

Incorporation of dietary nitrogen from fish meal and pea meal (*Pisum sativum*) in muscle tissue of Pacific white shrimp (*Litopenaeus vannamei*) fed low protein compound diets

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Abstract

Stable isotope analyses were applied to explore the relative dietary nitrogen contributions from fish meal and pea meal (*Pisum sativum*) to muscle tissue of Pacific white shrimp postlarvae (141 ± 31 mg) fed low protein diets having different proportions of both ingredients as the sole dietary protein sources. A negative control diet was formulated to contain 100% pea meal and six more isoproteic diets to have decreasing levels of pea meal-derived nitrogen: 95%, 85%, 70%, 55%, 40% and 0% of the initial level. Growth rates were negatively correlated to dietary pea protein inclusion due to progressive essential amino acid deficiencies (sulphur amino acids, threonine, lysine, histidine). The nitrogen turnover rate significantly increased in muscle tissue of shrimps fed diets having high levels of pea meal; however, contrary to observations from a previous study using soy protein, the relative contributions of dietary nitrogen from pea meal to shrimp muscle tissue were equal or higher than expected contributions established by the dietary formulations. Results highlight the effectiveness of stable isotope analysis in assessing the nutritional contributions of alternative ingredients for aquaculture feeds and the potential suitability of pea as a source of protein (provided the diets are nutritionally balanced).

Keywords: *Litopenaeus vannamei*, *Pisum sativum*, stable isotopes, nutritional contributions

Introduction

In 2008, the aquaculture industry contributed 46% of the world production of crustaceans, and it supplied 73% of the Penaeid shrimps produced worldwide (FAO 2010). The Pacific white shrimp *Litopenaeus vannamei* has dominated as the main farmed crustacean species since 2003. Increased shrimp production has progressively demanded higher amounts of compound feeds and the required ingredients for their manufacture. Due to its nutritional properties, fishmeal has long been the primary protein source in high protein aquafeeds and will probably continue to be used as a supplement in plant-based formulas in the near future (Hardy 2006). It is estimated that in 2006, 68% of the global fish meal production was utilized by the aquaculture industry (Tacon & Metian 2008). Replacing fish meal with plant-derived proteins in aquaculture diets represents economical and ecological advantages. Intensive research and new processing technologies have contributed to increase the nutritional suitability of plant-proteins used as partial replacements for marine animal proteins in compound diets for Penaeid shrimps (Cruz-Suarez, Ricque-Marie, Tapia-Salazar, McCallum & Hickling 2001; Amaya, Davis & Rouse 2007; Suárez, Gaxiola, Mendoza, Cadavid, Garcia, Alanis, Suárez, Faillace & Cuzon 2009; Enami 2011) and thus convey a high potential to be used at increasingly higher levels of substitution. The feed pea (*Pisum sativum*) has been successfully

mass produced in Europe, Canada, Australia and USA, and its seeds are a good source of digestible carbohydrates (mainly starches, 40–50% as is) and protein (21–25% as is), but contain relatively low protein and methionine levels when compared with soy and fish meal. Nevertheless, pea meal has been a highly recommended feed for animals as it contains high digestible energy level and protein content (Cruz-Suarez *et al.* 2001; Hickling 2003). The round-shaped varieties contain low concentrations of anti-nutritional compounds (e.g. tannins and proteases inhibitors); therefore, they have been selected for intensive agricultural production (Castell, Guenter & Igbasan 1996). Over the last decade, pea meals have been successfully tested as nutritional sources of energy and protein replacing animal- and other plant-derived proteins in aquaculture diets intended for different species of marine shrimp, such as the black tiger prawn *Penaeus monodon* (Smith, Allan, Williams & Barlow 2000; Bautista-Teruel, Eusebio & Welsh 2003), the Pacific blue shrimp *L. stylirostris* (Cruz-Suarez *et al.* 2001; Hickling 2003) and the Pacific white shrimp *L. vannamei* (Davis, Arnold & McCallum 2002). These studies have evaluated the effect of dehulled, extruded, dehulled and extruded, and micronized feed pea on digestibility and shrimp growth. As in the case of other plant-derived products, extrusion and micronizing processes have improved the digestibility of energy and protein of pea meals (Cruz-Suarez *et al.* 2001).

The use of stable isotopes in nutritional studies has been applied as an additional tool to assess the incorporation of nutrients from specific dietary ingredients into animal tissue (Beltrán, Fernández-Borrás, Médale, Pérez-Sánchez, Kaushik & Blasco 2009; Gamboa-Delgado & Le Vay 2009b). Information obtained using isotopic techniques can be interpreted in conjunction with growth data and chemical profiles of diets and tissues to infer on the performance and suitability of new diets and feeding regimes for marine species (Gamboa-Delgado, Le Vay, Manchado, Ponce, Fernandez-Diaz, Zerolo & Cañavate 2011). The natural isotope ratio of an element can be used to infer trophic linkages when different food items have different isotopic signatures (Vander Zanden, Shuter, Lester & Rasmussen 1999). Isotopic values in animal tissue are not constant and shift due to growth, metabolic turnover and ontogenetic changes (e.g. metamorphosis) (Tibbets, Wheelless & Del Rio 2008). The stable isotope ratios of nitrogen ($\delta^{15}\text{N}$

in delta notation) have been used as indicators of trophic position (D'Avanzo, Alber & Valiela 1991; Vander Zanden *et al.* 1999), as nutritional tracers and as a mean to estimate dietary contributions in aquatic organisms after applying mass-balance isotopic mixing models (Su, Ma, Tian & Dong 2008; Gamboa-Delgado & Le Vay 2009b; Matsuda, Takenouchi, Tanaka & Watanabe 2009). Dietary resources found in aquatic and terrestrial ecosystems usually show contrasting isotopic values due to characteristically different nutrient flows and metabolic pathways. The nutritional allocation of such food sources can thus be quantified after the isotopic signatures in sources and consuming organisms have been determined. In this context, controlled feeding experiments have allowed to determine time periods in which diet-elicited isotopic changes occur (Jomori, Ducatti, Carneiro & Portella 2008; Gamboa-Delgado & Le Vay 2009a; Matsuda *et al.* 2009). Simple, one-compartment exponential models of isotopic change as those proposed by Fry and Arnold (1982) and Hesslein, Hallard and Ramlal (1993) associate isotopic changes to time or biomass increase, allowing differentiating and quantifying the isotopic change due to either growth or metabolic turnover. To date, relatively few nutritional studies have adopted this approach, which has been particularly useful in assessing tissue turnover rates of carbon and nitrogen in several fish and crustacean species (Houlihan, Carter & McCarthy 1995; Waterlow 2006; Le Vay & Gamboa-Delgado 2011). The present study aimed to assess the relative incorporation of dietary nitrogen in muscle tissue of shrimps fed low protein compound diets formulated with pea meal and/or fish meal as the only available nitrogen sources. In addition, nitrogen turnover rates and half times in muscle tissue were estimated.

Materials and methods

Experimental animals

Pacific white shrimp (*L. vannamei*) postlarvae were obtained from a commercial farm (Langostinos y Camarones de Oriente) located in Veracruz, Mexico. On reception, animals were acclimated to a bioassay room under the following conditions: water temperature $29.9 \pm 0.7^\circ\text{C}$, salinity $33.4 \pm 0.7 \text{ g L}^{-1}$, pH 8.4 ± 0.1 and saturated dissolved oxygen. A photoperiod was set up to provide a

light:dark ratio of 10:14 h. Shrimps were exclusively fed on a crumbled commercial compound diet (32% protein, as fed, Grupo Costamar, Mexico) previously analysed for nitrogen content and $\delta^{15}\text{N}$ value. To establish a known isotopic baseline in shrimp tissue before the start of the experiment, this diet was fed for 15 days as it has been demonstrated that this time period is sufficient for fast-growing postlarval Penaeid shrimps to achieve isotopic equilibrium with their respective diets (Gamba-Delgado & Le Vay 2009b).

Experimental diets

A series of seven isonitrogenous (21% crude protein, as fed) and isoenergetics (17.4 MJ kg⁻¹) experimental compound diets were formulated with different proportions of pea meal and fish meal (Table 1). Diets were not formulated to conduct an ingredient-substitution study; instead, they were formulated to

intentionally confer differing nitrogen stable isotope values to explore dietary nitrogen contributions as described below. A negative control diet (100P) was formulated using pea meal (24% crude protein, as is) as the only nitrogen source. From this reference diet, five diets were formulated using increasing levels of fish meal nitrogen substituting pea meal nitrogen: 5% (95P/5F), 15% (85P/15F), 30% (70P/30F), 45% (55P/45F), 60% (40P/60F), whereas a positive control diet (100F) was prepared using fish meal (sardine meal 69% crude protein, as is) as the sole nitrogen source. To formulate this diet with a protein level similar to those in the negative control and experimental compound diets (21% crude protein), the fish meal dietary nitrogen was “diluted” using wheat starch and cellulose. Micronutrients were weighed to the nearest mg and hand-mixed for 5 min and then added to the finely ground macronutrients, which in turn were homogenized for 15 min using a commercial blender. Lecithin

Table 1 Formulation profiles and proximal composition (as is) of seven experimental diets used to assess the dietary nitrogen contribution of fish meal and pea meal to muscle tissue of Pacific white shrimp *Litopenaeus vannamei*

Ingredients (g kg ⁻¹ diet)	Diets (Pea meal: Fish meal)						
	100:0	95:5	85:15	70:30	55:45	40:60	0:100
Wheat starch [†]	–	22.5	67.4	134.9	202.3	269.7	449.5
Fish meal prime [†]	–	16.2	48.5	96.9	145.4	193.9	323.1
Pea meal [‡]	849.1	806.7	721.8	594.4	467.0	339.7	–
Cellulose [§]	–	4.5	13.5	27.0	40.5	54.0	90.0
Fish oil [†]	47.7	47.6	47.5	47.2	47.0	46.7	46.0
Soy lecithin	33.7	33.3	32.5	31.2	30.0	28.8	25.5
Alginic acid [§]	15.0	15.5	16.5	18.0	19.5	21.0	25.0
Monosodium phosphate [¶]	29.2	28.7	27.6	26.0	24.4	22.8	18.6
Aquasavor [¶]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Calcium chloride [§]	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Cholesterol ^{**}	3.0	2.9	2.6	2.1	1.7	1.2	0.0
Vitamin mix [†]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mineral mix [†]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Antifungic [†]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Antioxidant (ethoxyquin) [†]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin E [†]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Supplied dietary nitrogen (pea meal: fish meal)	100:0	95:5	84:16	69:31	54:46	39:61	0:100
Moisture (g kg ⁻¹)	111	111	107	104	99.6	102	98.9
Protein (g kg ⁻¹)	202	202	212	215	222	227	244
Lipids (g kg ⁻¹)	79.2	78.5	77.1	85.9	84.1	82.6	76.5
Gross energy (MJ kg ⁻¹)	17.7	17.1	16.9	17.4	16.7	16.6	17.8
$\delta^{15}\text{N}$ (‰)	14.4	10.4	7.6	4.7	4.1	2.6	2.0

*Almidones y gluten S.A. (Monterrey, Mexico).

†Alimentos Costamar (Sonora, Mexico).

‡Alta calidad de semillas y Granos S.A de C.V. (D.F., Mexico).

§Sodium salt, Sigma-Aldrich (St. Louis, MO, USA).

¶Bentoli Inc. (Homestead, FL, USA).

|| Ragaza Industrias Proteínas Naturales S.A. de C.V. (Monterrey, Mexico).

**Solvay Pharmaceuticals (Houston, TX, USA).

was dissolved in warm fish oil and added to the mixture. The pH was measured in each ingredient mixture, and was adjusted to pH 8.0 using 1 M NaOH as reported by Lim (1993); NaOH was incorporated in distilled water also added to the mixture to form dough. The paste was extruded through a die plate having orifices of 1.4 mm in diameter. Strands were collected and placed into wire trays to be post-conditioned by 5 min autoclaving (18.5 psi, 125°C). Diets were dried in a convection oven for 8 min at 100°C and stored at 4°C until used. Proximal analyses of the conditioning compound diet (used to establish the pre-experimental isotopic baseline) and experimental diets included protein content (Dumas method, LECO), lipid content (Soxhlet system HT-1045, method AOAC 996.06) (Tecator 1983), fibre content (method 962.09B), moisture content (method 930.15) and ash content (method 942.05) (AOAC International 1997) and nitrogen-free extract (estimated by difference). The gross energy content of the experimental diets was estimated using a semi-micro bomb calorimeter (Parr 1425 PIC, Moline, IL, USA).

Experimental design and rearing system

Fifteen shrimps having an initial mean wet weight of 141 ± 31 mg were allocated to each of 21, 60-L capacity tanks individually fitted with air lifts. Artificial seawater (Fritz Industries Inc., Mesquite, TX, USA) was exchanged at a rate of 800% d⁻¹, and it was treated by recirculation through mechanical cartridge filters, UV filter, protein skimmers and a bubble bead biological filter. Total ammonia nitrogen, nitrite and nitrate were monitored using a commercial kit (FasTest; Aquarium Systems, Sarrebourg, France). Animals were fed *ad libitum* at 8:00, 14:00 and 18:00 for 29 days. Uneaten feed, faeces and moults were siphoned out daily before the first feeding ration and tank walls were scrubbed off every 3 days to avoid any possible biofilm growth. The individual wet weight of five animals per replicate was registered on six sampling days throughout the duration of the trial. The experimental time period and sampling points to collect samples for isotopic analysis were defined according to the exponential rate of isotopic change previously observed in experiments using small-sized Penaeid shrimp (Gamboa-Delgado & Le Vay 2009b). On experimental days 0, 2, 4, 8, 15, 22 and 29, one shrimp was randomly collected from each replicate tank,

killed in ice/water slurry, rinsed with distilled water and dissected to extract the abdominal muscle. The exoskeleton and hindgut were removed from the abdominal segments, and muscle tissue samples were stored in Eppendorf tubes at -80°C until isotopic analysis.

Sample pretreatment and stable isotope analyses

Samples of shrimp muscle tissue and compound diets were dehydrated at 60°C until constant weight and were manually ground using mortar and pestle to obtain a fine powder. Duplicate diet and muscle tissue samples of 900 to 1100 µg (yielding 40–150 µg N) were packed in tin cups (D1008 Elemental Microanalysis, Okehampton, UK) and organized in 96-well microplates. Samples were analysed at the Stable Isotope Facility of the Department of Plant Sciences, University of California (Davis, CA, USA) using a PDZ Europa Scientific Roboprep elemental analyser coupled to a PDZ Europa Hydra 20/20 stable isotope ratio mass spectrometer (Crewe, UK). Repeated measurements of two calibration standards indicated that instrument precision (SD) was 0.08‰ for δ¹⁵N values. Isotopic results are expressed in delta notation (δ), which is defined as part per thousand (‰) deviations from the δ¹⁵N value of the standard reference material (atmospheric nitrogen, δ¹⁵N = 0.0‰, 0.36‰ ¹⁵N). We use the term ‘discrimination factor’ following Cherel, Hobson and Hassani (2005) and Pearson, Levey, Greenberg and Martínez del Rio (2003) to describe changes in isotopic values between a consuming organism (whole body or specific tissue, in this case muscle) and its diet after having reached isotopic equilibrium (Δ¹⁵N). A 0.1‰ temporary fluctuation in δ¹⁵N values was accepted as a reasonable approximation of isotope equilibrium.

Estimation of nutrient contribution and nitrogen turnover rates

δ¹⁵N values and weight gain were monitored throughout the experimental period, and values were introduced into an exponential model of isotopic change (Hesslein *et al.* 1993) to allow estimating the metabolic nitrogen turnover rate in shrimp muscle tissue. The model provides a quantitative coefficient that allows distinguishing the isotopic change that is due to growth (*k*) and/or metabolic turnover (*m*). For nitrogen turnover rate assessments, the treatment-specific growth rate constant, *k*, was

estimated by fitting an exponential growth model to observed weight data, $k = \log(\text{final weight}/\text{initial weight})/\text{time}(\text{days})$, whereas parameter m was obtained from an exponential equation describing isotopic change and using iterative non-linear regression. The best estimate of m was the value that resulted in the least absolute sum of the differences between calculated and observed isotopic values. Coefficients k and m also 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 time, t_{50}):

$$t_{50} = \ln 2/m + k \quad (1)$$

The proportional dietary nitrogen contributions from fish meal and pea meal to shrimp growth were estimated using a two-source, one-isotope mixing model (Phillips & Gregg 2001). Estimation of isotopic discrimination factors ($\Delta^{15}\text{N}$) increases the accuracy of the estimated dietary contributions by integrating correction factors into the mixing model. Control negative and positive discrimination factors were obtained from the asymptotic isotopic differences between shrimps and diets supplying only dietary pea protein or only dietary fish protein respectively. Corrected $\delta^{15}\text{N}$ values from the different diets and shrimp muscle tissue were sequentially introduced into the model to estimate the relative proportion of dietary nitrogen incorporated from both ingredients and the 95% confidence intervals (truncated). Preliminary analysis indicated that nitrogen contents in pea meal and fish meal were significantly different ($N = 4.4 \pm 0.1$ and $11.9 \pm 0.7\%$, respectively, $t = 18.1$, $P < 0.0001$); therefore, to obtain an estimate of the relative dry matter (DM) contribution from both ingredients to growth, dry matter contributions were corrected for elemental concentration (N) using the equation proposed by Fry (2006).

$$\begin{aligned} f_{\text{total1}} &= f_1 \cdot W_2 / (f_1 \cdot W_2 + f_2 \cdot W_1) \quad \text{and} \\ f_{\text{total2}} &= 1 - f_{\text{total1}} \end{aligned} \quad (2)$$

where f_{total1} = is the total per cent contribution of source 1 in a two-source mixing model,

$$f_1 = (\delta^{15}\text{N}_{\text{sample}} - \delta^{15}\text{N}_{\text{source2}}) / (\delta^{15}\text{N}_{\text{source1}} - \delta^{15}\text{N}_{\text{source2}}) \quad \text{and} \quad f_2 = 1 - f_1$$

where $\delta^{15}\text{N}$ is the nitrogen isotopic value of diets and consumer, superscripts indicate the heavy isotope mass (N), and W_1 and W_2 represent the elemental content in each of the two sources.

Statistical analyses

Student's t -tests were used to compare nitrogen contents and $\delta^{15}\text{N}$ values in fish meal and pea meal. Dietary effects on $\delta^{15}\text{N}$ values of muscle tissue at different times, mean shrimp wet weight and survival were analysed using one way ANOVA after normal distribution and data homoscedasticity were verified. Tukey's pair wise comparisons were used to detect treatments significantly differing from each other. To detect statistical differences in the expected proportions of dietary nitrogen contributed by fish meal and pea meal and the observed estimated proportions of nitrogen incorporated in shrimp muscle tissue, Chi-square goodness of fit tests (χ^2) were applied. Parameter m (metabolic turnover rate) required by the exponential model of isotopic change was estimated using iterative non-linear regression. All tests were done using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) at a significance level of $P < 0.05$.

Results

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 as the previously described bioassay room conditions. Nitrite was not detected, and total ammonia nitrogen and nitrate concentrations remained below 0.09 ± 0.06 and $12.9 \pm 4.6 \text{ mg L}^{-1}$ respectively. Shrimps reared under the different compound diets showed significantly different growth parameters after 29 experimental days (Table 2). Growth rates were negatively correlated to dietary pea protein inclusion ($r^2 = -0.97$); however, animals in all dietary treatments reached isotopic equilibrium in 3 weeks. Shrimp survival rates were high and were not statistically different among dietary treatments (89–100%).

Isotopic shifts and discrimination factors

Due to their different ecological origins, fish meal and pea meal showed very contrasting $\delta^{15}\text{N}$ values (16.6 ± 0.1 and $1.5 \pm 0.4\%$ respectively). This significant difference allowed formulating isotopically contrasting diets that elicited a wide range of isotopic changes in muscle tissue (Fig. 1). Using a

Table 2 Final wet weight (FW), weight gain (WG), specific growth rate (SGR) and survival rate (S) of Pacific white shrimp *Litopenaeus vannamei* reared on diets having different levels of pea meal and fish meal ($n = 9$)

Diet	FW (mg)		WG (%)		SGR		S (%)	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
100P	233 ^a	±25	65 ^a	±18	1.71 ^a	±0.36	96	±6
95P/5F	252 ^a	±16	78 ^a	±12	1.99 ^a	±0.23	93	±6
85P/15F	249 ^a	±18	78 ^a	±13	1.97 ^a	±0.25	93	±13
70P/30F	313 ^b	±7	121 ^b	±5	2.74 ^b	±0.09	96	±6
55P/45F	359 ^{bc}	±34	156 ^{bc}	±23	3.23 ^{bc}	±0.31	93	±6
40P/60F	411 ^c	±56	193 ^c	±42	3.68 ^{cd}	±0.51	100	±0
100F	475 ^d	±38	236 ^d	±27	4.17 ^d	±0.28	89	±11

Initial body weight: 141 ± 31 mg. Shrimp were reared for 29 days.

Different superscripts indicate significant differences ($P < 0.05$) for that particular column.

mass-balance model for $\delta^{15}\text{N}$ values, we found that, over the experimental period, isotopic changes in shrimp muscle tissue were consistent with the isotopic dilution effect caused by tissue accretion; however, the isotopic change related to the metabolic turnover did not fully correspond to values estimated by the Hesslein model because parameter m in this model only provides a rough estimate of the isotopic change that is not explained by growth. The high range of isotopic trends facilitated both, assessment of nitrogen metabolic turnover rate and estimation of dietary contributions. Despite the low protein content, all seven diets exerted a rapid influence on the isotopic values of shrimp tissue and by day 22, animals in all treatments had reached isotopic equilibrium with their respective diets. $\delta^{15}\text{N}$ values in muscle of shrimps reared on mixed compound diets closely matched the isotopic values of the dietary nitrogen supplied by the fish meal and pea meal included at different proportions in the experimental diets. $\Delta^{15}\text{N}$ values between animals and their respective diets were very contrasting. $\Delta^{15}\text{N}$ values between muscle tissue of shrimps and diet 100F were small (0.5‰), whereas values observed in shrimp fed diet 100P were significantly larger (7.4‰). $\Delta^{15}\text{N}$ values in all treatments were strongly correlated ($r^2 = 0.94$) to pea meal inclusion level in diets.

Nitrogen turnover rates in muscle tissue

Estimation of parameter m by means of iterative non-linear regression (MacAvoy, Arneson & Bassett 2006) indicated that nitrogen turnover rates in muscle tissue of shrimp fed the different diets showed a high variability (0.014–0.078 day^{-1} , Table 3), and although values were

not correlated to dietary pea meal inclusion, turnover rates were consistently high (0.053–0.078 day^{-1}) in shrimp fed diets having only pea meal (100P) or high proportions of it as nitrogen source (85P/15F and 95P/5F). Lower turnover values were observed in shrimp fed on the rest of the diets, which contained increasingly higher amounts of fish meal (0.014–0.027 day^{-1}). The lowest value was determined in muscle tissue of shrimp fed on diet 70P/30F. Estimated nitrogen half times in tissue ranged from 7.4 days in shrimp fed diet 85P/15F to 17.6 days in shrimp fed on diet 70P/30F (Table 3).

Dietary nitrogen and dry matter contribution from pea meal and fish meal

Changes in $\delta^{15}\text{N}$ values observed over the experimental period and inclusion of asymptotic values into the isotopic mixing model indicated that the contributions of dietary nitrogen from pea meal and fish meal to the growth of shrimps were very similar to (or slightly higher than) the expected contributions indicated by the respective proportions of dietary nitrogen established by the dietary formulation (Table 4, Fig. 2a). The only exception was observed in shrimps fed diet 70P/30F where the dietary nitrogen contribution from pea meal to muscle tissue (78.0%) was significantly higher ($\chi^2 = 7.67$, $P = 0.0056$) than the dietary nitrogen supplied by pea meal in this diet (69.1%). Diet 55P/45F supplied a pea meal:fish meal proportion of dietary nitrogen of 54:46 and the estimated contributions were statistically similar (59:41). After correcting for differential dietary nitrogen content, expected dry matter contributions from diets to abdominal muscle tissue followed a similar

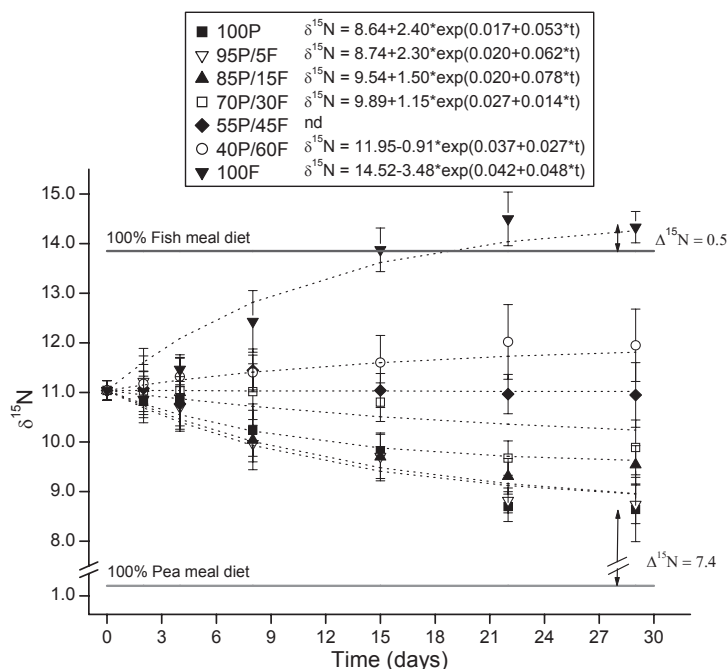


Figure 1 Changes in nitrogen stable isotope values (‰) in abdominal muscle tissue of shrimp *Litopenaeus vannamei* after a dietary shift from a conditioning diet to experimental diets having different proportions of pea meal and fish meal as the only nitrogen sources. Equations represent predicted values generated by an exponential model and show the best fits to observed data (dotted lines). Arrows indicate isotopic discrimination factors between shrimps fed only 100% pea meal diet (negative isotopic control) and 100% fish meal diet (positive isotopic control). Means of 6–12 samples ± SD.

Table 3 Growth rates (*k*), estimated nitrogen metabolic turnover rates (*m*) and half times (*t*₅₀) in muscle tissue of Pacific white shrimp *Litopenaeus vannamei* fed diets formulated with different levels of pea meal (*Pisum sativum*) and fish meal. Δ¹⁵N represents the isotopic difference between diets and muscle tissue after isotopic equilibrium was reached.

Diet	<i>k</i> (day ⁻¹)		<i>m</i> (day ⁻¹)		<i>t</i> ₅₀ (days)		Δ ¹⁵ N (‰) Mean
	Mean	± SD	Mean	±SD	Mean	±SD	
100P	0.017 ^c	±0.004	0.053 ^a	±0.011	10.1 ^{ab}	±2.2	7.4
95P/5F	0.020 ^c	±0.002	0.062 ^a	±0.008	8.5 ^b	±1.0	6.3
85P/15F	0.020 ^c	±0.003	0.078 ^a	±0.024	7.4 ^b	±2.1	6.1
70P/30F	0.027 ^b	±0.001	0.014 ^b	±0.013	17.6 ^a	±6.0	3.6
55P/45F	0.032 ^{ab}	±0.003	0.021 ^{ab}	±0.006	12.8 ^{ab}	±0.8	3.2
40P/60F	0.037 ^a	±0.005	0.027 ^a	±0.028	11.3 ^{ab}	±4.5	3.0
100F	0.042 ^a	±0.003	0.048 ^a	±0.012	7.7 ^b	±1.3	0.5

**m* data were estimated from expected isotopic values generated by iterative non-linear regression.

pattern as those observed for dietary nitrogen (Fig. 2b).

Discussion

Growth and survival

Presence of anti-nutritional factors affecting growth has been reported in pea meals (Gomes,

Corraze & Kaushik 1993; Hickling 2003; Tacon 1997); however, in the present experiment, possible negative effects on shrimps were avoided or attenuated through diet post-conditioning. The high negative correlation ($r^2 = -0.94$) observed between the level of dietary pea meal and growth rate indicated that the nutritional profile of pea meal does not fully satisfy the nutritional requirements of Penaeid shrimps (Cruz-Suarez *et al.*

Table 4 Estimated relative proportions of dietary nitrogen and total dry matter supplied from pea meal and fish meal and contributing to the muscle growth of *Litopenaeus 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				
95P/5F				
Pea meal	94.8 ^a	90.5	98.2 ^a	100
Fish meal	5.2	0.0	1.8	9.5
85P/15F				
Pea meal	84.5 ^a	78.3	84.2 ^a	90.1
Fish meal	15.5	9.9	15.8	21.7
70P/30F				
Pea meal	69.1 ^a	73.7	78.0 ^b	82.4
Fish meal	30.9	17.6	22.0	26.3
55P/45F				
Pea meal	54.0 ^a	50.0	59.4 ^a	68.8
Fish meal	46.0	31.2	40.6	50.0
40P/60F				
Pea meal	39.0 ^a	32.3	41.8 ^a	51.4
Fish meal	61.0	48.6	58.2	67.7
Dry matter*				
95P/5F				
Pea meal	98.0 ^a	97.6	99.4 ^a	100
Fish meal	2.0	0.0	0.6	2.4
85P/15F				
Pea meal	93.7 ^a	87.7	93.6 ^a	99.5
Fish meal	6.3	0.5	6.4	12.3
70P/30F				
Pea meal	86.0 ^a	86.3	90.7 ^b	95.6
Fish meal	14.0	4.4	9.3	13.7
55P/45F				
Pea meal	76.3 ^a	70.6	80.0 ^a	89.4
Fish meal	23.7	10.6	20.0	29.4
40P/60F				
Pea meal	63.7 ^a	56.8	66.3 ^a	75.8
Fish meal	36.3	24.2	33.7	43.2

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).

2001; Davis *et al.* 2002). In general, it is considered that a dietary protein level above 32% is optimal for early juveniles of this species (Kureshy & Davis 2002). Bautista-Teruel *et al.* (2003) replaced up to 25% of the protein supplied by soybean meal with pea meal in practical diets for *P. monodon* containing fish meal without any detrimental effects on growth. The lower growth rates observed in shrimp fed diets having higher levels of pea meal could thus be related to the comparatively lower level of digestible methionine in this

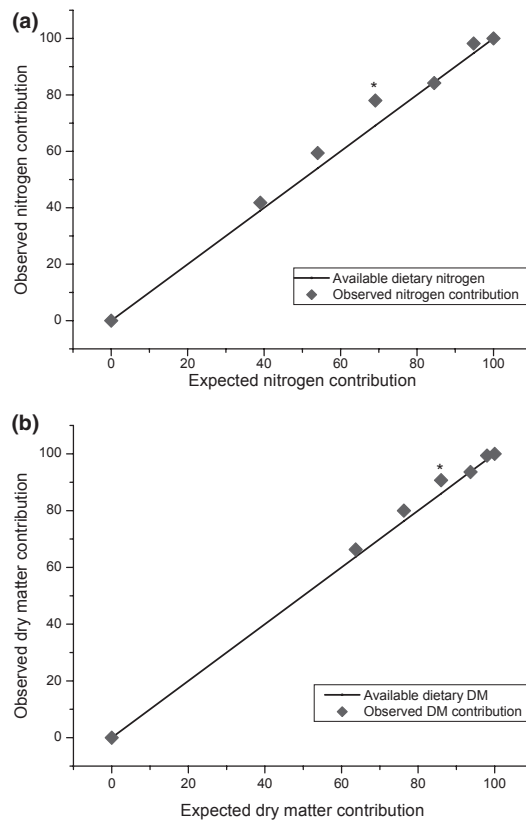


Figure 2 Expected and observed proportions (%) of dietary nitrogen (a) and dry matter (b) contributed by pea meal to the growth of abdominal muscle tissue of Pacific white shrimp *Litopenaeus vannamei* fed diets formulated with varying proportions of pea meal and fish meal. Contributions were estimated using a one-isotope, two-source mixing model (Table 4 indicates the confidence intervals, *n* = 12). *Significant differences at *P* < 0.05.

ingredient. In addition, it is suggested that as dietary proportions of fish meal increased, higher levels of sulphur-containing amino acids were available, thereby increasing the nitrogen accretion capacity in shrimps when compared with those fed diets having high proportions of pea meal. Although the experimental diets used in the present study were formulated to have a relatively low protein level and high dietary pea protein levels, the observed growth rate, in conjunction with the nitrogen turnover rates elicited by the different diets was sufficient for the dietary $\delta^{15}\text{N}$ values to be reflected in muscle tissue and reach isotopic equilibrium. Abdominal muscle tissue comprises more than 60% of the body weight in Penaeid shrimp, and previous studies in crustaceans have

shown only small differences in nitrogen isotopic ratios between muscle and whole body samples (Stenroth, Holmqvist, Nystrom, Berglund, Larsson & Granell 2006; Gamboa-Delgado & Le Vay 2009b), therefore indicating that isotopic routing effects were not significant. The isotopic routing represents the sum of metabolic pathways mobilizing specific nutrients to specific pools as the different nutritional elements of a diet are not completely homogenized in the animal before synthesis of new tissue (Schwarcz 1991; Martínez del Rio & Wolf 2005; Wolf, Carleton & Martínez del Rio 2009). In this context, muscle tissue, as the main nitrogen reservoir, represents a good target sample in studies exploring isotope dynamics, while also allowing comparison to other studies.

Isotopic shifts and discrimination factors

The contrasting nitrogen isotopic difference between pea meal and fish meal allowed formulating experimental diets having a wide range of $\delta^{15}\text{N}$ values, which in turn allowed exploring the nutritional effects of low protein diets on the isotopic shifts in shrimp muscle tissue. $\delta^{15}\text{N}$ value of fish meal ($16.6 \pm 0.1\text{‰}$) was not directly reflected by diet 100F ($14.4 \pm 0.6\text{‰}$). We presume that this difference was caused by the presence of non-protein nitrogen compounds present in this diet. The isotopic values of the compound diets were rapidly reflected in shrimp muscle tissue, and isotopic equilibrium between diets and animals was reached between experimental days 15 and 20. Shrimps fed on diets 100P, 95P/5F and 85P/15F increased their body weights only between 65% and 78%; however, these animals also reached isotopic equilibrium, but evidently not through biomass accretion; instead, they must have reached isotopic equilibrium as a consequence of tissue metabolic turnover. At the end of the experiment, $\Delta^{15}\text{N}$ values between shrimp and diets were very contrasting and ranged from 0.5‰ (diet 100F) to 7.4‰ (diet 100P). $\Delta^{15}\text{N}$ values in all dietary treatments were strongly correlated ($r^2 = 0.94$) to pea meal inclusion in diets. Studies on isotopic dynamics consider that different $\Delta^{15}\text{N}$ values between consuming organisms and diet might be related to the quality of the available dietary protein (Roth & Hobson 2000; Robbins, Felicetti & Sponheimer 2005), but on the other hand, results from other studies suggest that protein quantity is what elicits different $\Delta^{15}\text{N}$ values

(Pearson *et al.* 2003). Regardless of cause (protein quantity or quality), there is increasing evidence indicating that high $\Delta^{15}\text{N}$ values indicate a higher demand for specific nutrients, in particular, when the growth rate of consuming animals is high, as those observed during the early life stages (Le Vay & Gamboa-Delgado 2011). Martínez del Rio and Wolf (2005) consider that a nutritional deficiency of specific dietary nutrients may increase the feeding rate, in turn causing additional metabolic cycling of non-essential nutrients and increasing the $\Delta^{15}\text{N}$ values between animal tissue and diet. Evidence from a study conducted on juvenile blue crab (Fantle, Dittel, Schwalm, Epifanio & Fogel 1999) showed that the metabolism of individual non-essential amino acids elicits higher discrimination factors (between dietary and tissue amino acids) when compared with those observed in the metabolism of essential amino acids, which are directly incorporated into tissue. Recent advances leading to the refinement of isotopic techniques (Morrison, Taylor & Preston 2010) might help to elucidate the origin, metabolism and fate of specific compounds, as well as their role in causing differing isotopic discrimination factors.

Nitrogen turnover rates in muscle tissue

Although somatic growth can be compromised at restricted levels of dietary protein, the majority of the required amino acids are still supplied by increasing the consumption rates and by increasing the protein breakdown in tissue, which exerts a significant effect modifying the nitrogen turnover rates (Waterlow 2006). Nitrogen turnover rates in muscle tissue were lower in shrimps fed on diets 70P/30F, 55P/45F and 40P/60F, the latter being the only compound diet promoting growth as high as that elicited by the diet containing only fish meal. Values were higher in muscle tissue of shrimp fed diets containing higher proportions of dietary pea protein, which also elicited low growth rates. Shrimps fed diets 100P and 100F showed similar high nitrogen turnover rates (0.053 and 0.048 day^{-1} respectively), but significantly different biomass gain. The extent at which rapid nitrogen turnover would limit growth depends on the demand for amino acids and energy, in turn determined by the rates of protein synthesis (Millward, Garlick, Stewart, Nnanyelugo & Waterlow 1975). In postlarval and juvenile Penaeid shrimps, the rates of protein synthesis are characteristically

high, for example, Mente, Coutteau, Houlihan, Davidson and Sorgeloos (2002) determined an efficiency of retention of synthesized protein as growth of 94% when *L. vannamei* is reared on nutritionally optimal diets, suggesting very low protein turnover rates occurring under these conditions ($k > m$). Although the energy cost of high metabolic turnover rates and protein synthesis is substantial (Waterlow 2006), in the present experiment, the dietary energy supplied to shrimp was not limiting as all diets were formulated to have high caloric yield by supplying carbohydrates known to be highly digestible for this shrimp species (Cousin, Cuzon, Guillaume & Aquacop 1996). Estimated nitrogen half times (t_{50}) in tissue ranged from 7.4 (diet 95P/5F) to 17.6 days (diet 70P/30F) and were not correlated to growth rate. High metabolic turnover rates usually translate into short t_{50} values (Hobson & Clark 1992) and values determined in muscle tissue in the present study were relatively high when compared with other tissues (e.g. hepatopancreas, haemolymph proteins) having shorter t_{50} values for nitrogen due to higher metabolism. Tissue-specific turnover rates can allow researchers to tailor the sampled tissue to an appropriate time scale for the study's objective (Buchheister & Latour 2010).

Dietary nitrogen and dry matter contributions from pea meal and fish meal

Although estimated proportions of dietary nitrogen and total dry matter supplied by pea meal and fish meal were very similar to the expected contributions indicated by the different dietary formulations, differing growth rates provide a good indicator of the different nutritional properties of the experimental diets. The lower growth rate observed in animals fed diets containing only pea meal or high proportions of it, is consistent with studies reporting poor growth in Penaeid shrimps fed diets containing high levels of (or only) plant protein sources (Galgani, Ceccaldi & AQUACOP 1988; Paripatananont, Boonyaratpalin, Pengseng & Chotipuntu 2002; Molina-Poveda & Morales 2004). The amino acid profile of fish meal is considered nutritionally suitable for Penaeid shrimp, whereas diets containing high proportions of plant-derived proteins (e.g. canola, soy and pea meal) do not support similar high growth rates due to their lower biological value (lower levels of sulphur amino acids) in comparison to fish

meal-based diets. In this context, it has been pointed out that animal tissue does not often 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, O'Brien & Martínez del Rio 1997). Although overall growth rates were low due to the relative protein restriction, dietary nitrogen contributions from pea meal to muscle tissue were high and consistent with the amounts of nitrogen available in the respective compound diets, with the exception of shrimps fed on diet 70P/30F. Animals under the latter treatment incorporated significantly higher amounts of dietary nitrogen (78%) and dry matter (91%) from pea meal than those established in the diet (69% and 86% respectively). As pea meal contains lower dietary nitrogen level than fish meal, a higher amount of nutrients from the former had to be physiologically incorporated to reach the estimated contributions of nitrogen to tissue growth (or turnover). In a previous experiment exploring dietary nitrogen contributions to *L. vannamei* postlarvae and juveniles, Gamboa-Delgado and Le Vay (2009b) observed significant differences when fish meal (72% contribution) and soy protein isolate (28% contribution) were included in compound diets designed to supply similar proportions of dietary nitrogen (50:50, at 46% crude protein). Results thus indicated a lower nutritional contribution from soy protein isolate to growth and a disproportionately high contribution of fish meal at this protein level. The observed higher proportions of incorporated dietary nitrogen from *Pisum sativum* meal suggests its potential use not only as protein source, as confirmed in the present study, but also as a dietary energy source due to the high level of starch naturally present in pea meal, which is well utilized by shrimp (Catacutan 1991; Cruz-Suárez, Ricque-Marie, Pinal-Mansilla & Wesche-Ebellung 1994). Feed peas contain high levels of lysine and could also replace other plant protein sources (Bautista-Teruel *et al.* 2003) in nutritionally balanced diets containing low levels of fish meal. Moreover, thermal processes applied to gelatinize the starch apparently confer a higher digestibility to pea meal when used as dietary ingredient for *L. vannamei* (Davis & Arnold 1993; Cousin *et al.* 1996). Diets formulated with lower levels of pea meals and higher levels of crude protein have shown positive results. Cruz-Suarez *et al.* (2001) evaluated a series of compound diets formulated

with pea meal at a level of inclusion of 30% of the diet. Their results indicated that diets containing pea meal exhibit good digestibility coefficients for protein and dry matter in Penaeid shrimps. Although isotopic results indicated that dietary nitrogen contributions from fish meal and pea meal to shrimp muscle tissue closely resembled the dietary nitrogen levels established in the experimental diets, growth rates (*k*) suggest that substituting fish meal protein with pea meal protein at a level of 40% (diet 40P/60F) promotes growth and survival rates similar to those observed in shrimps fed only fish meal-based diets.

In the present study, the high levels of pea meal protein substituting fish meal protein and the relatively low dietary crude protein levels provided a different framework to assess dietary contributions from animal- and plant-derived nitrogen to the growth of shrimp. Increasing proportions of dietary nitrogen from pea meal in diets were reflected in higher nitrogen turnover rates in tissue and lower growth rates. From these results, it might be possible to infer that diets formulated with fully complemented amino acid profiles using fish meal and pea meal derivatives (isolated or concentrated protein) at higher protein levels might support higher growth while also contributing higher proportions of dietary nitrogen to postlarval and juvenile shrimps. The wider adoption of compound specific isotopic analysis (CSIA), particularly for amino acids, represents an opportunity to greatly improve the current knowledge of nutrient utilization, and further experiments might explain the diet-elicited physiological events leading to different degrees of metabolic cycling and nutrient allocation.

Acknowledgments

The authors acknowledge Julio César Beltrán-Rocha for conducting the proximal analyses of ingredients and diets and Langostinos y Camarones de Oriente for kindly donating the experimental animals. Thanks are due to an anonymous reviewer for their comments to improve the manuscript. This study was partially supported by the Mexican Council for Science and Technology (CONACYT, project CB-2009-1-128314).

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