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

Martínez-Rocha, Luis

Gamboa-Delgado, Julián*

Nieto-López, Martha

Ricque-Marie, Denis

Cruz-Suárez, Lucía Elizabeth

Programa Maricultura, Departamento de Ecología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Cd. Universitaria, Apdo. Postal F-67, San Nicolás de los Garza, Nuevo León 66450, México

*Corresponding author:

e-mail: julian.gamboad@uanl.mx jgam97@yahoo.com

Tel/Fax +52 (81) 8352 6380

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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 (sulfur 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 (Amaya, Davis & Rouse 2007; Cruz-Suarez, Ricque-Marie, Tapia-Salazar, McCallum & Hickling 2001; Enami 2011; Suárez, Gaxiola, Mendoza, Cadavid, Garcia, Alanis, Suárez, Faillace & Cuzon 2009) 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 to 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* (Bautista-Teruel, Eusebio & Welsh 2003; Smith, Allan, Williams & Barlow 2000), 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 in order 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, Wheeless & Del Rio 2008). The stable isotope ratios of nitrogen ($\delta^{15}N$ in delta notation) have been used as indicators of trophic position (Beltrán et al. 2009; D'Avanzo, Alber & Valiela 1991), as nutritional tracers and as a mean to estimate dietary contributions in aquatic organisms after applying mass-balance isotopic mixing

models (Gamboa-Delgado & Le Vay 2009b; Matsuda, Takenouchi, Tanaka & Watanabe 2009; Su, Ma, Tian & Dong 2008). 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; Matsuda et al. 2009; Gamboa-Delgado & Le Vay 2009a). Simple, one-compartment exponential models of isotopic change as those proposed by Fry & Arnold (1982) and Hesslein Hallard & 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; Le Vay & Gamboa-Delgado 2011; Waterlow 2006). 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. Additionally, nitrogen turnover rates and half times in muscle tissue were estimated.

Material 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 ±0.7 °C, salinity 33.4 ±0.7 g l^{-1}, pH 8.4 ±0.1 and saturated dissolved oxygen. A photoperiod was set up to provide a light:dark ratio of 10:14h. Shrimps were exclusively fed on a crumbled commercial compound diet (32% protein, as fed, 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 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 (Gamboa-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 in order 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), while a positive control diet (100F) was prepared using fish meal (sardine meal 69 % crude

protein, as is) as the sole nitrogen source. In order 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.

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 *L. vannamei*.

	Diets (Pea meal: Fish meal)						
Ingredients (g kg^{-1} diet)	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
$\delta^{15}N$ (‰)	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)
‖ Ragaza Industrias Proteínas Naturales S.A. de C.V. (Monterrey, Mexico)
¶ Bentoli Inc. (Homestead, FL, USA)
** Solvay Pharmaceuticals (Houston, TX, USA).

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 was dissolved in warm fish oil and added to the mixture. The pH was measured in each ingredient mixture and it was adjusted to pH 8.0 using 1M 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, Illinois, 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, Chemical Co., Texas, 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, France). Animals were fed *ad libitum* at 8:00, 14:00 and 18:00 for 29 days. Uneaten feed, feces and moults were siphoned out daily before the first feeding ration and tank walls were scrubbed off every three days in order 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 to 150 µg N) were packed in tin cups (D1008 Elemental Microanalysis Ltd., UK) and organized in 96-well microplates. Samples were analyzed at the Stable Isotope Facility of the Department of Plant Sciences, University of California, (Davis, CA, USA) using a PDZ Europa Scientific Roboprep elemental analyzer 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 $\delta^{15}N$ 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, $\delta^{15}N$= 0.0 ‰, 0.36% ^{15}N). We use the term "discrimination factor" following Cherel, Hobson & Hassani (2005) and Pearson, Levey, Greenberg & Martinez 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 ($\Delta^{15}N$). A 0.1‰ temporary fluctuation in $\delta^{15}N$ values was accepted as a reasonable approximation of isotope equilibrium.

Estimation of nutrient contribution and nitrogen turnover rates

$\delta^{15}N$ 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, $k = log$(final weight/initial weight)/time(d), while 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}):

$$t_{50} = In2 / m + k \quad (1)$$

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 ($\Delta^{15}N$) 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 $\delta^{15}N$ 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 ±0.1 and 11.9 ±0.7 %, respectively, t= 18.1, P<0.0001); therefore, in order 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).

$$f_{total1} = f_1 \cdot W_2 / (f_1 \cdot W_2 + f_2 \cdot W_1) \text{ and } f_{total2} = 1 - f_{total1}$$

(2)

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

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

where $\delta^{15}N$ 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 $\delta^{15}N$ values in fish meal and pea meal. Dietary effects on $\delta^{15}N$ values of muscle tissue at different times, mean shrimp wet weight and survival were analyzed by 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. In order 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 by iterative non-linear regression. All tests were done using SPSS 17.0 software (SPSS Inc.) 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 mg l^{-1} and 12.9 ±4.6 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 to100%).

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

	FW (mg)		WG (%)		SGR		S (%)	
Diet	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.

Isotopic shifts and discrimination factors

Due to their different ecological origins, fish meal and pea meal showed very contrasting $\delta^{15}N$ values (16.6 ±0.1 and 1.5 ±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 mass-balance model for $\delta^{15}N$ 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 7 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. $\delta^{15}N$ 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. $\Delta^{15}N$ values between animals and their respective diets were very contrasting. $\Delta^{15}N$ values between muscle tissue of shrimps and diet 100F were small (0.5 ‰), while values observed in shrimp fed diet 100P were significantly larger (7.4 ‰). $\Delta^{15}N$ values in all treatments were strongly correlated (r^2= 0.94) to pea meal inclusion level in diets.

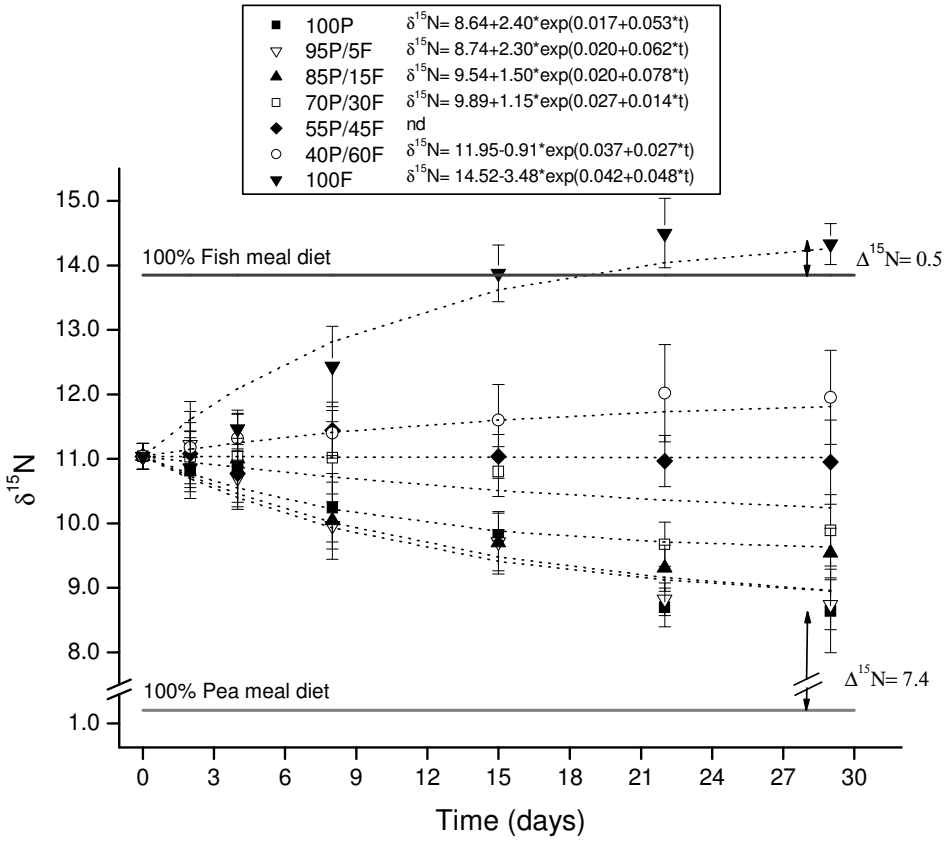

Figure 1. Changes in nitrogen stable isotope values (‰) in abdominal muscle tissue of shrimp *L. vannamei* after a dietary shift from a conditioning diet to experimental diets having different proportions of pea meal and fish meal as the only nitrogen sources. Equations represent predicted values generated 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.

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 d^{-1}, Table 3) and although values were not correlated to dietary pea meal inclusion, turnover rates were consistently high (0.053 to 0.078 d^{-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 to 0.027 d^{-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 d in shrimp fed diet 85P/15F to 17.6 d in shrimp fed on diet 70P/30F (Table 3).

Table 3. Growth rates (*k*), estimated nitrogen metabolic turnover rates (*m*) and half times (t_{50}) in muscle tissue of Pacific white shrimp *L. vannamei* fed diets formulated with different levels of pea meal (*P. sativum*) and fish meal. $\Delta^{15}N$ represents the isotopic difference between diets and muscle tissue after isotopic equilibrium was reached.

	k (d^{-1})		*m* (d^{-1})		t_{50} (d)		$\Delta^{15}N$ (‰)
Diet	Mean	±SD	Mean	±SD	Mean	±SD	Mean
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 and their fit on observed values, r^2= 0.63 to 0.98. Different superscripts indicate significant differences at $P<0.05$.

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

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 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 were the dietary nitrogen contribution from pea meal to muscle tissue (78.0 %) was significantly higher (χ^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 pattern as those observed for dietary nitrogen (Fig. 2b).

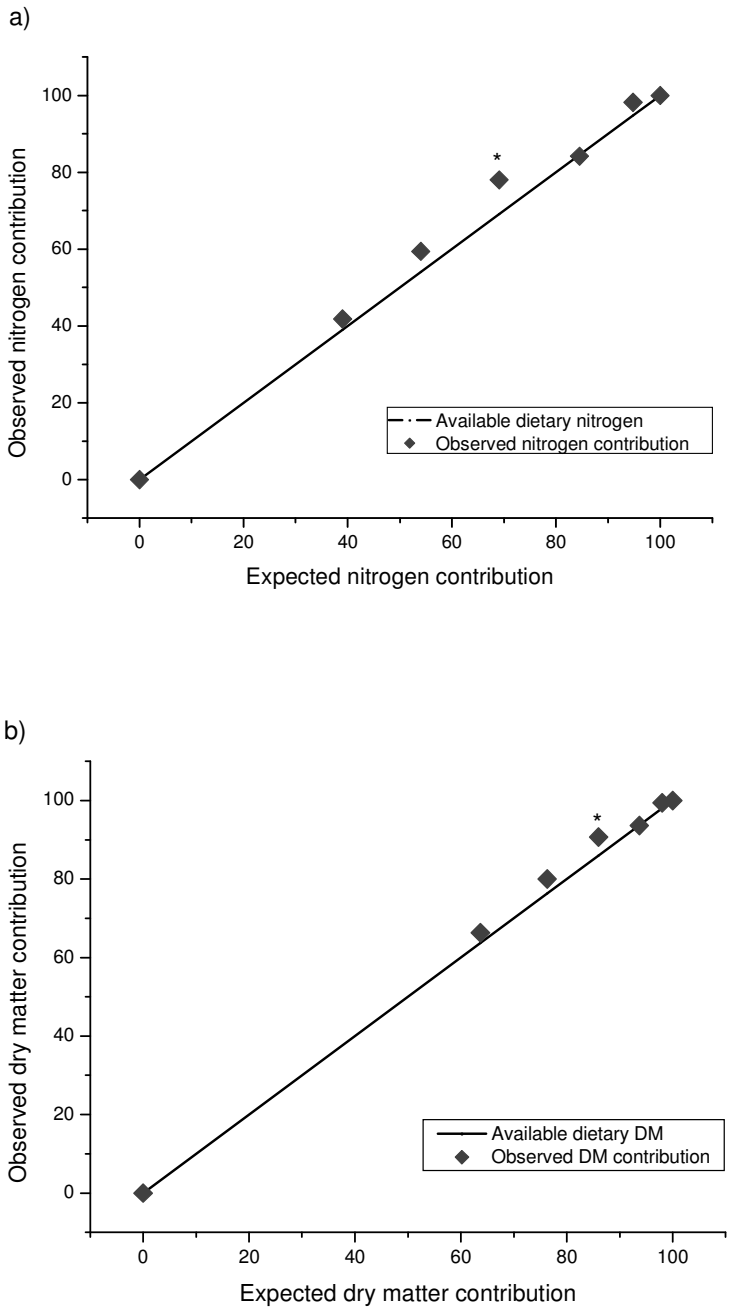

Figure 2. Expected and observed proportions (%) of dietary nitrogen (a) and dry matter (b) contributed by pea meal (P) to the growth of abdominal muscle tissue of Pacific white shrimp *L. vannamei* fed diets formulated with varying proportions of pea meal and fish meal. Contributions were estimated using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

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 *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				
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).

Discussion

Growth and survival

Presence of anti-nutritional factors affecting growth has been reported in pea meals (Tacon 1997; Gomes, Corraze & Kaushik 1993; McCallum 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. 2001; Davis et al. 2002). Generally, 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 mealwithout any detrimental effects on growth. The lower growth rates observed in shrimp fed diets having higher levels of pea meal, thus could be related to the comparatively lower level of digestible methionine in this ingredient. Additionally, 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 as compared to 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 $\delta^{15}N$ 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 (Gamboa-Delgado & Le Vay 2009b; Stenroth, Holmqvist, Nystrom, Berglund, Larsson & Granell 2006), 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 (Martínez del Rio & Wolf 2005; Schwarcz 1991; 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 $\delta^{15}N$ values, which in turn allowed exploring the nutritional effects of low protein diets on the isotopic shifts in shrimp muscle tissue. $\delta^{15}N$ value of fish meal (16.6 ±0.1‰) was not directly reflected by diet 100F (14.4 ±0.6 ‰). 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, $\Delta^{15}N$ values between shrimp and diets were very contrasting and ranged from 0.5 ‰ (diet 100F) to 7.4 ‰ (diet 100P). $\Delta^{15}N$ 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 $\Delta^{15}N$ values between consuming organisms and diet might be related to the quality of the available dietary protein (Robbins, Felicetti & Sponheimer 2005; Roth & Hobson 2000), but on the other hand, results from other studies suggest that protein quantity is what elicits different $\Delta^{15}N$ values (Pearson et al. 2003). Regardless of cause (protein quantity or quality), 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 the early life stages (Le Vay & Gamboa-Delgado 2011). Martínez del Rio & 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 $\Delta^{15}N$ 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) as compared to 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 d^{-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 & 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 d (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 to other tissues (*e.g.* hepatopancreas, hemolymph 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, while 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 sulfur amino acids) in comparison to fish meal-based 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, 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 in order 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 (Cruz-Suárez, Ricque-Marie, Pinal-Mansilla & Wesche-Ebelling 1994; Catacutan 1991). 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 of pea meal in *L. vannamei* (Cousin *et al.* 1996; Davis & Arnold 1993). Diets formulated with lower levels of pea meals and higher levels of crude

protein have shown positive results. Cruz-Suárez *et al*. (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 derivates (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 elucidate the diet-elicited physiological events leading to different degrees of metabolic cycling and nutrient allocation.

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References

Amaya E.A., Davis D.A. & Rouse D.B. (2007) Replacement of fish meal in practical diets for the Pacific white shrimp (*Litopenaeus vannamei*) reared under pond conditions. *Aquaculture,* **262**, 393-401.

AOAC International (1997) *Official methods of analysis of AOAC International,* AOAC International, Gaithersburg, MD.

Bautista-Teruel M.N., Eusebio P.S. & Welsh T.P. (2003) Utilization of feed pea, *Pisum sativum*, meal as a protein source in practical diets for juvenile tiger shrimp, *Penaeus monodon*. *Aquaculture,* **225**, 121-131.

Beltrán M., Fernández-Borrás J., Médale F., Pérez-Sánchez J., Kaushik S. & Blasco J. (2009) Natural abundance of 15N and 13C in fish tissues and the use of stable isotopes as dietary protein tracers in rainbow trout and gilthead sea bream. *Aquaculture Nutrition,* **15**, 9-18.

Buchheister A. & Latour R.J. (2010) Turnover and fractionation of carbon and nitrogen stable isotopes in tissues of a migratory coastal predator, summer flounder (*Paralichthys dentatus*). *Canadian Journal of Fisheries and Aquatic Sciences,* **67**, 445-461.

Castell A.G., Guenter W. & Igbasan F.A. (1996) Nutritive value of peas for nonruminant diets. *Animal Feed Science Technology,* **60**, 209-227.

Catacutan M.R. (1991) Apparent digestibility of diets with various carbohydrate levels and the growth response of *Penaeus monodon*. *Aquaculture,* **95**, 89-96.

Cherel Y., Hobson K.A. & Hassani S. (2005) Isotopic discrimination between food and blood and feathers of captive penguins: implications for dietary studies in the wild. *Physiological and Biochemical Zoology,* **78**, 106-115.

Cousin M., Cuzon G., Guillaume J. & Aquacop (1996) Digestibility of starch in *Penaeus vannamei*: in vivo and in vitro study on eight samples of various origin. *Aquaculture,* **140**, 361-372.

Cruz-Suarez L., Ricque-Marie D., Tapia-Salazar M., McCallum I. & Hickling D. (2001) Assesment of differently processed feed pea (*Pisum sativum*) meals and canola meal *(Brassica sp.)* in diets for blue shrimp (*Litopenaeus stylirostris*). *Aquaculture,* **196**, 87-104.

Cruz-Suárez L.E., Ricque-Marie D., Pinal-Mansilla J.D. & Wesche-Ebelling P. (1994) Effect of different carbohydrate sources on the growth of *Penaeus vannamei*: economical impact. *Aquaculture,* **123**, 349-360.

D'Avanzo C., Alber M. & Valiela I. (1991) Nitrogen assimilation from amorphous detritus by two coastal consumers. *Estuarine, Coastal and Shelf Science,* **33**, 203-209.

Davis D.A. & Arnold C.R. (1993) Evaluation of five carbohydrate sources for *Penaeus vannamei*. *Aquaculture,* **114**, 285-292.

Davis D.A., Arnold C.R. & McCallum I. (2002) Nutritional value of feed peas (*Pisum sativum*) in practical diet formulations for *Litopenaeus vannamei*. *Aquaculture Nutrition,* **8**, 87-94.

Enami H.R. (2011) A review of using canola/rapeseed meal in aquaculture feeding. *Journal of Fisheries and Aquatic Science,* **6**, 22-36.

Fantle M.S., Dittel A.I., Schwalm S.M., Epifanio C.E. & Fogel M.L. (1999) A food web analysis of the juvenile blue crab, *Callinectes sapidus*; , using stable isotopes in whole animals and individual amino acids. *Oecologia,* **120**, 416-426.

FAO (2010) -SOFIA 2010 Fisheries and Aquaculture Department, Rome, Italy. 242 pp..

Fry B. (2006) Stable Isotope Ecology. Springer*,* 308 pp.

Fry B. & Arnold C. (1982) Rapid $^{13}C/^{12}C$ turnover during growth of brown shrimp (*Penaeus aztecus*). *Oecologia,* **54**, 200-204.

Galgani F., Ceccaldi H.J. & AQUACOP (1988) Effet de l'incorporation de farines de soja et de poisson dans l'aliment sur la croissance et les enzymes digestives de *Penaeus vannamei*. *Aquatic Living Resources,* **1**, 181-187.

Gamboa-Delgado J. & Le Vay L. (2009a) Artemia replacement in co-feeding regimes for mysis and postlarval stages of Litopenaeus vannamei: Nutritional contribution of inert diets to tissue growth as indicated by natural carbon stable isotopes. *Aquaculture,* **297**, 128-135.

Gamboa-Delgado J. & Le Vay L. (2009b) Natural stable isotopes as indicators of the relative contribution of soy protein and fish meal to tissue growth in Pacific white shrimp (*Litopenaeus vannamei*) fed compound diets. *Aquaculture,* **291**, 115-123.

Gamboa-Delgado, J., Le Vay, L., Manchado, M., Ponce, M., Fernandez-Diaz, C., Zerolo, R. Cañavate, J.P.(2011) Effect of larval feeding regime on trypsinogen gene expression, proteolytic enzyme activity and dietary carbon and nitrogen assimilation in Senegalese sole (*Solea senegalensis*) larvae. *Comparative Biochemistry and Physiology Part B*, **158**, 251-258

Gannes L.Z.O'Brien D.M. & Martínez del Rio C.M. (1997) Stable isotopes in animal ecology: assumptions, caveats and a call for more laboratory experiments *Ecology,* **78**, 1271-1276.

Gomes E.F., Corraze G. & Kaushik S.J. (1993) Effects of dietary incorporation of a co-extruded plant protein (rapeseed and peas) on growth, nutrient utilization and muscle fatty acid composition of rainbow trout *Oncorhynchus mykiss*. *Aquaculture,* **113**, 339–353.

Hardy R.W. (2006) Worlwide fish meal production outlook and the use of alternative protein meals for aquaculture. In: *Avances en nutricion acuicola VIII. Memorias del VIII Simposio Internaicional de Nutricion Acuicola* (ed. by L.E. Cruz Suarez, D. Ricque Marie , M. Nieto Lopez, M. Tapia Salazar, D. Villareal, A.C.Puello Cruz & A. Garcia Ortega). Universidad Autonoma de Nuevo Leon, Mazatlan, Sinaloa, Mexico*,* pp. 410-419.

Hesslein R.H., Hallard K.A. & Ramlal P. (1993) Replacement of Sulfur, Carbon, and Nitrogen in Tissue of Growing Broad Whitefish (*Coregonus nasus*) in Response to a Change in Diet Traced by $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$. *Canadian Journal of Fisheries and Aquatic Sciences,* **50**, 2071–2076

Hickling D. (2003) *Canadian Feed Peas Industry Guide.,* Pulse Canada, Winnipeg, Manitoba.

Hobson K.A. & Clark R.G. (1992) Assessing Avian Diets Using Stable Isotopes I: Turnover of ^{13}C in Tissues. *The Condor,* **94**, 181-188

Houlihan D., Carter C.G. & McCarthy I.D. (1995) Protein turnover in animals. In: *Nitrogen metabolism and excretion* (ed. by Walsh PJ, Wright P). CRC Press, Boca raton, FL, USA, pp. 1-31.

Jomori R.K., Ducatti C., Carneiro D.J. & Portella M.C. (2008) Stable carbon (δ13C) and nitrogen (δ15N) isotopes as natural indicators of live and dry food in *Piaractus mesopotamicus* (Holmberg, 1887) larval tissue. *Aquaculture Research,* **39**, 370-381.

Kureshy N. & Davis D.A. (2002) Protein requirement for maintenance and maximum weight gain for the Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture,* **204**, 125-143.

Le Vay L. & Gamboa-Delgado J. (2011) Naturally-occurring stable isotopes as direct measures of larval feeding efficiency, nutrient incorporation and turnover. *Aquaculture,* **315**, 95-103.

Lim C. (1993) Effect of dietary pH on amino acid utilization by shrimp (*Penaeus vannamei*). *Aquaculture,* **114**,293-303

MacAvoy S., Arneson L. & Bassett E. (2006) Correlation of metabolism with tissue carbon and nitrogen turnover rate in small mammals. *Oecologia,* **150**, 190-201.

Martínez del Rio C. & Wolf B.O. (2005) Mass-balance models for animal isotopic ecology. In: *Physiological and ecological adaptations to feeding in vertebrates* (ed. by Starck JM, WangT.). Science Publishers, Enfield, NH, pp. 141-174.

Matsuda H., Takenouchi T., Tanaka S. & Watanabe S. (2009) Relative contribution of Artemia and mussel as food for cultured middle-stage *Panulirus japonicus* phyllosomata as determined by stable nitrogen isotope analysis *New Zealand Journal of Marine and Freshwater Research,* **43** 217 - 224.

McCallum I. (1997) Opportunities and constraints for feeds and chick peas in aquaculture feeds. In: *Notes for Presentation to the Sasketchewan Pulse Growers Association.,* pp. 24.

Mente E., Coutteau P., Houlihan D., Davidson I. & Sorgeloos P. (2002) Protein turnover, amino acid profile and amino acid flux in juvenile shrimp *Litopenaeus vannamei*: effects of dietary protein source. *Journal of Experimental Biology,* **205**, 3107-3122.

Millward D.J., Garlick P.J., Stewart R.J., Nnanyelugo D.O. & Waterlow J.C. (1975) Skeletal-muscle growth and protein turnover. *Biochemical Journal* **150**, 235-243.

Molina-Poveda C. & Morales M.E. (2004) Use of a mixture of barley-based fermented grains and wheat gluten as an alternative protein source in practical diets for *Litopenaeus vannamei* (Boone). *Aquaculture Research,* **35**, 1158-1165.

Morrison D.J., Taylor K. & Preston T. (2010) Strong anion-exchange liquid chromatography coupled with isotope ratio mass spectrometry using a Liquiface interface. *Rapid Communications in Mass Spectrometry,* **24**, 1755-1762.

Paripatananont T., Boonyaratpalin M., Pengseng P. & Chotipuntu P. (2002) Substitution of soy protein concentrate for fishmeal in diets of tiger shrimp *Penaeus monodon*. *Aquaculture Research,* **32**, 369-374.

Pearson S.F., Levey D.J., Greenberg C.H. & Martínez del Rio C. (2003) Effects of elemental composition on the incorporation of dietary nitrogen and carbon isotopic signatures in an omnivorous songbird. *Oecologia,* **135**, 516-523.

Phillips D.L. & Gregg J.W. (2001) Uncertainty in source partitioning using stable isotopes. *Oecologia* **127**, 171-179.

Robbins C., Felicetti L. & Sponheimer M. (2005) The effect of dietary protein quality on nitrogen isotope discrimination in mammals and birds. *Oecologia,* **144**, 534-540.

Roth J.D. & Hobson K.A. (2000) Stable carbon and nitrogen isotopic fractionation between diet and tissue of captive red fox: implications for dietary reconstruction. *Canadian Jornal of Zoology,* **78**, 848-852.

Schwarcz H.P. (1991) Some theoretical aspects of isotope paleodiet studies. *Journal of Archaeological Science,* **18**, 261-275.

Smith D.M., Allan G.L., Williams K.C. & Barlow C.G. (2000) Fishmeal replacement research for shrimp feed in Australia. In: *Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola* (ed. by L.E. Cruz-Suárez, D. Ricque-Marie, , M.Tapia-Salazar, , M.A. Olvera-Novoa & R. Civera-Cerecedo). Universidad Autónoma de Nuevo León, Mérida, Yucatán, México*,* pp. 277-286.

Stenroth P., Holmqvist N., Nystrom P., Berglund O., Larsson P. & Granell W. (2006) Stable isotopes as an indicator of diet in omnivorous crayfish (*Pacifastacus leniusculus*): the influence of tissue, sample treatment, and season. . *Canadian Journal of Fisheries and Aquatic Sciences* **67**, 821–831

Su Y., Ma S., Tian X. & Dong S. (2008) A Study on the contribution of different food sources to shrimp growth in an intensive *Fenneropenaeus chinensis* pond. *Journal of Ocean University of China (English Edition),* **7**, 453-456.

Suárez J.A., Gaxiola G., Mendoza R., Cadavid S., Garcia G., Alanis G., Suárez A., Faillace J. & Cuzon G. (2009) Substitution of fish meal with plant protein sources and energy budget for white shrimp *Litopenaeus vannamei* (Boone, 1931). *Aquaculture,* **289**, 118-123.

Tacon A.G.J. (1997) Fishmeal replacers: review of antinutrients within oilseeds and pulses — a limiting factor for the aquafeed green revolution? . In: *Feeding Tomorrow's Fish* (ed. by Tacon A, Basurco B). Cahiers Options Méditerranéennes, Institut Agronomique Méditerranéen de Zaragoza, Zaragoza, Spain*,* pp. 153-182.

Tacon A.G.J. & Metian M. (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture,* **285**, 146-158.

Tecator (1983) Fat Extraction on Feeds with the Soxtec System HT—The Influence of Sample Preparation and Extraction Media. Application note AN 67/83 (1983.06.13). In: *Soxtec System HT Manual Tecator*, AB Sweden.

Tibbets T.M., Wheeless L.A. & Del Rio C.M. (2008) Isotopic enrichment without change in diet: an ontogenetic shift in δ15N during insect metamorphosis. *Functional Ecology,* **22**, 109-113.

Vander Zanden M.J., Shuter B.J., Lester N. & Rasmussen J.B. (1999) Patterns of Food Chain Length in Lakes: A Stable Isotope Study. *The American Naturalist,* **154**, 406-416.

Waterlow J.C. (2006) *Protein Turnover,* CAB International, Oxfordshire, UK

Wolf N., Carleton S.A. & Martínez del Rio C. (2009) Ten years of experimental animal isotopic ecology. *Functional Ecology,* **23**, 17-26.