Simultaneous estimation of the nutritional contribution of fish meal, soy protein isolate and corn gluten to the growth of Pacific white shrimp (*Litopenaeus vannamei*) using dual stable isotope analysis

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Abstract

The nutritional contribution of the dietary nitrogen, carbon and total dry matter supplied by fish meal (FM), soy protein isolate (SP) and corn gluten (CG) to the growth of Pacific white shrimp *Litopenaeus vannamei* was assessed by means of isotopic analyses. As SP and CG are ingredients derived from plants having different photosynthetic pathways which imprint specific carbon isotope values to plant tissues, their isotopic values were contrasting. FM is isotopically different to these plant meals in regards to both, carbon and nitrogen. Such natural isotopic differences were used to design experimental diets having contrasting isotopic signatures. Seven isoproteic (36% crude protein), isoenergetic (4.7 Kcal gr^{-1}) diets were formulated; three diets consisted in isotopic controls manufactured with only one main ingredient supplying dietary nitrogen and carbon: 100 % FM (diet 100F), 100% SP (diet 100S) and 100% CG (diet 100G). Four more diets were formulated with varying mixtures of these three ingredients, one included 33% of each ingredient on a dietary nitrogen basis (diet 33FSG) and the other three included a proportion 50:25:25 for each of the three ingredients (diets 50FSG, 50SGF and 50GFS). At the end of the bioassay there were no significant differences in growth rate in shrimps fed on the four mixed diets and diet 100F (k = 0.215-0.224). Growth rates were significantly lower (k = 0.163-0.201) in shrimps grown on diets containing only plant meals. Carbon and nitrogen stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) were measured in experimental diets and shrimp muscle tissue and results were incorporated into a three-source, two-isotope mixing model. The relative contributions of dietary nitrogen, carbon and total dry matter from FM, SP and CG to growth were statistically similar to the proportions established in most of the diets after correcting for the apparent digestibility coefficients of the ingredients. Dietary nitrogen

available in diet 33FSG was incorporated in muscle tissue at proportions representing 24, 35 and 41% of the respective ingredients. Diet 50GSF contributed significantly higher amounts of dietary nitrogen from CG than from FM. When the level of dietary nitrogen derived from FM was increased in diet 50FSG, nutrient contributions were more comparable to the available dietary proportions as there was an incorporation of 44, 29 and 27% from FM, SP and CG, respectively. Nutritional contributions from SP were very consistent to the dietary proportions established in the experimental diets.

Keywords: Stable isotopes, nutrient contribution, fish meal, soy protein, corn gluten, *Litopenaeus vannamei*

1. Introduction

Information gathered from traditional nutritional assays in conjunction with data from chemical analyses of diets and animal tissues provides valuable information to infer on the dietary performance of specific ingredients. Among these chemical analyses, the use of stable isotopes represents an additional tool for nutritional studies conducted on aquatic species. The integration of isotopic data into isotopic mixing models has made possible to convert the isotopic values of consumers and their different trophic elements to dietary contributions (Phillips, 2012). In the fields of ecology and nutrition, the isotopic techniques have provided an improved understanding of how organisms incorporate the elements they consume. 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 dietary

components from which the tissue was biosynthesized (Gannes et al., 1997; Newsome et al., 2011). In aquaculture nutrition, the natural isotope ratios of nitrogen and carbon ($^{15}N/^{14}N$ and $^{13}C/^{12}C$, respectively measured and reported in delta notation as $\delta^{15}N$ and $\delta^{13}C$) have been used as natural biomarkers to estimate dietary contributions in organisms fed either on different types of live food and inert diets, or raised on formulated diets having ingredients with contrasting isotopic signatures (Gamboa-Delgado et al., 2008; Jomori et al., 2008; Gamboa-Delgado & Le Vay, 2009a, 2009b; Matsuda et al., 2009; Martínez-Rocha et al., 2012).

Partial or total replacement of fish meal in aquaculture diets represents important advantages in economical and ecological terms. The progressively higher production of several aquaculture species is in turn exerting a higher demand for aquafeeds. Among these mass-produced marine animals, the Pacific white shrimp *Litopenaeus vannamei* has become the main shrimp species produced through aquaculture practices since 2003 (FAO, 2007). Hence, numerous nutritional studies conducted on this species have focused on testing different plant-derived meals and purified, isolated plant proteins as dietary ingredients to replace fish meal (*e.g.* Amaya et al., 2007; Harter et al., 2011; Liu et al., 2012; Oujifard et al., 2012). Different dietary resources found in the aquatic and terrestrial ecosystems frequently show distinct isotopic values due to the effect of characteristic nutrient flows and metabolic pathways. This natural isotopic labeling allows conducting studies aimed to elucidate the nutritional contribution of specific dietary sources to the growth of a consuming organism. Plants exhibit three different photosynthetic pathways (C3, C4 and CAM), which imprint different isotopic values to vegetal tissues. For example, soy is a C3 or Calvin cycle plant (called C3 because during photosynthesis, the first product

of CO_2 fixation is a 3-carbon compound), while corn is a C4 or Hatch-Slack cycle plant (Leegood, 2002). The reaction kinetics of these photosynthetic pathways has a significant influence on the carbon isotopic values ($\delta^{13}C$) of each type of plant. C3 plants have a mean $\delta^{13}C$ value of -29‰, while C4 plants show a more isotopically-enriched, mean $\delta^{13}C$ value of -13‰ (O'Leary, 1988; Ehleringer and Cerling, 2002). In the case of $\delta^{15}N$ values, most plants have isotopic values ranging from 2 to 6‰; however, the nitrogen isotope values of most traditional crops are strongly influenced by the $\delta^{15}N$ values of the inorganic fertilizers used to grow them. As the isotopic mixing models are able to estimate dietary contributions at higher resolution when the nutrient sources are isotopically distinct (Phillips, 2012), the isotopic values of primary producers have been systematically manipulated using specific fertilizers (Gamboa-Delgado et al., 2009, 2011). The present study employed the natural isotopic differences found in soy protein isolate, corn gluten and fish meal, to simultaneously assess the relative incorporation of dietary nitrogen, carbon and total dry matter supplied by these three sources to the muscle tissue of Pacific white shrimp. In addition, the nitrogen and carbon half times in muscle tissue of shrimps fed on the different experimental diets were estimated.

2. Material and methods

2.1. Experimental animals

Pacific white shrimp (*Litopenaeus vannamei*) postlarvae were obtained from a commercial hatchery (Maricultura del Pacífico) located in Mazatlán, Mexico. After reception, animals were placed in 500 L tanks and acclimated for 20 d to a bioassay room under the following

conditions: seawater temperature 30.2 ±0.7 °C, salinity 35.4 ±0.7 g l^{-1}, pH 8.4 ±0.1 and saturated dissolved oxygen. Total ammonia nitrogen (0.09 ± 0.06 mg/L), nitrite (not detected), and nitrate (12.9 ± 4.6 mg/L) were monitored using a commercial kit (FasTest; Aquarium Systems, Sarrebourg, France). A photoperiod was set up to provide a light:dark ratio of 10:14h. During the acclimation period, shrimps were exclusively fed a crumbled commercial compound diet (35% protein, Grupo Costamar, Hermosillo, Mexico) that established a known isotopic baseline in shrimp tissue before the start of the experiment. It has been demonstrated that fast-growing postlarval Penaeid shrimps achieve isotopic equilibrium with their respective diets in 15 to 20 d (Gamboa -Delgado and Le Vay, 2009; Gamboa -Delgado et al., 2011). The commercial diet was analyzed for nitrogen and carbon content and their respective isotopic values ($\delta^{15}N$ and $\delta^{13}C$) before the experimental feeding trial.

2.2. Experimental diets

Seven isonitrogenous (36% crude protein) and isoenergetic (4.7 kcal g^{-1}) experimental compound diets were formulated with different proportions of fish meal (FM), soy protein isolate (SP) and corn gluten (CG) (Table 1). The software Nutrion (Nutrion Software, Chapala, Mexico) was used to assist with the formulation of experimental diets. Diets were not manufactured to conduct an ingredient-substitution study; instead, they were formulated with ingredients having contrasting isotopic values to explore their nutritional contributions to shrimp growth as described below.

Table 1. Nutritional (g 1000 g^{-1} diet dry weight) and isotopic ($\delta^{15}N$, $\delta^{13}C$, ‰) composition of formulated diets fed to *Litopenaeus vannamei* to estimate the nutritional contribution of fish meal (F), soy protein isolate (S) and corn gluten (G) to shrimp muscle tissue.

Ingredient / Diet	**100F**	**100S**	**100G**	**33FSG**	**50FSG**	**50SFG**	**50GFS**
Fish meal[a]	556.2	0.0	0.0	180.0	278.0	139.0	139.0
Soy protein isolate[b]	0.0	449.5	0.0	149.0	112.0	224.9	112.1
Corn gluten[c]	0.0	0.0	600.2	199.0	150.7	150.0	300.5
Wheat starch[d]	348.8	390.7	253.5	348.1	348.1	354.3	323.4
Lecithin[e]	35.0	54.1	54.5	45.4	40.6	47.4	47.5
Fish oil[a]	24.0	34.6	10.4	26.3	27.9	28.3	22.2
Disodium phosphate[f]	-	20.2	41.0	10.8	-	12.5	17.7
Cellulose[f]	7.0	20.6	10.0	12.0	13.7	14.1	7.9
Alginate[f]	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin premix[a]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[a]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Choline chloride[a]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Cholesterol[g]	-	1.2	1.5	0.4	-	0.6	0.7
Vitamin C[a]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Antioxidant[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antifungic agent[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000	1000	1000	1000
Proximal and isotopic analysis							
Crude protein (g kg-1)	362	356	365	356	367	353	362
Lipids (g kg-1)	83	80	84	83	80	81	84
Gross energy (Kcal g-1)	4.6	4.8	4.6	4.7	4.7	4.8	4.7
$\delta^{15}N$ (‰)	16.5	0.6	3.1	6.4	9.0	5.1	5.9
$\delta^{13}C$ (‰)[h]	-19.5	-25.1	-16.6	-20.1	-20.4	-21.7	-19.3

[a]Alimentos Costamar (Sonora, Mexico).
[b]American Soybean Association (St. Louis, MO, USA).
[c]Trow Nutrition International (Putten, The Netherlands).
[d]Almidones y gluten S.A. (Monterrey, Mexico).
[e]Sodium salt, Sigma-Aldrich (St. Louis, MO, USA).
[d]Bentoli Inc. (Homestead, FL, USA).
[e]Ragaza Industrias Proteínas Naturales S.A. de C.V. (Monterrey, Mexico).
[f]Sigma-Aldrich (St. Louis, MO, USA).
[g]Solvay Pharmaceuticals (Houston, TX, USA).
[h]After lipid extraction and uncorrected for isotopic discrimination factors.

Three diets were formulated with only one ingredient supplying dietary nitrogen: 100% FM (diet 100F), 100% SP (diet 100S) and 100% CG (diet 100G). These diets were used as isotopic controls to correct for the isotopic differences between diets and consumers (isotopic discrimination factors) after having reached dietary equilibrium. The other four diets were formulated with varying mixtures of FM, SP and CG, one included 33% of each ingredient on a dietary nitrogen basis (diet 33FSG) and the other three included a proportion of 50:25:25 for each of the respective three ingredients (diets 50FSG, 50SFG and 50GFS). Before manufacturing the diets, macronutrients were finely ground using a Pulvex 200 grinder fitted with a size #35 mesh. Micronutrients were weighed to the nearest mg, hand-mixed for 5 min and added to the macronutrients, which in turn were homogenized for 15 min using a commercial blender. Lecithin was dissolved in pre-weighed, warm fish oil and added to the mixture. The dough was extruded through a die plate having orifices of 1.4 mm in diameter. Strands were collected on wire trays and post-conditioned by 5 min autoclaving (18.5 psi, 125 °C) to reduce nutrient leaching rates. Diets containing plant meals as the only protein source were sprayed-coated with a hydrolyzed protein to improve palatability. Diets were dried in a convection oven for 8 min at 100 °C and stored at 4 °C. Proximal analyses of the experimental diets included moisture content (method AOAC 930.15), protein content (Dumas method, LECO) and lipid content (Soxhlet system HT-1045, method AOAC 996.06) (Tecator, 1983). The energy content of the ingredients was estimated using a semi-micro bomb calorimeter (Parr 1425 PIC, Illinois, USA).

2.3. Experimental design and rearing system

Shrimps having an initial mean wet weight of 162 ± 36 mg were distributed in 21, 60-L capacity tanks. Twenty animals were placed in triplicate tanks after conducting a pre-selection aimed to distribute animals with the same size distribution pattern in each unit. The experimental tanks having built-in air lifts are connected to a recirculation system holding artificial seawater (Fritz, Chemical Co., Texas, USA). Seawater was exchanged in every tank at a rate of 800% d^{-1} and it was treated by mechanical cartridge filters, UV filter, protein skimmers and a bubble bead biological filter. The experimental tank array is designed so that possible water quality variations affect all tanks simultaneously. Animals were fed the experimental compound diets at daily amounts representing 10 to 15% of the animal biomass. Feed was delivered in four rations at 8:00, 12:00, 16:00 and 20:00 hours for 29 days. Before the first feeding ration, uneaten feed, feces and moults were siphoned out daily. Tank walls were periodically scrubbed off with a rough fiber to avoid any possible biofilm growth. The experimental time period and sampling points to collect muscle 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 et al., 2011; Martínez-Rocha et al., 2012). In order to verify isotopic values shifting in time to isotopic equilibrium, on experimental days 0, 2, 4, 8, 15 and 22, one shrimp was randomly collected from each replicate tank, killed in ice/water slurry and dissected to isolate the abdominal muscle. The exoskeleton and hind gut were removed, muscle tissue samples were rinsed with distilled water and stored in Eppendorf tubes at −80 °C until sample pretreatment. As an estimate of growth (k) is required for the exponential model of isotopic change, the individual wet weight of five animals per replicate was determined on the sampling days using a digital balance. Animals were captured with nets and weighed after blotting off excess water with a moist cloth. At the end of the experiment

(day 29), all the remaining animals were killed and three shrimps per replicate tank (9 per treatment) were also weighed, sacrificed and dissected to obtain abdominal muscle tissue.

2.4. Sample pretreatment and stable isotope analyses

Samples of shrimp muscle tissue and compound diets were dehydrated at 50 °C until constant weight in a convection oven. Dry samples were manually ground using mortar and pestle to obtain a fine powder. In order to avoid sample loss, small muscle samples (*e.g.* those belonging to shrimps sampled on the first experimental week or showing slow growth) were not ground, instead, fragments of dry muscle tissue were obtained for isotopic analysis. Lipids are usually depleted in ^{13}C relative to carbohydrates and protein (De Niro & Epstein, 1978, Stenroth et al., 2006); therefore, in order to reduce the variability of $\delta^{13}C$ values and allow further comparisons, diet samples (8% lipid content) were lipid extracted following Beaudoin et al. (2001) by suspending the ground material in a 50:50 solution of chloroform–methanol for 12 h. Samples were solvent-treated twice over this period of time. After lipid extraction samples were oven-dried (50 °C until constant weight), homogenized again, and kept in a desiccator. Muscle tissue samples were not lipid extracted as part of the pre-treatment as shrimp muscle contains low lipid levels and it has been shown that $\delta^{15}N$ values in muscle tissue of decapod crustaceans undergo minimal, no significant changes after solvent treatment (Stenroth et al., 2006; Bodin et al., 2007). Diet and muscle tissue samples of 900 to 1100 µg 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 $\delta^{15}N$ and 0.14 ‰ for $\delta^{13}C$. Isotopic results are expressed in delta notation (δ), which is defined as part per thousand (‰) deviations from the $\delta^{15}N$ and $\delta^{13}C$ values of the isotopic standard reference materials (atmospheric nitrogen and Pee Dee belemnite, respectively). We employ the term "discrimination factor" following Cherel et al. (2005) and Dennis et al. (2010) to describe changes in isotopic values between a consuming organism (whole body or specific tissue) and its diet after having reached isotopic equilibrium ($\Delta^{15}N$ or $\Delta^{13}C$).

2.5. Estimation of nutrient contribution and elemental residency times in tissue

A three-source, two-isotope mixing model (Phillips & Koch, 2002) was applied to estimate the relative contribution of dietary nitrogen, carbon and total dry matter supplied by FM, SP and CG to the muscle tissue of shrimps under the different dietary treatments. The model considers the isotopic differences between the sources (in this particular study represented by the ingredients FM, SP and CG) and the mixture (shrimp muscle tissue). One of the model assumptions indicates that the consuming organism is in isotopic equilibrium with its diet, this assumption was verified by measuring the isotopic values in shrimp muscle throughout the experimental period and until asymptotic values were reached. Additional assumptions associated to the use of isotopic mixing models and the validation of results (Gannes et al., 1997; Martínez del Rio and Wolf, 2005; Post, et al., 2007; Martinez del Rio et al., 2009) were also met or taken into consideration in interpreting the results. These include similar (or known, in order to correct for) elemental composition of the food sources (dietary ingredients), estimation of discrimination factors

and consideration of isotopic routing and dietary assimilation efficiencies. Estimation of isotopic discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$) increases the accuracy of the estimated dietary contributions by integrating correction factors into the mixing model (Martínez del Rio et al., 2009; Phillips, 2012). In the present study, measured isotope values were corrected for discrimination factors by introducing into the model three reference isotopic values determined in muscle tissue of shrimps fed exclusively on diets containing only FM, SP or CG. Previous studies have shown that FM, SP and CG have different apparent digestibility coefficients (ADC) for protein (0.78, 0.96 and 0.81, respectively) and dry matter (0.66, 0.92 and 0.83, respectively) when fed to *L. vannamei* (Cruz-Suárez et al., 2009; Terrazas et al., 2010; Villarreal, 2011). Therefore, expected dietary proportions of dietary nitrogen, carbon and total dry matter were corrected for ADC before comparisons to observed proportions determined in muscle tissue were conducted. Considering that the carbon and nitrogen isotopes found in amino acids reflect both, diet composition and metabolic processes (Boecklen et al., 2011) and the majority of deposited carbon in muscle tissue is derived from amino acids, corrections applied for the ADC of dietary protein (nitrogen) were also applied to dietary carbon. $\delta^{13}C$ and $\delta^{15}N$ values measured in shrimp muscle tissue were sequentially introduced into the model to estimate the relative proportion of dietary nitrogen, carbon and dry matter incorporated from the three main ingredients. An indicator of the variability of nutritional contributions was generated by introducing into the isotopic mixing model isotope values measured in individual animals and not averaged values. Preliminary analysis indicated that elemental contents in FM, SP and CG were significantly different (N= 10.5±0.4, 13.8±0.3 and 10.7±0.7%, respectively, and C= 39.3±0.4, 47.2±0.7 and 52.1±1.7%, respectively). Elemental values are also considered by the mixing model to obtain estimates of the relative contribution of dry

matter from the food sources to growth. $\delta^{15}N$ and $\delta^{13}C$ values were determined at different times of the experimental period and these values were introduced into an exponential model of isotopic change (Hesslein et al., 1993) as described in Gamboa-Delgado et al. (2011). The model provides a quantitative coefficient (*m*) that allows distinguishing the isotopic change that is due to growth (*k*) and/or metabolic turnover (*m*). Coefficients *k* and *m* in turn provide an indicator of the residency time (Equation 1), the time period necessary for half of the muscle nitrogen or carbon to be replaced after animals consume a new diet (half time, t_{50}) (MacAvoy et al,. 2005).

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

2.6. Statistical analyses

Nitrogen and carbon contents of dietary ingredients and their respective $\delta^{15}N$ and $\delta^{13}C$ values, dietary effects on isotopic values of muscle tissue at different times, and mean shrimp wet weight were analyzed by one way ANOVA after normal distribution and data homoscedasticity were verified. Tukey pair wise comparisons were used to detect treatments significantly differing from each other. Survival data lacked homoscedasticity after transformation and comparisons were done using a Kruskal-Wallis test. Chi-square goodness of fit tests (χ^2) were applied to compare expected (dietary proportions of nutrients contributed by FM, SP and CG after correcting for ADC) and observed (estimated proportions incorporated in muscle tissue) dietary proportions of incorporated nutrients. Parameter *m* in 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 $P<0.05$.

3. Results

3.1. Shrimp growth and survival

During the experimental feeding period, water quality parameters remained within the recommended optimal values for this species. Temperature, pH, salinity, dissolved oxygen and nitrogenous waste concentrations were maintained as the previously described conditions for the bioassay room. At the end of the experiment, shrimps reared under the seven experimental treatments showed similar survival rates (93±6%) but significantly different mean final wet weights (Table 2). Shrimp fed diet 100F and those fed the four mixed diets containing the three ingredients at different inclusion levels showed statistically similar final weights. From these treatments, animals fed on diets 50SFG and 50GFS showed lower growth rates. Growth rates were significantly lower in shrimps fed diets containing only SP or only CG as nitrogen source.

Table 2. Final wet weight (FW), weight gain (WG), specific growth rate (SGR) and survival rate of Pacific white shrimp *L. vannamei* reared under diets having different dietary proportions of fish meal, soy protein isolate and corn gluten.

Diet	FW (mg)	WG (%)	SGR (% d^{-1})	Survival (%)
100F	804 ± 348^{a}	404 ± 96^{a}	5.58 ± 0.61^{a}	96 ± 6^{a}
100S	508 ± 189^{b}	214 ± 49^{bc}	3.95 ± 0.54^{b}	93 ± 6^{a}
100G	276 ± 79^{c}	72 ± 32^{c}	1.87 ± 0.64^{c}	93 ± 13^{a}
33FSG	839 ± 301^{a}	419 ± 53^{a}	5.68 ± 0.34^{a}	96 ± 6^{a}
50FSG	816 ± 310^{a}	407 ± 101^{a}	5.60 ± 0.63^{a}	93 ± 6^{a}
50SFG	724 ± 256^{a}	343 ± 58^{ab}	5.14 ± 0.41^{ab}	100 ± 0^{a}
50GFS	682 ± 233^{ab}	317 ± 20^{ab}	4.93 ± 0.16^{ab}	89 ± 11^{a}

Different superscripts indicate significant differences for that particular column.

3.2. Isotopic shifts and discrimination factors

SP and CG showed very contrasting $\delta^{15}N$ (0.6 ± 0.2 and 3.0 ± 0.1‰, respectively) and $\delta^{13}C$ values (-25.5±0.4 and -13.5±0.1‰, respectively). Isotopic values in plant sources were also significantly different when compared to the isotopic values of FM ($\delta^{15}N$=16.6±0.2‰ and $\delta^{13}C$= -16.9±0.4‰). These significant differences allowed formulating diets having ingredients with isotopically contrasting values that in turn elicited a wide range of nitrogen and carbon isotope changes in muscle tissue (Figs. 1a and 1b). All mixed experimental diets exerted a rapid influence on the isotopic values of shrimp tissue and by day 22, animals in all treatments (including those in the three isotopic control diets) had reached isotopic equilibrium with their feed. $\Delta^{15}N$ and $\Delta^{13}C$ values between animals and their respective diets were significantly different (Table 3). $\Delta^{15}N$ values between muscle tissue and diet 100F were small (0.3‰), while values observed in shrimps fed on diets 100S and 100G were significantly larger (5.3 and 5.8‰, respectively).The mixed diets caused $\Delta^{15}N$ values ranging from 2.5 to 3.3‰. In contrast, overall $\Delta^{13}C$ values were less variable and ranged from -0.1 to 2.8 ‰. The high range of isotopic values in the three main dietary ingredients being reflected in shrimp muscle tissue increased the resolution when assessing total dry matter contributions and nitrogen and carbon residency times in tissue. However, after applying correction factors for isotopic discriminations, the $\delta^{15}N$ values of some diets approached to the $\delta^{15}N$ values of shrimp tissue and isotopic changes did not describe exponential trends (Fig. 1a).

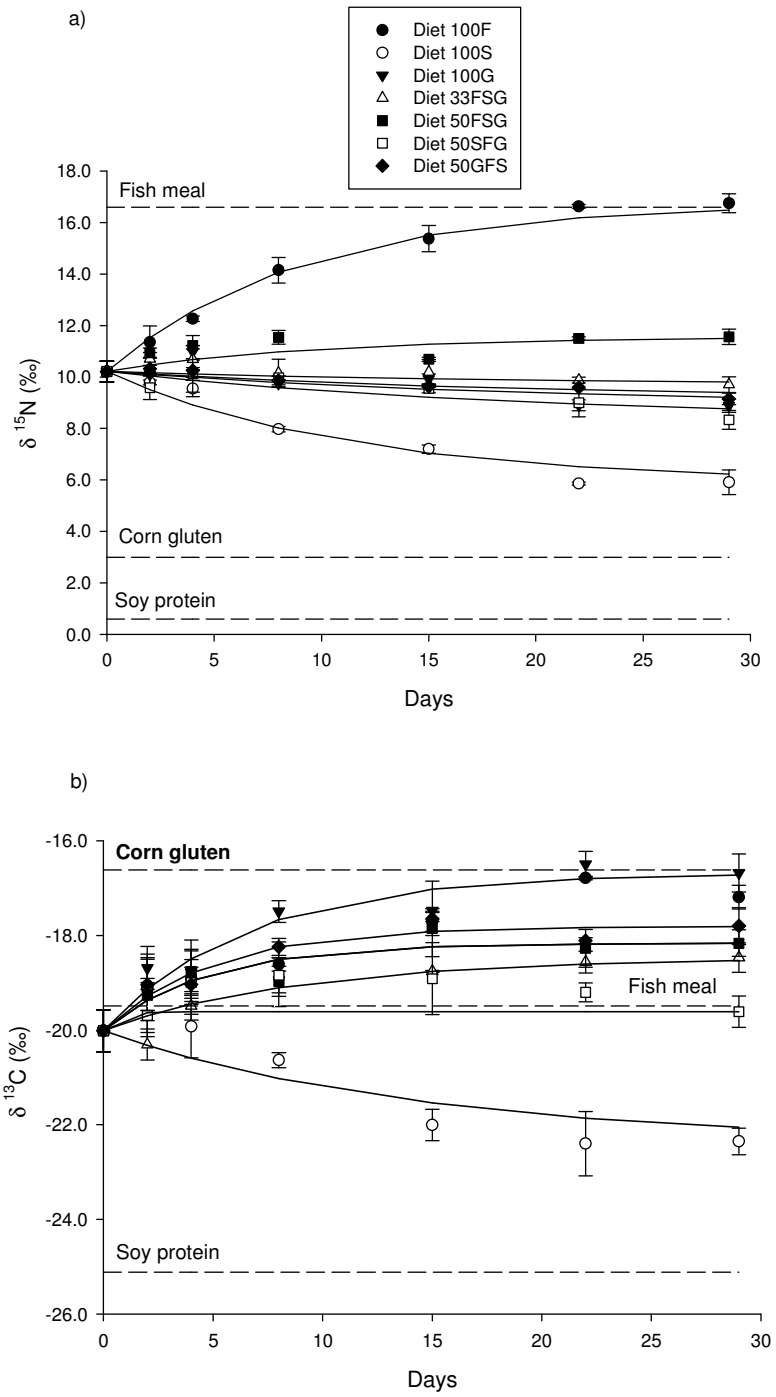

Fig. 1. Changes in nitrogen (a) and carbon (b) stable isotope values in muscle tissue of white shrimp *L. vannamei* reared on experimental diets having different proportions of fish meal, soy protein isolate and corn gluten. Dotted lines represent the isotopic values of control diets containing only one nitrogen source. Solid lines indicate predicted isotopic values generated by an exponential model of isotopic change (Hesslein et al., 1993) and show the best fit to observed data. Mean of 3 to 9 animals per sample ±SD.

3.3. Nitrogen and carbon half times in tissue

Nitrogen and carbon isotopic shifts followed an expected pattern characterized by an exponential trend caused by the isotopic values of the experimental diets being reflected in shrimp muscle tissue. For most treatments, predicted isotopic values fitted well on the observed data and from these data, parameter *m* (metabolic turnover) was estimated by means of iterative non-linear regression. Although isotopic differences between the conditioning diet and the experimental diets were significant, it was not possible to estimate the nitrogen half times for all diets because after applying corrections for isotopic discrimination factors, isotopic differences between shrimp and diets were narrowed down.

Table 3. Mean growth rates (*k*) and estimated half times of nitrogen and carbon in muscle tissue of Pacific white shrimp *L. vannamei* reared under diets having different dietary proportions of fish meal, soy protein and corn gluten. $\Delta^{15}N$ and $\Delta^{13}C$ represents the isotopic difference between diets and muscle tissue after isotopic equilibrium was reached.

Diet		Nitrogen			Carbon		
	k (d^{-1})	half time (d)	R^2	$\Delta^{15}N$	half time (d)	R^2	$\Delta^{13}C$
100F	0.056 ± 0.004^{a}	6.2 ± 0.7^{a}	99	0.3	6.4 ± 0.9^{ab}	83	2.3
100S	0.039 ± 0.007^{b}	7.8 ± 1.1^{a}	95	5.3	9.8 ± 2.1^{b}	74	2.8
100G	0.019 ± 0.006^{c}	-	-	5.8	4.5 ± 0.4^{a}	93	-0.1
33FSG	0.057 ± 0.003^{a}	-	-	3.3	6.2 ± 1.2^{ab}	86	1.7
50FSG	0.056 ± 0.004^{a}	6.6 ± 1.3^{a}	49	2.5	3.3 ± 0.7^{a}	87	2.2
50SFG	0.051 ± 0.005^{a}	-	-	3.2	-	-	2.1
50GFS	0.049 ± 0.008^{ab}	-	-	3.2	3.4 ± 0.5^{a}	93	1.5

Different superscripts indicate significant differences for that particular column. R^2 values indicate the degree of fitness of data generated by the exponential model of isotopic change and isotopic values measured in shrimp muscle tissue.

Due to this, diets 50SFG and 50GFS did not cause exponential nitrogen isotopic shifts in shrimps (Fig. 1a). Parameters *m* and *k* indicated that estimated nitrogen half times in tissue ranged from 6.2 d in shrimp fed diet 100F to 7.8 d in shrimp fed on diet 100S (Table 3). Estimated carbon half times in muscle tissue ranged from 3.3 d (diet 50FSG) to 9.8 d (diet 100S).

3.4. Nutritional contributions from fish meal, soy protein and corn gluten

Isotopic changes observed over the experimental period and inclusion of asymptotic values into the isotopic mixing model (Fig. 2) indicated that, in most cases, the contributions of dietary nitrogen and carbon from FM, SP and CG to the growth of shrimps were statistically similar to the expected nutritional contributions available in the dietary formulations after correcting for ADC (Tables 1 and 4). Although differences were small, shrimps fed on diet 50GFS incorporated significantly higher amounts of dietary nitrogen (58%), dietary carbon (66%) and total dry matter (66%) from CG (χ^2=6.2, *P*= 0.044) and significantly less from FM. Shrimps fed on diet 50SFG also incorporated significantly higher amounts of dietary carbon and dry matter from CG (χ^2=7.7, *P*= 0.020). Nutritional contributions from SP to muscle tissue were very consistent with the proportions pre-established in the respective dietary formulations.

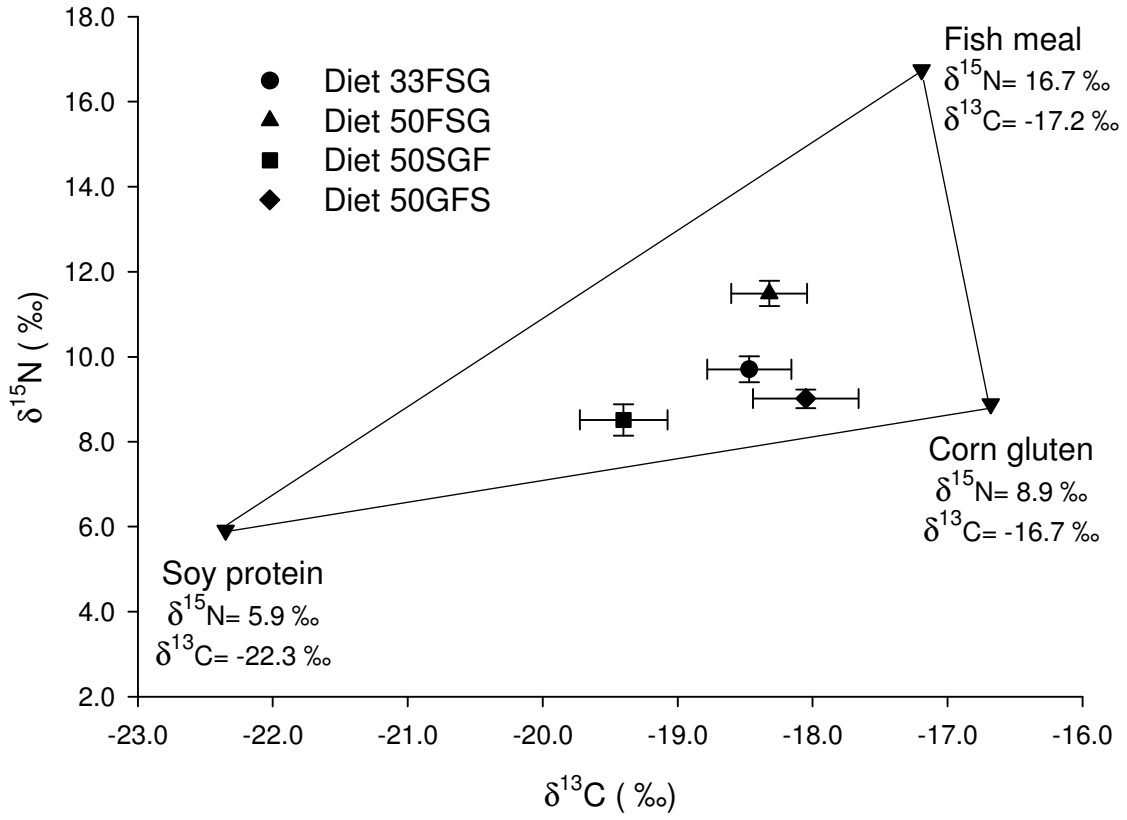

Fig. 2. Carbon and nitrogen isotope values of experimental diets (corrected for isotopic discrimination factors) and isotope values of muscle tissue of shrimps fed on four experimental diets having varying levels of fish meal, soy protein isolate and corn gluten. Mean of 9 animals per sample ±SD.

Table 4. Estimated relative proportions of dietary nitrogen, carbon and total dry matter supplied from fish meal, soy protein isolate and corn gluten to muscle growth of Pacific white shrimp *L. vannamei* as indicated by a three-source, two-isotopes mixing model (mean ±SD, n= 9 per diet).

Diet	Expected contributions		Observed contributions
	Bulk diet	Corrected for ADC*	Muscle tissue
Nitrogen			
33FSG			
Fish meal	33.2	30.5[a]	23.9 ± 0.4[a]
Soy protein	33.4	37.6	35.4 ± 1.8
Corn gluten	33.4	31.9	40.7 ± 1.2
50FSG			
Fish meal	50.4	47.2[a]	44.1 ± 0.4[a]
Soy protein	24.7	28.5	29.1 ± 1.6
Corn gluten	24.9	24.3	26.8 ± 1.2
50SFG			
Fish meal	25.3	22.5[a]	15.5 ± 0.8[a]
Soy protein	49.8	54.4	53.5 ± 1.8
Corn gluten	24.9	23.1	31.0 ± 1.0
50GFS			
Fish meal	25.3	23.4[a]	12.7 ± 0.2[b]
Soy protein	24.9	28.4	29.2 ± 2.4
Corn gluten	49.8	48.2	58.1 ± 2.6
Carbon			
33FSG			
Fish meal	29.8	27.4[a]	21.8 ± 0.4[a]
Soy protein	30.3	34.3	29.6 ± 1.8
Corn gluten	39.9	38.3	48.6 ± 1.4
50FSG			
Fish meal	46.5	43.6[a]	41.7 ± 0.4[a]
Soy protein	22.9	26.4	25.2 ± 1.6
Corn gluten	30.6	30.0	33.1 ± 1.2
50SFG			
Fish meal	23.6	21.1[a]	14.8 ± 0.8[b]
Soy protein	46.9	51.4	46.6 ± 1.8
Corn gluten	29.5	27.5	38.6 ± 1.0
50GFS			
Fish meal	21.6	20.1[a]	11.0 ± 0.2[b]
Soy protein	21.5	24.6	23.1 ± 2.0
Corn gluten	56.9	55.3	65.9 ± 2.4
Total DM**			
33FSG			
Fish meal	34.1	28.3[a]	26.3 ± 0.4[a]
Soy protein	28.2	32.5	29.7 ± 1.8
Corn gluten	37.7	39.2	44.0 ± 1.4
50FSG			
Fish meal	51.4	44.6[a]	42.5 ± 0.4[a]
Soy protein	20.7	25.0	25.3 ± 1.4
Corn gluten	27.9	30.4	32.2 ± 1.0
50SFG			
Fish meal	27.0	21.7[a]	12.8 ± 0.8[b]
Soy protein	43.8	48.9	46.9 ± 1.8
Corn gluten	29.2	29.4	40.3 ± 1.0
50GFS			
Fish meal	25.2	20.7[a]	10.3 ± 0.2[b]
Soy protein	20.4	23.3	23.3 ± 2.0
Corn gluten	54.4	56.0	66.4 ± 2.4

* ADC: Apparent digestibility coefficients. **Total dry matter contributions were estimated after correcting for elemental concentrations (C and N) available in the main ingredients. Different superscripts indicate significant differences between mean expected and observed dietary contributions.

4. Discussion

4.1. Growth and survival

In the present study, significant differences observed in growth rates among treatments cannot be attributed to the protein level of the experimental diets (36%) as it has been reported that a dietary protein level above 32% is optimal for early juveniles of this species (Kureshy & Davis, 2002). Likewise, all diets were supplemented with fish oil in order to avoid deficiencies of fatty acids induced in marine animals by ingredients containing low lipid levels such as CG and SP (Lewis & Kohler, 2008). Lower growth rates observed in animals fed on diets having only plant-derived protein can be explained by the nutritionally unsuitable amino acid profile of SP and CG for marine shrimp. Additionally, presence of anti-nutritional factors in plant meals affecting growth has been previously documented, and although diets were post-conditioned, the possibility of a residual presence of protease inhibitors, lectins, phytic acid or saponins should not be discarded (Francis et al., 2001). Over the experimental period, shrimps increased their body weight in 3 to 4-fold, except animals fed on diets having only SP or CG as protein sources. These observations are in agreement to other studies that have remarked that the nutritional profile of SP and CG does not fully satisfy the nutritional requirements of Penaeid shrimps when supplied at high inclusion levels (Cruz-Suarez et al., 2001; Davis et al., 2002). The observed growth rate, in conjunction with the nitrogen turnover rates elicited by the different diets was sufficient for the dietary $\delta^{15}N$ and $\delta^{13}C$ values to be reflected in muscle tissue and reach isotopic equilibrium. Results from previous studies in which FM has been replaced with plant-derived ingredients in diets for Pacific white shrimp, indicate that lower growth and survival rates are observed when the dietary levels of plant meals are high or represent the

only source of protein (Galgani et al., 1988; Paripatananont et al., 2002; Molina-Poveda and Morales, 2004), such observations are consistent with the observed growth rate in shrimps fed diets containing only plant-derived meals. SP and CG are ingredients that have been previously used to replace FM in aquaculture diets for crustaceans and fish. Most studies indicate that better results in terms of biomass production, survival and hematological parameters are achieved when these ingredients have been used at low to medium replacement levels and in conjunction with other ingredients derived from plant or animal sources (Robaina et al., 1997; Kikuchi, 1999; Regost et al., 1999; Lewis and Kohler, 2008; Ye et al., 2011; Li et al., 2012; Sookying and Davis, 2012). However, successful substitution of high levels of dietary FM using plant proteins has also been reported for some marine organisms. For example, Alvarez et al. (2007) replaced up to 75% of FM with soybean meal in diets for shrimp *L. schmitti* without compromising weight gain, feed conversion ratio and protein efficiency ratio. In a study conducted on Senegalese sole (*Solea senegalensis*), Cabral et al. (2011) reported that a diet in which 75% of FM was replaced by a mixture of plant proteins, promoted similar weight gain and protein efficiency ratio than a diet containing FM as the main protein source.

4.2. Isotopic shifts and discrimination factors

SP and CG were selected for their potential to replace FM and also for having highly contrasting $\delta^{15}N$ and $\delta^{13}C$ values in comparison to FM. Such differences allowed formulating experimental diets with ingredients having a wide range of isotopic values, which in turn allowed exploring dietary effects on the isotopic shifts in shrimp. The

isotopic values of the experimental diets were rapidly reflected in shrimp muscle tissue and isotopic steady state between diets and animals was reached between experimental days 22 and 29. As different growth rates were observed, it is assumed that animals reached isotopic equilibrium through both, tissue accretion and metabolic turnover rates. Shrimps fed on diets 100G and 100S increased their body weights by only 72 and 214 %, respectively; however, these animals also reached isotopic equilibrium through tissue metabolic turnover. Besides comprising more than 60% of the Penaeid shrimp body weight, abdominal muscle tissue was selected because previous studies conducted on crustaceans have shown only small differences in nitrogen isotopic ratios between muscle and whole body samples (Stenroth et al., 2006; Gamboa-Delgado and Le Vay, 2009b; Gamboa-Delgado et al., 2011). Such similarity also indicates that isotopic routing effects were not significant (*e.g.* dietary elements were not differently allocated to muscle tissue). At the end of the experiment, $\Delta^{15}N$ and $\Delta^{13}C$ values between shrimp and their respective experimental diets were very contrasting and ranged from 0.3 to 5.8‰ for nitrogen and from -0.1 to 2.7‰ for carbon. Even though the $\Delta^{15}N$ and $\Delta^{13}C$ values are necessary to apply correction factors in the mixing models, these isotopic discrimination factors are frequently unknown or difficult to estimate when conducting nutritional or ecological studies. Under these situations, average values are taken from the literature despite the fact that wide variations in the $\Delta^{15}N$ and $\Delta^{13}C$ values have been reported (Caut et al., 2009). There is an ongoing discussion about what causes these isotopic discrimination factors, and although increasing evidence indicates that high $\Delta^{15}N$ values are related to a higher demand for specific nutrients (Le Vay and Gamboa-Delgado, 2011), is still not known if $\Delta^{15}N$ values are more affected by the quality of the available dietary protein (Roth and Hobson, 2000; Robbins et al., 2005) or by the protein quantity (Pearson et al., 2003). For example, in juvenile blue

crabs *Callinectes sapidus*, high $\Delta^{15}N$ values have been associated to diets containing high C:N ratios (Fantle et al., 1999) and some authors consider that when organisms face nutritional deficiencies, they respond by increasing the metabolic cycling of nonessential nutrients, which might increase the $\Delta^{15}N$ values between animal tissue and diet (Martínez del Rio and Wolf, 2005). In this context, the higher discrimination factors observed in shrimps fed diets having only SP or only CG as protein source, could be related to the comparatively lower availability of some essential amino acids (*i.e.* lysine, methionine) in plant-derived ingredients.

4.3. Nitrogen and carbon half times in muscle tissue

Estimated half times of nitrogen and carbon in muscle tissue ranged from 3.3 to 9.8 d and differences were attributed to diet type. As observed in the isotopic discrimination factors, diets containing only plant-derived protein elicited different responses in the half times in tissue. Diet 100S caused longer nitrogen and carbon half times in tissue (7.8 and 9.8 d, respectively), while diets containing higher levels of FM (diets 100F and 50FSG) elicited shorter half times (3.3 to 6.6 d), which were probably associated to higher metabolic rates caused by higher growth rates. It has been reported that the rates of protein synthesis are characteristically high in postlarval and juvenile Penaeid shrimps (Mente et al., 2002). 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. Carbohydrates known to be highly digestible for this shrimp species were supplied (Cousin

et al., 1996) and it is thus suggested that a restriction of specific amino acids (*e.g.* lysine and methionine in SP and CG) in diets formulated with plant-derived proteins caused longer nitrogen and carbon half times and lower growth rates.

4.4. Nutrient contribution from fish meal, soy protein isolate and corn gluten

The contrasting isotopic values of the main dietary ingredients and their introduction into the isotopic mixing model in conjunction with the isotopic values measured in shrimps, allowed estimating the relative proportional contributions of dietary nitrogen, carbon and total dry matter. Although most of the nutritional contributions to shrimp growth were statistically similar to the proportions of available nutrients in the dietary formulations, there were some differences. For example, under lower FM availability (diets 50SFG and 50GFS), there was a higher incorporation of nutrients from CG (7 to 11% more) than from FM. Diet 33FSG supplied amounts of dietary nitrogen and carbon that were similar to the proportions established in the formulated diets. Estimated proportions of assimilated total dry matter were not statistically different to the dietary proportions available in diets 33FSG and 50FSG, but there was a higher incorporation of total dry matter from CG than from FM in diets 50SFG and 50GFS. Dietary nitrogen contributions from SP to muscle tissue were high and consistent with the amounts of nitrogen available in the respective compound diets. In previous experiments applying isotopic techniques to explore dietary nitrogen contributions from plant meals to shrimp growth, high contributions of plant-derived nitrogen have been observed, although not necessarily in proportions matching the dietary availability. For example, Gamboa-Delgado and Le Vay (2009b) reported

significant differences in the incorporation of FM (73% contribution) and SP (27% contribution) when both ingredients were included in diets supplying similar proportions of dietary nitrogen (50:50, at 46% crude protein) for juvenile *L. vannamei* (414 mg). In contrast, Martínez-Rocha et al. (2012) observed that postlarval shrimp *L. vannamei* (141 mg) incorporated similar amounts of dietary nitrogen from pea meal (*Pisum sativum*) and FM when fed on formulated diets having varying proportions of dietary nitrogen supplied from both ingredients. It is very likely that the different nutritional contributions might be explained by differences in the amino acid profiles of FM, SP and CG. While FM contains higher amounts of the essential amino acids methionine and lysine than SP (Cruz-Suárez et al., 2009), CG contains leucine at levels that are up to 2-fold higher than the levels available in FM (Terrazas et al., 2010; Villarreal, 2011). Besides its importance as a branch chained amino acid, studies conducted on mammals have shown that leucine is the only dietary amino acid that has the capacity to stimulate the muscle protein synthesis, hence slowing down the degradation of muscle tissue (Etzel, 2004). Higher levels of the amino acid phenylalanine are also found in CG than in FM (Terrazas et al., 2010; Villarreal, 2011), which might further explain the higher contributions of dietary nitrogen and carbon contributed by CG to the muscle of shrimp fed on some of the experimental formulations. It has been demonstrated in crustaceans and fish that different amino acids may significantly differ in their $\delta^{15}N$ (Schmidt et al., 2004) and $\delta^{13}C$ values (McCullagh et al., 2008) in a range of up to 20 units (‰). Therefore, future nutritional studies might use this natural isotopic labeling to explore the transfer of dietary amino acids by applying compound specific isotopic analysis (CSIA) of amino acids in diets and shrimp tissues. In the present study, the estimated incorporation of nutrients into muscle tissue and the growth rates thus suggest that when FM is replaced with SP and CG at a level between 50 to 66% (diets

33FSG and 50FSG), growth and survival rates are similar to those observed in shrimps fed on fish meal-based diets. The isotopic techniques can yield valuable information on the rates of incorporation of specific dietary nutrients in marine organisms, and results from the present study highlight the nutritional and economical benefits that plant-derived ingredients represent when supplied at dietary levels that promote complementary nutritional effects on shrimp growth.

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