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Recent Advances in the Application of Stable Isotopes as Nutritional Tools in Aquaculture

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Abstract

From an ecological point of view, aquaculture systems consist of simple food webs having a limited number of nutritional sources. These characteristics facilitate the application of stable isotope ratios of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) to assess the flow of dietary components. Due to rapid and measurable bioaccumulation of the heavier stable isotopes, such isotopic shifts can be tracked at different times and at each trophic step to provide an indicator of what dietary components are being incorporated into animal tissue and how fast. The present manuscript presents results from recent, controlled nutritional experiments designed to quantify the relative contribution of dietary carbon and nitrogen supplied by different dietary items. Stable isotopes ratios were measured in a range of food sources and experimental animals. In a first experiment, juvenile shrimp *Litopenaeus vannamei* were reared on co-feeding regimes having different proportions of live biomass of the green macroalgae *Ulva clathrata* and inert feed in order to identify nutritional contributions to tissue growth using dual stable isotope analysis. In another trial, nitrogen stable isotopes were measured to explore the relative dietary nitrogen contributions from fish meal and pea meal (*Pisum sativum*) to the growth

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of white shrimp postlarvae fed low protein diets having different proportions of both ingredients. In a third, multidisciplinary experiment, Senegalese sole (*Solea senegalensis*) larvae were used as a model to evaluate the effect of different larval feeding regimes on (1) trypsinogen gene expression (*ssetryp1*), (2) trypsin and chymotrypsin activities and (3) changes in stable isotope composition to estimate the assimilation of dietary carbon from the larval diets.

Keywords: *L. vannamei, stable isotopes, nutritional contributions*

Introduction

The isotopic signature of a consuming organism reflects the isotopic profile of the assimilated dietary material and since different food items have different isotopic signatures, they can be used to infer trophic linkages (Van der Zanden, Hulshof, Ridgway & Rasmussen, 1998). Isotopic estimations provide an integration of feeding over time (Peterson & Fry, 1987) and also allow using mixing and mass balance models to estimate the relative contribution of different food sources to growth (Burford, Sellars, Arnold, Keys, Crocos & Preston, 2004). The application of these techniques and models has also supported research on the nutrition of aquatic animals in studies using either naturally-occurring or artificially-enriched stable isotopes in compound diets (Parker, Anderson & Lawrence, 1989; D'Avanzo, Alber & Valiela, 1991; Preston, Smith, Kellaway & Bunn, 1996). In an extension of ecological food web studies, naturally-occurring stable isotopes have been used to investigate the fate of nutrients in aquaculture systems where several food sources are present (Schlechtriem, Focken & Becker, 2004; Jomori, Ducatti, Carneiro & Portella, 2005; Gamboa-Delgado, Cañavate, Zerolo & Le Vay, 2008; Gamboa-Delgado & Le Vay, 2009b). The use of this approach assists in the design of nutritional experiments under normal feeding and environmental conditions. Simple isotopic mixing models (Phillips & Gregg 2001, 2003; Fry 2006) have been used under laboratory conditions to trace nutrients and to define the relative utilisation of inert diets, live feeds and individual ingredients of compound feeds. Such experiments have also investigated the effectiveness of co-feeding regimes, optimal timing of live food transitions (*e.g.* from rotifers to Artemia), optimal size/age for weaning and the incorporation of specific dietary components (Gamboa-Delgado et al., 2008, Jomori et al., 2008; Gamboa-Delgado & Le Vay 2009a,b; Matsuda, Takenouchi & Tanaka, 2009). Due to their natural abundance in animal tissue, carbon and nitrogen are elements frequently analyzed to determine their isotopic proportions ($\delta^{13}C$ y $\delta^{15}N$). In the studies reviewed herein, isotopic values were estimated in fish meal, pea meal, compound formulated feeds, live biomass of macroalgae *Ulva clathrata* and several larval feeds in order to estimate the incorporation of dietary

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carbon and nitrogen supplied by experimental diets and feeding regimes for pacific white shrimp (*Litopenaeus vannamei*) and Senegal sole (*Solea senegalensis*).

Material and Methods

Experimental animals and experimental design

For experiment 1, juvenile shrimps (188 ±28 mg mean wet weight) were reared on co-feeding regimes supplying live biomass of macroalgae *Ulva clathrata* and inert feed. Under these experimental co-feeding regimes, 75, 50 and 25% of the daily consumed macroalgal biomass was substituted by inert feed (regimes 75F/25U, 50F/50U and 25F/75U, respectively). Negative and positive control feeding protocols consisted only of macroalgae biomass (100U) and inert feed (100F), respectively. Animals were reared in duplicate experimental tanks (60 l, air lift-fitted and connected to a recirculation system) under the following conditions: temperature 30.1±0.5 °C, salinity 34.5±0.9 g l^{-1}, pH 8.4±0.1 and saturated dissolved oxygen. Total ammonia nitrogen (0.09 ±0.06 mg l^{-1}), nitrite (not detected) and nitrate (12.9 ±4.6 mg l^{-1}) were monitored using a commercial kit. Shrimp samples were taken throughout the duration of the trial and processed as described below. In experiment 2, pea meal and fishmeal were used as ingredients representing the only dietary nitrogen sources to formulate five mixed diets having decreasing levels of pea meal-derived nitrogen: 95, 85, 70, 55 and 40%. Diets were supplied to shrimp (141 ± 31 mg mean wet weight) held in triplicate tanks. 15 individuals were placed per tank. Negative and positive control diets were formulated using only pea meal and fish meal, respectively. All compound diets were isonitrogenous (21% crude protein) and isoenergetic (17.4 kJ g^{-1}). In experiment 3, Senegal sole larvae (22 µg mean dry weight) were placed in 300 l-capacity rearing tanks at an initial fish density of 50 ind l^{-1}. Rearing tanks were double-walled to maintain water temperature at 19-21°C. If necessary, spaces between walls were synchronically flooded with thermostatically-controlled water pumped from a chiller. Moderate bubbling and water circulation were provided by diffused air pumped through a ring-shaped tube attached to the tank bottom. At a larval age of 3 days after hatching (DAH), fish were reared on five experimental larval feeding regimes consisting of live

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rotifers, *Artemia* nauplii, rotifers followed by *Artemia*, rotifers co-fed with inert diet and inert diet alone. Diets were supplied for 20 days and larval fish were sampled at different times to estimate the following parameters: Trypsinogen gene expression (*ssetryp1*) as described in Manchado, Infante, Ascencio, Crespo, Zuasti & Cañavate (2008), trypsin and chymotrypsin activities and changes in larval carbon stable isotope ($\delta^{13}C$) composition to estimate dietary carbon assimilation from Artemia, rotifers and inert feed (Gamboa-Delgado, Le Vay, Manchado, Ponce, Fernandez-Diaz, Zerolo & Cañavate, 2011).

Stable isotope analysis and sample pre-treatment

Samples of dietary items, shrimp and fish whole bodies and muscle tissue were oven-dried (24 h, 60 °C) and manually ground using mortar and pestle. As lipids are usually depleted in $\delta^{13}C$ relative to carbohydrates and protein (DeNiro & Epistein, 1978, Stenroth, Holmqvist, Nyström, Berglund, Larsson & Granéli, 2006), the formulated diets, the macroalgal biomass, whole shrimp bodies and muscle tissue samples were lipid extracted in order to reduce the variability of $\delta^{13}C$ values. Lipid extraction was done by suspending ground dietary items or minced tissue in a solution of chloroform:methanol for 12 h. Sub-samples of 1.0 mg were loaded into tin cups (Elemental Microanalysis Ltd, UK) to be analyzed for elemental nitrogen and carbon and their respective stable isotope ratios at natural abundance levels. Samples were analyzed using a PDZ Europa Scientific Roboprep elemental analyzer coupled to a PDZ Europa Hydra 20/20 stable isotope ratio mass spectrometer (Crewe, UK) at the Department of Plant Sciences of the University of California, Davis. $\delta^{13}C$ and $\delta^{15}N$ values are expressed in delta notation (δ), which is defined as parts per thousand deviations from the stable isotope ratios of their the respective standard reference materials: Pee Dee belemnite and atmospheric nitrogen. Every 12 measurements, two validation samples were run. Proportions of nitrogen and carbon in animal tissue derived from any two dietary sources were estimated using a two-source, one-isotope mixing model (Phillips and Gregg, 2001). The model considers the isotopic differences between the sources (dietary items and ingredients) and the mixture (animal tissue). The use of mixing models requires assumptions prior to use (see review by

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Martínez del Rio, Wolf, Carleton & Gannes 2009). One of these assumptions indicates that the consuming organism should be in isotopic equilibrium with its diet. In the present studies isotope values were monitored to ensure that isotopic equilibrium had been reached. Observed isotopic values in equilibrium were corrected for discrimination factors by introducing into the model reference isotopic values determined in animals fed exclusively on positive and negative control diets (*i.e.* 100% macroalgae biomass, 100% inert feed, 100% pea meal diet, 100% rotifers).

Statistical analysis

In order to examine differences, carbon and nitrogen contents and isotopic values of *U. clathrata* biomass and inert feed were compared by means of Student's *t*-tests. Similar comparisons were done for pea meal and fishmeal and for rotifers and larval inert feeds. Tissue isotopic values at different times, mean shrimp and fish wet weights and survival rates observed in different treatments were analyzed by one way ANOVA after variance homogeneity was verified by Levene's tests. When needed, Tukey's pairwise comparisons were used to detect treatments significantly differing from each other. Chi-square tests were applied to determine statistical differences in the expected (known dietary carbon and nitrogen proportions supplied by co-feeding regimes or experimental diets) and observed estimated proportions of dietary carbon and nitrogen incorporated in animal whole body and muscle tissue.

Results & Discussion

Experiment 1: Assessment of nutrient allocation in Pacific white shrimp co-fed live macroalgae *Ulva clathrata* and inert feed: dual stable isotope analysis

Higher weight gain was observed in shrimps reared on regime 75F/25U (1067 ±364 mg ww), followed by shrimps fed only inert feed (100F, 995 ±289 mg ww). Animals fed only on *U. clathrata* biomass (100U) showed minimal growth (221 ±49 mg ww) (Table 1) and very high metabolic turnover rates of carbon and nitrogen (data not shown). Isotopic

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values measured in inert feed ($\delta^{13}C$= -23.0 ‰, $\delta^{15}N$= 9.7‰) and macroalgae ($\delta^{13}C$= -13.1‰, $\delta^{15}N$= -3.5‰) were very contrasting and had a rapid influence on the isotopic values of shrimp. Animals reached full isotopic equilibrium through growth and/or fast metabolic turnover in only 2 weeks, except shrimps fed only macroalgae.

Table 1. Growth, survival rate and estimated food consumption (dry weight) by juvenile *Litopenaeus vannamei* reared on five different feeding regimes for 28 days (n= 8-20, mean values ±SD).

Feeding regime	Survival (%)	Final wet weight (mg)	Weight increase (%)	Consumed inert feed (g)	Consumed *U. clathrata* (g)
100A	95 ± 13[a]	995 ± 289[a]	429	0.94	-
75F/25U	93 ± 11[a]	1067 ± 364[a]	467	0.81	0.40
50F/50U	78 ± 11[ab]	768 ± 273[ab]	308	0.43	0.44
25F/75U	60 ± 21[b]	424 ± 207[b]	125	0.14	0.65
100U*	23 ± 4[c]	221 ± 49[c]	18	-	1.32

Initial wet weight = 188 ±28 mg.
Different superscripts indicate significant differences at $p<0.05$.
* Parameters in animals from feeding regime 100U were estimated at experimental day 21.
Tomado de Gamboa-Delgado et al., (2011b)

Fast isotopic changes in shrimp tissue have been previously reported (Al Maslamani 2006; Le Vay & Gamboa-Delgado, 2011), which led to fast isotopic equilibriums between diets and shrimps (15 to 20 days). At the end of the experiment, $\delta^{13}C$ and $\delta^{15}N$ values in shrimp reared on all co-feeding regimes were strongly biased towards the isotopic value of *U. clathrata*. Results hence indicated that shrimps in the co-feeding regimes incorporated significantly higher amounts of dietary carbon and nitrogen from the macroalgal biomass (Table 2). Shrimps in treatments 75F/25U incorporated 52% of carbon from the inert feed and 48% from the macroalgae. Animals under feeding regimes 50F/50U and 25F/75U incorporated higher amounts of dietary carbon from *U. clathrata* (65-89%) when compared to carbon proportions supplied by both co-feeding regimes (33-70%) and also incorporated the majority of nitrogen from the macroalgae. However, a high incorporation of nitrogen was not reflected in high growth in the latter treatments as metabolic turnover rates were

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very high and accounted for most of the observed isotopic change in tissue (not caused by tissue accretion).

Table 2. Estimated relative proportions of total dry matter and dietary carbon and nitrogen supplied from inert feed and live *U. clathrata* and contributing to the growth of juvenile *L. vannamei* as indicated by a two-source, one-isotope mixing model (mean ± CI, n = 9).

Feeding regime	Expected	Observed					
		Whole bodies			Muscle tissue		
		min.	mean	max.	min.	mean	max.
Carbon							
75F/25U							
Inert feed	79.9[a*]	59.8	68.2[b]	76.7	69.7	73.0[a]	76.3
Ulva biomass	20.1	23.3	31.8	40.2	23.7	27.0	30.3
50F/50U							
Inert feed	66.5[a]	44.3	50.9[b]	57.5	55.7	60.3[a]	64.9
Ulva biomass	33.5	42.5	49.1	55.7	35.1	39.7	44.3
25F/75U							
Inert feed	30.5[a]	8.8	20.2[b]	31.6	21.5	30.5[a]	39.6
Ulva biomass	69.5	68.4	79.8	91.2	60.4	69.5	78.5
Nitrogen							
75F/25U							
Inert feed	79.6[a]	13.1	26.7[b]	40.2	22.2	33.3[b]	44.3
Ulva biomass	20.4	59.8	73.3	86.9	55.7	66.7	77.8
50F/50U							
Inert feed	66.1[a]	0	4.2[b]	13.3	5.4	12.6[b]	19.7
Ulva biomass	33.9	86.6	95.8	100	80.3	87.4	94.6
25F/75U							
Inert feed	30.1[a]	0	2.0[b]	16.8	0.7	6.1[b]	11.4
Ulva biomass	69.9	83.2	98.0	100	88.6	93.9	99.3
Total DM**							
75F/25U							
Inert feed	66.9[a]	19.3	30.3[b]	41.3	23.1	30.3[b]	37.5
Ulva biomass	33.1	58.7	69.7	80.7	62.5	69.7	76.9
50F/50U							
Inert feed	49.4[a]	8.5	20.2[b]	32.0	8.7	19.4[b]	30.2
Ulva biomass	50.6	68.0	79.8	91.5	69.8	80.6	91.3
25F/75U							
Inert feed	17.7[a]	0	6.9[b]	20.0	0	8.4[b]	20.3
Ulva biomass	82.3	80.0	93.1	100	79.7	91.6	100

*Superscripts indicate significant differences between expected and mean observed dietary contributions.
**Total dry matter contributions were estimated after correcting for carbon and nitrogen concentrations measured in both food sources using the equation proposed by Fry (2006).
Tomado de Gamboa-Delgado et al. (2011b)

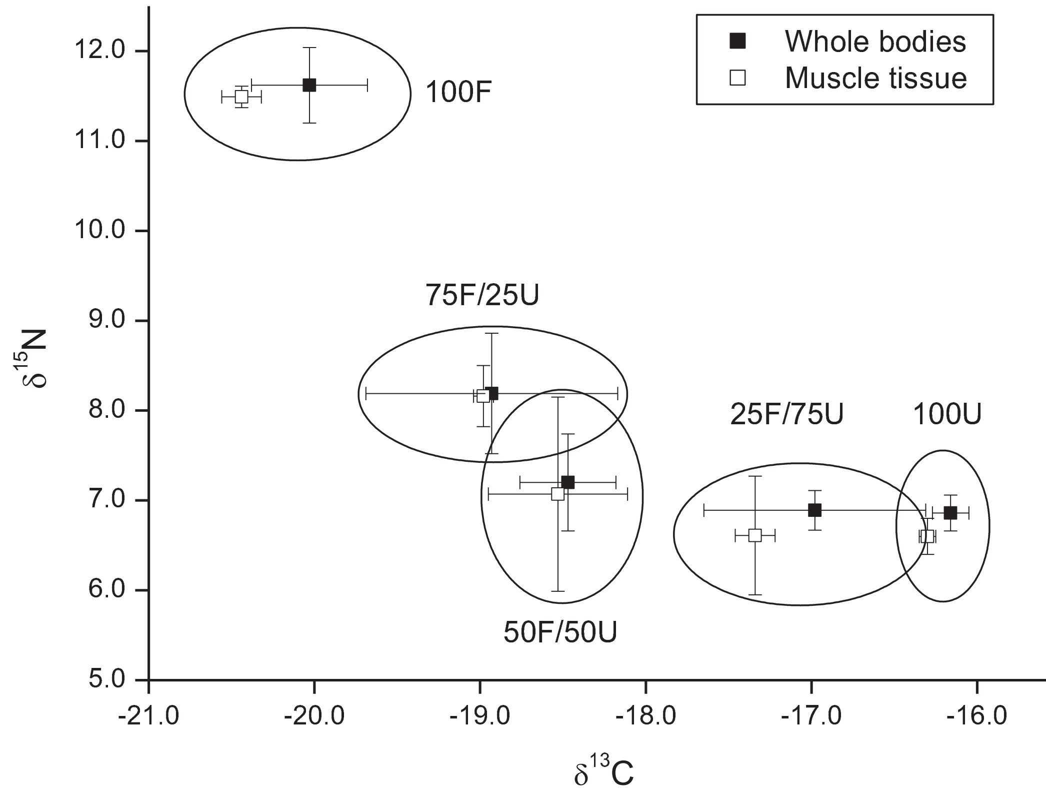

Figure 1. Carbon and nitrogen dual isotope (‰) plot of whole bodies and muscle tissue of white shrimp *L. vannamei* fed on different proportions of inert feed and live *U. clathrata* biomass. 100F and 100U correspond to values measured in shrimps fed exclusively on inert feed or macroalgae and are thus considered as the isotopic discrimination-corrected values of both food sources. Muscle tissue values for treatment 100U were estimated for day 28 from values in whole bodies. n= 2-4, mean values ±SD.
(Gamboa-Delgado *et al*., 2011b)

Figure 1 combines $\delta^{13}C$ and $\delta^{15}N$ values of shrimp whole bodies and muscle tissue and provides a graphic indication of the total organic matter contributed by inert feed and macroalgae. The higher than expected contributions of dietary carbon (32 – 80%) and nitrogen (73 – 98%) to the shrimp whole bodies and muscle tissue are possibly related to the higher digestibility of *U. clathrata* and its continuous availability in the experimental tanks. Only two daily rations of inert diet were supplied to shrimp in the co-feeding

regimes. Despite lower nutrient concentration as compared to inert feeds, live food contains higher water content (~80%) which contributes to their higher digestibility (Conceição, Yúfera, Makridis, Morais, Dinis, 2010). In contrast, inert feed can contribute nutrients that are scarce or absent in the natural productivity or live food, but the incorporation of such nutrients is limited by low feed digestibility or unsuitable formulation.

Experiment 2: Incorporation of dietary nitrogen from fish meal and pea meal (*Pisum sativum*) in muscle tissue of *L. vannamei* fed low protein compound diets

While there were no significant differences in survival rate among treatments, shrimp growth rates were negatively correlated to dietary pea meal inclusion (Table 3). Animals in all dietary treatments reached isotopic equilibrium in 3 weeks. Pea meal and fish meal showed very contrasting nitrogen stable isotope values (16.6 ±0.1 and 1.5 ±0.4 ‰, respectively) and the experimental diets had different isotopic values that started to be reflected in shrimp muscle tissue over the first days of the trial.

Table 3. 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.

	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

Different superscripts indicate significant differences (P<0.05) for that particular column.
Tomado de Martínez-Rocha et al. (2012)

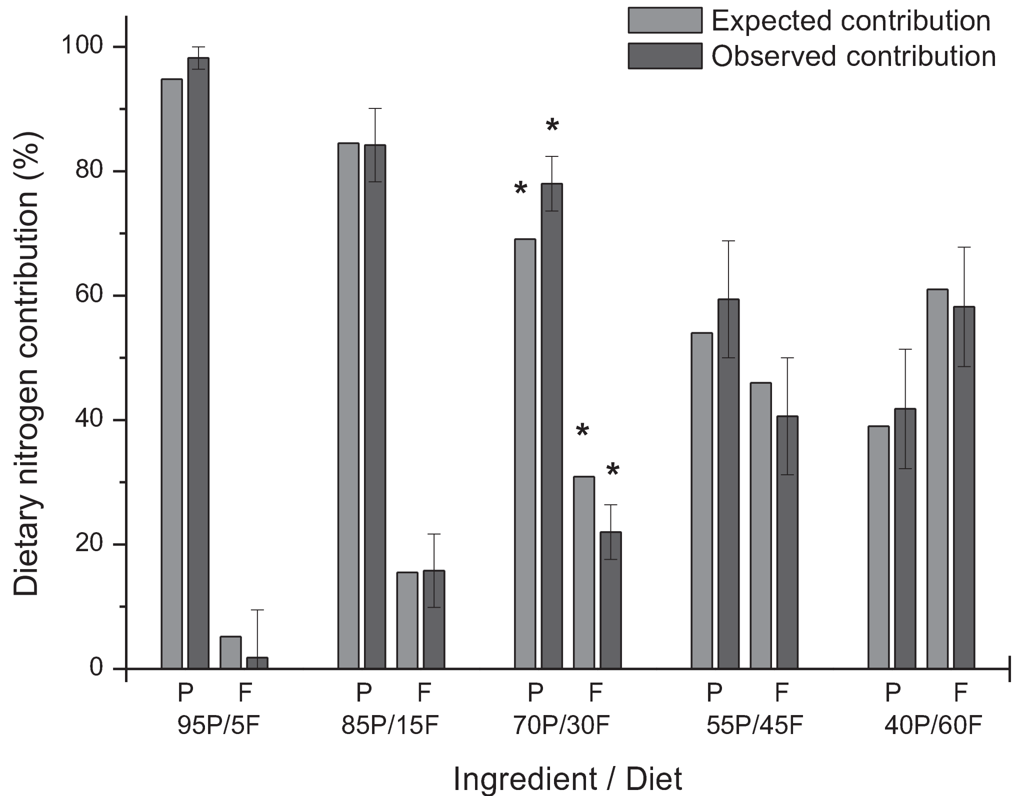

Figure 2. Expected and observed proportions of dietary nitrogen contributed by pea meal and fish meal to the growth of abdominal muscle tissue of Pacific white shrimp *L. vannamei* fed diets formulated with varying proportions of both ingredients. Contributions were estimated using a one-isotope, two-source mixing model (Means ±Confidence Intervals, n = 12). * denotes significant differences at $P<0.05$. (Martínez-Rocha *et al*., 2012).

Results from an isotope mixing model indicated that the relative contributions of dietary nitrogen from pea meal and fish meal to shrimp muscle tissue were similar to the expected contributions indicated by the dietary nitrogen proportions established in the dietary formulations (Fig. 2). The only exception was observed in shrimps fed on diet containing 70% pea meal, where the dietary nitrogen contribution from pea meal to muscle tissue was significantly higher (78.0 %) than the dietary nitrogen established by the formulation in this diet (69.1 %). Results highlight the effectiveness of stable isotope analysis to assess nutritional contributions of specific ingredients as alternatives to the use of fish meal in

aquaculture feeds. Plant proteins are ideally suited for nutritional evaluations using stable isotopes at natural abundance levels as they have very contrasting nitrogen and carbon isotopic values when compared to feedstuffs from marine sources. Moreover, the consistent isotopic pattern of variation in plants among amino acids that are essential for animals suggests its use as useful biomarkers in animals for plant-derived protein (O'Brien, Boggs & Fogel 2005). The increasing demand for aquaculture feeds and their rising costs have stressed the need to evaluate ingredients based on domestic plant crops and recent advances in processing technologies have provided more selected products with improved nutritional value for marine organisms (Gouveia & Davies, 2000).

Experiment 3: Effect of different diets on proteolytic enzyme activity, trypsinogen gene expression and dietary carbon assimilation in Senegalese sole larvae

At the end of the experiment, there were significant differences among treatments in mean final dry weight, survival and metamorphosis but not in total length. Fish fed rotifers and rotifers followed by Artemia showed higher dry mean weights at the end of the experiment (Fig. 3). Higher survival was observed in both latter treatments and in the rotifer/inert feed treatment. $\delta^{13}C$ values in fish tissue were quickly influenced by their respective dietary regimes (Fig. 4a). From 3 DAH onwards, isotopic changes were very fast in fish fed on rotifers, Artemia, rotifers followed by Artemia and co-fed inert diets and rotifers. However, larvae fed only Artemia showed a delay in isotopic influence on tissue as compared to the other treatments. Carbon isotopic shifts were not distinguishable between fish and inert diet because $\delta^{13}C$ values in the latter were similar to those observed in starved fish. Further changes in $\delta^{13}C$ values influenced by the dietary shifts to Artemia (6 DAH in the Rotifers/Artemia treatment and 11 DAH in all other treatments) were also clearly observed as steep isotopic changes in fish tissue (Fig. 4b). $\delta^{13}C$ values in diet and fish from the Co-feeding group allowed for estimating
the contribution of each dietary source to tissue growth. $\delta^{13}C$ values indicated that when rotifers and inert diet are co-fed in similar dry weight proportions, they supply significantly different levels of dietary carbon to the growth of fish larval tissue. Results from the

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isotopic mixing model indicated that by 10 DAH, from 84 to 96% of the incorporated dietary carbon in tissue was derived from rotifers and only 4 to 14% was provided by the inert diet.

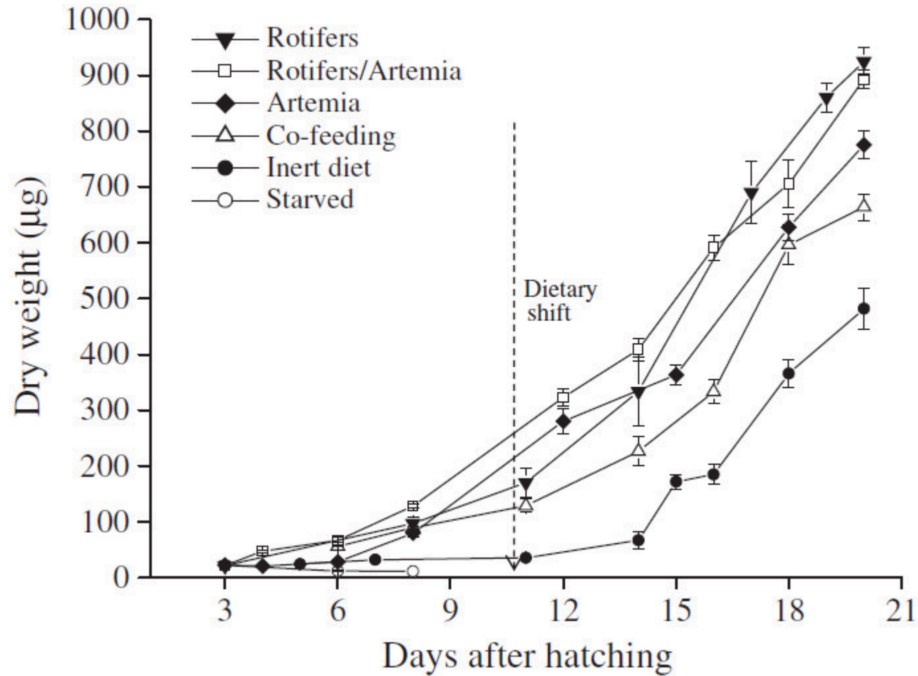

Figure 3. Larval growth of *Solea senegalensis* reared on different diets supplied from 3 to 10 DAH followed by a dietary shift to enriched *Artemia* metanauplii from 11 to 20 DAH (except treatment Rotifers-Artemia, 6 DAH). Mean of 15 samples, vertical bars indicate standard deviations. (Gamboa-Delgado *et al.,* 2011a).

The lower incorporation of carbon from the inert diet suggests a strong selection of live food and a low digestibility and/or assimilation of nutrients from the former. From a nutritional perspective, the faster the animal isotopic composition resembles the dietary isotope values, the greater is the nutrient retention from the diet (Buchheister & Latour, 2010; German & Miles, 2010). Fish larvae fed only Artemia showed reduced growth and survival, most likely due to less efficient prey capture and low availability of specific nutrients. Trypsin and chymotrypsin activities

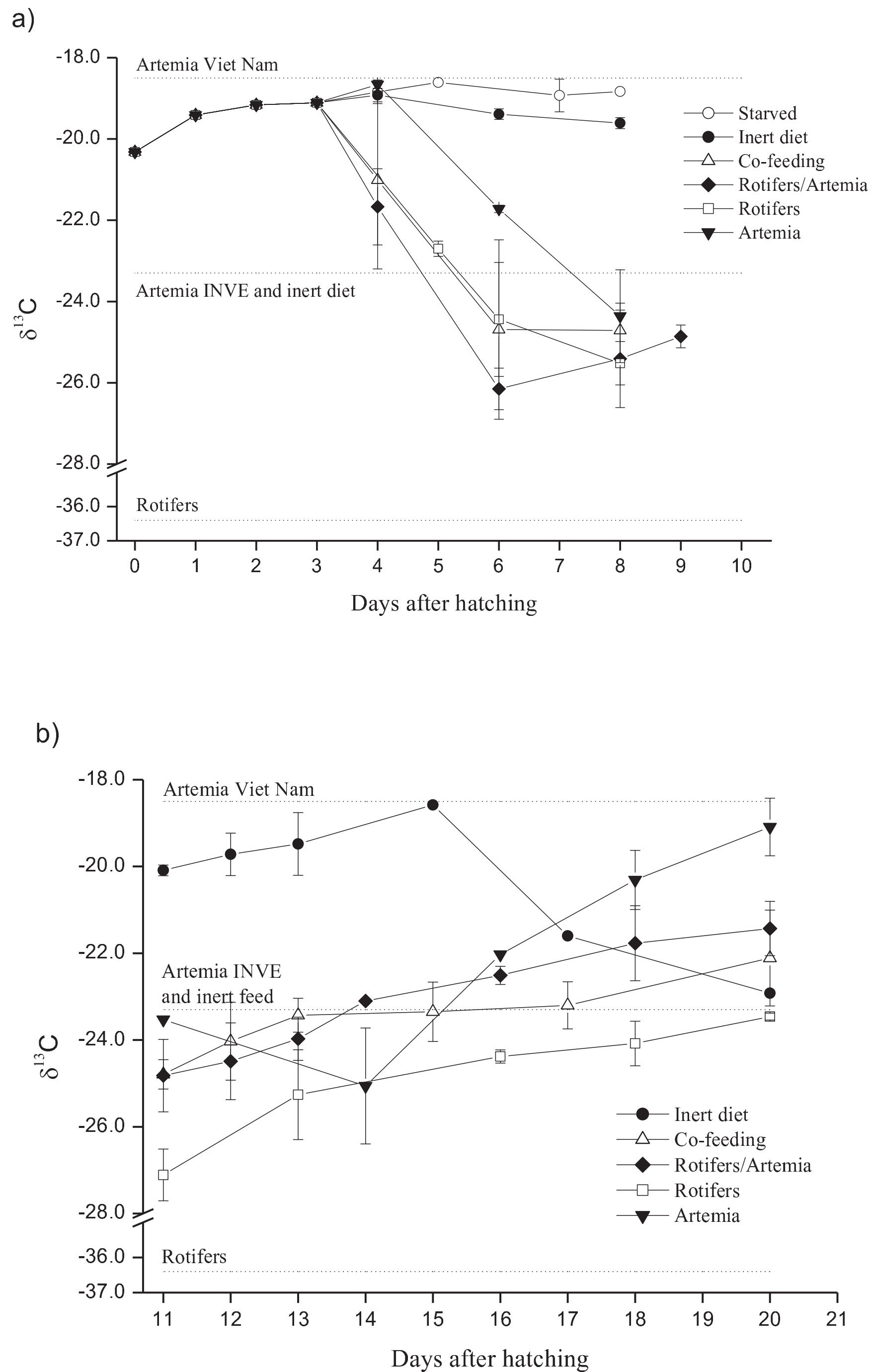

Figure 4. Changes in carbon isotope values (‰) in body tissue of *Solea senegalensis* reared on five different diets from 0 to 10 DAH (a) and from 11 to 20 DAH (b). Diets were shifted to enriched *Artemia* metanauplii from 11 to 20 DAH, except treatment Rotifers-Artemia (6

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DAH). Mean of 15 samples, vertical bars indicate standard deviations. (Gamboa-Delgado *et al.*, 2011a).

increased from 3-4 days after hatching (DAH) in all dietary treatments, while *ssetryp1* transcripts increased at 4-5 DAH only in larvae fed live prey. *ssetryp1* gene expression was activated later in larvae fed only *Artemia* and this corresponded with *Artemia* $\delta^{13}C$ values being reflected in larval tissue (Fig. 5).

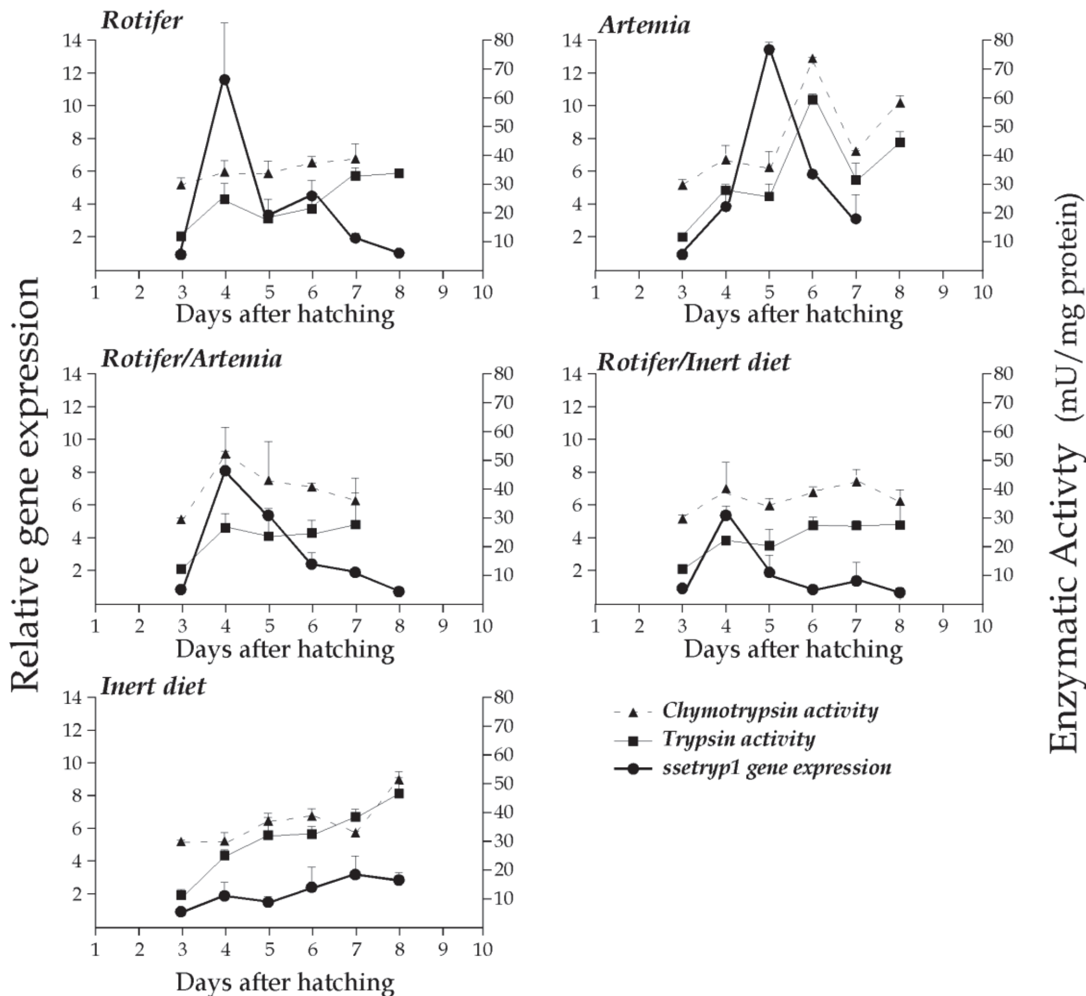

Figure 5. Specific activity of trypsin and chymotrypsin (mU mg protein^{-1}, right axis) in whole *Solea senegalensis* larvae fed different feeding regimes (means ± SD, n = 3) and relative *ssetryp1* expression (left axis). Data were expressed as the mean fold change from a calibrator group (3 DAH). Mean values ± SD, n = 3.

(Gamboa-Delgado *et al*., 2011a)

Supplying Artemia as the only food source was reflected in lower survival at the end of the experiment, indicating limited ability to capture or ingest Artemia nauplii at these early larval stages. This hypothesis is also supported by *ssetryp1* gene expression and trypsin enzymatic assays. In larvae fed only rotifers, *ssetryp1* transcripts increased rapidly with a peak at 4 DAH. In contrast, fish from the Artemia group showed mRNA levels and trypsin activity peaking at 5 and 6 DAH, respectively, probably associated to the ability of slightly older larvae to capture Artemia nauplii. Early sole larvae (3 DAH) have an average mouth opening size of 400 μm (R. Zerolo, personal communication), while the length of the Artemia strains used in the present experiment averaged 530 μm in nauplii and 615 μm in metanauplii. Prey capture is influenced by different factors including visual acuity, prey contrast, shape, mobility or concentration, with the relationship between mouth size and prey size being the most critical factor (Cunha and Planas, 1999). Although inert diet was consumed, as indicated by microscopic observations, fish larvae in the Inert diet and co-feeding groups exhibited significantly lower weight than fish from the other feeding regimes at 20 DAH. These results are in agreement with studies reporting reduced larval performance when high proportions of inert diets are provided to marine fish (Teshima, Ishikawa & Koshio 2000; Engrola, Figueira, Conceição, Gavaia, Ribeiro & Dinis 2009). The poor performance of larval artificial diets has been related to the inadequate incorporation of nutrients by the fish due to poor ingestion, digestion and/or assimilation (see review by Kolkovski, 2001). In this context, although fish larvae co-fed live and inert diets were significantly smaller in weight at 20 DAH, survival rates were high. The use of co-feeding regimes from mouth opening has been reported to produce better quality postlarvae at weaning (68 DAH) in sole (Engrola et al., 2009). Possible explanations for improved larval performance and increased nutrient assimilation observed when live and dry diets are co-fed are the contribution of digestive enzymes from the live food organisms and an increased supply of more suitable nutrients (Kolkovski, Tandler, Kissil & Gertler, 1993; Rosenlund, Stoss & Talbot, 1997). Moreover, modulation of trypsinogen gene

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expression by feeding regime is another factor to be taken into account. *Ssetryp1* gene expression (but not trypsin) was modulated by feeding regimes showing a clear relationship with growth and survival. Dietary treatments supporting optimal growth and survival increased expression levels rapidly, whereas no activation was detected in larvae fed inert diet. Moreover, larvae co-fed on rotifers and inert feed showed lower *ssetryp1* transcripts than larvae fed live preys, while those fed on Artemia increased mRNAs one day later. Together, these results suggest that quantification of *ssetryp1* transcripts could be used as a biomarker for evaluation of digestive capacity and growth in early life stages of sole during the planktonic and benthic phases. Analysis of digestive enzymes also provides valuable information on nutrient utilization over time and under different nutritional conditions, while stable isotope analysis represents an additional nutritional tool to assess nutrient incorporation from different dietary sources and to estimate elemental turnover rates in tissue.

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