Nutritional contribution of torula yeast and fish meal to the growth of shrimp *Litopenaeus vannamei* as indicated by natural nitrogen stable isotopes

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Highlights

- Torula yeast and fish meal were used to formulate diets for Pacific white shrimp.
- Isotopic measurements were applied to ingredients and muscle tissue.
- Relative dietary contributions from both ingredients to growth were similar.
- Torula yeast can be used to replace up to 60% of fish meal.

Abstract

Torula yeast (*Candida utilis*) and fish meal were used to formulate six experimental diets for Pacific white shrimp *Litopenaeus vannamei.* The dietary nitrogen supplied by fish meal was replaced by increasing dietary proportions of torula yeast (0, 7.5, 15, 30, 60 and 100%). Nitrogen stable isotope values ($\delta^{15}N$) were measured in ingredients, diets and muscle tissue of experimental animals in order to estimate the relative contributions of dietary nitrogen and dry matter supplied by both ingredients. At the end of a 29 d bioassay, there were no significant differences in survival rate among treatments. Shrimps fed on all diets containing torula yeast and fish meal had higher growth rates (k = 0.059-0.064) than animals fed on diets containing only fish meal or only torula yeast (k = 0.041-0.054). Incorporation of $\delta^{15}N$ values of ingredients and muscle tissue into an isotopic mixing model indicated that the relative incorporation of dietary nitrogen and total dry matter from torula yeast to growth consistently increased in relation to increasing proportions of this ingredient in the experimental diets. The only exception was the diet formulated with the highest yeast content (60%, diet 40F/60T) where fish meal contributed a

higher proportion of dietary nitrogen to growth. Dietary nitrogen from torula yeast available in diets 93F/7T, 85F/15T, 70F/30T and 40F/60T was incorporated in muscle tissue at proportions of 6.5, 13.7, 27.1 and 50.5 %, respectively. Estimated nitrogen residency time in tissue (t_{50}) was relatively shorter in shrimps fed on diets 85F/15T and 70F/30T (3 d), indicating higher metabolic turnover rates in these animals than those fed on diet containing only fish meal. Growth and survival rates were statistically similar in shrimp fed on all mixed diets, therefore indicating the suitability of this single cell protein as dietary ingredient in diets containing up to 60% of torula yeast.

Keywords: Stable isotopes, nutrient contribution, single cell protein, torula yeast, fish meal, *Litopenaeus vannamei*

1. Introduction

The Pacific white shrimp *Litopenaeus vannamei* has become the main shrimp species produced through aquaculture practices since 2003 (FAO, 2007). The progressively higher production of this and several other aquaculture species is in turn exacerbating the high demand for aquaculture feeds and the ingredients required for their manufacture. The commercialization of marine-derived proteins maintains increasing economic and ecological concerns mainly due to overfishing of small pelagic fish from which fish meal is obtained. Several alternative sources of plant and microbial protein have drawn attention and are being currently used either to replace fish meal in aquaculture diets or tested as ingredients. Defatted microalgae biomass from biorefineries, yeast and bacterial aggregates from highly controlled production systems that

employ agricultural byproducts as substrates are some of the emerging alternative feed ingredients for manufacturing and improving the highly demanded aquaculture feeds. Due to their good nutritional and immunostimulatory properties, single cell proteins (SCP), including yeasts, have been studied in the past as feed ingredients for terrestrial and aquatic animals (Perera et al., 1995; Poulose, 2013; Macias-Sancho et al-. 2014; Martins et al., 2014). In addition to the good nutritional properties, sources of SCP are becoming more affordable. Bob-Manuel and Erondu (2010) carried out an economic evaluation of the use of yeast in tilapia diets. Result showed that, from a range of substitution levels, a substitution of 50% of fish meal with yeast had the lowest profit index and cost-benefit ratio. Nowadays, several enterprises have achieved massive production of different microorganisms for commercial purposes and among these; torula yeast (*Candida utilis*) has been commercially available for decades. This particular type of yeast has been tested as protein source in diets for aquatic animals and it has been found that feed acceptance and survival are not affected when used as partial fish meal replacement. Olvera-Novoa et al. (2002) used torula yeast as a dietary protein source for tilapia fry and replaced different proportions of animal-derived protein. Highest growth responses were observed for fish on diets containing 30% torula yeast. Britz (1996) fed abalone *Haliotis midae* on diets containing different sources of protein. A diet containing only torula yeast as protein source elicited lower feed conversion ratios than than those observed in control abalone fed on seaweed, although the growth rate was lower.

Measurements of nitrogen stable isotope ratios ($\delta^{15}N$) have been applied as nutritional tools to explore the transference and deposition of dietary protein in several aquatic species. The integration of isotopic data into mass-balance mixing models has made possible to convert the isotopic values of consumers and their different trophic elements to dietary contributions

(Phillips, 2012). In aquaculture nutrition, the isotopic values of carbon and nitrogen have been used as natural biomarkers to estimate dietary contributions in organisms fed on experimental dietary formulations having ingredients with contrasting isotopic signatures (Martínez-Rocha et al., 2013; Gamboa-Delgado et al., 2013, 2014). The different dietary resources found in aquatic and terrestrial ecosystems frequently show distinct $\delta^{15}N$ values due to the effect of characteristic nutrient flows and metabolic pathways. However, this natural flow of nutrients (and isotopes) is altered under the controlled, artificial systems promoting the growth of commercial valuable microorganisms from specific substrates. The present study employed the isotopic differences found in torula yeast and fish meal to assess the relative incorporation of dietary nitrogen and total dry matter supplied by these sources to the growth of Pacific white shrimp by means of an isotope mixing model.

2. Material and methods

2.1. Experimental diets

A batch of inactive torula yeast *Candida utilis* (Uniprot®) was obtained from FerMex (Toluca, Mexico). Torula yeast (TY) contained 41% crude protein and it represented one of two protein sources used for diet formulation. The second protein source was fish meal (FM, prime Mexican sardine, 68% protein). From these main ingredients, six isonitrogenous (40% crude protein) and isoenergetic (4.6 kcal/gr) experimental diets were formulated using the software Nutrion® (Nutrion, Chapala, Mexico). FM and TY represented the only nitrogen sources, both having contrasting $\delta^{15}N$ values. Four mixed experimental diets were formulated with different proportions of FM substituted with different levels of TY, at 7.5 (diet 93F/7T), 15 (diet 85F/15T),

30 (diet 70F/30T) and 60% (diet 40F/60T) of the available dietary nitrogen. Dietary substitutions were done based on available dietary nitrogen in ingredients (Table 1). Diets containing only one source of protein were used as positive (100F) and negative (100T) controls in order to estimate and correct for isotopic discrimination factors ($\Delta^{15}N$). Experimental diets were not manufactured to conduct an ingredient substitution study; instead, they were formulated with ingredients having contrasting $\delta^{15}N$ values in order to explore their nutritional contributions to shrimp growth. TY contained lower protein content than FM; therefore its dietary bulk inclusion was higher; a concomitant, progressive reduction of dietary starch was applied in order to maintain isoenergetic diets. Micronutrients were weighed to the nearest mg and hand-mixed for 5 min before mixing these with finely ground macronutrients. The mixture was homogenized for 10 min using a commercial blender. Supplemental cholesterol was added only to diet containing 100% TY, as cholesterol content in FM covered the nutritional requirement in the rest of diets. Lecithin was dissolved in warm fish oil and the content was slowly added to the main mixture. Distilled water was added until the dough was formed. The resulting paste was extruded through a die plate (1.6 mm orifices). Diets were dried in a convection oven for 8 min at 100 °C and stored at 4 °C. Proximal analyses of experimental diets, included protein content (Dumas method, LECO), lipid content (Soxhlet system HT-1045, method AOAC 996.06) (Tecator 1983), fibre content (AOAC 962.09B), moisture content (AOAC 930.15) and ash content (AOAC 942.05) were performed. The nitrogen-free extract was estimated as the difference of the latter assays subtracted from 100.

Table 1. Nutritional (g 1000 g^{-1} diet dry weight) and isotopic ($\delta^{15}N$, ‰) composition of formulated diets fed to Pacific white shrimp *Litopenaeus vannamei* to estimate nutritional contributions of fish meal and torula yeast to shrimp muscle tissue.

Ingredient / Diet	**100F**	**93F/7T**	**85F/15T**	**70F/30T**	**40F/60T**	**100T**
Fish meal[a]	475.5	439.7	404.4	332.8	190.6	0.0
Torula yeast[b]	0.0	64.0	127.0	255.0	509.0	849.5
Wheat starch[c]	424.7	393.8	363.3	301.5	163.3	0.0
Lecithin[d]	35.5	35.5	35.5	35.5	40.7	40.0
Fish oil[a]	16.3	19.1	21.8	27.3	33.1	37.2
Disodium phosphate[e]	0.0	0.0	0.0	0.0	16.5	34.8
Calcium chloride[e]	0.0	0.0	0.0	0.0	0.0	7.0
Cellulose[e]	19.0	19.0	19.0	19.0	17.9	1.3
Alginate[e]	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin premix[a]	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[a]	2.5	2.5	2.5	2.5	2.5	2.5
Choline chloride[a]	2.0	2.0	2.0	2.0	2.0	2.0
Cholesterol[f]	0.0	0.0	0.0	0.0	0.0	1.2
Vitamin C[a]	1.0	1.0	1.0	1.0	1.0	1.0
Antioxidant[a]	0.5	0.5	0.5	0.5	0.5	0.5
Antifungic agent[a]	0.5	0.5	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000	1000	1000
Proximal and isotopic analysis						
Crude protein (g kg-1)	403	391	391	403	397	412
Lipids (g kg-1)	87	94	94	91	93	80
Ash (g kg-1)	107	109	100	98	111	120
Gross energy (Kcal g-1)	4.7	4.7	4.6	4.6	4.5	4.3
$\delta^{15}N$ (‰)	16.9	15.4	14.4	11.8	6.6	1.1

[a]Alimentos Costamar (Sonora, Mexico).
[b]Fermex (Toluca, Mexico).
[c]Almidones y gluten S.A. (Monterrey, Mexico).
[d]Ragaza Industrias Proteínas Naturales S.A. de C.V. (Monterrey, Mexico).
[e]Sigma-Aldrich (St. Louis, MO, USA).
[f]Solvay Pharmaceuticals (Houston, TX, USA).

2.2. Experimental design and rearing system

Juvenile shrimp, *L. vannamei* were obtained from a commercial hatchery (Gran Mar) located in Baja California Sur, Mexico. After reception, animals were placed in two 500 L tanks and acclimated to local conditions: water temperature 28.9 ±0.8 C, salinity 34.4 ±0.6 g/L, pH 8.4 ±0.1 and saturated dissolved oxygen. Total ammonia nitrogen (0.08 ±0.04 mg/L), nitrite (not detected), and nitrate (10.9 ±3.9 mg/L) were monitored using a commercial kit (FasTest; Aquarium Systems, Sarrebourg, France). A photoperiod was set up as to provide a 10:14h light-dark regime. Shrimps were exclusively fed on a crumbled commercial diet (35% crude protein, Grupo Costamar, Mexico) previously analyzed for nitrogen content and $\delta^{15}N$ value. In order to establish a known isotopic baseline in shrimp tissue before the start of the experiment, this diet was supplied for 15 days. Twelve shrimps having initial mean wet weight of 589 ±80 mg (mean ±S.D.) were allocated to 12, 60-L capacity tanks individually fitted with air lifts. Each dietary treatment was applied to duplicate tanks. Artificial seawater (Fritz, Chemical Co., Texas, USA) was exchanged at a rate of 800%/d in every unit. Re-circulated seawater was treated by mechanical cartridge filters, UV filter, protein skimmers and a bubble bead biological filter. The experimental diets were delivered in excess to every tank at 6:00, 9:00, 12:00, 15:00 and 18:00 h. Uneaten feed, feces and moults were siphoned out daily before first feeding. Tank walls were periodically scrubbed off in order to avoid any possible biofilm growth contributing as food. Feeding rations were adjusted in relation to observed survival and number of sampled animals. The experimental time period, sampling and weighing points were defined according to the exponential rate of isotopic shift previously observed in experiments using small-sized Penaeid shrimp (Gamboa Delgado et al., 2014). On days 0, 4, 8, 15 and 22, one or two shrimps (depending on individual weight) were randomly collected from every replicate tank, killed in

ice/water slurry, rinsed with distilled water and dissected to extract abdominal muscle. On day 29, all remaining animals were scarified. The exoskeleton and hind gut were removed from the abdominal segments and samples were kept in labeled vials at −80 C until pretreatment for isotopic analysis.

2.3. Sample pretreatment and stable isotope analyses

Samples of diets and shrimp muscle tissue were dehydrated at 60 °C until samples reached constant weight. Subsamples were manually ground using mortar and pestle to obtain a fine powder. Samples of 900 to 1100 µg were packed in tin cups (D1008 Elemental Microanalysis Ltd., UK) and organized in 96-well microplates. Isotopic analysis (natural abundance levels) was carried out at the Stable Isotope Facility of the Department of Plant Sciences, University of California, (Davis, CA, USA) using a Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Repeated measurements of a calibration standard indicated that instrument precision (S.D.) was 0.12 ‰ for $\delta^{15}N$ values. Isotopic results are expressed in delta notation (δ), which is defined as part per thousand (‰) deviations from the $\delta^{15}N$ value of the standard reference material (atmospheric nitrogen, 0.36% of "heavy" nitrogen, ^{15}N). These values were defined by equation 1.

$$\delta^{H}X = (R_{sample}/R_{standard} - 1) \cdot 1000 \quad (1)$$

where X is nitrogen, superscript H indicates the heavy isotope mass for that element and R = $^{15}N/^{14}N$.

The term "discrimination factor" ($\Delta^{15}N$) is used in the present study to describe differences in isotopic values between a consuming organism (whole body or specific tissue, in this case muscle) and its diet after having reached isotopic equilibrium.

2.4. Estimation of nutrient contribution and nitrogen half lives (t_{50})

Preliminary analysis indicated that elemental N contents in TY and FM were significantly different (N= 6.6 ± 0.5, and 10.7 ± 0.6 %, respectively). Elemental values are considered in the data generated by the mixing model, and allow estimating the relative contribution of dry matter from the food sources to growth. The proportional dietary nitrogen contributions from FM and TY to shrimp growth were estimated using a two-source, one-isotope mixing model (Phillips and Gregg 2001). Previous studies have shown that, when fed to *L. vannamei*, fish meal and yeast have similar apparent digestibility coefficients (0.66 and 0.71, respectively, dry matter) (Cruz-Suárez et al., 2009; Villarreal-Cavazos, 2011; Liu et al., 2013). Therefore, expected dietary proportions of dietary nitrogen and total dry matter were not corrected for apparent digestibility coefficients. Estimation of $\Delta^{15}N$ is desirable to integrate correction factors into the model as these improve the accuracy of estimations of nutrient contributions (Post, 2002). Values were obtained from the isotopic differences between shrimps fed exclusively on diets 100% FM and 100% TY. Corrected $\delta^{15}N$ values were drawn from the $\delta^{15}N$ values of animals fed control diets. $\delta^{15}N$ values measured in shrimps fed mixed diets were introduced into the model to estimate the proportional dietary nitrogen incorporation from TY and FM (and their truncated 95% confidence intervals). Total dry matter contributions were estimated after correcting for nitrogen concentrations measured in both ingredients using the equation proposed by Fry (2006). $\delta^{15}N$ values measured through the experimental period were integrated in an exponential model of isotopic change (Hesslein et al., 1993) to obtain an estimate of the metabolic nitrogen turnover rate in shrimp

muscle tissue. The model allows distinguishing the isotopic change that is due to growth (k) or metabolic turnover (m). The growth rate constant, k, was estimated by fitting an exponential growth model to observed weight data, $k = log$(final weight/initial weight)/time(d), while parameter m was obtained using iterative non-linear regression. Coefficients k and m provide an indicator of the time period necessary for half of the muscle nitrogen to be replaced by new nitrogen after animals consume a new diet (half life, t_{50}) (MacAvoy et al. 2006).

$$t_{50} = In2 / m+k \quad (2)$$

where $In2$ represents the natural logarithm of 2.

2.5. Statistical Analysis

Student's t-tests were used to compare nitrogen contents and $\delta^{15}N$ values in FM and TY. Dietary effects on $\delta^{15}N$ values of muscle tissue at different times, mean shrimp final weight and survival were analyzed by Kruskal-Wallis tests and ensuing pair comparisons by Mann-Whitney tests. In order to detect statistical differences in the expected proportions of dietary nitrogen (contributed by FM and TY) and the observed proportions of dietary nitrogen incorporated in shrimp muscle tissue, Chi-square goodness of fit tests (χ^2) were applied. Parameters required by the Hesslein model were estimated by iterative non-linear regression. All tests were conducted using SPSS 17.0 software (SPSS Inc.) at a significance level of $P<0.05$.

3. Results

3.1. Growth and survival rates

During the experimental period, water conditions remained within the recommended optimal values for this species. Temperature, pH, salinity and dissolved oxygen were maintained during the trial as the previously described bioassay room conditions. Overall shrimp survival rates were high (89 to100%) and these were statistically similar among dietary treatments. Although the variability was high at the end of the experiment, shrimps reared under the different experimental diets showed significantly different mean final weights (Table 2). All diets containing a mixture of FM and TY outperformed the control diets (100F and 100T) in terms of growth (3122 to 2822 mg). Shrimps raised on diet 85F/15T showed significantly higher final mean weight (3822 mg) than diet containing only FM (2992 mg). The lowest final growth was observed in diet containing only TY (1873 mg).

Table 2 . Final mean wet weight (FW), weight gain (WG), specific growth rate (SGR) and survival rate (S) of Pacific white shrimp *L. vannamei* reared under diets having different dietary proportions of fish meal and torula yeast. Mean values ± S.D.

Diet	FW (mg)	WG (%)	SGR	S (%)
100F	2992 ± 917^{b}	408 ± 97^{b}	5.60	92 ± 12^{a}
93F/7T	3320 ± 709^{ab}	464 ± 115^{ab}	5.96	96 ± 6^{a}
85F/15T	3822 ± 705^{a}	549 ± 88^{a}	6.45	88 ± 6^{a}
70F/30T	3122 ± 911^{ab}	430 ± 107^{ab}	5.75	92 ± 12^{a}
40F/60T	3423 ± 813^{ab}	481 ± 101^{ab}	6.07	88 ± 6^{a}
100T	1873 ± 603^{c}	218 ± 33^{c}	3.99	100 ± 0^{a}

Different superscripts indicate significant differences for that particular column

3.2. Isotopic shifts and discrimination factors

FM and TY showed very contrasting $\delta^{15}N$ values (16.9 ±0.1 and 1.1 ±0.1 ‰, respectively). This significant difference allowed formulating isotopically contrasting diets that elicited a wide range of trends in the isotopic changes occurring in shrimps under the different treatments (Fig. 1). The high range of isotopic values in diets and ingredients facilitated both, assessment of nitrogen metabolic turnover rate and estimation of dietary contributions to growth. All diets exerted a rapid influence on the isotopic values of muscle tissue and by day 22, shrimps in all treatments reached isotopic equilibrium with their respective diets. $\delta^{15}N$ values in muscle of shrimps reared on mixed compound diets closely matched the isotopic values of the experimental diets. $\Delta^{15}N$ values between animals and their respective diets were very contrasting but correlated (r^2= 0.94) to the increasing dietary inclusion levels of TY. Average $\Delta^{15}N$ values reported in the literature are close to 3.0 ‰ (McCutchan et al. 2003, Caut et al. 2009) and are frequently applied to correct for $\Delta^{15}N$ values when estimating nutritional contributions in a wide range of animal taxa. However, as observed in the present study, this average might not be representative for all different taxa/species or life stages. $\Delta^{15}N$ values between muscle tissue of shrimps and diet 100F were small (0.2 ‰), while values observed in shrimp fed diet 100T were significantly larger (5.6 ‰).

3.3. Nitrogen half lives in tissue

Changes in $\delta^{15}N$ values in shrimp muscle elicited by the change from the conditioning diet to the experimental diets followed an expected, exponential trend. For most treatments, predicted isotopic values fitted well on the observed data (r^2= 90 to 99). From each of these data groups,

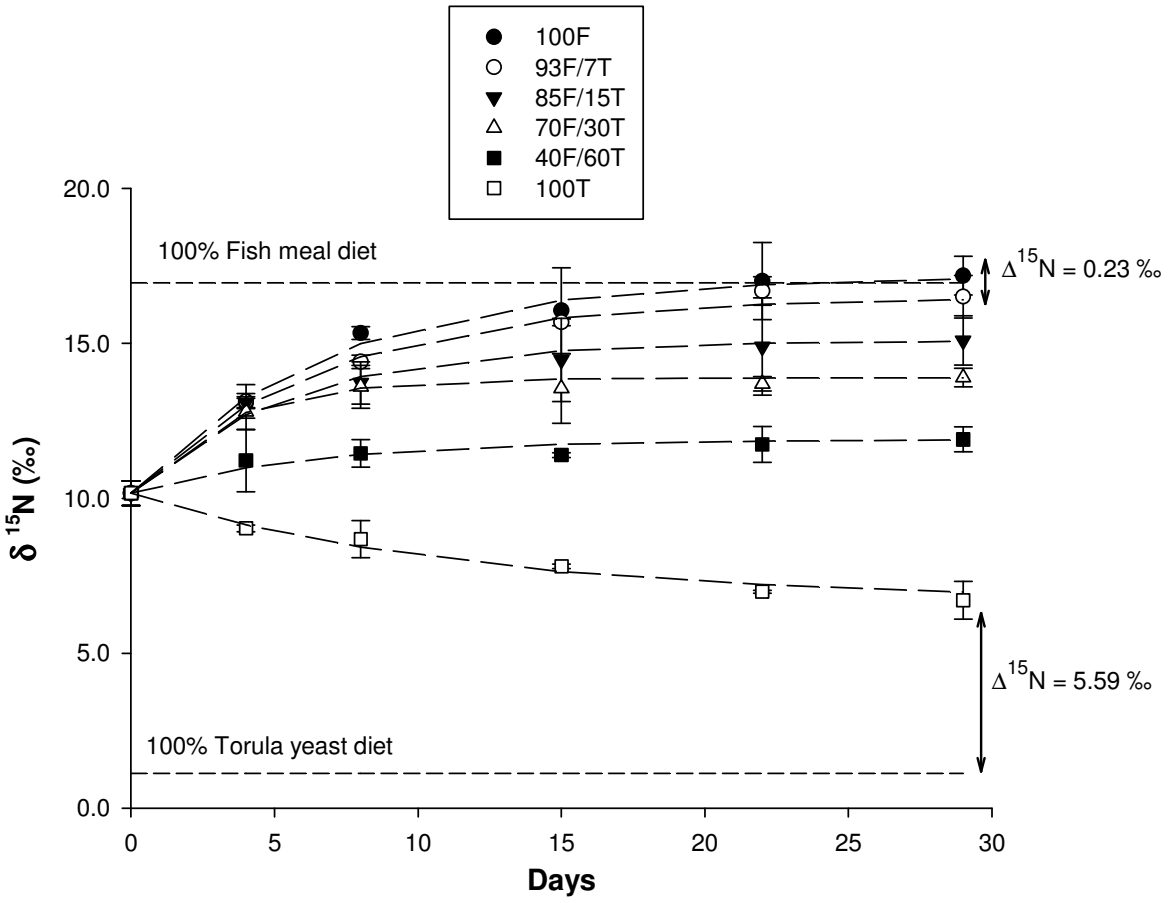

Fig. 1. Changes in nitrogen stable isotope values (‰) in muscle tissue of Pacific white shrimp *Litopenaeus vannamei* after a dietary shift from a conditioning diet to six experimental diets containing different proportions of torula yeast *Candida utilis* and fish meal. Lines represent predicted values generated by a model of isotopic change and show the best fit to observed data. Arrows indicate nitrogen isotopic discrimination factors between control diets and shrimps. n= 2 individuals, 12 on final point (mean values ± S.D.).

parameter *m* (metabolic turnover) was estimated by means of iterative non-linear regression. Parameters *m* and *k* indicated that estimated nitrogen half lives in tissue (t_{50}) ranged from 2.3 d in shrimp fed diet 70F/30T to 8.0 d in shrimp fed on diet 100T (Table 3). t_{50} values decreased from diet 100F (4.8 d) to diet 70F/30T (2.3 d), while the effect of diets 40F/60T and 100T on t_{50} values did not fit a specific pattern.

Table 3. Mean growth rates (*k*) and estimated half lives (t_{50}) of nitrogen in muscle tissue of Pacific white shrimp *L. vannamei* reared under diets having different dietary proportions of fish meal and torula yeast. $\Delta^{15}N$ represents the isotopic difference between diets and muscle tissue after isotopic equilibrium was reached. Mean values ± S.D.

Diet	Nitrogen			
	k (d^{-1})	half life (d)	r^2	$\Delta^{15}N$
100F	0.054 ± 0.009^{b}	4.8 ± 0.7	99	0.2
93F/7T	0.060 ± 0.006^{ab}	4.7 ± 1.1	99	1.1
85F/15T	0.064 ± 0.006^{a}	3.8 ± 0.6	98	0.6
70F/30T	0.056 ± 0.009^{ab}	2.3 ± 0.9	98	2.1
40F/60T	0.059 ± 0.008^{ab}	4.2 ± 1.3	90	5.3
100T	0.041 ± 0.009^{c}	8.0 ± 1.5	97	5.6

Different superscripts indicate significant differences for that particular column.

3.4. Dietary nitrogen and dry matter contribution from fish meal and torula yeast

Changes in $\delta^{15}N$ values observed over the experimental period and inclusion of asymptotic values into the isotopic mixing model indicated that the contributions of dietary nitrogen from FM and TY to the growth of shrimps were very similar to the expected contributions indicated by the

respective proportions of dietary nitrogen established in the dietary formulations (Tables 1 and 4). The only exception was observed in shrimps fed on diet 40F/60T where the dietary nitrogen contribution from TY to muscle tissue (50.5 %) was significantly lower (χ^2=3.84, *P*= 0.049) than the dietary nitrogen available in this diet (60.1 %), therefore indicating a higher contribution of dietary nitrogen from FM. Dietary nitrogen from torula yeast available in diets 93F/7T, 85F/15T, 70F/30T and 40F/60T was incorporated in muscle tissue at proportions of 6.5, 13.7, 27.1 and 50.5 %, respectively. After correcting for the different dietary nitrogen content in FM and TY, expected dry matter contributions from TY slightly increased as the dietary inclusion of this ingredient (containing less dietary nitrogen) was added at a higher levels than FM. Observed dry matter contributions from TY in tissue also increased, suggesting that estimated dry matter contributions to muscle tissue followed a similar pattern as those observed for dietary nitrogen (Table 4).

Table 4. Estimated relative proportions of dietary nitrogen and total dry matter supplied from torula yeast (TY) and fish meal (FM) and contributing to the growth of *L. vannamei* as indicated by a two-source, one-isotope mixing model (mean ± CI, n = 12).

Diet	Expected	Observed in muscle tissue		
		min.	mean	max.
Nitrogen				
93F/7T				
FM	92.4[a]	86.3	93.5[a]	100
TY	7.6	0.0	6.5	13.7
85F/15T				
FM	85.0[a]	78.1	86.3[a]	94.4
TY	15.0	5.6	13.7	21.9
70F/30T				
FM	69.8[a]	69.2	72.9[a]	76.6
TY	30.2	23.4	27.1	30.8
40F/60T				
FM	39.9[a]	45.2	49.5[b]	53.8
TY	60.1	46.2	50.5	54.8
Dry matter				
93F/7T				
FM	87.3[a]	83.9	89.9[a]	95.9
TY	12.7	4.1	10.1	16.1
85F/15T				
FM	76.1[a]	72.5	79.5[a]	86.5
TY	23.9	13.5	20.5	27.5
70F/30T				
FM	56.6[a]	59.4	62.4[a]	65.4
TY	43.4	34.6	37.6	40.6
40F/60T				
FM	27.2[a]	33.6	37.7[b]	41.8
TY	72.8	58.2	62.3	66.4

Superscripts indicate significant differences between expected and mean observed dietary contributions. *Total dry matter contributions were estimated after correcting for nitrogen concentrations measured in both ingredients using the equation proposed by Fry (2006).

4. Discussion

4.1. Growth and survival

The protein level and composition of the experimental diets promoted high survival and growth rates. The rapid tissue accretion caused a fast transference of dietary $\delta^{15}N$ values to muscle tissue. The dietary inclusion of TY had a significant effect on the final mean weight of shrimps under different treatments. Although it has been shown that TY has low methionine levels (1.40 *vs.* 2.80 % protein^{-1} in FM), results allow inferring that the presence of FM in diets containing both protein sources complemented this and other possible deficiencies. Different types of yeast (*Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Candida utilis*) and its extracts have been used in aquaculture diets at different inclusions levels, either as an additive or as fish meal replacement. For example, Zhao et al. (2015) recently reported that up to 45% of FM in diets for shrimp *L. vannamei* can be replaced with a yeast extract without affecting growth and digestibility. Biswas et al. (2012) and Deng et al. (2013) reported improved growth and immune-stimulant effects of yeast-based products in kuruma shrimp, *Marsupenaeus japonicus* and white shrimp, *L. vannamei,* respectively. Studies suggest that fish might be less tolerant to yeasts as ingredient. In a trial with juvenile cobia (*Rachycentron canadum*), Lunger et al. (2006) concluded that only 25% of FM could be replaced with yeast-based protein without detrimental impacts on growth rates, and feed efficiency. In contrast, when compared to fish fed on control diets, Ribeiro et al. (2014) and Luzzana et al. (2005) reported lower growth in Red-Stirling tilapia (*O. niloticus*) fed on a diet having 15% of yeast (*K. marxianus*) and in grey mullet *Mugil cephalus* fed a torula yeast-based diet.

4.2. Isotopic shifts and discrimination factors

Given that previous studies in crustaceans have shown only small differences between the $\delta^{15}N$ values in muscle and whole body samples (Stenroth et al., 2006; Gamboa-Delgado et al., 2011), muscle tissue is considered a good target sample in studies exploring isotope dynamics. The isotopic values of the experimental diets were rapidly reflected in shrimp muscle tissue and isotopic equilibrium between diets and animals was reached between experimental days 15 and 22. These observations are consistent with previous studies conducted on Penaeid shrimp. Shrimps fed on diets containing TY and FM increased their body weights between 430 and 549 %. In contrast, animals fed on diet containing only TY increased in weight by 218% over the experimental period. These animals reached isotopic equilibrium but evidently not through biomass accretion, but mainly as a consequence of tissue metabolic turnover. The wide range of observed $\Delta^{15}N$ values between shrimp and diets is consistent with observations reported in nutritional studies employing ingredients having different dietary quality (Martínez-Rocha et al., 2013; Gamboa-Delgado et al., 2014). Diet containing only TY as protein source is considered nutritionally restricted for shrimp and the estimated $\Delta^{15}N$ value estimated between diet and shrimp was 5.6 ‰. Isotopic discrimination occurs because organisms have an affinity to incorporate heavy isotopes rather than the lighter and more common isotopes. This is the result of heavy isotopes concentrating in molecules were the bond strengths are greatest, the effect being amplified in every chemical and enzymatic transformation (Peterson and Fry, 1987). Studies on isotopic dynamics consider that different $\Delta^{15}N$ values between consuming organisms and diet might be related to the quality of the available dietary protein as there is increasing evidence indicating that high $\Delta^{15}N$ values indicate a higher demand for specific nutrients, in particular

when the growth rate of consuming animals is high, as those observed during earlier life stages (Martínez del Rio and Wolf, 2005; Le Vay and Gamboa-Delgado 2010).

4.3. Nitrogen half lives in muscle tissue

In the present study, estimated t_{50} values measured in muscle tissue were relatively short when compared to studies where nutritionally restricted diets (low dietary levels of plant derived proteins) were fed to *L. vannamei* (t_{50}=7.7-12.8 d; Martínez-Rocha et al., 2012). Unlike $\Delta^{15}N$ values and estimated nutrients contributions, t_{50} values were not clearly correlated to the dietary inclusion of TY. However, a slight decrease from 4.8 d to 2.3 d was observed from diet 100F to diet 70F/30T. High metabolic turnover rates usually translate into short t_{50} values (Hobson and Clark, 1992). As t_{50} values are estimated from the parameters of growth constant k and the metabolic turnover m, in the present study it can be stated that, with the exception of diet 100T, all diets elicited fast growth and metabolic turnover in muscle tissue. Results are consistent with previous studies conducted on the same species and life stage. Gamboa-Delgado and Le Vay (2009) reported t_{50} values ranging from 2.8 to 4.0 d in shrimps fed on high protein diets having different proportions of fish meal and soy protein isolate. It has been reported that juvenile Penaeid shrimps present a high efficiency of retention of synthesized protein as growth (94%) when reared on nutritionally optimal diets (Mente et al., 2002). Estimation of metabolic turnover rate (m) and t_{50} values in other crustacean species has shown that these parameters significantly vary among different tissues (*i.e.* metabolic turnover rates are much higher in hemolymph than those determined in muscle and exoskeleton). This information has allowed defining sample schemes for exploring nutritional effects on specific types of tissue (Suring and Wing, 2009).

4.4. Nutrient contribution from torula yeast and fish meal

The relatively similar contribution of dietary nitrogen supplied by FM and TY to shrimp growth indicates that both protein sources were nutritionally suitable or that nutrients from both sources complemented well. Diet containing up to 60% of TY promoted similar growth as diet containing only FM. Supplying the diet having TY as the only protein source, did not support similar high growth rates probably due to their lower biological value and palatability. Although it has been considered that the cell walls of unicellular organisms are difficult to digest by a range of species (Becker, 2007), results from the present study indicate that a high proportion of dietary nitrogen was incorporated from TY in animals fed on all mixed diets. In this context, it has been pointed out that animal tissue often does not reflect the bulk isotopic composition of the diet, but the isotopic composition of the constituents of the diet from which the tissue was biosynthesized (Gannes et al., 1997). Growth and survival rates were statistically similar in shrimp fed on all mixed diets, therefore indicating the suitability of this SCP ingredient to replace FM in diets containing up to 60% of TY. Nevertheless, it should be stated that at this high dietary level, isotopic results indicated a higher allocation of dietary nitrogen from FM. Diet containing only FM at this protein level is considered nutritionally suitable for Penaeid shrimp as it presents favorable amino acid profile. It is suggested that even low levels of FM in the diet, compensate for nutritional deficiencies caused by alternative protein sources (plant- or microbial-derived). Differential retention of essential and non- essential amino acids from both sources might explain the different allocation of dietary nitrogen observed in diet containing the lowest FM level. This differential retention can also explain the similar growth and survival rates elicited by all diets containing both protein sources. Studies employing compound specific isotopic analysis (CSIA) have reported on the isotopic transformations leading to the final allocation of specific amino

acids in invertebrates (Fantle et al., 1999; O'Brien et al., 2003). Future studies employing CSIA might elucidate which dietary amino acids are preferentially incorporated, while also identifying the source ingredients supplying these amino acids. In conclusion, results from the present study demonstrate that the dietary inclusion of TY improved the final growth of shrimp, while also contributing high proportions of dietary nitrogen to growth when it is used to replace up to 60% of dietary FM.

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Nutritional contribution of torula yeast and fish meal to the growth of shrimp *Litopenaeus vannamei* as indicated by natural nitrogen stable isotopes

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Highlights

- Torula yeast and fish meal were used to formulate diets for Pacific white shrimp.
- Isotopic measurements were applied to ingredients and muscle tissue.
- Relative dietary contributions from both ingredients to growth were similar.
- Torula yeast can be used to replace up to 60% of fish meal.

Abstract

Torula yeast (*Candida utilis*) and fish meal were used to formulate six experimental diets for Pacific white shrimp *Litopenaeus vannamei.* The dietary nitrogen supplied by fish meal was replaced by increasing dietary proportions of torula yeast (0, 7.5, 15, 30, 60 and 100%). Nitrogen stable isotope values ($\delta^{15}N$) were measured in ingredients, diets and muscle tissue of experimental animals in order to estimate the relative contributions of dietary nitrogen and dry matter supplied by both ingredients. At the end of a 29 d bioassay, there were no significant differences in survival rate among treatments. Shrimps fed on all diets containing torula yeast and fish meal had higher growth rates (k = 0.059-0.064) than animals fed on diets containing only fish meal or only torula yeast (k = 0.041-0.054). Incorporation of $\delta^{15}N$ values of ingredients and muscle tissue into an isotopic mixing model indicated that the relative incorporation of dietary nitrogen and total dry matter from torula yeast to growth consistently increased in relation to increasing proportions of this ingredient in the experimental diets. The only exception was the diet formulated with the highest yeast content (60%, diet 40F/60T) where fish meal contributed a

higher proportion of dietary nitrogen to growth. Dietary nitrogen from torula yeast available in diets 93F/7T, 85F/15T, 70F/30T and 40F/60T was incorporated in muscle tissue at proportions of 6.5, 13.7, 27.1 and 50.5 %, respectively. Estimated nitrogen residency time in tissue (t_{50}) was relatively shorter in shrimps fed on diets 85F/15T and 70F/30T (3 d), indicating higher metabolic turnover rates in these animals than those fed on diet containing only fish meal. Growth and survival rates were statistically similar in shrimp fed on all mixed diets, therefore indicating the suitability of this single cell protein as dietary ingredient in diets containing up to 60% of torula yeast.

Keywords: Stable isotopes, nutrient contribution, single cell protein, torula yeast, fish meal, *Litopenaeus vannamei*

1. Introduction

The Pacific white shrimp *Litopenaeus vannamei* has become the main shrimp species produced through aquaculture practices since 2003 (FAO, 2007). The progressively higher production of this and several other aquaculture species is in turn exacerbating the high demand for aquaculture feeds and the ingredients required for their manufacture. The commercialization of marine-derived proteins maintains increasing economic and ecological concerns mainly due to overfishing of small pelagic fish from which fish meal is obtained. Several alternative sources of plant and microbial protein have drawn attention and are being currently used either to replace fish meal in aquaculture diets or tested as ingredients. Defatted microalgae biomass from biorefineries, yeast and bacterial aggregates from highly controlled production systems that

employ agricultural byproducts as substrates are some of the emerging alternative feed ingredients for manufacturing and improving the highly demanded aquaculture feeds. Due to their good nutritional and immunostimulatory properties, single cell proteins (SCP), including yeasts, have been studied in the past as feed ingredients for terrestrial and aquatic animals (Perera et al., 1995; Poulose, 2013; Macias-Sancho et al-. 2014; Martins et al., 2014). In addition to the good nutritional properties, sources of SCP are becoming more affordable. Bob-Manuel and Erondu (2010) carried out an economic evaluation of the use of yeast in tilapia diets. Result showed that, from a range of substitution levels, a substitution of 50% of fish meal with yeast had the lowest profit index and cost-benefit ratio. Nowadays, several enterprises have achieved massive production of different microorganisms for commercial purposes and among these; torula yeast (*Candida utilis*) has been commercially available for decades. This particular type of yeast has been tested as protein source in diets for aquatic animals and it has been found that feed acceptance and survival are not affected when used as partial fish meal replacement. Olvera-Novoa et al. (2002) used torula yeast as a dietary protein source for tilapia fry and replaced different proportions of animal-derived protein. Highest growth responses were observed for fish on diets containing 30% torula yeast. Britz (1996) fed abalone *Haliotis midae* on diets containing different sources of protein. A diet containing only torula yeast as protein source elicited lower feed conversion ratios than than those observed in control abalone fed on seaweed, although the growth rate was lower.

Measurements of nitrogen stable isotope ratios ($\delta^{15}N$) have been applied as nutritional tools to explore the transference and deposition of dietary protein in several aquatic species. The integration of isotopic data into mass-balance mixing models has made possible to convert the isotopic values of consumers and their different trophic elements to dietary contributions

(Phillips, 2012). In aquaculture nutrition, the isotopic values of carbon and nitrogen have been used as natural biomarkers to estimate dietary contributions in organisms fed on experimental dietary formulations having ingredients with contrasting isotopic signatures (Martínez-Rocha et al., 2013; Gamboa-Delgado et al., 2013, 2014). The different dietary resources found in aquatic and terrestrial ecosystems frequently show distinct $\delta^{15}N$ values due to the effect of characteristic nutrient flows and metabolic pathways. However, this natural flow of nutrients (and isotopes) is altered under the controlled, artificial systems promoting the growth of commercial valuable microorganisms from specific substrates. The present study employed the isotopic differences found in torula yeast and fish meal to assess the relative incorporation of dietary nitrogen and total dry matter supplied by these sources to the growth of Pacific white shrimp by means of an isotope mixing model.

2. Material and methods

2.1. Experimental diets

A batch of inactive torula yeast *Candida utilis* (Uniprot®) was obtained from FerMex (Toluca, Mexico). Torula yeast (TY) contained 41% crude protein and it represented one of two protein sources used for diet formulation. The second protein source was fish meal (FM, prime Mexican sardine, 68% protein). From these main ingredients, six isonitrogenous (40% crude protein) and isoenergetic (4.6 kcal/gr) experimental diets were formulated using the software Nutrion® (Nutrion, Chapala, Mexico). FM and TY represented the only nitrogen sources, both having contrasting $\delta^{15}N$ values. Four mixed experimental diets were formulated with different proportions of FM substituted with different levels of TY, at 7.5 (diet 93F/7T), 15 (diet 85F/15T),

30 (diet 70F/30T) and 60% (diet 40F/60T) of the available dietary nitrogen. Dietary substitutions were done based on available dietary nitrogen in ingredients (Table 1). Diets containing only one source of protein were used as positive (100F) and negative (100T) controls in order to estimate and correct for isotopic discrimination factors ($\Delta^{15}N$). Experimental diets were not manufactured to conduct an ingredient substitution study; instead, they were formulated with ingredients having contrasting $\delta^{15}N$ values in order to explore their nutritional contributions to shrimp growth. TY contained lower protein content than FM; therefore its dietary bulk inclusion was higher; a concomitant, progressive reduction of dietary starch was applied in order to maintain isoenergetic diets. Micronutrients were weighed to the nearest mg and hand-mixed for 5 min before mixing these with finely ground macronutrients. The mixture was homogenized for 10 min using a commercial blender. Supplemental cholesterol was added only to diet containing 100% TY, as cholesterol content in FM covered the nutritional requirement in the rest of diets. Lecithin was dissolved in warm fish oil and the content was slowly added to the main mixture. Distilled water was added until the dough was formed. The resulting paste was extruded through a die plate (1.6 mm orifices). Diets were dried in a convection oven for 8 min at 100 °C and stored at 4 °C. Proximal analyses of experimental diets, included protein content (Dumas method, LECO), lipid content (Soxhlet system HT-1045, method AOAC 996.06) (Tecator 1983), fibre content (AOAC 962.09B), moisture content (AOAC 930.15) and ash content (AOAC 942.05) were performed. The nitrogen-free extract was estimated as the difference of the latter assays subtracted from 100.

Table 1. Nutritional (g 1000 g^{-1} diet dry weight) and isotopic ($\delta^{15}N$, ‰) composition of formulated diets fed to Pacific white shrimp *Litopenaeus vannamei* to estimate nutritional contributions of fish meal and torula yeast to shrimp muscle tissue.

Ingredient / Diet	**100F**	**93F/7T**	**85F/15T**	**70F/30T**	**40F/60T**	**100T**
Fish meal[a]	475.5	439.7	404.4	332.8	190.6	0.0
Torula yeast[b]	0.0	64.0	127.0	255.0	509.0	849.5
Wheat starch[c]	424.7	393.8	363.3	301.5	163.3	0.0
Lecithin[d]	35.5	35.5	35.5	35.5	40.7	40.0
Fish oil[a]	16.3	19.1	21.8	27.3	33.1	37.2
Disodium phosphate[e]	0.0	0.0	0.0	0.0	16.5	34.8
Calcium chloride[e]	0.0	0.0	0.0	0.0	0.0	7.0
Cellulose[e]	19.0	19.0	19.0	19.0	17.9	1.3
Alginate[e]	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin premix[a]	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[a]	2.5	2.5	2.5	2.5	2.5	2.5
Choline chloride[a]	2.0	2.0	2.0	2.0	2.0	2.0
Cholesterol[f]	0.0	0.0	0.0	0.0	0.0	1.2
Vitamin C[a]	1.0	1.0	1.0	1.0	1.0	1.0
Antioxidant[a]	0.5	0.5	0.5	0.5	0.5	0.5
Antifungic agent[a]	0.5	0.5	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000	1000	1000
Proximal and isotopic analysis						
Crude protein (g kg-1)	403	391	391	403	397	412
Lipids (g kg-1)	87	94	94	91	93	80
Ash (g kg-1)	107	109	100	98	111	120
Gross energy (Kcal g-1)	4.7	4.7	4.6	4.6	4.5	4.3
$\delta^{15}N$ (‰)	16.9	15.4	14.4	11.8	6.6	1.1

[a]Alimentos Costamar (Sonora, Mexico).
[b]Fermex (Toluca, Mexico).
[c]Almidones y gluten S.A. (Monterrey, Mexico).
[d]Ragaza Industrias Proteínas Naturales S.A. de C.V. (Monterrey, Mexico).
[e]Sigma-Aldrich (St. Louis, MO, USA).
[f]Solvay Pharmaceuticals (Houston, TX, USA).

2.2. Experimental design and rearing system

Juvenile shrimp, *L. vannamei* were obtained from a commercial hatchery (Gran Mar) located in Baja California Sur, Mexico. After reception, animals were placed in two 500 L tanks and acclimated to local conditions: water temperature 28.9 ±0.8 C, salinity 34.4 ±0.6 g/L, pH 8.4 ±0.1 and saturated dissolved oxygen. Total ammonia nitrogen (0.08 ±0.04 mg/L), nitrite (not detected), and nitrate (10.9 ±3.9 mg/L) were monitored using a commercial kit (FasTest; Aquarium Systems, Sarrebourg, France). A photoperiod was set up as to provide a 10:14h light-dark regime. Shrimps were exclusively fed on a crumbled commercial diet (35% crude protein, Grupo Costamar, Mexico) previously analyzed for nitrogen content and $\delta^{15}N$ value. In order to establish a known isotopic baseline in shrimp tissue before the start of the experiment, this diet was supplied for 15 days. Twelve shrimps having initial mean wet weight of 589 ±80 mg (mean ±S.D.) were allocated to 12, 60-L capacity tanks individually fitted with air lifts. Each dietary treatment was applied to duplicate tanks. Artificial seawater (Fritz, Chemical Co., Texas, USA) was exchanged at a rate of 800%/d in every unit. Re-circulated seawater was treated by mechanical cartridge filters, UV filter, protein skimmers and a bubble bead biological filter. The experimental diets were delivered in excess to every tank at 6:00, 9:00, 12:00, 15:00 and 18:00 h. Uneaten feed, feces and moults were siphoned out daily before first feeding. Tank walls were periodically scrubbed off in order to avoid any possible biofilm growth contributing as food. Feeding rations were adjusted in relation to observed survival and number of sampled animals. The experimental time period, sampling and weighing points were defined according to the exponential rate of isotopic shift previously observed in experiments using small-sized Penaeid shrimp (Gamboa Delgado et al., 2014). On days 0, 4, 8, 15 and 22, one or two shrimps (depending on individual weight) were randomly collected from every replicate tank, killed in

ice/water slurry, rinsed with distilled water and dissected to extract abdominal muscle. On day 29, all remaining animals were scarified. The exoskeleton and hind gut were removed from the abdominal segments and samples were kept in labeled vials at −80 C until pretreatment for isotopic analysis.

2.3. Sample pretreatment and stable isotope analyses

Samples of diets and shrimp muscle tissue were dehydrated at 60 °C until samples reached constant weight. Subsamples were manually ground using mortar and pestle to obtain a fine powder. Samples of 900 to 1100 µg were packed in tin cups (D1008 Elemental Microanalysis Ltd., UK) and organized in 96-well microplates. Isotopic analysis (natural abundance levels) was carried out at the Stable Isotope Facility of the Department of Plant Sciences, University of California, (Davis, CA, USA) using a Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Repeated measurements of a calibration standard indicated that instrument precision (S.D.) was 0.12 ‰ for $\delta^{15}N$ values. Isotopic results are expressed in delta notation (δ), which is defined as part per thousand (‰) deviations from the $\delta^{15}N$ value of the standard reference material (atmospheric nitrogen, 0.36% of "heavy" nitrogen, ^{15}N). These values were defined by equation 1.

$$\delta^{H}X = (R_{sample}/R_{standard} - 1) \cdot 1000 \quad (1)$$

where X is nitrogen, superscript H indicates the heavy isotope mass for that element and R = $^{15}N/^{14}N$.

The term "discrimination factor" ($\Delta^{15}N$) is used in the present study to describe differences in isotopic values between a consuming organism (whole body or specific tissue, in this case muscle) and its diet after having reached isotopic equilibrium.

2.4. Estimation of nutrient contribution and nitrogen half lives (t_{50})

Preliminary analysis indicated that elemental N contents in TY and FM were significantly different (N= 6.6 ± 0.5, and 10.7 ± 0.6 %, respectively). Elemental values are considered in the data generated by the mixing model, and allow estimating the relative contribution of dry matter from the food sources to growth. The proportional dietary nitrogen contributions from FM and TY to shrimp growth were estimated using a two-source, one-isotope mixing model (Phillips and Gregg 2001). Previous studies have shown that, when fed to *L. vannamei*, fish meal and yeast have similar apparent digestibility coefficients (0.66 and 0.71, respectively, dry matter) (Cruz-Suárez et al., 2009; Villarreal-Cavazos, 2011; Liu et al., 2013). Therefore, expected dietary proportions of dietary nitrogen and total dry matter were not corrected for apparent digestibility coefficients. Estimation of $\Delta^{15}N$ is desirable to integrate correction factors into the model as these improve the accuracy of estimations of nutrient contributions (Post, 2002). Values were obtained from the isotopic differences between shrimps fed exclusively on diets 100% FM and 100% TY. Corrected $\delta^{15}N$ values were drawn from the $\delta^{15}N$ values of animals fed control diets. $\delta^{15}N$ values measured in shrimps fed mixed diets were introduced into the model to estimate the proportional dietary nitrogen incorporation from TY and FM (and their truncated 95% confidence intervals). Total dry matter contributions were estimated after correcting for nitrogen concentrations measured in both ingredients using the equation proposed by Fry (2006). $\delta^{15}N$ values measured through the experimental period were integrated in an exponential model of isotopic change (Hesslein et al., 1993) to obtain an estimate of the metabolic nitrogen turnover rate in shrimp

muscle tissue. The model allows distinguishing the isotopic change that is due to growth (k) or metabolic turnover (m). The growth rate constant, k, was estimated by fitting an exponential growth model to observed weight data, $k = log$(final weight/initial weight)/time(d), while parameter m was obtained using iterative non-linear regression. Coefficients k and m provide an indicator of the time period necessary for half of the muscle nitrogen to be replaced by new nitrogen after animals consume a new diet (half life, t_{50}) (MacAvoy et al. 2006).

$$t_{50} = In2 / m+k \quad (2)$$

where $In2$ represents the natural logarithm of 2.

2.5. Statistical Analysis

Student's t-tests were used to compare nitrogen contents and $\delta^{15}N$ values in FM and TY. Dietary effects on $\delta^{15}N$ values of muscle tissue at different times, mean shrimp final weight and survival were analyzed by Kruskal-Wallis tests and ensuing pair comparisons by Mann-Whitney tests. In order to detect statistical differences in the expected proportions of dietary nitrogen (contributed by FM and TY) and the observed proportions of dietary nitrogen incorporated in shrimp muscle tissue, Chi-square goodness of fit tests (χ^2) were applied. Parameters required by the Hesslein model were estimated by iterative non-linear regression. All tests were conducted using SPSS 17.0 software (SPSS Inc.) at a significance level of $P<0.05$.

3. Results

3.1. Growth and survival rates

During the experimental period, water conditions remained within the recommended optimal values for this species. Temperature, pH, salinity and dissolved oxygen were maintained during the trial as the previously described bioassay room conditions. Overall shrimp survival rates were high (89 to100%) and these were statistically similar among dietary treatments. Although the variability was high at the end of the experiment, shrimps reared under the different experimental diets showed significantly different mean final weights (Table 2). All diets containing a mixture of FM and TY outperformed the control diets (100F and 100T) in terms of growth (3122 to 2822 mg). Shrimps raised on diet 85F/15T showed significantly higher final mean weight (3822 mg) than diet containing only FM (2992 mg). The lowest final growth was observed in diet containing only TY (1873 mg).

Table 2 . Final mean wet weight (FW), weight gain (WG), specific growth rate (SGR) and survival rate (S) of Pacific white shrimp *L. vannamei* reared under diets having different dietary proportions of fish meal and torula yeast. Mean values ± S.D.

Diet	FW (mg)	WG (%)	SGR	S (%)
100F	2992 ± 917^{b}	408 ± 97^{b}	5.60	92 ± 12^{a}
93F/7T	3320 ± 709^{ab}	464 ± 115^{ab}	5.96	96 ± 6^{a}
85F/15T	3822 ± 705^{a}	549 ± 88^{a}	6.45	88 ± 6^{a}
70F/30T	3122 ± 911^{ab}	430 ± 107^{ab}	5.75	92 ± 12^{a}
40F/60T	3423 ± 813^{ab}	481 ± 101^{ab}	6.07	88 ± 6^{a}
100T	1873 ± 603^{c}	218 ± 33^{c}	3.99	100 ± 0^{a}

Different superscripts indicate significant differences for that particular column

3.2. Isotopic shifts and discrimination factors

FM and TY showed very contrasting $\delta^{15}N$ values (16.9 ±0.1 and 1.1 ±0.1 ‰, respectively). This significant difference allowed formulating isotopically contrasting diets that elicited a wide range of trends in the isotopic changes occurring in shrimps under the different treatments (Fig. 1). The high range of isotopic values in diets and ingredients facilitated both, assessment of nitrogen metabolic turnover rate and estimation of dietary contributions to growth. All diets exerted a rapid influence on the isotopic values of muscle tissue and by day 22, shrimps in all treatments reached isotopic equilibrium with their respective diets. $\delta^{15}N$ values in muscle of shrimps reared on mixed compound diets closely matched the isotopic values of the experimental diets. $\Delta^{15}N$ values between animals and their respective diets were very contrasting but correlated (r^2= 0.94) to the increasing dietary inclusion levels of TY. Average $\Delta^{15}N$ values reported in the literature are close to 3.0 ‰ (McCutchan et al. 2003, Caut et al. 2009) and are frequently applied to correct for $\Delta^{15}N$ values when estimating nutritional contributions in a wide range of animal taxa. However, as observed in the present study, this average might not be representative for all different taxa/species or life stages. $\Delta^{15}N$ values between muscle tissue of shrimps and diet 100F were small (0.2 ‰), while values observed in shrimp fed diet 100T were significantly larger (5.6 ‰).

3.3. Nitrogen half lives in tissue

Changes in $\delta^{15}N$ values in shrimp muscle elicited by the change from the conditioning diet to the experimental diets followed an expected, exponential trend. For most treatments, predicted isotopic values fitted well on the observed data (r^2= 90 to 99). From each of these data groups,

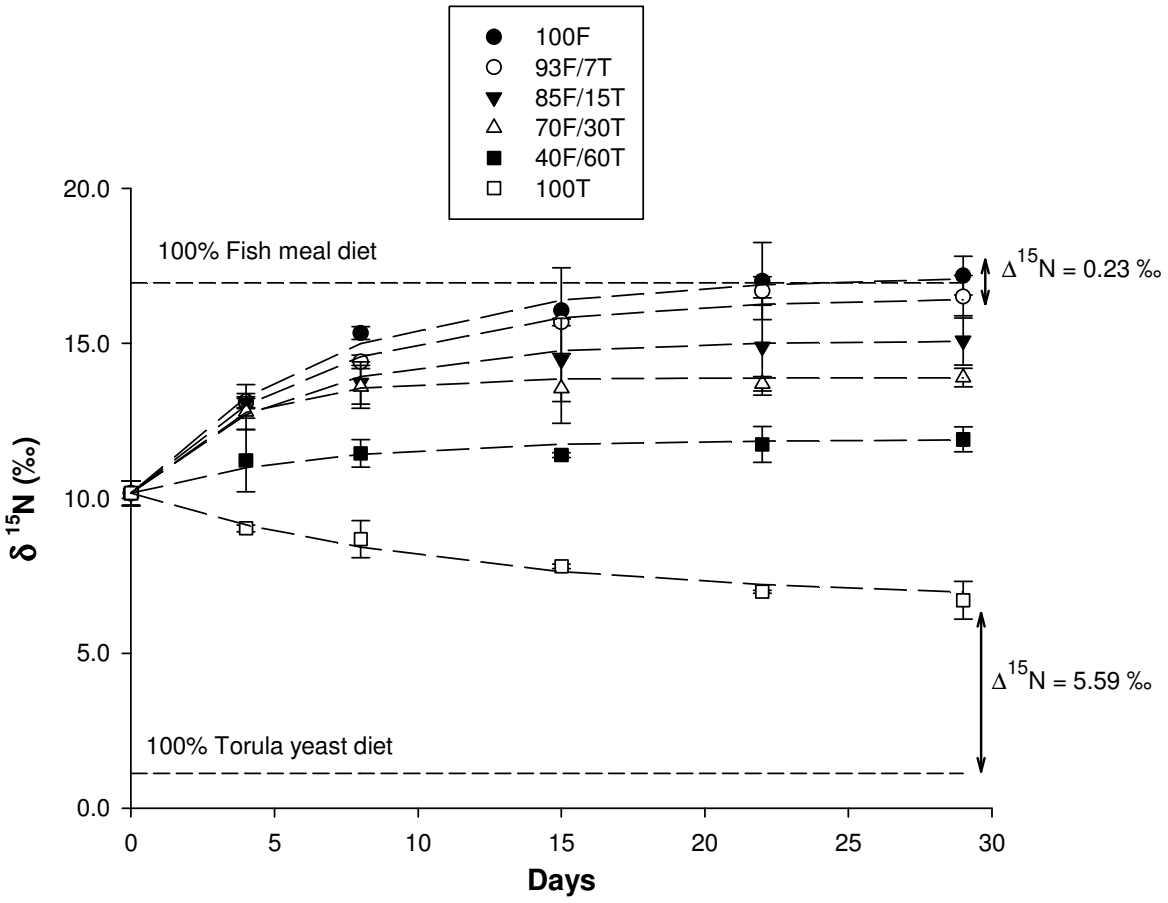

Fig. 1. Changes in nitrogen stable isotope values (‰) in muscle tissue of Pacific white shrimp *Litopenaeus vannamei* after a dietary shift from a conditioning diet to six experimental diets containing different proportions of torula yeast *Candida utilis* and fish meal. Lines represent predicted values generated by a model of isotopic change and show the best fit to observed data. Arrows indicate nitrogen isotopic discrimination factors between control diets and shrimps. n= 2 individuals, 12 on final point (mean values ± S.D.).

parameter *m* (metabolic turnover) was estimated by means of iterative non-linear regression. Parameters *m* and *k* indicated that estimated nitrogen half lives in tissue (t_{50}) ranged from 2.3 d in shrimp fed diet 70F/30T to 8.0 d in shrimp fed on diet 100T (Table 3). t_{50} values decreased from diet 100F (4.8 d) to diet 70F/30T (2.3 d), while the effect of diets 40F/60T and 100T on t_{50} values did not fit a specific pattern.

Table 3. Mean growth rates (*k*) and estimated half lives (t_{50}) of nitrogen in muscle tissue of Pacific white shrimp *L. vannamei* reared under diets having different dietary proportions of fish meal and torula yeast. $\Delta^{15}N$ represents the isotopic difference between diets and muscle tissue after isotopic equilibrium was reached. Mean values ± S.D.

Diet	Nitrogen			
	k (d^{-1})	half life (d)	r^2	$\Delta^{15}N$
100F	0.054 ± 0.009^{b}	4.8 ± 0.7	99	0.2
93F/7T	0.060 ± 0.006^{ab}	4.7 ± 1.1	99	1.1
85F/15T	0.064 ± 0.006^{a}	3.8 ± 0.6	98	0.6
70F/30T	0.056 ± 0.009^{ab}	2.3 ± 0.9	98	2.1
40F/60T	0.059 ± 0.008^{ab}	4.2 ± 1.3	90	5.3
100T	0.041 ± 0.009^{c}	8.0 ± 1.5	97	5.6

Different superscripts indicate significant differences for that particular column.

3.4. Dietary nitrogen and dry matter contribution from fish meal and torula yeast

Changes in $\delta^{15}N$ values observed over the experimental period and inclusion of asymptotic values into the isotopic mixing model indicated that the contributions of dietary nitrogen from FM and TY to the growth of shrimps were very similar to the expected contributions indicated by the

respective proportions of dietary nitrogen established in the dietary formulations (Tables 1 and 4). The only exception was observed in shrimps fed on diet 40F/60T where the dietary nitrogen contribution from TY to muscle tissue (50.5 %) was significantly lower (χ^2=3.84, *P*= 0.049) than the dietary nitrogen available in this diet (60.1 %), therefore indicating a higher contribution of dietary nitrogen from FM. Dietary nitrogen from torula yeast available in diets 93F/7T, 85F/15T, 70F/30T and 40F/60T was incorporated in muscle tissue at proportions of 6.5, 13.7, 27.1 and 50.5 %, respectively. After correcting for the different dietary nitrogen content in FM and TY, expected dry matter contributions from TY slightly increased as the dietary inclusion of this ingredient (containing less dietary nitrogen) was added at a higher levels than FM. Observed dry matter contributions from TY in tissue also increased, suggesting that estimated dry matter contributions to muscle tissue followed a similar pattern as those observed for dietary nitrogen (Table 4).

Table 4. Estimated relative proportions of dietary nitrogen and total dry matter supplied from torula yeast (TY) and fish meal (FM) and contributing to the growth of *L. vannamei* as indicated by a two-source, one-isotope mixing model (mean ± CI, n = 12).

Diet	Expected	Observed in muscle tissue		
		min.	mean	max.
Nitrogen				
93F/7T				
FM	92.4[a]	86.3	93.5[a]	100
TY	7.6	0.0	6.5	13.7
85F/15T				
FM	85.0[a]	78.1	86.3[a]	94.4
TY	15.0	5.6	13.7	21.9
70F/30T				
FM	69.8[a]	69.2	72.9[a]	76.6
TY	30.2	23.4	27.1	30.8
40F/60T				
FM	39.9[a]	45.2	49.5[b]	53.8
TY	60.1	46.2	50.5	54.8
Dry matter				
93F/7T				
FM	87.3[a]	83.9	89.9[a]	95.9
TY	12.7	4.1	10.1	16.1
85F/15T				
FM	76.1[a]	72.5	79.5[a]	86.5
TY	23.9	13.5	20.5	27.5
70F/30T				
FM	56.6[a]	59.4	62.4[a]	65.4
TY	43.4	34.6	37.6	40.6
40F/60T				
FM	27.2[a]	33.6	37.7[b]	41.8
TY	72.8	58.2	62.3	66.4

Superscripts indicate significant differences between expected and mean observed dietary contributions. *Total dry matter contributions were estimated after correcting for nitrogen concentrations measured in both ingredients using the equation proposed by Fry (2006).

4. Discussion

4.1. Growth and survival

The protein level and composition of the experimental diets promoted high survival and growth rates. The rapid tissue accretion caused a fast transference of dietary $\delta^{15}N$ values to muscle tissue. The dietary inclusion of TY had a significant effect on the final mean weight of shrimps under different treatments. Although it has been shown that TY has low methionine levels (1.40 *vs.* 2.80 % protein^{-1} in FM), results allow inferring that the presence of FM in diets containing both protein sources complemented this and other possible deficiencies. Different types of yeast (*Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Candida utilis*) and its extracts have been used in aquaculture diets at different inclusions levels, either as an additive or as fish meal replacement. For example, Zhao et al. (2015) recently reported that up to 45% of FM in diets for shrimp *L. vannamei* can be replaced with a yeast extract without affecting growth and digestibility. Biswas et al. (2012) and Deng et al. (2013) reported improved growth and immune-stimulant effects of yeast-based products in kuruma shrimp, *Marsupenaeus japonicus* and white shrimp, *L. vannamei,* respectively. Studies suggest that fish might be less tolerant to yeasts as ingredient. In a trial with juvenile cobia (*Rachycentron canadum*), Lunger et al. (2006) concluded that only 25% of FM could be replaced with yeast-based protein without detrimental impacts on growth rates, and feed efficiency. In contrast, when compared to fish fed on control diets, Ribeiro et al. (2014) and Luzzana et al. (2005) reported lower growth in Red-Stirling tilapia (*O. niloticus*) fed on a diet having 15% of yeast (*K. marxianus*) and in grey mullet *Mugil cephalus* fed a torula yeast-based diet.

4.2. Isotopic shifts and discrimination factors

Given that previous studies in crustaceans have shown only small differences between the $\delta^{15}N$ values in muscle and whole body samples (Stenroth et al., 2006; Gamboa-Delgado et al., 2011), muscle tissue is considered a good target sample in studies exploring isotope dynamics. The isotopic values of the experimental diets were rapidly reflected in shrimp muscle tissue and isotopic equilibrium between diets and animals was reached between experimental days 15 and 22. These observations are consistent with previous studies conducted on Penaeid shrimp. Shrimps fed on diets containing TY and FM increased their body weights between 430 and 549 %. In contrast, animals fed on diet containing only TY increased in weight by 218% over the experimental period. These animals reached isotopic equilibrium but evidently not through biomass accretion, but mainly as a consequence of tissue metabolic turnover. The wide range of observed $\Delta^{15}N$ values between shrimp and diets is consistent with observations reported in nutritional studies employing ingredients having different dietary quality (Martínez-Rocha et al., 2013; Gamboa-Delgado et al., 2014). Diet containing only TY as protein source is considered nutritionally restricted for shrimp and the estimated $\Delta^{15}N$ value estimated between diet and shrimp was 5.6 ‰. Isotopic discrimination occurs because organisms have an affinity to incorporate heavy isotopes rather than the lighter and more common isotopes. This is the result of heavy isotopes concentrating in molecules were the bond strengths are greatest, the effect being amplified in every chemical and enzymatic transformation (Peterson and Fry, 1987). Studies on isotopic dynamics consider that different $\Delta^{15}N$ values between consuming organisms and diet might be related to the quality of the available dietary protein as there is increasing evidence indicating that high $\Delta^{15}N$ values indicate a higher demand for specific nutrients, in particular

when the growth rate of consuming animals is high, as those observed during earlier life stages (Martínez del Rio and Wolf, 2005; Le Vay and Gamboa-Delgado 2010).

4.3. Nitrogen half lives in muscle tissue

In the present study, estimated t_{50} values measured in muscle tissue were relatively short when compared to studies where nutritionally restricted diets (low dietary levels of plant derived proteins) were fed to *L. vannamei* (t_{50}=7.7-12.8 d; Martínez-Rocha et al., 2012). Unlike $\Delta^{15}N$ values and estimated nutrients contributions, t_{50} values were not clearly correlated to the dietary inclusion of TY. However, a slight decrease from 4.8 d to 2.3 d was observed from diet 100F to diet 70F/30T. High metabolic turnover rates usually translate into short t_{50} values (Hobson and Clark, 1992). As t_{50} values are estimated from the parameters of growth constant k and the metabolic turnover m, in the present study it can be stated that, with the exception of diet 100T, all diets elicited fast growth and metabolic turnover in muscle tissue. Results are consistent with previous studies conducted on the same species and life stage. Gamboa-Delgado and Le Vay (2009) reported t_{50} values ranging from 2.8 to 4.0 d in shrimps fed on high protein diets having different proportions of fish meal and soy protein isolate. It has been reported that juvenile Penaeid shrimps present a high efficiency of retention of synthesized protein as growth (94%) when reared on nutritionally optimal diets (Mente et al., 2002). Estimation of metabolic turnover rate (m) and t_{50} values in other crustacean species has shown that these parameters significantly vary among different tissues (*i.e.* metabolic turnover rates are much higher in hemolymph than those determined in muscle and exoskeleton). This information has allowed defining sample schemes for exploring nutritional effects on specific types of tissue (Suring and Wing, 2009).

4.4. Nutrient contribution from torula yeast and fish meal

The relatively similar contribution of dietary nitrogen supplied by FM and TY to shrimp growth indicates that both protein sources were nutritionally suitable or that nutrients from both sources complemented well. Diet containing up to 60% of TY promoted similar growth as diet containing only FM. Supplying the diet having TY as the only protein source, did not support similar high growth rates probably due to their lower biological value and palatability. Although it has been considered that the cell walls of unicellular organisms are difficult to digest by a range of species (Becker, 2007), results from the present study indicate that a high proportion of dietary nitrogen was incorporated from TY in animals fed on all mixed diets. In this context, it has been pointed out that animal tissue often does not reflect the bulk isotopic composition of the diet, but the isotopic composition of the constituents of the diet from which the tissue was biosynthesized (Gannes et al., 1997). Growth and survival rates were statistically similar in shrimp fed on all mixed diets, therefore indicating the suitability of this SCP ingredient to replace FM in diets containing up to 60% of TY. Nevertheless, it should be stated that at this high dietary level, isotopic results indicated a higher allocation of dietary nitrogen from FM. Diet containing only FM at this protein level is considered nutritionally suitable for Penaeid shrimp as it presents favorable amino acid profile. It is suggested that even low levels of FM in the diet, compensate for nutritional deficiencies caused by alternative protein sources (plant- or microbial-derived). Differential retention of essential and non- essential amino acids from both sources might explain the different allocation of dietary nitrogen observed in diet containing the lowest FM level. This differential retention can also explain the similar growth and survival rates elicited by all diets containing both protein sources. Studies employing compound specific isotopic analysis (CSIA) have reported on the isotopic transformations leading to the final allocation of specific amino

acids in invertebrates (Fantle et al., 1999; O'Brien et al., 2003). Future studies employing CSIA might elucidate which dietary amino acids are preferentially incorporated, while also identifying the source ingredients supplying these amino acids. In conclusion, results from the present study demonstrate that the dietary inclusion of TY improved the final growth of shrimp, while also contributing high proportions of dietary nitrogen to growth when it is used to replace up to 60% of dietary FM.

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