Assessment of Nutrient Allocation and Metabolic Turnover Rate in Pacific White Shrimp *Litopenaeus vannamei* Co-Fed Live Macroalgae *Ulva clathrata* and Inert Feed: Dual Stable Isotope Analysis

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ASSESSMENT OF NUTRIENT ALLOCATION AND METABOLIC TURNOVER RATE IN PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI* CO-FED LIVE MACROALGAE *ULVA CLATHRATA* AND INERT FEED: DUAL STABLE ISOTOPE ANALYSIS

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ABSTRACT The current study quantified the relative contribution of dietary carbon and nitrogen supplied by live biomass of the green macroalgae *Ulva clathrata* and a commercial inert feed to the growth of juvenile shrimp *Litopenaeus vannamei*. The stable isotope ratios of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) were analyzed in both food sources, whole bodies, and muscle tissue of shrimp reared on co-feeding regimes where 75%, 50%, and 25% of daily consumed macroalgal biomass was substituted by inert feed (regimes 75F/25U, 50F/50U, and 25F/75U, respectively). Higher growth rates were observed in shrimp fed regime 75F/25U ($k = 0.062$), followed by shrimp fed only inert feed (100F, $k = 0.060$). Animals reared only on *U. clathrata* (100U) showed minimal growth ($k = 0.008$) and very high metabolic turnover rates of carbon and nitrogen. Isotopic 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 highly contrasting and both had a rapid influence on the isotopic values of shrimp. Animals reached full isotopic equilibrium through growth and fast metabolic turnover in only 2 wk, except shrimp fed macroalgae only. 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 toward the isotopic values of *U. clathrata*. Total dry matter contributions to growth were estimated using an isotope mixing model, and considered the elemental concentration of both nutritional sources. Results indicated that shrimp in the co-feeding regimes incorporated significantly higher amounts of dietary carbon and nitrogen from the macroalgal biomass. Shrimp in treatment 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 with 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 larger growth in the latter treatments because metabolic turnover rates were very high. Estimated turnover rates ranged from 0.049–0.191/day for carbon and from 0.013–0.100/day for nitrogen, and values followed an increasing trend as a function of macroalgae consumption. Nitrogen halftimes in tissue consistently decreased throughout the different treatments from 9.5 days (100F) to 6.4 days (100U). Proportions of incorporated nutrients in muscle tissue followed similar patterns as those observed in whole bodies.

KEY WORDS: Pacific white shrimp, *Litopenaeus vannamei*, *Ulva clathrata*, stable isotopes, nutritional contributions, turnover rates

INTRODUCTION

The assessment of isotopic signatures in dietary sources and consuming organisms has allowed tracing the origin and fate of nutrients in field and laboratory studies. As a result of their high natural abundance, the stable isotope ratios of carbon and nitrogen ($^{13}C/^{12}C$ and $^{15}N/^{14}N$, respectively measured as $\delta^{13}C$ and $\delta^{15}N$) are the most commonly used in identifying nutrient and energy sources, and have been a very effective tool in assessing nutrient flows in aquatic systems (Michener & Schell 1994). Isotopes of the same element take part in the same chemical reactions, but different isotopes contain different numbers of neutrons and they react at different rates; thus, enzymatic discrimination and differences in kinetic characteristics can result in metabolic products that are isotopically different than their precursor substrates. Mass-balance mixing models can be applied to estimate dietary contributions under the assumption that the isotopic composition of a consuming organism equals the weighted average of the isotopic composition of its diet constituents (Martínez del Rio & Wolf 2005). The use of mixing models has been of special assistance to conduct dietary reconstructions in animals living in marine and littoral environments (Riera & Richard 1996, Dang et al. 2009, Granek et al. 2009), and to develop experimental studies aimed at quantifying nutrient incorporation in farmed marine species (Schroeder 1983, Burford et al. 2004, Gamboa-Delgado & Le Vay 2009b, Matsuda et al. 2009). The use of preanalyzed dietary components with contrasting isotopic values allows distinguishing and quantifying the contribution of resources after they have been allocated physiologically (Fischer et al. 2004, Gamboa-Delgado & Le Vay 2009a). The dietary contributions to growth can be expressed as elemental concentrations, bulk organic matter, or incorporated energy. On the other hand, diet-elicited isotopic shifts can be integrated with time or biomass increase through the use of exponential models (Fry & Arnold 1982, Hesslein et al. 1993, Carleton & Martínez del Rio 2010) that allow discriminating the isotopic changes caused either by diet (isotopic dilution through somatic growth) or by metabolic turnover.

The Pacific white shrimp *Litopenaeus vannamei* has become the main crustacean species produced through aquaculture since 2003 (FAO 2007). The continued growth of shrimp aquaculture is demanding increasingly higher amounts of feeds and, in this context, improved feeds and feeding strategies would optimize feed utilization. Because of their nutritional properties, several seaweed meals have been used as dietary supplements in shrimp feeds (Briggs & Funge-Smith 1996, Peñaflorida & Golez 1996, Cruz-Suárez et al. 2009a). The chemical composition of macroalgae

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varies among species and environmental conditions; however, most are rich in nonstarch polysaccharides, vitamins, and minerals (Mabeau & Fleurence 1993, Wong & Cheung 2000). In general, green macroalgae (Chlorophyceae) may have 2–3 times more protein content than brown seaweeds (Burtin 2003). The green macroalgae *Ulva clathrata*, (Roth) C. Agardh 1811 (= *Enteromorpha clathrata* (Roth) Greville 1830) has been cultured during past years under a patented technology developed by Aonori Aquafarms Inc. (formerly Sinaloa Seafields). Under this methodology, macroalgae biomass is grown on the water surface to accelerate the production without eliciting detrimental effects to the environment (Moll & Deikman 1995, Moll 2004, Moll et al. 2006). Although it has been observed that use of macroalgae biomass alone as feed do not cover the nutritional requirements for optimal growth in marine shrimp (Marinho-Soriano et al. 2007, Cruz-Suárez et al. 2010), coculture of *U. clathrata* and Pacific white shrimp *L. vannamei* (Boone 1931) has been conducted with positive results in terms of lower feed utilization and improvement of shrimp nutritional and organoleptic (flesh color and texture) quality. Nevertheless, the direct nutritional contribution of *U. clathrata* to shrimp in coculture conditions was not clearly demonstrated as a result of the interaction with the natural productivity in the experimental tanks (Cruz-Suárez et al. 2010). We hypothesized that although the live macroalgae by itself is not nutritionally complete for white shrimp, it supplies a significant proportion of structural carbon and nitrogen when it is co-fed with inert feed. Therefore, taking advantage of the contrasting natural carbon and nitrogen stable isotope values measured in a commercial inert feed and in live macroalgal biomass of *U. clathrata*, the current study aimed to quantify the relative contribution of dietary carbon and nitrogen to the growth of Pacific white shrimp fed different proportions of both food sources. In addition, carbon and nitrogen turnover rates in whole shrimp bodies were also estimated and compared among dietary treatments.

MATERIALS AND METHODS

Experimental Organisms

Thalli of farmed, live *U. clathrata* was donated by Aonori Aquafarms Inc. (San Diego, CA). To produce locally macroalgal biomass having known $\delta^{13}C$ and $\delta^{15}N$ values, 400 g (wet weight (WW)) thalli were separated into smaller batches and attached individually to a rope. Series of "seeded" ropes were set in plastic trays containing artificial seawater (Fritz, Chemical Co., Mesquite, TX) enriched with a commercial fertilizer (NPK 30-10-10). The fertilized seawater was completely renewed every 15 days, sterilized using a solution of sodium hypochlorite, and neutralized after 12 h using sodium thiosulfate and strong aeration. Macroalgae biomass grew rapidly in this system under the following conditions: temperature, 17.1 ± 1.5°C; salinity, 35.0 ± 0.5 g/L; pH, 8.0 ± 0.2; light/dark cycle, 12 h/12 h, with the dark cycle established from an array of fluorescent tubes. Dissolved oxygen and carbon dioxide concentration in seawater were not monitored as the macroalgae takes up atmospheric gases when it grows close to or above the water surface. Periodic microscopic observations of the algal biomass were conducted to verify that no epibionts were present on the rapidly growing filaments. Only a few free-living diatom cells were observed on the days previous to the total water exchange. Pacific white shrimp (*L. vannamei*) late postlarvae were donated by a commercial facility (Langostinos y Camarones de Oriente, Veracruz, Mexico). On reception, animals were acclimated in 400-L fiberglass tanks for 20 day and, to confer a known isotopic baseline to shrimp tissue before the start of the experiment, they were fed exclusively on a crumbled commercial diet (35% protein, Grupo Costamar, Hermosillo, Mexico). Proximal analysis of this feed was determined as described in Cruz-Suárez et al. (2009b). Feed was analyzed for elemental carbon, nitrogen, and their respective isotopic values. This inert feed was also supplied during the experimental feeding period.

Experimental Design and Sampling

The feeding trial was carried out in an indoor array of ten 60-L-capacity tanks fitted individually with airlifts. Twenty juvenile shrimp weighing 188 ± 28 mg WW were transferred to each experimental tank. Tanks received a water exchange of 800% per day ^{1} and were connected to a recirculation system consisting of double mechanical cartridge filters (50 μm), a UV filter, three individual protein skimmers, and a bubble bead biological filter. The following mean experimental conditions were maintained throughout the duration of the trial: temperature, 30.1 ± 0.5°C; salinity, 34.5 ± 0.9 g/L; pH, 8.4 ± 0.1; and saturated dissolved oxygen levels. 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 cycle of 12 h/12 h from fluorescent lamps. Five different feeding regimes were designed to supply similar amounts of dry matter by estimating the dry weight of macroalgae biomass and by correcting for the moisture content in the inert feed (10.3%). Feeding regimes were assigned to duplicate tanks and consisted of 100% inert feed (treatment 100F, positive isotopic control), 100% macroalgae (treatment 100U, negative isotopic control), and 3 co-feeding regimes where 75%, 50%, and 25% of the daily consumed macroalgal biomass was substituted by inert feed (regimes 75F/25U, 50F/50U, and 25F/75U, respectively) on a dry weight basis. Two grams of live *U. clathrata* were supplied to shrimp by attaching the algal biomass to 9 × 9-cm sandwich-type, plastic mesh units from which the algal filaments were constantly available and easily nibbled upon by the shrimp. Two units were placed in each respective tank. Shrimp receiving inert feed were fed twice daily at 0800 HR and 1600 HR. Feeding rations and proportions were progressively adjusted on a daily basis in relation to the amount of macroalgal biomass consumed, observed survival, and number of sampled animals. Figure 1 depicts the experimental feeding methodology applied in the current experiment.

Amounts and proportions of inert feed and macroalgae supplied to shrimp were estimated on a dry weight basis and as total biomass consumption. Values in these proportions were converted to dietary carbon and nitrogen supplied to compare elemental concentration values observed in shrimp tissue. Two shrimp from every tank were randomly sampled on experimental days 0, 3, 7, 14, 21, and 28. To minimize the amount of food present in the gut, sampled animals were starved overnight in individual containers holding filtered seawater. It has been estimated that complete gut evacuation time in this species is approximately 3 h (Beseres et al. 2006). Animals were killed in ice/water slurry, rinsed with tap water, weighed to the nearest

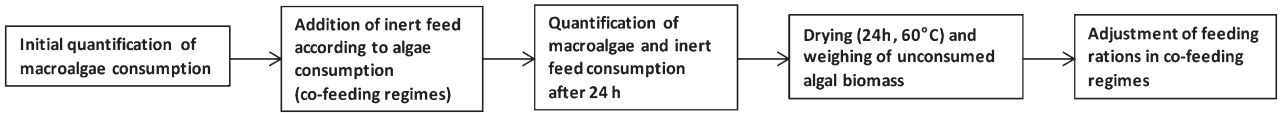

Figure 1. Feeding methodology used to rear *L. vannamei* on co-feeding regimes consisting of different proportions of pelleted inert feed and live biomass of macroalgae *Ulva clathrata*.

milligram, and kept in labeled vials at –80°C until pretreatment for isotopic analysis.

Stable Isotope Analysis and Estimation of Nutrient Contribution

Samples of macroalgae biomass, inert feed, whole shrimp, and muscle tissue were desiccated (60°C until constant weight) and ground manually in a mortar to obtain a fine powder. Lipids are usually depleted in ^{13}C relative to carbohydrates and protein (De Niro & Epstein 1978, Stenroth et al. 2006); therefore, to reduce the variability of $\delta^{13}C$ values in both feeding sources and animal tissue, samples of inert feed, macroalgae, and whole shrimp bodies were lipid extracted using the method of Beaudoin et al. (2001) by suspending the ground material in a 50:50 solution of chloroform–methanol for 2 h. Samples were solvent treated twice, oven-dried (60°C until constant weight), homogenized again, and kept in a desiccator. A series of aliquots and muscle tissue samples were left untreated to interpret results on $\delta^{15}N$ values. Samples from 900–1,100 µg were packed in tin cups (D1008; Elemental Microanalysis Ltd., Okehampton, UK). Elemental and isotopic analyses were conducted as described in Gamboa-Delgado & Le Vay (2009b). Isotopic results are expressed in delta notation (δ), which is defined as part per thousand (‰) deviations from the $\delta^{13}C$ and $\delta^{15}N$ values of the standard reference materials (Pee Dee belemnite and atmospheric nitrogen, respectively). Repeated measurements of a calibration standard indicated that instrument precision (SD) was 0.15‰ ($\delta^{13}C$) and 0.08‰ ($\delta^{15}N$). Relative proportions of dietary carbon and nitrogen contributing to shrimp growth and originating either from *U. clathrata* biomass or the inert diet were estimated using a 2-source, 1-isotope mixing model (Phillips & Gregg 2001). Estimation of isotopic discrimination factors ($\Delta_{consumer\ diet}$) increases the output accuracy of the mixing models and, in the current study, control values were taken from the isotopic differences between whole tissue of shrimp fed only inert feed (positive control) and *U. clathrata* biomass (negative control) after reaching isotopic equilibrium. Estimated relative dietary carbon and nitrogen incorporation values from both sources are expressed as means and their 95% confidence intervals (truncated to 0% and 100%). To obtain an estimate of the relative dry matter contribution to growth from *U. clathrata* biomass and inert feed, mean dual stable isotope values for each feeding source and shrimp (from the 3 co-feeding regimes) were corrected for isotopic discrimination factors and plotted. Euclidean distances between plotted dual isotopic values of *U. clathrata*, inert feed and shrimp (*S*) were calculated by $Z = \sqrt{X^2 + Y^2}$ and compared. Distances between *U. clathrata* (*A'*) and inert feed (*B'*) to shrimp are inversely related to the relative dry weight organic matter contribution of *U. clathrata* and inert feed to the shrimp's diet (i.e., the shorter the distance, the greater the contribution). Because of this inverse relationship, the relative dry matter contribution of each food source was calculated as

$$\%\ X \text{ in diet} = \left(SX'^{-1} / SA'^{-1} + SB'^{-1}\right)100 \quad (1)$$

where X' is A' or B'.

Preliminary analysis indicated that carbon and nitrogen contents in *U. clathrata* biomass and inert feed were significantly different (C = 20.9 ± 1.4% and 41.2 ± 0.9%, respectively, $t = 23.3$, $P < 0.0001$; N = 3.0 ± 0.4 and 5.6 ± 0.1%, respectively, $t = 14.7$, $P < 0.0001$); therefore, organic matter contributions estimated by dual isotopic values were corrected for elemental concentration (C and N) using the equation proposed by Fry (2006).

Estimation of Carbon and Nitrogen Turnover Rates

Isotopic changes were monitored throughout the experimental period in shrimp following a dietary shift from the basal diet to the experimental feeding regimes. Predicted isotopic values generated by an exponential model describing isotopic change (Hesslein et al. 1993) were fitted to observed values to obtain an estimate of the metabolic carbon and nitrogen turnover rates in whole shrimp bodies. The coefficients of this model indicate the magnitude of the isotopic rate of change in relation to growth (k) and metabolic turnover (m), both coefficients also provide an indicator of the time necessary for half of the body tissue to be replaced after consuming a new diet (residency halftime, t_{50}):

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

Nutritional sources that are isotopically distinct represent an advantage in estimating turnover rates and nutrient incorporation as output resolution of the mixing models is improved when isotopic values of dietary components are contrasting.

Statistical Analysis

To examine differences, carbon contents and $\delta^{13}C$ values of *U. clathrata* biomass and inert feed were compared by means of Student's *t*-tests. Tissue isotopic values at different times, mean shrimp WW, and survival rates in shrimp from the different treatments were analyzed by 1-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-squared goodness of fit tests were applied to determine statistical differences in the expected (known dietary carbon and nitrogen proportions supplied by the co-feeding regimes) and observed estimated proportions of dietary carbon and nitrogen incorporated in shrimp whole bodies and muscle tissue. For nutrient turnover rate assessments, the treatment-specific growth rate constant, k, was estimated by fitting an exponential growth model to observed weights, $k = log$(final weight/initial weight)/time (days), whereas the unknown parameter m in Eq (2) was estimated by

TABLE 1.

Growth, survival rate, and estimated food consumption (dry weight) by juvenile *Litopenaeus vannamei* reared on 5 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]	1,067 ± 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 on experimental day 21.

iterative nonlinear regression using the Hesslein et al. (1993) model. All tests were done using SPSS 17.0 software (SPSS Inc., Chicago, IL) at a significance level of $P < 0.05$.

RESULTS

Growth and Survival

There was a high variability in the final growth of shrimp in the different dietary treatments; however, a clear tendency for a higher growth rate was observed in shrimp reared on regime 75F/25U ($k = 0.062$, 1,067 ± 364 mg final mean WW), followed by shrimp fed inert feed only ($k = 0.060$, 995 ± 289 mg WW). Shrimp from both feeding regimes increased their WW more than 400% (Table 1). Animals fed only on *U. clathrata* biomass showed a very low growth rate ($k = 0.008$, 221 ± 49 mg WW), and only 23% of the animals in this treatment survived by day 21, when remaining animals were collected for biometric and isotopic analysis. Higher survival rates (93–95%) were observed in shrimp reared on feeding regimes 100F and 75F/25U, whereas shrimp in dietary treatments 50F/50U and 25F/75U had respective mean survival rates of 78% and 60%.

Isotopic Shift and Discrimination Factors

The inert feed and the live macroalgal biomass showed very contrasting $\delta^{13}C$ (–23.0 ± 0.1‰ and –13.1 ± 0. ‰, respectively) and $\delta^{15}N$ values (9.7 ± 0.2‰ and –3.5 ± 0.4‰, respectively). $\delta^{13}C$ and $\delta^{15}N$ values in live algal biomass varied slightly throughout the experimental period as a result of different growth stage and cold storage; however, this variability was accounted for in the isotopic mixing models applied to estimate relative dietary contributions. Both food sources had a rapid influence on the $\delta^{13}C$ and $\delta^{15}N$ values of shrimp in all treatments. At the end of the experiment, isotopic values of shrimp tissue reared on co-feeding treatments were strongly biased toward the isotopic values of the *U. clathrata* biomass, with the effect being more pronounced for $\delta^{15}N$ values. On experimental day 3, there was an increase in $\delta^{13}C$ values in whole body tissue of shrimp in dietary treatments 50F/50U and 25F/75U (Fig. 2), and it was probably associated with a nutritional adaptation period. Isotopic changes were more pronounced for $\delta^{15}N$ values than for $\delta^{13}C$ values. The mean range of $\delta^{13}C$ and $\delta^{15}N$ values in all treatments was 3.9‰ and 4.5‰, respectively. Shrimp in all dietary treatments reached full isotopic equilibrium with their respective new diets as fast as 14 days (Figs. 2 and 3), except shrimp fed on macroalgal biomass only, which presented minimal growth and high mortality. As surviving animals in this treatment were sampled before the end of the experiment, isotopic equilibrium was not fully achieved; therefore, estimated isotopic equilibrium values were calculated for day 28 using predicted values from the model of Hesslein et al (1993). The estimated asymptotic $\delta^{13}C$ value for this treatment was –16.17‰, which was very similar to mean values observed in shrimp tissue on day 21 (–16.30‰), whereas the

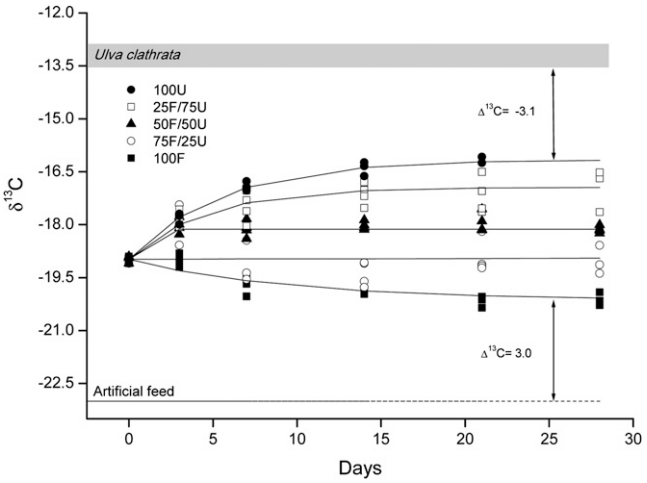

Figure 2. Changes in carbon stable isotope values (measured in parts per thousand) in whole bodies of white shrimp *L. vannamei* reared on feeding regimes having different proportions of inert feed and live biomass of macroalgae *Ulva clathrata*. Lines represent predicted values generated by the model of Hesslein et al. (1993) and show the best fit to observed data. Arrows represent isotopic discrimination factors between both feeding sources and shrimp fed inert feed and macroalgae only.

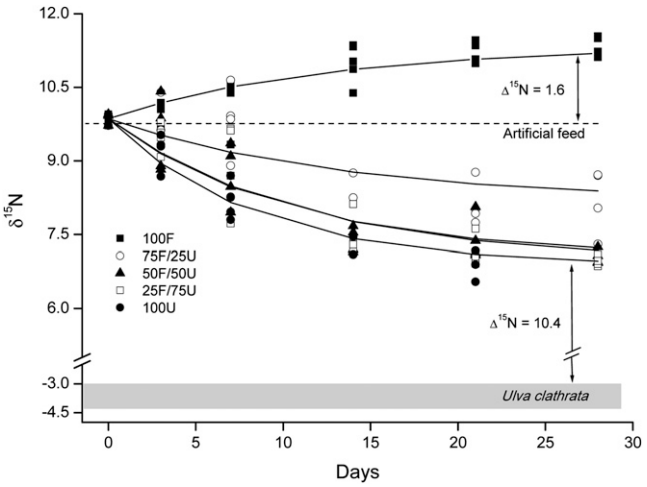

Figure 3. Changes in nitrogen stable isotope values (measured in parts per thousand) in whole bodies of white shrimp *L. vannamei* reared on feeding regimes consisting of different proportions of inert feed and live biomass of macroalgae *Ulva clathrata*. Lines represent predicted values generated by the model of Hesslein et al. (1993) and show the best fit to observed data. Arrows represent isotopic discrimination factors between both feeding sources and shrimps fed inert feed and macroalgae only.

TABLE 2.

Growth rates (k), estimated carbon and nitrogen metabolic rates (m), and half-times (t_{50}) in whole tissue of Pacific white shrimp *L. vannamei* reared under co-feeding regimes having different levels of *U. clathrata* biomass and inert feed.

		Carbon			Nitrogen		
Feeding Regime	k Value (/day)	m Value (/day)*	t_{50} (d)	$\Delta^{13}C$ (‰)	m Value (/day)	t_{50} (day)	$\Delta^{15}N$ (‰)
100F	0.060 ± 0.011^{a}	0.049 ± 0.014^{a}	6.4 ± 1.0^{b}	3.0	0.013 ± 0.009^{a}	9.5 ± 1.9^{a}	1.6
75F/25U	0.062 ± 0.011^{a}	nd	nd	—	0.013 ± 0.015^{a}	9.2 ± 2.4^{a}	—
50F/50U	0.050 ± 0.015^{a}	nd	nd	—	0.048 ± 0.015^{b}	7.1 ± 1.5^{ab}	—
25F/75U	0.029 ± 0.015^{ab}	0.191 ± 0.042^{b}	3.2 ± 0.7^{a}	—	0.062 ± 0.012^{b}	7.6 ± 1.9^{ab}	—
100U	0.008 ± 0.019^{b}	0.176 ± 0.008^{b}	6.4 ± 0.6^{b}	–3.1	0.100 ± 0.013^{c}	6.4 ± 0.9^{b}	10.4

* m Values were estimated from expected values fitted on observed values using the exponential equation proposed by Hesslein et al. (1993), $R^2 =$ 0.63–0.98.
Different superscripts indicate significant differences at $P < 0.05$.
nd, no data.

estimated $\delta^{15}N$ value was 6.60‰ for day 28 as compared with the measured mean value of 6.86‰ for day 21. Predicted isotopic values for this treatment were used as negative isotopic control values to correct for the discrimination factors between shrimp and *U. clathrata*. Discrimination factors between shrimp and diets were also very contrasting. The $\Delta^{13}C$ value between shrimp and inert feed after reaching isotopic equilibrium was 3.0‰, whereas shrimp fed on *U. clathrata* only showed an negative $\Delta^{13}C$ value (–3.1‰; Fig. 2). On the other hand, the mean $\Delta^{15}N$ value between shrimp bodies and diets was 1.6‰ in shrimp reared on inert feed only, whereas a large mean $\Delta^{15}N$ value of 10.4‰ was observed in shrimp fed live macroalgae biomass (Fig. 3).

Carbon and Nitrogen Turnover Rates

Table 2 shows the estimated carbon and nitrogen turnover rates and halftimes in tissue. Estimated metabolic turnover rates ranged from 0.049–0.191/day for carbon and from 0.013–0.100/day for nitrogen. Because nitrogen turnover rates were slower than carbon, it took slightly longer for $\delta^{15}N$ values in shrimp tissue to reach a steady state (Fig. 3).Turnover rates increased significantly as a function of macroalgae consumption, being most conspicuous for dietary nitrogen. Nitrogen t_{50} values in whole tissue consistently decreased throughout the different treatments from 9.5 days (100F) to 6.4 days (100U). It was not possible to estimate carbon turnover rates in animals from treatments 75F/25U and 50F/50U because isotopic values elicited by the respective dietary regimes throughout the experimental period did not describe an exponential trend in relation to values established by the basal diet (Fig. 2), thus preventing the use of the model by Hesslein et al. (1993), which relies on exponential isotopic changes.

Dietary Contributions from Macroalgae and Inert Feed

Results from the isotopic mixing model indicated that shrimp in the three co-feeding regimes incorporated significantly higher amounts of dietary carbon and nitrogen from *U. clathrata* biomass than from the inert feed (chi-squared = 23.7, $P < 0.0001$ for the smallest observed difference; Table 3). At the end of the experiment, shrimp in treatment 75F/25U incorporated 68% of carbon from the inert feed and 32% from the macroalgae. Shrimp under feeding regimes 50F/50U and 25F/75U incorporated significantly higher amounts of dietary carbon from *U. clathrata* (49% and 80%, respectively) when compared with the expected dietary carbon proportions supplied in the co-feeding regimes (34% and 70%, respectively). Shrimp grown in co-feeding regime 75F/25U incorporated 27% of nitrogen from the inert feed and the remaining 73% from the macroalgal biomass, whereas animals reared on regimes 25F/75U and 50F/50U incorporated the majority of their dietary nitrogen (98% and 96%, respectively) from the macroalgae. The smaller growth attained by these animals indicated that a very high proportion of the isotopic change was a result of high nitrogen metabolic turnover and not tissue accretion. Estimated dietary contributions to muscle tissue followed similar patterns as the nutritional contributions incorporated in whole bodies. Carbon incorporated from the inert feed in muscle tissue was 5–10% higher than proportions estimated in whole bodies (lipid extracted), whereas nitrogen deposition was 4–8% higher in muscle tissue compared with untreated whole bodies (Table 3). None of the differences was statistically significant (chi-squared = 3.3, $P = 0.69$ for the highest observed difference). Figure 4 combines $\delta^{13}C$ and $\delta^{15}N$ values measured in shrimp whole bodies and muscle tissue, and provides a graphic indication of the total organic matter contributed by both the inert feed and macroalgae. Although Euclidean distances indicated that shrimp reared on treatments 75F/25U and 50F/50U incorporated similar amounts (~50%) of dry matter from both sources, contribution values corrected for elemental concentration (Table 3) showed a higher dry matter incorporation from the macroalgae biomass (70–80%) in these treatments. Because of its lower carbon and nitrogen contents, the macroalgal biomass had to be consumed at higher amounts to supply the observed elemental contributions to shrimp whole bodies and muscle tissue.

DISCUSSION

Growth and Survival

Final WW and survival rates increased in relation to the amount of inert feed supplied. Shrimp in regime 25F/75U had a final mean WW of 424 mg and 60% of the animals survived. Shrimp fed regimes 50F/50U and 75F/25U had respective survival rates of 78% and 93%, and weighed, on average, 768 mg and 1,067 mg, respectively. The higher growth rate observed in shrimp raised on co-feeding regime 75F/25U is consistent with recent observations made by Cruz-Suárez et al. (2010) after

TABLE 3.

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 2-source, 1-isotope mixing model (mean ± CI, $n = 9$).

		Observed					
		Whole Bodies			Muscle Tissue		
Feeding Regime	Expected	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).

DM, dry matter; Max., maximum; Min., minimum.

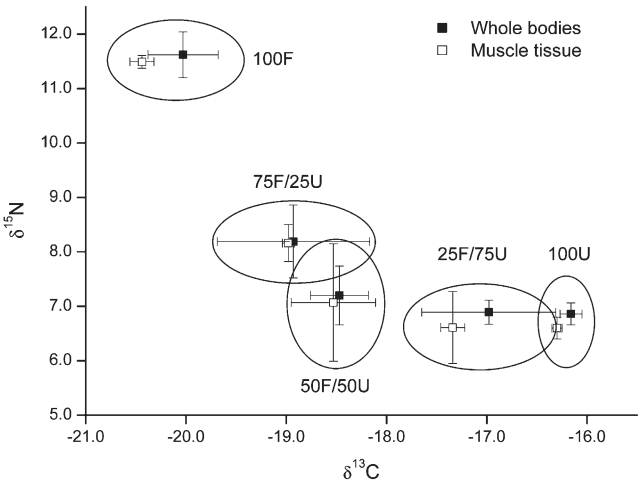

Figure 4. Carbon and nitrogen dual isotope (measured in parts per thousand) plot of isotopic values measured in whole bodies and muscle tissue of white shrimp *L. vannamei* fed different proportions of inert feed and live *U. clathrata* biomass. 100F and 100U correspond to values measured in shrimp fed inert feed or macroalgae exclusively and are thus considered as the isotopic discrimination-corrected values for 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.

they conducted an experiment on shrimp *L. vannamei* reared in experimental ponds prestocked with *U. clathrata* biomass. Higher growth rates in farmed shrimp have been associated with the presence and ingestion of different potential food items available in the natural productivity of ponds (Hunter et al. 1987, Nunes et al. 1997, Gamboa-Delgado et al. 2003). However, specific nutrients such as fatty acids and essential amino acids are present in low concentrations in some types of live food, and can be supplemented by the co-fed inert feed. This fact is highlighted by the poor growth and survival observed in shrimp fed on macroalgal biomass only. The positive effect of supplying both live feed and an inert diet has been observed recurrently in larval and juvenile crustaceans (Teshima et al. 2000, Thompson et al. 2002, Gamboa-Delgado & Le Vay 2009a) and fish (Kolkovski et al. 1993, Rosenlund et al. 1997, Curnow et al. 2006).

Isotopic Values in Food Sources and Isotopic Changes in Shrimp Tissue

$\delta^{13}C$ values measured in the inert feed (–23.0 ± 0.2‰) reflected the isotopically depleted values of the bulk ingredients used in its manufacture (fish, krill, soybean, and wheat meals). Conversely, isotopic signatures measured in live biomass of cultured *U. clathrata* (–13.1 ± 0.6‰) indicated a relative enrichment in the heavier isotope ^{13}C, which is consistent with data reported by Mercado et al. (2009) for *U. clathrata* collected from the natural environment (–14.4 ± 1.0‰). $\delta^{13}C$ values of macroalgae are strongly influenced by the metabolic pathways associated with the photosynthesis and uptake of dissolved inorganic carbon (CO_2 or HCO_3^-) and/or atmospheric CO_2, which imprints consistent isotopic signatures on different seaweed classes and species (Fry 2006, Mercado et al. 2009).

In contrast, $\delta^{15}N$ values in macroalgae are subject to wide variations because they are influenced directly by variable nitrogen sources in the littoral and sublittoral environments ($\delta^{15}N$ = 2.3–7.8‰ for *Ulva* sp. (Rogers 1999)). In laboratory conditions, the $\delta^{15}N$ values of micro- and macroalgae have been manipulated systematically through the use of different fertilizers in the culture media (Cohen & Fong 2004, Teichberg et al. 2008, Le Vay & Gamboa-Delgado 2010), as was also the case in the current study, in which the use of a commercial fertilizer elicited a mean, negative $\delta^{15}N$ value of –3.5‰ in the algal biomass. Shrimp in most treatments reached isotopic equilibrium with their respective diets in 2 wk, which is similar to that reported by Fry and Arnold (1982) and Gamboa Delgado and Le Vay (2009b) after conducting feeding trials to explore isotopic dynamics on penaeid shrimp.

The very low growth observed in shrimp fed *U. clathrata* biomass only indicates that animals under this treatment reached isotopic equilibrium through tissue catabolic turnover and not

through biomass accretion. When plotted, the dual isotopic values of muscle tissue slightly mismatched those of lipid-extracted whole bodies as a result of small ^{13}C and ^{15}N depletions in muscle (average of 0.14‰ for both isotopes). Stenroth et al. (2006) also failed to observe significant differences in $\delta^{13}C$ values of muscle tissue and whole bodies of crayfish (*Pacifastacus leniusculus*), although $\delta^{15}N$ values were slightly higher in muscle tissue (0.9‰) than in lipid-extracted whole bodies. Muscle tissue has been referred to as a good target tissue to infer isotopic dynamics occurring in whole bodies. Negative carbon discrimination factors ($\Delta^{13}C$) observed between shrimp and *U. clathrata* indicate a relative depletion of the heavier isotope (^{13}C) that occurs as nutrients are metabolized selectively and incorporated. In general, animals tend to get enriched in the heavier isotope as a result of preferential incorporation of ^{13}C and excretion of $^{12}CO_2$ (De Niro & Epstein 1978). Although unusual, negative $\Delta^{13}C$ values have been observed in most taxa and it is considered that, depending on the isotopic routing of the nutritional resources (Martínez del Rio & Wolf 2005), respired carbon may have a relatively high ^{13}C content (in comparison with diet) when it results from metabolic utilization of carbohydrates (enriched in ^{13}C), whereas the case is the opposite when lipids (depleted in ^{13}C) are preferentially metabolized (Breteler et al. 2002). *U. clathrata* contains very low lipid levels (1.5% (Cruz-Suárez et al. 2010)); therefore, isotopic results indicate a possible use of starch polysaccharides because there are no reports on the ability to digest nonstarch polysaccharides in penaeid shrimp.

Different field studies indicate average enrichment values of 2.6–3.4‰ in animal $\Delta^{15}N$ values when compared with diet (De Niro & Epstein 1981, Post 2002, McCutchan et al. 2003). These values are frequently used as assumed discrimination factors in estimating dietary contributions using mixing models (see review by Caut et al. (2009)). There is increasing evidence that shows that $\Delta^{15}N$ values are species specific and highly dependent on life stage and tissue sampled (Yokoyama et al. 2005, Stenroth et al. 2006). In the current experiment, $\Delta^{15}N$ values observed between shrimp whole bodies and inert feed ($\Delta^{15}N = 1.6$‰) were 6-fold higher than values observed in shrimp fed macroalgae ($\Delta^{15}N = 10.4$‰). The latter value is more than 3-fold higher than enrichment values frequently assumed in studies aimed at estimating nutritional contributions (Caut et al. 2009), hence highlighting the need for experimentally estimating isotopic equilibrium points with regard to time and/or biomass gain, to refine the output of the mixing models when estimating nutritional contributions.

It has been hypothesized that different discrimination factors are the result of different dietary assimilation and excretion rates (Olive et al. 2003, Gamboa-Delgado et al. 2008), and are influenced by dietary protein quality (Roth & Hobson, 2000, Waddington & MacArthur 2008) and quantity (Fantle et al. 1999, Pearson et al. 2003). Results from the current study indicate that nitrogen content, and ingestion and excretion rates might play a role in the degree of $\Delta^{15}N$ in shrimp and their diets. To satisfy their protein requirements, shrimp fed macroalgae only foraged continuously on the macroalgal filaments. The protein content of the inert feed was greater (35.2%) than the protein content in *U. clathrata* biomass (18.6%). High $\Delta^{13}C$ and $\Delta^{15}N$ values elicited by limiting nutritional conditions have been reported, and it has been hypothesized that greater discriminatory factors are the result of the higher metabolic turnover of animals that are allocating scarce resources physiologically (Martínez del Rio & Wolf 2005), as in the case of shrimp consuming greater proportions of algal biomass in the current study. After conducting a nutritional study on killifish (*Fundulus heteroclitus*), McMahon et al. (2010) suggested that differences in amino acid abundance in the diet relative to consumer muscle possibly requires varying the degree of biosynthesis and catabolism to meet the muscle composition demand, which may explain corresponding shifts in amino acid $\Delta^{13}C$ values.

Carbon and Nitrogen Turnover Rates

Predicted isotopic values generated by the model by Hesslein et al. (1993) fitted reasonably well to observed values ($r^2 = 0.63$–0.98). Metabolic turnover rates (m) and full isotopic equilibrium values were estimated from these equations. Relatively high carbon and nitrogen turnover rates suggested a greater recycling of nutrients occurring in shrimp reared on co-feeding regimes receiving lower amounts of inert feed, hence consuming greater amounts of macroalgal biomass. Isotopic changes driven by metabolic turnover account for most of the observed isotopic change in slow-growing organisms, and it has been demonstrated that animals reared on nutritionally restricted diets increase their metabolic turnover rates as a strategy to incorporate insufficient nutrients such as amino acids (Fantle et al. 1999). In the current experiment, nitrogen turnover rates in shrimp fed inert feed only were several orders of magnitude lower (0.013/day) than those observed in animals fed macroalgae biomass only (0.100/day). The high metabolic turnover rates estimated in muscle tissue of animals under co-feeding regimes accounted for 20–95% of the observed isotopic change. High nitrogen metabolic turnover rates have also been observed in postlarval *L. vannamei* (2.0 mg) fed high-protein diets containing soy protein isolate only as a nitrogen source (0.117/day), whereas shrimp fed fish meal-based diets showed lower nitrogen turnover rates (0.060/day^1) and larger growth (Gamboa-Delgado & Le Vay 2009b). When *L. vannamei* is fed nutritionally optimal diets, an efficiency of retention of synthesized protein as growth of 94% has been reported (Mente et al. 2002), suggesting a very low protein turnover rate occurring under ideal nutritional conditions. Such observation is also supported by the lower nitrogen turnover rates (0.013/day) and higher growth rates (0.060–0.062/day) estimated in the current study in shrimp reared under treatments 100F and 75F/25U. t_{50} Values integrate parameters k (growth) and m (metabolic turnover rates); therefore, estimated t_{50} values in the current study increased as a function of the growth promoted by greater levels of supplied inert feed. Low nitrogen t_{50} values observed in shrimp fed on *U. clathrata* (6.4 days) only suggest shorter protein residency times in the tissue of these slow-growing animals. Values increased to 7.1 days in shrimp reared under co-feeding regime 50F/50U, and up to 9.2 days and 9.5 days in shrimp fed regime 75F/25U and inert feed only, respectively.

Dietary Contributions from U. clathrata *and Inert Feed to Growth*

Results indicate that dietary carbon and nitrogen supplied by the 2 sources were allocated in different ways in shrimp. This effect has been termed "isotopic routing" (Schwarcz 1991, Martínez del Rio & Wolf 2005, Wolf et al. 2009) and 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. In the current experiment, this effect can be discerned by comparing the different dietary carbon and nitrogen contributions and the dual isotopic values of whole bodies and muscle tissue, which are slightly overlapped. However, there were no significant differences in the latter measurements, and nutritional contributions were estimated using isotopic values in both muscle tissue and whole shrimp bodies, thus canceling any isotopic routing effect in the interpretation of results.

Digestibility of both feeding sources for *L. vannamei* has been assessed and is similarly high. Villareal-Cavazos (unpubl. results) determined apparent digestibility coefficients of dry matter and crude protein of 82% and 91%, respectively, in *L. vannamei* fed diets formulated with *U. clathrata* meal. On the other hand, digestibility of inert feeds containing high proportions of fishmeal is greater than 80% for penaeid shrimp and it has been reported elsewhere (Lemos et al. 2004, Terrazas-Fierro et al. 2010, Villareal-Cavazos 2011). 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 high digestibility of *U. clathrata* and its continuous availability for shrimp. In contrast, only 2 daily rations of inert diet were supplied to shrimp in the co-feeding regimes. Despite their lower nutrient concentration, live food contains a higher water content (~80%), which contributes to their greater digestibility (Conceição et al. 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. Co-feeding experiments conducted on postlarval shrimp and larval fish have shown that available live food (zooplankton, biofilm) frequently contributes greater proportions of nutrients than those supplied by co-fed inert feeds to the growth of the consuming animals (Abreu et al. 2007, Gamboa-Delgado et al. 2008, Jomori et al. 2008, Gamboa-Delgado & Le Vay, 2009a). In the current experiment, the dietary carbon contributions from the inert feed to muscle tissue were significantly greater when compared with dietary carbon incorporation to whole bodies. Considering the muscle tissue as the main body protein reservoir, this difference can be attributed to preferential incorporation of amino acids (and their carbon skeletons) from the inert feed. On the other hand, dietary nitrogen contributions from the macroalgae biomass to growth were very high and accounted for more than 95% of the incorporated nitrogen in co-feeding regimes 50F/50U and 25F/75U, which supplied nitrogen proportions of 66:34 and 30:70 from inert feed and *U. clathrata*, respectively. Such disproportion in the relative incorporation of dietary nitrogen from the macroalgae might be the result of greater amino acid digestibility and assimilation, in addition to the continuous availability of the macroalgal biomass to shrimp. Results thus suggest that dietary carbon and nitrogen from both sources were differentially metabolized.

In this context, Gannes et al. (1997) point out that animal tissue often does not reflect the bulk isotopic composition of the diet; instead, it reflects the isotopic composition of the constituents of the diet from which the tissue was biosynthesized. Total organic matter contributions were estimated using both carbon and nitrogen isotopic values, and the elemental concentration in food sources. Because carbon and nitrogen contents in inert feed were higher, results indicate that dry matter contributions from the macroalgae were significantly higher (i.e., had to be consumed and incorporated in greater amounts to supply the estimated elemental concentrations) than contributions from inert feed in the three co-feeding regimes, and proportional contributions were similar for whole bodies and muscle tissue. Chemical analyses of *U. clathrata* have shown that it typically contains low to medium protein levels (20–30%) and very low lipid levels (1.5% (Cruz-Suárez et al. 2010)), and although the cell wall polysaccharides might represent up to 54% of dry algal matter (Lahaye & Kaeffer 1997), a tentative role of the latter as an energy source is unlikely because specific enzymatic activities (ulvanase, fucoidanase) for these polysaccharides have not been reported for penaeid shrimp. The low levels of energy, amino acids, and fatty acids in the macroalgae biomass available to shrimp were compensated through greater ingestion, which caused the observed higher incorporation values estimated in shrimp tissue.

On the other hand, it is very likely that in the co-feeding regimes, the carbohydrates and lipids supplied by the inert feed (37% and 8.8%, respectively) contributed significantly to the energy requirements of shrimp. Although the macroalgae can also complement the dietary supply of vitamins, minerals, and fiber (Mabeau & Fleurence 1993) in shrimp, it only supports maintenance levels when offered alone. The importance of the natural productivity to shrimp grown in semi-intensively managed ponds has been widely documented (Rubright et al. 1981, FAO 1989, Nunes & Parsons 2000, Azim et al. 2004). The systematic use of macroalgae in production ponds can provide a significant nutritional supply to cultured organisms, but also offers substrate for periphyton growth and refuge for shrimp in recent pre- and postmolting stages. In addition, it has been demonstrated that *U. clathrata* and other species show greater growth rates (3.3%/day), and are efficient removers of the main dissolved inorganic nutrients, hence maintaining good water quality levels in aquaculture ponds and effluents (Wang et al. 2007, Copertino et al. 2008). Feeding regime 75F/25U promoted higher survival and growth, whereas the estimated carbon metabolic turnover rate in this treatment was similar to those estimated in shrimp fed on inert feed only. Therefore, results from the current study indicate that a feeding strategy allowing a supply of at least 25% of the weight of the administered feed as fresh macroalgae biomass improves the overall response of shrimp compared with shrimp fed inert feed or macroalgal biomass only. Future studies will focus on assessing the nutritional contributions from *U. clathrata* to other shrimp species and will examine the incorporation of nutrients from this macroalgae species included as dry meal in formulated diets for shrimp.

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