lisina y metionina) (Ganga R., Montero D, Bell J.G, Atalah E, Ganuza E, Vega-Orellana O, Tort L, Acerete L, Afonso J.M. Benitez-Santana T, Fernández V.A. Izquierdo M 2011), por medio de la actividad de factores antitrípsicos o inhibidores de proteasas los cuales son compuestos termolábiles de naturaleza proteica que inhiben o modifican las enzimas digestivas como la tripsina o quimotripsina, alterando la digestibilidad de la proteína e incrementando las secreciones digestivas del páncreas (acetilcolina, gastrina e histamina) (Norton, 1991; Francis *et al*., 2001; Merrifield D.L, Olsen R.E, Myklebust R, Ringø E 2010).

Por su parte, la soya es un ingrediente empleado de manera importante en la industria acuícola debido a la composición de las proteínas que la conforman, ya que la estructura de sus aminoácidos es muy similar a las proteínas de origen animal (Watanabe. 2002); sin embargo, es un producto que posee elementos desfavorables como 22% de oligosacáridos, inhibidores de proteasas que genera cambios morfológicos en el intestino (vacuolización supra nuclear de las células de absorción, acortamiento del plegamiento de la mucosa y presencia de células inflamatorias), lo que afecta la digestión de la proteína y la asimilación de los aminoácidos (2006, Murray H.M., Lall S.P., Rajaselvam R., Boutilier L.A., Blanchard B., Flight R.M., Colombo S., Mohindra V., Douglas S.E 2010). No obstante dichos elementos pueden ser desactivados durante el proceso de manufactura por medio de temperaturas elevadas (Aragão C, Conceição L.E.C, Dias J, Marquez A.C, Gomez E, Dinis M.T 2003; Vucelić-Radović V, Barić M, Stanojević S, Pešić M, Hristić M, Miladinović J, Prijić Lj.,Srebrić M 2005, Merrifield D.L, Olsen R.E, Myklebust R, Ringø E 2011). De esta manera, la mayoría de las semillas contienen inhibidores que protegen sus componentes de la degradación no intencional, ejemplo de esto, son los inhibidores para la hidrólisis de la proteína y lípidos, así como la absorción de minerales. Estos inhibidores pueden ser proteínas simples o complejas y son desnaturalizadas por medio de tratamientos con calor, extracción de alcohol y fermentación (Hardy 2010). El mecanismo de acción de

Galaviz, M. 2015. Avances en Estudios Sobre Expresión y Actividad de Enzimas Digestivas en Juveniles de Totoaba (*Totoaba macdonaldi*, Gilbert 1890) Alimentados con Proteínas de Origen Vegetal. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Veja, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 377-401.

los inhibidores en los organismos comienza con la unión de éstos a la enzima blanco (tripsina y en menor medida quimotripsina) formando complejos estequiométricos estables lo que en consecuencia inhibe su actividad. Al inhibir la actividad de estas enzimas se estimula la secreción de pancreocimina-colecistoquinina de la pared intestinal las cuales estimulan la secreción de tripsina del tejido pancreático (Krogdahl *et al.* 2003); sin embargo, después de un tiempo prolongado de haber obligado al páncreas a producir una gran cantidad de enzimas, se produce una hipertrofia pancreática (Hardy 2010, Silva M.R y Silva M.A 2000), la cual consiste en el agrandamiento de las células pancreáticas y por lo tanto del órgano.

Las proteasas son enzimas que actúan sobre proteínas o sobre ellas mismas, rompiendo los enlaces peptídicos produciendo fragmentos de proteína de menor tamaño (eg. tripeptidos, dipeptidos) o incluso hasta aminoácidos libres. Las proteasas en general tienen diversas funciones como: resistencia inmunitaria, apoptosis, replicación celular y digestión de los alimentos (Werner. 2008). Es así que la proteína animal consiste de cadenas de L-amino ácidos ligados a otros aminoácidos a través de enlaces peptídicos (-NH·CO-). Para permitir que las proteínas sean biodisponibles, éstas deben ser hidrolizadas por endopeptidasas extracelulares, las cuales rompen los enlaces péptidos a lo largo de la cadena proteínica, y por exopeptidasas que rompen los aminoácidos terminales. La acción de estas enzimas libera oligopeptidos, dipeptidos y aminoácidos. En este aspecto, los oligopéptidos y dipéptidos son además hidrolizados por otras enzimas contenidas en las células epiteliales intestinales para ser absorbidas posteriormente. Dentro de estas enzimas se encuentran: la pepsina, tripsina, quimiotripsina. Los peces poseen proteasas de tipo ácido (pepsina) segregadas en el estómago y proteasas de tipo básico o neutro (tripsina, quimiotripsina, carboxipeptidasas, etc.). Al igual que en los vertebrados, las enzimas proteolíticas pancreáticas en peces son secretadas como proenzimas inactivas y es en el intestino medio el único sitio de activación de éstas enzimas (Conceição L., Aragão C., Rønnestad I 2011). Para medir las enzimas presentes en los tejidos de los animales, existen diferentes metodologías que se han desarrollado durante décadas, por ejemplo técnicas histoquímicas, bioquímicas y más recientes las moleculares. Esta última técnica ha tomado

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gran importancia debido a que se logra un panorama más certero de la síntesis de proteínas que se están utilizando con respecto a la nutrición del animal. Sin embargo, pocos estudios han aplicado las técnicas de estudios bioquímicos y moleculares para describir la relación entre la transcripción de una enzima digestiva y su correspondiente actividad enzimática (Mario A. Galaviz, Lus M. López, Alejandra García Gasca, Carlos Alfonso Álvarez González, Conal D. True, Enric Gisbert 2015).

La expresión y actividad de enzimas digestivas que son secretadas por el páncreas sufren modificaciones durante el desarrollo y maduración de órganos que forman el sistema digestivo en los peces (Péres A. Zambonino Infante.J.L, Cahu, C 1998). Así mismo, la síntesis de estas enzimas puede ser modulada por genes, hormonas, condiciones ambientales y la nutrición de los organismos (Peres *et al*. 1998). La secreción y el contenido de proteasas, lipasas y amilasas en los organismos, sufren cambios en respuesta a la cantidad de sustratos empleados en relación a la técnica utilizada (Lhoste *et al*. 1994, Wang C, Xie S, Zhu, X, Lei W, Yang Y, Liu J 2006).

De esta manera, la aplicación de las técnicas de bioquímica, biología celular y molecular durante la nutrición de los juveniles de peces marinos puede ser una herramienta útil, ya que puede ayudar a determinar si los cambios en la cantidad de enzimas digestivas reflejan el control en el nivel de la transcripción o de traducción de los genes implicados, así como, permite la identificación de genes específicos que participan en la regulación del desarrollo gastrointestinal (Zambonino Infante y Cahu. 2001). En los últimos años, el número de estudios realizados donde se llevan a cabo la relación de expresión y actividad de enzimas digestivas en peces han sido escasos (Douglas S.E, Gawlicka A., Mandlam, S, Gallant, J.W 1998, Péres *et al*., 1999; García-Gasca A, Galaviz M, Gutiérrez JN, García-Ortega A 2006; Galaviz M, García-Ortega A, Gisbert E, López LM, García-Gasca A 2012, Galaviz *et al.* 2015). La regulación de la expresión de enzimas digestivas es especie-especifica, y esta modulada por la edad. Por lo tanto, la expresión génica de estas enzimas dependientes de células acinares están reguladas por al menos dos señales fisiológicas complejas que son hormonas y dieta (Moal, J, Daniel, J.Y, Sellos, D, Van Wormhoudt, A,

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Samain, J.F 2000, Wang, C.F, Xie, S.Q, Zheng, K.K, Zhu, X.M, Lei, W, Yang, Y.X, Liu, J.K 2006). Es por ello que los estudios de expresión y actividad de enzimas digestivas en juveniles de peces marinos representan una fuente de información importante sobre el funcionamiento de la fisiología digestiva de peces marinos, con el propósito de formular dietas adecuadas durante las diferentes etapas de crecimiento.

La totoaba se encontraba clasificada en el género *Cynoscion*, género en el que la totoaba fuera la especie que alcanzaba la mayor talla dentro de su familia (Sciaenidae) con tallas cercanas a los dos metros de longitud (Berdegué. 1955) y pesos superiores a los 135 kg (Cannon. 1966). Actualmente se encuentra clasificada como *Totoaba macdonaldi*, en un género y especie única para esta familia y es un pez endémico del Golfo de California (Cisneros-Mata, M., Botsford, L.W, Quinn, J.F 1997). A partir de 1975 ha sido incluida en la lista de especies en peligro de extinción (CITES, 2005). A principios de 1900 se exportaba su vejiga gaseosa a oriente para ser utilizado como ingrediente principal en una sopa gourmet (Berdegué. 1955). En 1942, la captura de esta especie alcanzó un máximo de 2,261 toneladas. Sin importar el incremento en el esfuerzo pesquero, la producción anual fluctuó hasta llegar a capturar sólo 58 toneladas en 1975 (Flanagan y Hendrickson 1976).

Aunque se han postulado diversas causas probables que afectan a las poblaciones de este importante recurso, la información que existe sobre la totoaba sigue siendo muy limitada. Se han llevado a cabo estudios sobre su desarrollo embrionario (Morales-Ortiz 1999), desarrollo larval (Sandoval-Garibaldi 2001) y requerimientos nutricionales. En éstos últimos, se ha investigado el efecto de diferentes niveles de ácidos grasos, dietas isoprotéicas, niveles de alimentación, etc. (López, L.M., Durazo, E., Rodríguez, M.A., True, C., Viana, M.T 2006, Solórzano-Salazar 2006; Vizcaíno-Pérez 2008, Bañuelos-Vargas, I, López, L.M, Pérez-Jiménez, A, Peres, H 2014).

Actualmente la Unidad de Biotecnología en Piscicultura de la Facultad de Ciencias Marinas de la Universidad Autónoma de Baja California, lleva a cabo el desarrollo de la biotecnia de cultivo de ésta especie, mismo que se encuentra enfocado a logar el inicio de

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un programa de repoblamiento a partir de reproductores mantenidos en cautiverio (True C.D, A. Silva Loera y N. Castro Castro 1997, True C.D, N. Castro-Castro, G. Sandoval-Garibaldi y C. Morales-Ortiz 2001). Aunque ya se ha logrado el cultivo de esta especie, existen limitantes para escalarlo a nivel comercial. En vista de lo anterior, investigación básica y aplicada recientes se centran en la obtención de información detallada sobre la nutrición y fisiología digestiva durante la engorda de esta especie cuando se sustituye la harina de pescado por proteína de origen vegetal como la soya. El propósito de esta investigación es analizar el efecto de la proteína y harina de soya sobre la expresión y actividad de las enzimas digestivas de totoaba, con la intención de poder diseñar dietas económicamente viables, sin afectar el desarrollo, crecimiento y salud de los peces en cultivo.

Materiales y Métodos

Manejo de peces

Se realizaron dos experimentos con *T. macdonaldi* para evaluar el concentrado de proteína de soya (CPS) y harina de soya (HS) como fuentes de proteínas. Los peces utilizados en los dos estudios fueron obtenidos de la Unidad de Biotecnología en Piscicultura (UBP) de la Facultad de Ciencias Marinas de la Universidad Autónoma de Baja California, México. Los peces fueron transferidos a las instalaciones experimentales las cuales consistieron de un sistema de recirculación termo regulado equipado con 24 tanques de fibra de vidrio de 120 L de capacidad para el estudio con CPS y 12 tanques de las mismas características que el estudio anterior para el estudio de HS. Durante el ensayo de ambos experimentos, la temperatura fue mantenida a 23.2 ± 0.4°C y el fotoperiodo fue programado a 12:12 h (luz:oscuridad). La concentración promedio de la salinidad fue de 34‰ y el oxígeno fue mantenido arriba del 6mg/l durante todo el experimento.

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Formulación de dietas

Para el primer experimento que consistió en la utilización de CPS, fueron formuladas ocho dietas experimentales siendo isoprotéicas, incrementado la sustitución de harina de pescado por CPS (0, 15, 30, 45, 60, 75, 90 y 100%), mientras que para el estudio con HS, se formularon cuatro dietas experimentales isoprotéicas (proteína cruda: 50%, materia seca) e isolipídicas (lípidos crudos: 12%, materia seca) con un incremento en la sustitución de harina de pescado por harina de soya: 33, 66 and 100% (HS-33, HS-66 y HS-100, respectivamente); y una dieta control de 100% harina de pescado (HS-0).

Para ambos estudios, todos los ingredientes fueron mezclados en un procesador de alimentos (Hobart, Troy, OH, USA). La mezcla húmeda fue peletizada a través de un molde de 3 mm de diámetro en un moledor de carne comercial, posteriormente los pellets fueron secados durante 18 horas en un horno de convección con salida de humedad a 70 ± 5°C. Los pellets secos fueron fraccionados a un tamaño menor y posteriormente tamizados para eliminar el polvo fino, posteriormente fueron almacenados a -20ºC hasta su uso.

Previo al comienzo del experimento, los peces fueron aclimatados a las instalaciones y a la dieta control (AS-0) por un periodo de dos semanas. Al finalizar la aclimatación, 480 (50.5 ±1.0 g) y 420 (4.03 ±0.1 g) (experimento 1 y 2) juveniles de totoaba fueron seleccionadas al azar y asignadas a sus respectivos tanques a una densidad de 20 y 35 peces/tanque. Cada dieta experimental fue asignada al azar en tres diferentes tanques (triplicados), la alimentación fue a aparente saciedad dos veces al día (09:00 y 16:00 h) durante 60 días.

Biometrías y obtención de muestras

Los peces fueron pesados al inicio, después de 4 semanas y al final del experimento bajo el efecto de anestesia (100 mg L^{-1} de aceite de clavo). Del stock inicial se tomaron muestras de peces para su análisis correspondiente, y al final del experimento se seleccionaron 4 peces al azar de cada tanque los cuales fueron almacenados a -20°C para

subsecuentemente realizar análisis proximales de pez entero. Se seleccionaron otros 10 peces de cada tanque para tomar muestras de hígado y músculo los cuales fueron congelados a -20°C para realizar futuros análisis. El peso húmedo de los organismos y sus respectivos hígados fueron registrados para la determinación del índice hepatosomático.

Actividad enzimática digestiva

Se extrajo el sistema digestivo (estómago e intestino) de 3 organismos de cada tanque los cuales fueron congelados inmediatamente después de la disección en hielo seco y almacenados a -80°C. Los análisis iniciaron con la preparación del homogenizado de las dos primeras partes del intestino obteniendo 3 homogenizados de cada tanque (9 por tratamiento). La actividad de las enzimas se cuantificó en un espectrofotómetro modelo DR 5000 UV-Vis marca HACH. Las enzimas que se analizaron para estudiar la hidrólisis de proteínas fueron proteasas alcalinas totales, tripsina, quimotripsina, leucina aminopeptidasa, además de medir la actividad de fosfatasa alcalina y α-amilasa.

Análisis molecular

Extracción de ARN

El análisis molecular se realizó según la metodología descrita por García-Gasca *et al*. (2006). El ARN total fue extraído de tejido de usando reactivo Trizol (Invitrogen), seguido por dos tratamientos de DNAsa para eliminar completamente el ADN genómico. La síntesis de ADNc se llevó a cabo con 5 μg de ARN total y la enzima trancriptasa reversa MMLV (Promega) en presencia de random primers.

PCR Cuantitativo (qPCR).

La expresión de genes de tripsina, quimotripsina, fosfatasa alcalina y 18s ribosomal fue cuantificada de manera relativa con un termociclador de tiempo real CFX96 BioRad usando SYBER GREEN®. Las muestras de ADNc de totoaba de los diferentes

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tratamientos fueron analizadas por triplicado y se utilizó como control interno el gen 18s ribosomal (los primers para qPCR se muestran en la Tabla 4). Las reacciones de PCR se realizaron bajo las siguientes condiciones: 95 °C por 2.5 minutos, y 40 ciclos a 95 °C por 30s, 60 °C por 30s y 72 °C por 30s. La elaboración de la curva estándar de cada uno de los genes se realizó por medio de diluciones seriadas del ADNc amplificado con los primers de tripsina, quimotripsina, fosfatasa alcalina y 18s ARNr. Se utilizaron los valores del ciclo umbral (CT) y el número de copias en escala logarítmica (log copy number) obtenidos del análisis de dilución serial para realizar un análisis de regresión lineal y calcular cada una de las curvas estándar.

Para el cálculo del número de copias (C0) en muestras no conocidas se empleó el modelo de regresión lineal:

$$y = a + b\ (CT)$$

Donde:

y= nivel de expresión de cada gen, a= intercepto, b= pendiente de la curva estándar y CT= ciclo umbral.

Finalmente para normalizar la C0 de cada muestra se dividió la C0 de cada uno de los genes por el C0 de 18s rRNA y por último cada muestra normalizada se dividió por el calibrador que en este caso fue la muestra de la dieta control.

Análisis estadístico

Los resultados son presentados como media ± error estándar. La normalidad de los datos fue determinada por la prueba de Kolmogorov-Smirnov test y la homocedasticidad con la prueba de Levene. Se realizó un análisis de varianza de una vía (ANOVA) para procesar los resultados, seguida de una prueba de Tukey de comparaciones múltiples para comparar el comportamiento de las dietas (HS-0, HS-33, HS-66 y HS-100). Todos los análisis se llevaron a cabo usando el programa Sigma Stat versión 3.5 y se empleó un nivel de significancia de 0.05.

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Resultados

Expresión y actividad de enzimas digestivas

Primer experimento con CPS

Los niveles de actividad de proteasas digestivas de juveniles de totoaba presentaron diferencias significativas ($P<0.05$) en respuesta a los diferentes niveles de inclusión de CPS en la dieta. Los niveles de actividad de tripsina osciló entre 0.17 ±0.00 a 0.073 ±0.00 mU x 10^{-3} mg protein^{-1}, la actividad más alta fue observada en juveniles alimentados con la dieta control (CD) siendo significativamente diferente ($P<0.001$) con respecto al resto de los tratamientos. Por otra parte los niveles de quimiotripsina fueron variando con respecto a la sustitución de CPS en la dieta, observándose valores de 4.69 a 9.31 mU x 10^{-3} mg proteína^{-1}, la actividad más alta se presentó en CD, SP15 y SP30 siendo significativamente diferentes ($P<0.001$) al resto de los tratamientos. En cuanto a la actividad de la fosfatasa alcalina los valores obtenidos fueron de 1.89 a 4.18 mU x 10 mg protein^{-1}, mostrando la mejor respuesta en las dietas CD, SP15 y SP30, observándose diferencias significativas ($P<0.001$) con respecto al resto de las tratamientos. La actividad de leucina aminopeptidasa presentó diferencias significativas por la inclusión de SPC ($P<0.001$), en esta enzima se obtuvieron valores de 0.13 ±0.01 a 0.24 ±0.00 mU x 10^{-3} mg proteina^{-1}, donde la actividad más alta se presentó en SP15 y SP30, con respecto a las dietas con mayor inclusión de SPC.

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Tabla 1. Actividad de enzimas digestivas de juveniles de *Totoaba macdonaldi* alimentados con diferentes niveles concentrado de harina de soya en la dieta. Los datos están representados en mU x 10^{-3} mg proteína.

	Tratamientos							
	DC	**CPS15**	**CPS30**	**CPS45**	**CPS60**	**CPS70**	**CPS90**	**CPS100**
Tripsina	0.073±0.0^{a}	0.037±0.0^{b}	0.039±0.0^{b}	0.025±0.0^{c}	0.021±0.0^{cd}	0.020±0.0^{cd}	0.018±0.0^{d}	0.017±0.0^{d}
Quimotripsina	9.31^{a}	8.08^{b}	8.58^{ab}	6.19^{c}	5.99^{c}	4.92^{d}	4.72^{d}	4.69^{d}
Fosfatasa alcalina	4.13^{a}	4.18^{a}	4.14^{a}	3.45^{b}	2.51^{c}	2.03^{d}	1.92^{d}	1.89^{d}
L-aminopeptidasa	0.24±0.00^{b}	0.25±0.01^{a}	0.26±0.01^{a}	0.21±0.01^{bc}	0.20±0.00^{c}	0.14±0.00^{d}	0.15±0.01^{d}	0.13±0.01^{d}

Segundo experimento con HS

La presencia de harina de soya en las dietas, modificó la actividad de la mayoría de las enzimas digestivas analizadas en éste estudio. La proteasa alcalina disminuyó significativamente su actividad en presencia en los tratamientos que contenían arriba del 33% de HS. Contrario a esto, la enzima Leucina aminopeptidasa incrementó significativamente su actividad en la dieta HS-100 comparativamente con el resto de las dietas. La tripsina mostró su mayor actividad en ausencia de harina de soya (HS-0), y presentó valores significativamente menores en el resto de las dietas. La quimotripsina no presenta diferencias significativas en su actividad. La enzima α-amilasa presenta su mayor actividad en la dieta HS-0 y disminuye conforme se incrementa la harina de soya en las dietas, mostrando su menor valor en la dieta HS-100. Tabla 2.

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Tabla 2. Actividad de enzimas digestivas de juveniles de *Totoaba macdonaldi* alimentados con diferentes niveles de harina de soya en la dieta.

Dieta	HS-0	HS-33	HS-66	HS-100
Proteasa Alcalina U mg prot^{-1}	19.29 ± 0.3^{a}	20.5 ± 0.45^{a}	13.8 ± 1.90^{b}	0.8 ± 0.38^{b}
Leucina Aminopeptidasa mU x 10^{-3} mg proteína^{-1}	0.71 ± 0.03^{b}	0.70 ± 0.03^{b}	0.64 ± 0.04^{b}	0.96 ± 0.05^{a}
Tripsina mU x 10^{-3} mg proteína^{-1}	5.20 ± 0.17^{a}	2.69 ± 0.14^{b}	1.16 ± 0.15^{c}	0.78 ± 0.38^{c}
Quimotripsina mU x 10^{-3} mg proteína^{-1}	2.27 ± 0.5	1.46 ± 0.42	1.19 ± 0.3	1.40 ± 0.37
Alfa amilasa mU x 10^{-3} mg proteína^{-1}	5.26 ± 0.18^{a}	3.43 ± 0.35^{b}	2.91 ± 0.40^{b}	1.39 ± 0.22^{c}

Con respecto a la expresión de enzimas digestivas, actualmente se cuenta con las secuencias y primers específicos para tripsina, quimotripsina, pepsina y fosfatasa alcalina, mismas que se han estudiado para larvas y ahora en juveniles de *T. macdonaldi* (Tabla 3).

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Tabla 3. Secuencias de genes de enzimas digestivas evaluadas en el presente estudio.

> 18s RIBOSOMAL (443 nucleótidos) GenBank HM754483

ATAAAATTCCAGCTGCCATAGCGGATCTTGATATCGCTGCAGTTAAACAACTCG
TAGTTGGATCTCGGGATCGAGCCGTCGAACAGCCGCCGGGCCGACCACCGTCT
GTCCCAGCCCCTGCCTCTCGGCGCCCCTACGATGCTCTTAGCTGAGTGTGCCGC
GCGGTCCGAAGCGTCTACTTTGAAAAAATTAGAGTGTTCAAACCAGGCGCCGT
CGCCAGTAAACCGCAGCTACGAATATTGGAATAGAACTCCGGTTCTATTTTGTG
GGTTTTCTTCTCTGAACTGGGGCCATGATTAAGAGGGACGCCCGGGGGCATTCG
TATTGTGCGGCTAGAGGTGAAATTCTTGACCCGGCAGAAGACGGACGAAAGCG
AAAGCATTTGCCAAGAATGTTTTCATAATTCAAGAACGAAAGTCGGAGGTTCG
AAGACGATCAGATACTGTCGTAGTTCCGATCATCA

> TRIPSINA (314 nucleótidos) Genbank HM754480

ACATTGACATCATGCTGATCAAGCTGAGCAAGCCCGCCACCCTGAACAGCTAC
GTCCGCACCGTGTCCCTGGCCTCCAGCTGTGCAGCTGCTGGCACCCGCTGTCTG
ATCTCTGGATGGGGCAACACCAGCAGCTCTGGAAGCAACTACCCTGATCGTCT
GAGGTGCCTGGATGCCCCCATCCTGAGCGACAGCAGCTGCAGGACTGCCTACT
GTGGACAGATCACTCACAACATGTTCTGTTCTGGATTTGTCGAGAGAGGCAAA
GACTCCTGGCGCGGTGGCGCTGTTGTCTCCGGGGTGTGCGTTGGTCGG

> QUIMOTRIPSINA (477 nucleótidos) GenBank HM754481

CTGGCCATGGCAGGTGTCTCTGCAGCAATCCAATGGCTTCTACTTCTGTGGAGG
ATCTCTGATCAACGAGAACTGGGTGGTGACCGCCGCTCACTGTAACGTCAGGA
CCTACCACCGTGTGGTCGCTGGAGAACACATCAAGGGCTACGGCTCCAACGAG
CACGTCAAGGTTCTGAAGCCCGCCAAAGTGTTCACCCACCCCTACTGGAACCCC
CACACAATCAACAACGACATCTCCCTCATCAAGTTGTCCACCCCCGCCCGCCTG
GGCACAAACGTGTCCCCTGTCTGCCTCGCCGAGTCCCCCGATGTCTTTTCCGCC
GGATGGACCTGCGTCAACTCCGGCTGGGGTCTGACCCGCTACAACGCTCCCAG
TACTCCCAACACACTCCAGCAGGGCGCCCTGCCCCTGCTGTCCAACGAGCAGT
GCAAGAAACACTGGGGCAGCAACATCTCCGGAATCATGATTTGTGCTGG

> PEPSINA (450 nucleótidos) GenBank HM754482

GGGGCgACCAGCCTCTGTCCATCCAGTACGCAGCTGgCAGCATGAccgGATATCT
GGGCAGCGACATTGTTGAGGTGCGACGCATCTCTGTGAACAACCAGGTGTGTG
GTTTCAGCGACTCAGAGGCTCTCCTACATGCTCACATGCACGCTGATGGTATCC
TGGGACTGGCTCTCCAGTCCAATGCCTCTGACGATGTTGTGCCAGTCTTTGACA
ACATGATCAGCCAGCACCTGGTGTCACAGACCCTGTTCTCTGTCTACCTGAGCA
GCAACAGTCAGCAGGGCAATGAGGTGCTCTCCGGTGGTATTGACAGCAACTAC
TACACTGGACAAATTACCTGGATCCCTCTGACCTCTGCCACCTACTGGCAGATC
AAAATGGACAGTGTTACCATCAATGGACAGACTGTGGCCTGCTCTGATGGTTG
CGAGGCCATCATCGACAATC

> FOSFATASA ALCALINA (428 BASES) (En proceso de registro)
CTGCGACGCCTGCTCGTATACTGAAGGGTCAGCTAAACGTGCAGAGTGGAGAG
GAGACCCAGCTGGAGATGGACAAGTTCCCCTTTGTGTCTTTGGCCAAGACATAC
AACACTAACGCGCAGGTGCCAGACAGCGCCGGCACCGCCACAGCTTATCTCTG
CGGGGTCAAGGCCAATGAGGGCACGGTGGGAGTGAGTGCAGCTGCTGTCCGAT
CCCAGTGTAACACCACACAGGGCAATGTAGTCACCTCCATACTCAGATGGGCT
AAGGACGCAGGCAAGTCAGTGGGAATAGTGACAACAACCCGTGTCAACCATGC
GACTCCCAGTGCTGCCTACGCCCACAGCGTGGACAGAGACTGGTACTCCGACA
ATGAGATGCCACGTGAAGCTCTGCAGTCCGGCTGCAAAGACATCGCCAGACAA
CTC

A partir de estas secuencias se diseñaron los siguientes oligonucleótidos específicos por medio del programa PRIMER3, para amplificarlas por tiempo real (Tabla 4).

Tabla 4. Primers específicos de genes de enzimas digestivas evaluadas en el presente estudio.

Nombre		**Primers específicos**	**Numero de GENBANK**
TotoTryp F		5´ACC CGC TGT CTG ATCT CTG GAT 3	**HM754480**
TotoTryp R		5´TTG TCG AGA GAG GCA AAG ACT CCT 3´	
TotoChemo	F	5´CGC TCA CTG TAA CGT CAG GAC CTA 3´	**HM754481**
TotoChemo R		5´GGT GGA CAA CTT GAT GAG GGA GAT 3´	
TotoPepsin	F	5´CTC TGA CGA TGT TGT GCC AGT CTT 3´	**HM754482**
TotoPepsin R		5´CAG AGG TCA GAG GGA TCC AGG TAA 3´	
Toto18sRib-F		5´CTG AAC TGG GGC CAT GAT TAA GAG 3´	**HM754483**
Toto18sRib-R		5´GTC TTC GAA CCT CCG ACT TTC GTT 5´	
Toto-FA-F		GGA GAT GGA CAA GTT CCC CTT TG	**En proceso**
Toto-FA-R		GGT GTT ACA CTG GGA TCG GAC AG	

Hasta el momento se cuenta con la expresión de los genes de enzimas digestivas del experimento 1 que consiste en la sustitución de la harina de pescado por concentrado de proteína de soya en la dieta para juveniles de totoaba. mRNA de tripsina se detectó a niveles muy bajos durante el presente estudio. El nivel de expresión fue disminuyendo conforme se aumentaba el nivel de CPS en la dieta, lo cual podría indicar una inhibición de esta enzima por los factores antinutricionales contenido en el CPS (Figura 1A).

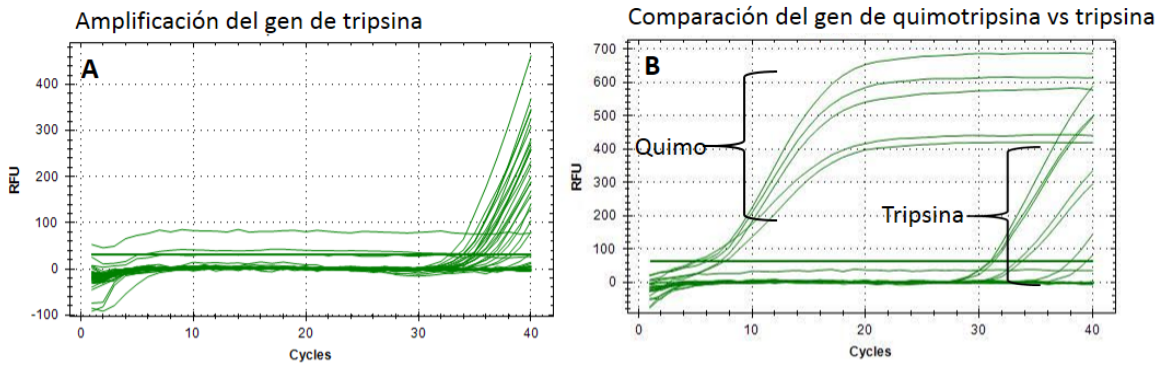

Figura 1. Amplificación de genes de tripsina (A) y quimotripsina *vs* tripsina (B) en muestras de intestino de juveniles de totoaba alimentados con dietas a base de CPS.

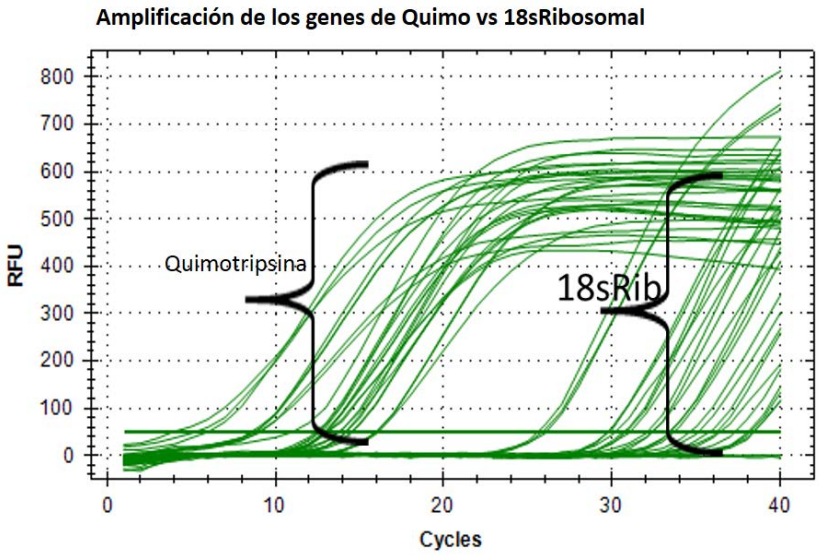

Figura 2. Amplificación de genes de quimotripsina y 18sRib en muestras de intestino de juveniles de totoaba alimentados con dietas a base de CPS.

El máximo nivel de mRNA de tripsina fue detectado en la dieta control, observándose que empieza a expresarse al ciclo 32, mientras que la expresión de las muestras de peces que fueron alimentados con la dieta que contenía 100% CPS fue detectado a niveles muy bajos al ciclo 38 aproximadamente. Al comparar los niveles de expresión de quimotripsina con respecto a tripsina se puede observar que esta enzima tiene niveles más elevados con respecto a tripsina, observándose que la expresión del gen en los juveniles alimentados con la DC empieza a expresarse al ciclo 8 y va disminuyendo conforme aumenta el nivel de sustitución de CPS por harina de pescado como fuente de proteína en la dieta (Figura 1B y 2).

Galaviz, M. 2015. Avances en Estudios Sobre Expresión y Actividad de Enzimas Digestivas en Juveniles de Totoaba (*Totoaba macdonaldi*, Gilbert 1890) Alimentados con Proteínas de Origen Vegetal. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Veja, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 377-401.

Discusión

Mediante estudios de fisiología digestiva en las diferentes etapas de desarrollo de peces marinos, se ha adquirido el conocimiento para entender el progreso de las enzimas digestivas y su papel en la digestión de los nutrientes contenidos en la dieta, por lo que se ha logrado la planificación de los modelos de alimentación y capacidades nutricionales de diversos peces marinos (Díaz-López, M, Moyano-López, F.J, García-Carreño, L.F, Alarcon, F,J., Sarasquete, M.C 1997; Zambonino-Infante & Cahu, C.L 2001; Álvarez-González, C.A, Moyano-López, F.J, Civera-Cerecedo, R, Carrasco-Chávez, V., Ortiz-Galindo, J.L, Dumas, S 2008). Es por ello, que actualmente los estudios se basan en el conocimiento detallado de actividad y expresión enzimática digestiva como proteasas, lipasas, amilasas, fosfatasas, entre otras. Por lo anterior, es importante el desarrollar investigación enfocada en la fisiología digestiva de los organismos determinada desde diferentes panoramas como la bioquímica y la biología molecular, ya que nos puede indicar el estado nutricional de los organismos en cultivo, de manera tal que los datos obtenidos pueden ser relevantes para establecer el momento preciso y el tipo de nutriente que el animal requiere y que será capaz de digerir de forma eficiente (Ueberschär. 1993, Alarcón & Martínez. 1998).

En la presente investigación observamos que los datos de los análisis enzimáticos, reflejan una alteración evidente en la actividad de las enzimas digestivas ya que con el incremento gradual del concentrado de proteína de soya y la harina de soya, la actividad se observó cada vez más disminuida, afectando directamente la digestión y asimilación de los nutrientes, especialmente de la proteína. De manera óptima, las enzimas realizan la hidrólisis de los nutrientes hasta su mínima expresión (aminoácidos libres) para posteriormente llevar a cabo el proceso de absorción a través de los enterocitos y ser transportados al hígado para su metabolismo (Rust. 2002). Sin embargo, si existe una falla en la actividad de éstas enzimas, los procesos de absorción y asimilación no pueden ser realizados de manera eficiente, afectando el estado de salud y el crecimiento de los organismos (Zambonino & Cahu. 2007). El concentrado de proteína de soya y la harina de soya son ingredientes que contiene factores antinutricionales los cuales anulan la actividad

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de algunas de éstas enzimas, por lo que la respuesta fisiológica que se podría generar en los organismos que consumen dietas formuladas con éstos factores son pancreatitis e hipertrofia pancreática los cuales han sido ampliamente relacionados con el uso de productos de la soya (Hardy *et al*. 2010, Krogdahl *et al.* 2003, Francis G, Makkar H.P.S, Becker K 2001). Su mecanismo de acción es inhibiendo la actividad de enzimas proteóliticas, principalmente tripsina y quimotripsina las cuales se unen a la enzima blanco (tripsina y en menor medida quimotripsina) formando complejos estequiométricos estables lo que en consecuencia inhibe su actividad estimulando la secreción de pancreocimina-colecistoquinina de la pared intestinal las cuales estimulan la secreción de tripsina del tejido pancreático; sin embargo, después de un tiempo prolongado de haber forzado al páncreas a producir una gran cantidad de enzimas, se produce una hipertrofia pancreática, lo que a su vez limita la producción de enzimas pancreáticas (Hardy *et al*. 2010; Silva & Silva 2000) y su severidad depende del tiempo y de la cantidad del nutriente suministrado (Berg-Lea *et al*. 1989). Lundstedt L.M., Bibiano Melo J. F. & Moraes G (2004), hacen mención que los cambios en los alimentos formulados tales como el tipo, fuente y cantidad de nutriente pueden llegar a alterar el perfil de las enzimas digestivas o la concentración de las mismas y en consecuencia el aprovechamiento de los nutrientes, de manera que la habilidad de los peces para utilizar los nutrientes ingeridos depende también de la actividad de las enzimas digestivas presentes a lo largo del tracto digestivo, así como de la calidad y tipo de ingrediente con el que se fabrica el alimento (Ali, Haque, Chowdhury & Shariful 2009). En el presente estudio en ambos experimentos se observó que la tripsina y quimotripsina fueron disminuyendo conforme se aumentaba la proteína derivada de la soya, posiblemente los inhibidores presentes en estos ingredientes impiden la síntesis y secreciones de gránulos de zimogenos en el intestino para que puedan ser activada mediante la estimulación química del alimento. Tramati C, Savona B & Mazzola (2005), argumentan que es fundamental relacionar los procesos digestivos de la especie con la composición del alimento que se le proporciona al organismo, ya que sin duda responderán las enzimas digestivas conforme a su capacidad de hidrolizar el alimento que se le proporcione. Por lo que conocer la respuesta de la actividad enzimática de totoaba hacia las fuentes vegetales es

Galaviz, M. 2015. Avances en Estudios Sobre Expresión y Actividad de Enzimas Digestivas en Juveniles de Totoaba (*Totoaba macdonaldi*, Gilbert 1890) Alimentados con Proteínas de Origen Vegetal. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Veja, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 377-401.

primordial y sobre todo de aquellas enzimas digestivas que pueden dar respuesta a un posible efecto por la incorporación de proteínas vegetal.

Conclusiones

Con base en los resultados generados a partir de la presente investigación de ambos experimentos, se concluye que la proteína derivada de soya es un ingrediente que puede sustituir de manera parcial a la harina de pescado en un porcentaje cercano al 30% sin afectar el crecimiento y desarrollo de juveniles de *Totoaba macdonaldi*. Los conocimientos obtenidos constituyen un avance importante para el desarrollo de la industria de alimentos destinados para la acuacultura en México, en especial para el cultivo de especies como totoaba. Así mismo, estos resultados son clave para apoyar de manera certera el cultivo de esta especie en Baja California, tal y como se realiza con otras especies de peces marinos en otros países, por lo que el éxito de dichos desarrollos tecnológicos se basa en un buen control y conocimiento exhaustivo del proceso de alimentación, digestión y nutrición durante el desarrollo fisiológico digestivo en peces.

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Criterios de Calidad de Huevos y sus Implicaciones en el Cultivo de Peces Marinos

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Resumen

El éxito del cultivo de peces marinos, recae en una producción constante de juveniles, lo cual permitirá reducir la dependencia de semilla silvestre, ya que la disponibilidad de ésta última es inconsistente y esta tendencia se ha incrementado en los últimos años. Para lograrlo, es necesaria la obtención de huevos en cantidades suficientes y de buena calidad que permitan la continuidad del programa de cultivo, ya sea como una alternativa económica sustentable, o como ámbito de investigación. Sin embargo, solo en algunos casos ha sido posible contar con una estable producción de juveniles a partir de desoves de reproductores silvestres o mantenidos en cautiverio.

Parte de ello se ha atribuido a las fluctuaciones en la calidad de los huevos que se obtienen durante la reproducción en cautiverio. La calidad del huevo se ha definido como la capacidad de un óptimo desarrollo embrionario y supervivencia de las larvas a la primera alimentación. Se han identificado una serie de parámetros que influyen directa ó indirectamente en la calidad de los huevos, que van desde el mecanismo de inducción al desove (manipulación foto-térmica o inyección hormonal), la calidad nutricional del alimento de los reproductores, las condiciones de incubación de los huevos o el estado fisiológico de los reproductores.
Históricamente, la tasa de eclosión y la supervivencia a la primera alimentación han sido los caracteres principales para evaluar la calidad de los desoves. Sin embargo, dichos parámetros no brindan información sobre los factores que puedan explicar ó mejorar la calidad de los huevos.

De esta forma, se ha reportado el uso de diferentes caracteres como criterios evaluadores ó indicadores de la calidad de los desoves de reproductores tanto silvestres como en cautiverio, con la finalidad de generar una estrategia que permita tomar decisiones precisas y confiables con respecto al desove que se ha obtenido.
El espectro de caracteres que se han utilizado para evaluar o predecir la calidad de los huevos es tan amplio, que abarca desde el porcentaje de fecundación ó el de flotabilidad de los huevos, hasta las “aberraciones” mitóticas en embriones, pasando por la actividad de enzimas metabólicas en embriones, el índice de actividad

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de larvas y la concentración de reservas alimenticias y energéticas (i.e. amino ácidos libres y ácidos grasos). A pesar de esta gama, la utilidad y confiabilidad de cada uno de estos criterios parece variar en forma inter-específica, lo que ha provocado que no exista un indicador “ideal” para evaluar la calidad de los desoves de peces. En el presente trabajo se exponen ventajas y desventajas de los diferentes criterios que han sido utilizados para evaluar la calidad de huevos de peces, ya sea con fines predictivos ó para mejorarla, y se sugieren aquellos que pudieran emplearse de manera rutinaria bajo un esquema productivo o científico, lo cual podría verse reflejado en una disminución de los costos durante el cultivo de peces marinos.

Palabras clave: Calidad de huevos, indicadores de calidad, desarrollo temprano, metabolismo energético, primera alimentación.

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1. Introducción

Un programa de cultivo de peces marinos que se considere exitoso, depende de una producción constante y sostenida de juveniles. Lo cual permitiría reducir los esfuerzos de captura de las poblaciones silvestres cuyas últimas tendencias muestran un descenso en la tasa de captura. Para poder lograrlo, es necesario obtener un número suficiente de huevos de buena calidad que permita mantener la continuidad de los esquemas productivos o de investigación.

Sin embargo, es común observar que durante el mismo ciclo reproductivo se presenten fluctuaciones tanto en el número de los desoves producidos como en la fecundidad, a pesar de que los reproductores se mantengan en óptimas condiciones ambientales y alimenticias (Kjorsvik *et al.*, 1984; Manning y Crim, 1998; Giménez *et al.*, 2006; Faulk y Holt, 2008; Aristizabal *et al.*, 2009; Rangel-Durán, 2014). De tal forma que la producción total no sea la óptima, y repercuta tanto en la actividad científica como en los rendimientos económicos de la actividad productiva. Dichas fluctuaciones han sido asociadas, en gran medida, a la variabilidad en la calidad de los huevos producidos por los reproductores.

La calidad de los huevos es un aspecto básico dentro de la acuacultura, el término fue introducido en los 80's pero fueron Kjorsvik *et al.* (1984) los primeros en definirla como el potencial de los huevos para presentar un desarrollo embrionario exitoso y la probabilidad de supervivencia de la larva vitelina hasta el momento de la primera alimentación; lo cual coincide con el consumo de las reservas alimenticias (vitelo y glóbulo de aceite) para dar inicio a la alimentación exógena, que es cuando las larvas deben iniciar con la captura de alimento.

Bajo este marco, resultado lógico considerar que dos criterios hayan sido tomados en cuenta como las principales características que deben presentar los huevos para ser de buena calidad, y que corresponden a dos etapas importantes durante el inicio del ciclo de

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vida de los peces: por un lado el porcentaje de eclosión, y por el otro, la supervivencia a la primera alimentación. A partir de entonces se han realizado numerosos estudios en los que se utilizan éstos criterios para estimar la calidad de los desoves. Sin embargo, algunos reportes indican que tanto el porcentaje de eclosión como la supervivencia inicial no necesariamente indican un buen desove, ya que se han reportado tasas de eclosión superiores al 90% (lo cual podría considerarse un desove de buena calidad) pero con un porcentaje de supervivencia a la primera alimentación con valores menores al 50% (Giménez *et al.*, 2006).

En ocasiones, bajo un esquema productivo, es deseable tomar decisiones de manera oportuna con respecto a la viabilidad de los desoves obtenidos y sobre la pertinencia de continuar el ciclo productivo ó si se detiene en espera de un desove de mejor calidad, y esta decisión debe tomarse lo antes posible con el objeto de evitar una inversión de tiempo y mano de obra en un desove que no garantice el éxito productivo. Si bien tanto la tasa de eclosión como la de supervivencia inicial han permitido tener obtener información sobre la calidad de los desoves, no brindan información sobre los posibles factores que la definen o sobre aquellos que pudieran influenciarla.

Con este fin, existen numerosos reportes en los que se han presentado toda una gama de criterios que han sido empleados, ya sea como indicadores ó predictores de la calidad de los desoves, así como para poder mejorarla y controlarla durante un ciclo productivo. No obstante, aún no ha sido posible generar un consenso sobre un criterio ideal para predecir ó estimar la calidad de los desoves.

En el presente trabajo se revisan aquellos factores que han sido reportados como influyentes en la calidad de los desoves y que ocurren con mayor regularidad en las actividades propias del cultivo de peces marinos, se excluyen las variables ambientales, ya que se asume que los desoves son incubados bajo las condiciones optimas, propias de cada especie (temperatura, salinidad, fotoperiodo, pH, etc), independientemente del método empleado para la obtención de los desoves ó de la nutrición de los reproductores, por lo que

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se reduce su posible efecto durante su cultivo, no así en el medio ambiente, donde juegan un papel preponderante en la regulación de las poblaciones silvestres.

Posteriormente se exponen aquellos criterios que han sido reportados para evaluar la calidad de los desoves, además de la tasa de eclosión y de la supervivencia a la primer alimentación. A pesar de la diversidad de caracteres utilizados, solo algunos de ellos han resultado útiles como indicadores de la calidad, por lo que se presenta una relación de aquellos criterios empleados con éxito para explicar la calidad de los desoves. Finalmente se exponen las implicaciones de estos criterios dentro de los esquemas de cultivo de peces marinos, y se propone una estrategia que pudiera ser empleada para desarrollar un protocolo de evaluación de la calidad de los desoves.

2. Factores que afectan la calidad de huevos

La calidad de un desove puede estar afectada por diferentes factores de naturaleza diversa. Algunos factores están directamente relacionados con las actividades propias de la producción en cautiverio mientras que otros ocurren de manera natural, afectando el reclutamiento en las poblaciones silvestres. En este sentido, las variables fisicoquímicas tienen un mayor impacto en el medio silvestre, por lo que no serán incluidas en el presente trabajo.

En un esquema de cultivo, los principales factores que pueden afectar la calidad de los desoves están íntimamente relacionados con los reproductores. En efecto, el huevo es el resultado del crecimiento del ovocito y todos los componentes internos, genéticos y nutritivos, deben ser incorporados durante la ovogénesis. Brooks *et al.* (1997) hicieron una revisión sobre el contenido del ovocito y sus implicaciones en la calidad del huevo. Es evidente que se trata de un complejo sistema que incluye procesos de biosíntesis, absorción, acumulación y transporte de biomoléculas indispensables para el correcto desarrollo embrionario y posterior diferenciación y crecimiento durante la etapa de larva vitelina. De esta forma, cualquier factor que interfiera, aún de forma parcial, en éste proceso, afectará la calidad del ovocito producido.

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Algunos de estos factores pueden ocurrir de manera natural como por ejemplo la edad de los reproductores, ya que algunos reportes muestran que hembras de edad media producen huevos con mayor calidad que hembras que desovan por primera vez, lo que ha sido asociado a la mayor capacidad de las primeras para sintetizar lípidos (Kjorsvik *et al.*, 1990). Dentro de un esquema de cultivo, los principales factores reportados que afectan la calidad de los huevos incluyen el mecanismo de inducción al desove, la alimentación y nutrición de los reproductores y el tiempo de colecta después de la ovulación ó sobre-maduración (over ripening).

2.1 Mecanismo de inducción al desove

El control del ciclo reproductivo bajo condiciones de cultivo es un aspecto fundamental en la industria de la acuacultura, por un lado brinda plasticidad al permitir manipular y planificar las etapas productivas, así como planear nuevas estrategias y sobre todo, puede permitir contar con una producción constante de huevos para la producción o la investigación.

Sin embargo, no todas las especies maduran y desovan de manera espontánea en condiciones de cultivo, por lo que se han desarrollado protocolos que permitan, por una lado, inducir la maduración de los reproductores; y por otro lado, lograr la ovulación y obtención del desove (Mylonas *et al.*, 2010). En algunos casos la manipulación foto-término ha logrado inducir la maduración y desove espontáneo de los reproductores (Rosales-Velázquez, 1997; Brandsen *et al.*, 2007; Faulk y Holt, 2008; Rangel-Durán, 2014). Sin embargo, en ocasiones es necesario inducir la maduración final de los reproductores. Para ello se ha utilizado la inducción hormonal. Los diferentes métodos reportados para inducir la maduración final y ovulación incluyen la inyección de gonadotropina (GTH), extractos de pituitaria que incluyen GTH, la hormona correncia humana (GTH) y hormonas liberadoras de gonadotropina (GnRH). Los análogos de la GnRH han sido más ampliamente utilizados y en años recientes, se ha extendido el uso de

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implantes de liberación lenta del análogo (D-Arg^6 - Pro^9 - Net salmon GnRH; Ovaplant® ; sGnRHa) que tienen la ventaja de aplicarse una sola vez y reducir el estrés sobre los reproductores por la manipulación al momento de la inyección (Ibarra-Castro y Ducan, 2007; Ibarra-Castro y Álvarez-Lajonchere, 2011; Duncan *et al.*, 2012). Sin embargo, se han reportado variaciones inter-específicas sobre la eficacia de la inducción hormonal, probablemente debidas a las diferencias en los estados de desarrollo de los ovocitos durante la inducción ó debidas al estatus fisiológico o de madurez de las hembras al momento de la inducción; y aún así, a pesar de ocurrir la ovulación, la liberación de los gametos no se lleva a cabo, por lo que es necesario obtenerlos manualmente mediante masaje abdominal, seguido de una fertilización artificial (Bourque y Phelps, 2007; Mommens *et al.*, 2015).

De esta forma, la inducción hormonal al desove es una práctica común durante el cultivo de especies que no ovulan espontáneamente en cautiverio, sin embargo, el impacto de la inducción hormonal en la calidad de los huevos no siempre es positivo. Varios estudios han demostrado que la inducción hormonal afecta negativamente algunos parámetros de la calidad de los huevos. Por ejemplo: la tasa de eclosión en *Dicentrarchus labrax* (Fornies *et al.*, 2001), el porcentaje de huevos flotantes en *Solea senegalensis* (Agulleiro *et al.*, 2006), produce un aumento en la mortalidad durante el desarrollo embrionario en *Limanda ferruginea* (Avery *et al.*, 2004), la actividad enzimática en *Lutjanus peru* (Moguel-Hernández *et al.*, 2013) y el tamaño de los huevos en *Lutjanus campechanus* (Papanikos, 2004) y *Argyrosomus regius* (Duncan *et al.*, 2012).

El principal inconveniente que se ha reportado con respecto a la inducción hormonal es el tiempo que transcurre entre la aplicación de la hormona, la ovulación de los ovocitos y la obtención de los mismos a través del masaje abdominal. Esto implica que se debe mantener un monitoreo cuidadoso de los ritmos ovulatorios en las hembras para minimizar el impacto del envejecimiento postovulatorio, también conocido como sobre maduración, el cual tiene un efecto directo sobre la calidad del desove.

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2.2 Sobre-maduración (Over-ripening)

La sobre maduración es un problema común que se ha reportado asociado a la inducción hormonal en los peces en cautiverio, principalmente en especies que son desovadores parciales, y cuando los gametos deben ser obtenidos manualmente y después fertilizados de manera artificial. Después de la ovulación, los ovocitos permanecen viables durante un corto periodo de tiempo antes de un proceso de descomposición natural. Este periodo varía de manera inter-específica y es termo-dependiente; después de este tiempo, la calidad de los ovocitos disminuye rápidamente. Es por ello que los huevos deben colectarse en un momento preciso después de la ovulación para evitar un posible efecto deletéreo en su calidad.

La sobre maduración provoca cambios visibles a simple vista en los huevos, los más comunes son cambios en la transparencia, los huevos se vuelven opacos y oscuros (McEvoy, 1984). Los cambios también incluyen una pérdida de peso y un incremento en el contenido de agua. Además, la composición bioquímica de los huevos cambia durante la sobre maduración y puede provocar una ruptura proteolítica de las proteínas en el vitelo y una pérdida de amino ácidos y péptidos a través de la membrana (Kjorsvik *et al.*, 1990).

La escala temporal de viabilidad de los huevos después de ser ovulados puede variar de 15-30 minutos en *Morone saxatilis* (Mylonas *et al.*, 1996) a 4-6 horas en *Hippoglossus hippoglossus* (Bromage *et al.* , 1994) y los efectos pueden ser tan drásticos como 0% de eclosión 10 h después de la ovulación (Kjorsvik *et al.*, 1990; Bromage *et al.* , 1994).

Existen varias formas de evaluar el estado de los ovocitos después de ser ovulados, pero deben ser verificados para cada especie. Algunos de los métodos más utilizados incluyen la observación y evaluación directa por canulación (Mansour *et al.*, 2007), el uso de ultrasonido (Shields *et al.*, 1993) o incluso, la evaluación de las propiedades bioquímicas del fluido ovárico (Rime *et al.*, 2004).

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2.3 Nutrición de los reproductores

Existe una innegable importancia de la nutrición de los reproductores sobre el desempeño reproductivo y la calidad de los desoves. Muchos reportes han demostrado que una inadecuada composición nutricional de la dieta puede provocar una reducción en la calidad de los huevos, afectando, por ejemplo, el porcentaje de eclosión y la tasa de desarrollo, provocando una baja supervivencia embrionaria y un aumento en las malformaciones embrionarias, así como una baja supervivencia larval (Izquierdo *et al.*, 2001).

Indudablemente, el efecto de la concentración de lípidos en la dieta de los reproductores es el aspecto más estudiado en nutrición de reproductores y calidad de huevos (Sargent, 1995). Particularmente importante es el nivel de ácidos grasos esenciales (EFA) debido al papel que juegan como componentes celulares y como fuente de energía (Mazorra *et al.*, 2003), y se ha reportado una clara correlación entre los ácidos grasos esenciales en la dieta de los reproductores y la calidad de los huevos en varias especies, por ejemplo, en *Dicentrarchus labrax* Navas *et al.*(1996) reportaron un incremento en la calidad de los huevos después de alimentar a los reproductores con una dieta enriquecida con aceite de pescado y en *Sparus aurata*, la composición de ácidos grasos de los huevos está afectada directamente por el contenido de n-3 HUFA de la dieta de los reproductores (Fernández-Palacios *et al.* , 1995) (Tabla 1). Sin embargo, un exceso de n-3 HUFA en la dieta puede provocar una mayor oxidación de lípidos durante el desarrollo embrionario, afectando la tasa de eclosión y la calidad de los huevos (Lavens *et al.*, 1999).

El nivel óptimo de n-3 HUFA en la dieta de los reproductores para promover la calidad de los huevos puede estar alrededor del 20% del total de ácidos grasos, independientemente de la especie (Fernández-Palacios *et al.*, 1995; Lavens *et al.*, 1999; Furuita *et al.*, 2000, 2002).

También se ha reportado el efecto de la fuente de proteína y el contenido de la misma en la

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dieta sobre la tasa de eclosión, cantidad de larvas normales y producción de semilla en *Pagrus major* (Watanabe, 1985). Otros componentes en la dieta como vitaminas E, C, A, B1 (tiamina), B6 (piridoxina) carotenoides (b-caroteno y astaxantina), han demostrado mejorar el desarrollo embrionario, el porcentaje de huevos flotantes (viables) y tasa de eclosión (Watanabe *et al.*, 1985; Mangor-Jensen *et al.*, 1994; Izquierdo *et al.*, 2001).

Tabla 1. Nivel de inclusión de diferentes nutrientes en la dieta de los reproductores y criterio de calidad de los desoves evaluado en peces marinos.

Especie	Nutriente en la dieta	Criterio evaluado	Referencia
Sparus aurata	1.5% - 2% n-3 HUFA	Desarrollo embrionario	Fernández-Palacios *et al.* , 1995
	1.8 % n-3 PUFA	Tasas de fertilización y eclosión	Rodríguez *et al.* , 1998
Dicentrarchus labrax	n-3 HUFA	Desarrollo embrionario, tasa de eclosión.	Navas *et al.* , 1996, Bruce *et al.* , 1999.
Gadus morhua	50 mg/kg Vitamin C	FAA concentration	Mangor-Jensen *et al.* , 1994
Hippoglossus hippoglossus	1.8 % ARA	Morfología de blastómeros, tasas de fertilización y eclosión.	Mazorra *et al.* , 2003
Paralychthys olivaceus	2.1 % n-3 HUFA	Morfología de blastómeros, huevos flotantes y tasa de eclosión.	Furuita *et al.* , 2002
Pagrus major	Proteína y fósforo	Eclosión y desarrollo de la larva	Watanabe, 1985.
Lutjanus campechanus	2.4 % DHA, 1.2 % ARA	Eclosión, fertilización.	Papanikos, 2004

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3. Criterios de evaluación de la calidad de los desoves

Además de la tasa de eclosión y la tasa de supervivencia a la primera alimentación, que son considerados los principales criterios para evaluar la calidad de los huevos, existe otros criterios que han sido utilizados con este fin, tanto durante el desarrollo embrionario como durante la etapa de larva vitelina. La tabla 2 muestra algunos de los diferentes tipos de criterios reportados en diferentes especies para evaluar la calidad de los desoves. Algunos de ellos han probado ser más útiles que otros, pero se han reportado diferencias inter-específicas que dificultan el uso generalizado de uno solo de ellos. Algunos como el nivel de actividad, la reacción cortical ó las aberraciones cromosómicas durante la mitosis solo han sido reportados en uno o dos estudios, por lo que su efectividad como criterio de evaluación de la calidad no se considera.

Tabla 2 Ejemplos de criterios reportados para evaluar la calidad de huevos en algunas especies de peces marinos.

Criterios	**Especie**	**Referencias**
Zootécnicos		
Tasa de fertilización	*Hippoglossus hippoglossus*, *Lates calcarifer*	Nocillado *et al.*, 2000; Mommens *et al.*, 2015.
Proporción de embriones flotantes	*Dentex dentex*, *Pagrus pagrus*, *Lutjanus campechanus*,	Giménez *et al.* , 2006; Aristizabal *et al.*, 2009; Bourque y Phelps, 2007.
Presencia de deformidades en las larvas	*Paralichthys olivaceus*	Furuita *et al.* , 2000.
Nivel de actividad	*Pagrus pagrus*	Aristizabal *et al.*, 2009.
Morfológicos / morfométricos		
Tamaño del huevo	*Gadus morhua*, *Scophthalmus maximus*,	McEvoy, 1984; Kjorsvik *et al.*, 1984; Salze *et al.* , 2005; Aristizabal

	Pagrus pagrus,	*et al.* 2009.
Morfometría del huevo y gota de aceite	*Sparus aurata*; *Diplodus puntazzo* *Dentex dentex*	Lahnsteiner y Patarnello, 2005; Lahnsteiner *et al* , 2008.
Número y distribución de gotas de aceite	*Pagrus major*, *Lutjanus campechanus*	Watanabe y Kiron, 1995; Bourque y Phelps, 2007.
Morfología de los blastómeros	*Gadus morhua*, *Scophthalmus maximus*, *Hippoglossus hippoglossus*,	McEvoy, 1984, Kjorsvik, 1994.Mommens *et al.* , 2015.
Aberraciones cromosómicas en la mitosis	*Gadus morhua*	Kjorsvik *et al.*, 1990.
Reacción cortical	*Gadus morhua*	Kjorsvik *et al.*, 1990;
Transparencia del huevo	*Scophthalmus maximus*	McEvoy, 1984.
Bioquímicos		
Contenido de ácidos grasos	*Dentex dentex*, *Hippoglossus hippoglossus*, *Gadus morhua, Lutjanus guttatus, Solea solea*.	Kjorsvik *et al.* , 1984; Salze *et al.*, 2005; Giménez *et al.*, 2006; Lund e al., 2008; Rangel-Duran, 2014.
Contenido de amino ácidos libres	*Hippoglossus hippoglossus, Rachycentron canadum*	Faulk y Holt, 2008; Mommens *et al.* , 2015.
Actividad enzimática	*Dentex dentex*, *Lutjanus peru*, *Sparus aurata*,	Giménez *et al.*, 2006; Lahnsteiner y Patarnello, 2004b; Moguel-Hernández *et al.*, 2015.
Concentración de metabolitos	*Dentex dentex*, *Lutjanus peru*, *Sparus aurata*	Giménez *et al.*, 2006; Lahnsteiner y Patarnello, 2004b; Moguel-Hernández *et al.*, 2015.

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3.1 Criterios zootécnicos

La principal ventaja del uso de criterios zootécnicos para evaluar la calidad de los desoves, es que son fáciles de estimar, no se requiere mucho tiempo ni experiencia para su evaluación. En contraparte, existen demasiados ejemplos sobre su uso, pero los resultados no han sido consistentes y ha sido difícil generalizar su uso como eficientes criterios de evaluación de la calidad.

3.1.1 Tasa de fertilización

La tasa de fertilización ha sido considerada como uno de los primeros criterios para evaluar la calidad de los huevos, sin embargo, se han reportado resultados muy variables (Kjorsvik *et al.*, 1984; 2003). En especies como *Gadus morhua*, *Scophthalmus maximus* y *Clupea harengus* no se reportó correlación entre éste criterio y la tasa de eclosión y supervivencia (McEvoy, 1984; Hay, 1986; Kjorsvik *et al.*, 1990). Sin embargo, Kjorsvik *et al.* (2003) reportaron una correlación positiva entre la tasa de fertilización y la viabilidad de larvas y juveniles de *S. maximus*.

3.1.2 Fracción de huevos flotantes

La fracción de huevos flotantes, ha sido reportado como criterio de calidad efectivo en *Pagrus major* (Sakai *et al.*, 1985), *S. maximus* (McEvoy, 1984), *Gadus morhua* (Kjorsvik *et al.*, 1984), *Pagrus pagrus* (Aristizabal *et al.*, 2009) y otras especies (Kjorsvik *et al* , 1990), pero no así en especies como *Dentex dentex*, donde la proporción de huevos que flotaban y no flotaban no funcionó para distinguir desoves de buena y mala calidad, ya que no se encontraron diferencias significativas en el porcentaje de eclosión entre ambos tipos de desove (Giménez *et al.*, 2006).

A pesar de esta falta de consistencia en la efectividad de los criterios zootécnicos para evaluar la calidad de los desoves entre diferentes especies, hay que mencionar que su

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mayor aporte ha sido como indicador de una muy pobre calidad de los desoves. Cuando la tasa de fertilización es muy baja o casi nula o la fracción de huevos muy elevada, se trata de un pobre desove que se descarta.

3.2 Criterios morfológicos y morfométricos

La evaluación de las características morfológicas y morfométricas de los huevos constituyen una serie de criterios que también demandan poco esfuerzo, y por lo tanto, al igual que los criterios zootécnicos, fueron de los primeros criterios en emplearse para evaluar la calidad de los desoves, como una alternativa a los porcentajes de eclosión y supervivencia inicial.

3.2.1 Tamaño del huevo y glóbulo de aceite

El tamaño del huevo fue uno de los primeros criterios utilizados para evaluar la calidad de los mismos. Era lógico asumir que mientras más grande el huevo, se producirían larvas más grandes con mayores posibilidades de sobrevivir. Sin embargo, existen reportes que contradicen tal suposición (Manning y Crim, 1998; Kjorsvik *et al.*, 1990; Moguel-Hernández, 2010).

Particularmente cuando el análisis del tamaño del huevo se ha realizado durante un ciclo reproductivo. Diferentes estudios demostraron que a lo largo del ciclo reproductivo existen diferencias significativas en el tamaño del huevo, tal variación puede estar asociada a la condición fisiológica de la hembra, a la magnitud del desove, al avance en la temporada reproductiva, al origen de la población, la latitud, la temperatura, la disponibilidad de alimento y la salinidad (Kamler, 2005), y más aún en especies con desoves parciales, que son la mayoría de las especies marinas, donde el diámetro del huevo depende de las reservas energéticas de la hembra y el número de desoves que ha tenido a lo largo de la temporada (Brooks *et al.*, 1997; Kamler, 2005).

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Como una alternativa de parámetros morfométricos Lahnsteiner y Patarnello (2005) y Lahnsteiner *et al.* (2008) reportaron el uso de diferentes parámetros morfométricos como criterios de evaluación de la calidad de huevos en *Sparus aurata*, *diplodus puntazzo* y *Dentex dentex*, respectivamente. Tomando como base los diámetros mínimo y máximo del huevo, del glóbulo de aceite y del vitelo, estimaron una serie de parámetros que se utilizaron como criterios para evaluar el porcentaje de supervivencia larvaria y el porcentaje de eclosión.

3.2.2 Simetría de los blastómeros

Uno de los criterios morfológicos que más ha sido utilizado para evaluar la calidad de los desoves, es la forma y los patrones de división de los blastómeros durante el inicio de la segmentación. Este criterio morfológico ha sido el más recurrido por la correlación positiva que presenta con los porcentajes de eclosión y la viabilidad de las larvas vitelinas en especies como *H. hippoglossus* (Shields *et al.*, 1997), *Melanogrammus aeglefinus* (Rideout *et al.*, 2004), *Sparus aurata, Diplodus puntazzo* (Lahnsteiner & Patarnello, 2005), *Gadus morhua* (Rani, 2005) y en el caso de *S. maximus* la asimetría celular temprana predijo la supervivencia de las larvas hasta la etapa de juveniles (Kjorsvik *et al.* , 2003).

No obstante, algunos autores han cuestionado su utilidad debido a que se ha reportado que el grado de malformación ó asimetría en la división de los blastómeros puede corregirse durante las etapas posteriores del desarrollo embrionario, de tal forma que no se detecten diferencias significativas en el porcentaje de eclosión de huevos con divisiones normales y huevos con divisiones asimétricas, (Vallin y Nissling, 1998; Avery *et al.*, 2005; 2009).

En este sentido, se han observado diferentes tipos de anormalidades de los blastómeros durante la segmentación (Shields *et al.*, 1997; Rideout *et al.*, 2004; Rani, 2005) como: disposición asimétrica, diferencia en el tamaño, poca adhesión, poca definición en el margen celular y presencia de vacuolas. De las cuales, la poca adhesión entre los

blastómeros es la anormalidad que provoca menores porcentajes de eclosión, indicando que ésta anormalidad interfiere severamente en la embriogénesis (Rideout *et al.*, 2004; Rani, 2005). Sin embargo, como las cuatro anormalidades tienden a coocurrir en un mismo desove, y la cantidad de embriones que mostraron ésta anomalía es baja, se ha considerado que el porcentaje de anormalidad en los blastómeros durante las divisiones tempranas puede ser un buen indicador del porcentaje de eclosión y la viabilidad de las larvas (Shields *et al.*, 1997; Rideout *et al.*, 2004).

A pesar de que este criterio es un buen indicador de calidad de los desoves, presenta una desventaja, ya que si se toman las muestras en tanques donde los reproductores tienen desoves espontáneos, los huevos recolectados estarán en diferentes estadios de desarrollo, lo cual restringe la aplicación de este método (Lahnsteiner & Patarnello, 2005).

3.3 Criterios bioquímicos

Gran parte del éxito que pueda tener un huevo en eclosionar y producir una larva viable, depende en gran medida de la cantidad, calidad y utilización de reservas energéticas y nutricionales que hayan sido acumulados durante la ovogénesis y que estén presentes en el vitelo y el glóbulo de aceite, cuyas proporciones difieren entre especies y varían en función de la edad, el peso y la dieta de los reproductores (Heming y Buddington, 1988; Bromage y Roberts, 1995). De manera general, las reservas energéticas y nutricionales que necesita el embrión durante su desarrollo son sintetizadas durante la ovogénesis de manera endógena y exógena, y son acumuladas en el vitelo y en el glóbulo de aceite de los ovocitos (Sarasquete *et al.*, 1993). Las fosfoglucolipoproteínas (vitelogenina) son sintetizadas por los hepatocitos y transportadas por el torrente sanguíneo e incorporadas en los oocitos mediante endocitosis, finalmente son hidrolizadas en proteínas vitelinas: lipovitelinas, glucovitelinas y fosfovitinas (Heming y Buddington, 1988; Carnevali *et al.*, 2001a; Kamler, 2008). El proceso final de maduración del ovocito está caracterizado morfológicamente por la coalición de las vesículas de vitelo y fisiológicamente por la hidratación del mismo (Lahnsteiner, 2006). Además de proporcionar insumos energéticos, el vitelo también cuenta

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con hormonas y enzimas (Brooks *et al.*, 1997). Por otro lado, el glóbulo de aceite (cuando está presente) se compone, en su mayoría, de triacilglicéridos que son una fuente de energía para las larvas, principalmente durante el proceso de la primera alimentación exógena (Kamler, 2008).

3.3.1 Concentración de lípidos y aminoácidos

De todos los componentes nutricionales en el vitelo, los lípidos y los aminoácidos han sido los más estudiados para evaluar la calidad de los desoves debido a su papel durante el crecimiento y desarrollo. Los lípidos en el vitelo funcionan como fuentes de energía, componentes estructurales para las células en desarrollo y como precursores de hormonas parácrinas (Tocher, 2003). Los triglicéridos (lípidos neutros) son la forma más común de almacenamiento de energía en los huevos pelágicos, mientras que los fosfolípidos (lípidos polares) tienen un importante papel como componentes estructurales de las biomembranas y están asociados con la fluidez de la membrana y funciones fisiológicas de las enzimas de membrana y funciones celulares en peces marinos (Bell *et al.*, 1986) además de ser una fuente de fósforo y colina (Sargent, 1995). Mientras que los amino ácidos funcionan como una importante fuente de energía, factores de señalización celular y sustratos para moléculas bioactivas y proteínas (Finn y Fyhn, 2010).

Es por lo anterior, que el uso de éstos criterios está íntimamente relacionado con la evaluación del efecto de la dieta de los reproductores sobre la calidad de los desoves (ver secc. 2.3) como una forma para estimar los requerimientos nutricionales de los reproductores; y en este caso, los resultados son muy variables precisamente por las diferencias en los requerimientos nutricionales de los reproductores de las diferentes especies.

En realidad son pocos los trabajos que reportan el uso de éstos criterios bioquímicos sin que esté involucrada la evaluación de diferentes tipos de dieta de los reproductores. Pickova *et al.* (1997), reportaron que el contenido de amino ácidos y la tasa DHA/EPA en

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la fracción de fosfolípidos en huevos del *Gadus morhua* están positivamente correlacionados son la simetría y la viabilidad de las larvas. Giménez *et al.* (2006) evaluaron la cantidad de lípidos totales, lípidos neutros y lípidos polares, así como las proporciones de ácidos grasos EPA, DHA, ARA en desoves de *Dentex dentex* obtenidos en una temporada reproductiva y no encontraron diferencia significativa entre huevos considerados de buena calidad (mortalidad < 10% a los 3 días después de la eclosión) y mala calidad (mortalidad > 35% al día 3). Lahnsteiner & Patarnello (2003) mencionan que los niveles de aminoácidos libres en huevos no viables son significativamente menores que en huevos viables, lo cual afecta su flotabilidad, así como la síntesis de proteínas y el estado energético de los embriones. Esto es importante, debido a que se ha reportado que en especies como *Scophthalmus maximus*, el 20% de los aminoácidos libres son utilizados en la síntesis proteica y el 80% se emplean en el metabolismo energético de los embriones y larvas vitelinas, siendo un proceso clave en la supervivencia de los organismos (Ronnestad & Fyhn, 1993). Salze *et al.* (2005) en huevos de *Gadus morhua* compararon la concentración de lípidos totales, ácidos grasos y carotenoide en huevos de reproductores silvestres, reproductores silvestres alimentados con una dieta artificial y huevos producidos de reproductores en cautiverio. La única diferencia significativa entre los tres tratamientos fue la concentración de fosfatidinositol que fue mayor en los huevos de reproductores silvestres y menor en los producidos en cautiverio. ARA se concentra en fosfatidinositol, sugiriendo que los menores niveles de este tipo de lípido afectan la calidad de los huevos y el desarrollo.

3.3.2 Componentes metabólicos

Ahora bien, durante la última década se han reportado estudios enfocados a evaluar diferentes aspectos metabólicos de los huevos como criterios de calidad. Mayor énfasis se ha puesto en aquellos componentes involucrados en la producción de energía, como son metabolitos y enzimas del metabolismo de carbohidratos, debido a su importante rol durante el desarrollo embrionario (Boulekbache, 1981). En efecto, la glucosa, además de ser utilizada como fuente de energía, es transformada en otros monosacáridos que son

necesarios para la síntesis de ácidos nucleicos y polisacáridos. Por su parte, la fructuosa es precursor en la formación de oligosacáridos los cuales juegan un papel importante durante la organogénesis (Lahnsteiner, 2006). En la síntesis y almacenamiento de estos metabolitos intervienen tres vías metabólicas, la glucolisis, la gluconeogénesis y la vía de las pentosas, los cuales se encuentran catalizados por muchas enzimas, entre las cuales se encuentran: fosfofructoquinasa y piruvatoquinasa en el primero; glucosa-6-fosfatasa (catalizando la reacción de glucosa-6-fosfato a glucosa) y lactato deshidrogenasa (reducción del piruvato en la vía anaeróbica) en el segundo; y transaldolasa (vía no oxidativa de la síntesis de pentosas para la formación de ácidos nucléicos) y glucosa-6-fosfato deshidrogenasa (vía oxidativa) para el último (Lahnsteiner, 2006). Dentro de ellos, la glucólisis ha sido propuesta como la vía más importante como fuente de energía en los huevos de peces durante las primeras etapas de desarrollo (Boulekbache, 1981; Lahnsteiner & Patarnello, 2003).

Algunos ejemplos de la evaluación de éstos componentes para evaluar la calidad de los desoves son los trabajos de Giménez *et al.* (2006) con *Dentex dentex*, Moguel-Hernández (2010) con *Lutjanus peru*, Lahnsteiner y Patarnello (2006) con *Sparus aurata Mullus, barbatus* y *Serranus cabrilla*. En ellos se evalúan las concentraciones de metabolitos como ATP, NADH, NAD, glucosa, ketosa, glucosa-6-fosfato, monosacáriso totales, ácido siálico, ribosa, fructosa-6-fosfato, y la actividad de enzimas como adenilato cinasa, piruvato cinasa, malato deshidrogenasa, glucosa-6-fosfato deshidrogenasa, transaldolasa, aspartato aminotransferasa, fosfatasa alcalina, fosfatasa ácida, glucosa-6-fosfatasa, ácido siálico y acetil coenzima A.

En *P. major*, los niveles de tirosina, fosfolípido y fosfocreatina estuvieron en mayor cantidad en los huevos que presentaron una mayor tasa de desarrollo de embriones que en los huevos con una baja tasa de desarrollo (Seoka *et al.*, 1997). En *Lutjanus peru*, los desoves con un mayor porcentaje de eclosión (>80%) presentaron menores niveles de actividad de transaldolasa y glucosa-6-fosfatasa que los desoves con porcentajes de eclosión menores a 50% (Moguel-Hernández *et al.* 2015). En *Sparus aurata*, reducidas

Peña, R. 2015. Criterios de Calidad de Huevos y sus Implicaciones en el Cultivo de Peces Marinos. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 402-434.

actividades enzimáticas transaldolasa (enzima reguladora de la vía de las pentosas) y glucosa-6-fosfatasa (enzima clave en la gluconeogénesis) son indicativos de huevos de mala calidad (Lahnsteiner & Patarnello, 2004a). En el caso de *Dentex dentex*, los desoves de mala calidad fue posible diferenciarlos por su mayor contenido de glucosa, ketosa, glucosa-6-fosfato y 6-desoxihexosa, indicando la acumulación de los metabolitos de la glucolisis (Giménez *et al.* 2006). Esto sugiere que los carbohidratos no estaban siendo utilizados como fuente de energía y que otros sustratos, probablemente lípidos, son utilizados. De igual forma, los desoves de mala calidad y con mayor tasa de mortalidad presentaban una mayor actividad de fosfatasa alcalina. Esta enzima está involucrada en el metabolismo de fosfolípidos y la defosforilación de fosvitiva en el vitelo (Sire *et al.*, 1994), lo cual podría indicar que el vitelo se consumía a una tasa mucho mayor y por lo tanto, las larvas presentaban una menor cantidad de reservas alimenticias antes del inicio de la alimentación exógena.

La actividad enzimática de catepsina, ha sido utilizada para evaluar la calidad de los desoves. La catepsina es una proteasa relacionada con la degradación de las proteínas vitelinas; como resultado de su acción, se producen nuevos aminoácidos libres, los cuales además de ser la mayor fuente de energía durante las primeras etapas del desarrollo, son esenciales para la multiplicación celular y para la formación de tejidos y órganos. También son importantes en el control osmótico y en la flotabilidad de los embriones, (Ronnestad & Fyhn, 1993; Kamler, 2008). En especies como *Sparus aurata* (Carnevali *et al.*, 1999; Carnevali *et al.*, 2001b) y *Dicentrarchus labrax* (Carnevali *et al.*, 2001a) se han detectado diferentes tipos de catepsinas (A, B, C, D, E y L) involucradas en distintas etapas del desarrollo embrionario. Se ha reportado que en huevos no viables se presenta una mayor actividad de catepsinas D y L con respecto a los huevos viables, sugiriendo un fallo en la proteólisis vitelina en los huevos de baja calidad.

4. Indicadores de la calidad de los desoves

La mayoría de los estudios reportados sobre calidad de huevos, han demostrado ser

útiles para detectar diferencias entre desoves de buena y mala calidad, o para evaluar el efecto del alimento de los reproductores, o el método de inducción al desove sobre alguno de los parámetros expuestos anteriormente y concluir si existe algún efecto en la calidad de los desoves. Sin embargo, no explican las razones subyacentes de los principales criterios que definen a un desove de buena calidad, es decir: el porcentaje de eclosión ó la supervivencia inicial de las larvas.

Esto ha llevado a proponer métodos de análisis de los resultados que aporten más información que la mera diferencia entre variables. De esta forma, se han reportado análisis de correlaciones y ajuste de modelos de regresión para intentar establecer qué parámetros participan, y en qué medida, para explicar la variabilidad de los principales criterios de calidad de los desoves (i.e. el porcentaje de eclosión y la supervivencia inicial de la larva). En este tipo de estudios rara vez se utilizan modelos simples debido a la variación de los criterios evaluados, por lo que se ha optado por utilizar modelos multivariados que pueden aportar mayor información.

Lahnsteiner y Patarnello (2004b) evaluaron la calidad de los desoves de *Sparus aurata* mediante el ajuste de modelos de regresión múltiple utilizando la concentración de metabolitos y la actividad de enzimas involucradas en el metabolismo energético como variables independientes y el porcentaje de eclosión como variable dependiente. Concluyeron que la actividad de fosfatasa ácida, adenilato cinasa y la concentración de ácido siálico son variables adecuadas como indicadores para explicar el porcentaje de eclosión de los huevos de *Sparus aurata*.

En este mismo sentido, Giménez *et al.* (2006) utilizaron la concentración de metabolitos asociados al metabolismo de carbohidratos y reportaron que los monosacáridos glucosa, ketosa y ribosa, y la enzima piruvato cinasa pudieran ser utilizadas para predecir el porcentaje de eclosión en *Dentex dentex,* y para predecir la viabilidad de las larvas (mortalidad a los 3 y 5 dde) las variables evaluadas fueron 6-desoxihexosa, glucosa, piruvato kinasa y fosfatasa alcalina.

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Para explicar la calidad en los desoves de *L. peru*, Moguel-Hernández (2010) propuso dos modelos para el porcentaje de eclosión y dos para el porcentaje de anormalidades en los blastómeros. Para ambos casos recomienda el modelo donde interviene la glucosa, por su papel en el metabolismo de los carbohidratos, el cual es utilizado en mayor medida para la obtención de energía durante el inicio del desarrollo embrionario (Ronnestad & Fyhn, 1993; Sveinsdóttir *et al.*, 2006; Kamler, 2008). Además, es precursora en la formación de monosacáridos necesarios para la producción de ácidos nucléicos (Lahnsteiner, 2006).

Recientemente Mommens *et al.* (2015) evaluaron el uso de las concentraciones de lípidos y aminoácidos en los huevos de *H. hippoglossus* como indicadores de la tasa de fertilización, la asimetría de los blastómeros y el porcentaje de eclosión de los desoves. Concluyeron que la tasa de fertilización se explicaba por la concentración de ácido oleico (OA, 18:1n9), ácido mirístico (MA, 14:0), ácido estearidónico (SA, 18:4n3) y ácido eicosadienoico (EDA, 20:2n6); la tasa de eclosión por ácido palmitoleicico (POA, 16:1n7) y ácido α-linolénico (LNA, 18:3n3); y la simetría de los blastómeros por las concentraciones de OA y EDA. Una mayor cantidad de ácidos grasos se correlacionaron con la tasa de eclosión que con la tasa de fertilización y la simetría de los blastómeros, lo cual refleja el importante rol que juegan estas moléculas en el metabolismo embrionario del *H. hippoglossus*.

En otros estudios, los ácidos grasos poliinsaturados se correlacionan positivamente con la tasa de fertilización y los n-3 con la tasa de eclosión. Por ejemplo en *Sparus aurata* (Fernández-Palacios *et al.*, 1995), *Gadus morhua* (Pickova *et al.*, 1997), *Paralichthys olivaceus* (Furuita *et al.*, 2000) y *Dicentrarchus labrax* (Bruce *et al.*, 1999). Uno de ellos, el ácido araquidónico (ARA, 20:4n6) ha sido sugerido como un importante ácido graso en la dieta de los reproductores para mejorar la calidad de los huevos de *H. hippoglossus* (Mazorra *et al.*, 2003). Sin embargo, Mommens *et al.* (2015) reportaron que no existe una correlación entre la concentración de ARA y la tasa de fertilización, la simetría de los blastómeros y la tasa de eclosión en ésta especie. También mostraron que las mayores

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correlaciones positivas con la tasa de eclosión fueron con ácido dihomo-ɣ-linoleico (DGLA, 20:3n6) y con ácido docosapentaenoico (DPA, 22:5n3). Estos ácidos grasos funcionan como moduladores del metabolismo de ecosanoides al competir con ARA por el acceso a ciclooxigenasa que convierte los ácidos grasos en prostaglandinas

Con respecto a los aminoácidos como reguladores de la calidad de los desoves, Mommens *et al.* (2015) demostraron que la tasa de fertilización se explicaba en un 45% por la concentración de alanina y valina en los huevos. La simetría de los blastómeros en un 68% por la concentración de ácido glutámico y tirosina; y la tasa de eclosión en 36% por la concentración de seria, arginina y valina. Es importante resaltar la importancia de valina, el cual es un amino ácido que además de ser utilizado como un sustrato de energía, participa en la síntesis de otros aminoácidos como ácido glutámico (Bak *et al.*, 2012) y proteína en músculos (Platell *et al.*, 2000). Esta función en el metabolismo muscular puede explicar su relevancia en durante la tasa de eclosión de *H. hippoglossus*.

5. Implicaciones en el cultivo de peces marinos

El principal objetivo del cultivo de peces marinos es el de producir la mayor cantidad de juveniles, en el menor tiempo posible y con el mayor rendimiento económico. Para ello debe contarse con desoves de calidad que sean producidos con regularidad y que presenten elevados porcentajes de eclosión y tasas de supervivencia inicial. Son pocas las especies a nivel mundial que cuentan con exitosos programas de cultivo y que se han consolidado como una alternativa económica capaz de cubrir la creciente demanda. Sin embargo, la gran mayoría de las especies que están siendo introducidas a la acuacultura se encuentran aún en incipientes programas de cultivo a nivel piloto e incluso experimental. Es en este caso en que la gran variabilidad de la calidad de los desoves cobra mayor importancia, ya que ha sido uno de los principales obstáculos en la introducción de especies alternativas en la maricultura.

Con lo expuesto en el presente trabajo, queda de manifiesto que existe una gran

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cantidad de criterios que pueden ser utilizados para evaluar la calidad de los desoves y que difícilmente puede encontrarse uno cuya utilidad como indicador de calidad en una especie, sea extrapolable a una nueva especie. Esto ha provocado que exista un pobre consenso sobre criterios confiables para establecer la calidad de los huevos, no obstante, en lo que sí se está de acuerdo es que el método debe ser simple y llevarse a cabo durante el desarrollo temprano, lo que permitirá tomar decisiones precisas y confiables sobre el desove que se ha obtenido y evitar el uso de las instalaciones y del personal en lo que podría ser un intento fallido ó en un desove de pobre calidad que no rinda la inversión realizada.

Resalta entonces la necesidad, y la importancia, de establecer un programa de monitoreo de la calidad de los desoves obtenidos en cautiverio. Para ello, se sugiere planificar estrategias y actividades que incorporen diferentes criterios de los que han sido expuestos con anterioridad. En efecto, encontrar un solo criterio que permita evaluar ó explicar la calidad de los desoves parece una labor complicada, en el mejor de los casos. Sin embargo, si se plantea el utilizar criterios que se complementen y se hace por etapas, el resultado puede aportar información más útil.

Un paso inicial en este esquema consistiría en evaluar (y en su caso, descartar) aquellos criterios que no requieran de mucho esfuerzo (por ejemplo, el diámetro del huevo ó la forma del glóbulo de aceite, el porcentaje de fertilización ó de flotabilidad), para elegir cuál, ó cuáles, se correlacionan mejor con las características más importantes que deben cumplir los desoves de buena calidad: el porcentaje de eclosión y la supervivencia a la primera alimentación. Esto permitirá tener un criterio confiable al momento de determinar la calidad del desove que se obtiene. Ahora bien, en caso de que ninguno de esos criterios iniciales permita estimar la calidad de los desoves, habrá que recurrir a criterios con un mayor grado de planificación. La simetría de los blastómeros podría ser utilizada como un criterio predictivo, sin embargo, la logística que implica colectar huevos en etapas tempranas de la segmentación, especialmente cuando se tienen desoves espontáneos ó inducidos por implantes hormonales, dificulta un poco su aplicación; pero los resultados reportados en varias especies lo sugieren como una alternativa que vale la pena explorar.

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Sin embargo, no solo es necesario poder evaluar la calidad, sino que un programa completo de monitoreo de la calidad requiere información confiable sobre aquellos factores que la definen, es decir, que provocan esas variaciones en calidad y que en un momento dado pueda mejorarse. En este sentido, es necesario poner particular atención a las condiciones en las que se mantienen los reproductores, y realizar evaluaciones de los desoves obtenidos en cada ciclo productivo.

Es un hecho que existe un vacío en el conocimiento sobre los factores determinantes de la calidad de los huevos, pero existen muchos que pueden fungir como causantes de los cuales, el origen de los reproductores, su condición (nutrición, edad, estado fisiológico) y su manipulación (manejo, inducción hormonal, nivel de estrés) pueden ser los más relevantes. Para poder evaluar los efectos de la nutrición de los reproductores en la calidad de los huevos, primero es necesario tener un conocimiento pleno de los requerimientos nutricionales de aquellos. Un enfoque inicial puede ser analizar la composición de los peces silvestres ya que pueden proveer información útil con respecto a la composición de la dieta de los reproductores. De igual forma, se debe producir mejor y más confiables estrategias de desove, invertir en la investigación enfocada a la obtención de desoves espontáneos, lo cual reduciría el efecto de la inducción hormonal sobre la calidad de los desoves.

En ocasiones, un solo criterio no ha sido el más útil para evaluar o predecir la calidad de los huevos a través de modelos de regresión simple, debido a la alta variabilidad de los criterios durante las estimaciones de los mismos. Es por ello que se han utilizado diferentes criterios, ya sean bioquímicos, morfométricos o metabólicos y se han desarrollado modelos de regresión múltiple con el objeto de explicar o predecir el porcentaje de eclosión o supervivencia durante los primeros días de desarrollo. Lo cual ha permitido obtener conclusiones importantes en especies como *Sparus aurata*, *Puntazzo puntazzo* (Lahnsteiner y Patarnello, 2004a), *Dentex dentex* (Giménez *et al.*, 2006), *Lutjanus peru* (Moguel-Hernández, 2010) y *Rachycentron canadum* (Nguyen *et al.*, 2012).

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Finalmente, este programa de evaluación de la calidad de los desoves no puede ser una tarea que corresponda completamente al sector productivo. Tanto la academia como la empresa deben desarrollar propuestas de trabajo en conjunto que permitan mejorar e incrementar la calidad de la investigación y los niveles productivos.

6. Conclusiones

Bajo este marco, en el que se presentan los diferentes caracteres que han sido empleados como criterios de evaluación de la calidad de los desoves, es prudente hacerse tres preguntas: Primero ¿Qué diferencia a un huevo de mala calidad de uno de buena calidad?. La respuesta más simple sería que los huevos de buena calidad son más grandes y eclosionan. Sin embargo, esos criterios no pueden considerarse confiables ya que no implican una mayor supervivencia durante la fase de larva vitelina.

Segundo ¿Cómo debe evaluarse la calidad de huevos?. Es evidente que no existe un criterio único que sea efectivo, por lo que es necesario realizar una evaluación que considere varios parámetros. Por ejemplo, incluir la evaluación de la supervivencia de la larva vitelina hasta la primera alimentación, y buscar variables en los primeros estadios de desarrollo embrionario (relaciones morfométricos del huevo ó del glóbulo de aceite, patrones de segmentación, concentración de nutrientes, etc.) que puedan correlacionarse con éste último parámetro y brinden la posibilidad de predecirla.

Y tercero ¿Qué hace que un huevo tenga buena calidad, y puede ésta mejorarse?. Es indudable que la respuesta está directamente relacionada a las condiciones de mantenimiento, características fisiológicas y aspectos nutricionales de los reproductores, por lo que se debe poner particular atención a estos aspectos. El incremento en la calidad de huevos será posible cuando se logre identificar aquellos parámetros que la definen, para ello, los estudios sobre los aspectos bioquímicos involucrados en el metabolismo de carbohidratos, las concentraciones de ácidos grasos y aminoácidos y su análisis mediante modelos de regresión son una herramienta útil que puede indicar la forma de mejorar la

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calidad de los desoves. Sin embargo, son preferibles los modelos que usan un número bajo de variables, ya que reducen el esfuerzo del análisis.

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