***Artemia* replacement in co-feeding regimes for mysis and postlarval stages of *Litopenaeus vannamei*: Nutritional contribution of inert diets to tissue growth as indicated by natural carbon stable isotopes.**

Gamboa-Delgado, Julián* and Le Vay, Lewis
School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey, Wales, United Kingdom, LL59 5AB
Tel +44 (0) 1248 351151
Fax +44 (0) 1248 716367
*corresponding author osp216@bangor.ac.uk jgam97@yahoo.com

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Abstract

The nutritional contribution from co-fed *Artemia* nauplii and inert diets to growth in mysis and early postlarval shrimp *Litopenaeus vannamei* was assessed by analyzing the carbon stable isotope ratios ($\delta^{13}C$) in diets and shrimp tissue. *Artemia* nauplii and inert diets showed significantly different $\delta^{13}C$ values but similar carbon contents and were supplied as single diets and also in three co-feeding regimes in which 25, 50 and 75% of the *Artemia* was replaced by inert diet on a dry weight basis, so that all feeding regimes provided similar amounts of dietary carbon. Shrimp $\delta^{13}C$ values were significantly influenced by the different feeding regimes and reached isotopic equilibrium with their respective diets as soon as 5 days. Survival was significantly higher in co-fed animals than in those fed either *Artemia* or inert diet alone. There was no significant difference in growth between shrimp fed on *Artemia* only and those co-fed *Artemia* with inert diet, although the variability was high. Growth and survival were very low in shrimp fed only inert diet. Results from an isotope mixing model suggest that observed nutrient contributions from *Artemia* nauplii were significantly higher than expected contributions indicated by proportions established in the co-feeding regimes. Nutrient contributions to growth in the dietary regime providing equal carbon amounts of each diet type ranged from 77 to 86% for *Artemia* and from 13 to 24% for inert diet. Shrimp fed the 25% *Artemia* replacement regime exhibited a significantly higher retention of dietary carbon from the inert diet than those fed inert diet alone. This may have resulted from greater ingestion and/or assimilation of the inert diet in the presence of *Artemia*, combined with the higher growth rate in the co-feeding treatment. The results demonstrate the effectiveness of up to 50% replacement of *Artemia* with inert diet for *L. vannamei* mysis and early postlarval stages, indicating also that the inert diet may provide specific nutrients that promote survival, while digestibility may limit its contribution to tissue growth. The

influence of maternal and dietary sources on $\delta^{13}C$ values in earlier larval stages was also described.

Keywords: *Litopenaeus vannamei*, larval diets, stable isotopes, carbon turnover, nutrient incorporation, growth.

1. Introduction

The Pacific White shrimp *Litopenaeus vannamei* has become the main crustacean species produced through aquaculture, with production exceeding that of the black tiger shrimp *Penaeus monodon* since 2003 (FAO, 2007). This is largely due to the advantages in terms of disease management and strain selection resulting from domestication of broodstock. The continued growth of shrimp aquaculture requires high numbers of good quality postlarvae and juveniles from commercial hatcheries, especially where captive broodstock are used. As is typical for penaeids, the larval stages of *L. vannamei* undergo complex trophic changes during development (Jones et al., 1997; Le Vay et al., 2001). The non-feeding naupliar stages are followed by the phytoplankton-feeding zoea and the carnivorous mysis stages, the latter being highly dependant on a constant supply of live feed, which is generally provided as rotifers and *Artemia* nauplii. Although live diets represent advantages such as high digestibility and stability in the water, rotifers and *Artemia* may lack essential nutrients for marine organisms (Léger et al. 1986; Webster and Lovell, 1990; Dhert et al., 2001). In this context, the development of nutritionally-complete microdiets that can partially substitute live feed in co-feeding regimes presents economic and environmental advantages. Shrimp larval rearing protocols in commercial shrimp hatcheries have been increasingly using higher proportions of inert diets to

partially replace live feeds in co-feeding regimes (see review by Teshima et al., 2000). Constant improvements through nutritional research have narrowed the dependency on live feeds and some studies have reported on the successful total replacement of microalgae and/or *Artemia* at different larval stages (Kanazawa, 1990; Cuzon and Aquacop, 1998; D'Abramo et al., 2006). Complete replacement of live foods with formulated diets through all the larval stages has also been demonstrated (Jones et al., 1979; Kurmaly et al., 1989; Le Vay et al., 1993) but while survival can be equivalent to that of larvae reared on live foods, growth and development may be slower (Le Vay et al., 1993; D'Abramo et al., 2006). Penaeid shrimp larvae show good acceptance of inert diets in co-feeding regimes and different methods have been applied to evaluate the physical, chemical and nutritional performance of new microdiets. Among the latter, larval growth, metamorphosis and survival rates, diverse condition indexes and stress tests are some of the assessment methods used to indirectly evaluate the nutritional quality of larval and postlarval diets (Gallardo et al., 2002; Robinson et al., 2005). Valuable information has also been gathered through the assessment of ingestion rates (Kurmaly et al., 1989a; Rosas et al., 1995; de Lima and Souza-Santos, 2007), digestion and enzyme activity (Le Vay et al., 1993; Kumlu and Jones, 1995; Brito et al., 2001) and estimation of diet assimilation (Sumule et al., 2003; Jiménez-Yan et al., 2006). Chemical analyses of diets and larval tissue have included the evaluation of nutrient composition and amino acid and fatty acid profiling of diets and larvae (Abdel-Rahman, 1996; Coutteau et al., 2000). Direct assessments of nutrient incorporation have advantages because they integrate not only what has been selected, ingested and digested, but also assimilated into growing tissue. The use of radioactive isotopes (^{14}C) as nutritional tracers has been successfully applied in early studies on crustacean nutrition in order to assess sterol biosynthesis (Teshima and Kanasawa, 1971; Teshima et al., 1976) and to evaluate the role of phospholipids as lipid-

transporters (Teshima et al., 1986, 1986a). More recently, radio-label compounds have been applied to trace nutrients in marine fish larvae (Koven et al., 1998; Rønnestad et al., 2001; Morais et al., 2007). However, the use of radioactive isotopes is limited to short-period studies and since radio-labels can be hazardous, they are subject to many regulations and restrictions; as a consequence, their use has been largely replaced by stable isotope tracers in studies of human and animal metabolism over the past 30 years (Schlechtriem et al., 2004; Conceição at al., 2007). The use of stable isotopes is a safe and useful technique that has been applied to determine nutrient incorporation from different dietary items in aquatic larval organisms (Schlechtriem et al., 2004; Jomori et al., 2008; Gamboa-Delgado et al., 2008). Estimation of nutrient retention in crustacean larvae is difficult due to their small size and the rapid and complex larval development, which increases the challenge in accurately assessing incorporation of nutrients into larval tissue. Carbon accounts for approximately 50% of the dry weight of most life forms; hence relationships between consumers and their diet, in terms of carbon stable isotope composition ($^{13}C/^{12}C$; $\delta^{13}C$ hereafter in the text), can be used to identify dietary components contributing to growth. In the present study, $\delta^{13}C$ values were determined in *Artemia* nauplii, inert diets and shrimp mysis and early postlarvae raised on different co-feeding regimes in order to estimate the relative contribution of dietary carbon provided by both feeding sources and to assess carbon metabolic turnover. Additionally, the influence of maternal and dietary sources on $\delta^{13}C$ values in earlier larval stages was also described.

2. Material and methods

2.1. Experimental animals

Pacific white shrimp *L. vannamei* larvae were obtained from domesticated broodstock reared at the School of Ocean Sciences, Bangor University, Anglesey, UK. For the

experiment, a batch of nauplii from an individual spawn was phototactically selected 10 h after hatching. Nauplii were reared in 100 l fibreglass tanks under the following environmental conditions: temperature 28.2 ±0.4°C, salinity 32.1 ±0.7 g l^{-1}, dissolved oxygen 6.4 ±0.4 mg l^{-1} and pH 8.2 ±0.3. A Palintest standard photometer (Palintest, Ltd., England, UK) was used to measure total ammonia nitrogen. Values remained below 0.7 mg l^{-1} during the experiment. An automatic photoperiod was set up to provide a light:dark ratio of 14:10 h. Zoea stages were exclusively fed on the microalgae *Chaetoceros gracilis* (130-150 cells ul^{-1}) and were thus isotopically influenced by the $\delta^{13}C$ value of the microalgal cells, in this way setting an isotopic reference value before starting the experiment at larval stage mysis 1 (M_1). Samples of shrimp eggs, nauplii and zoeas were collected and processed as described below.

2.2. Experimental design and larval rearing system

After 90% of zoea larvae had moulted to M_1 stage, animals were counted, size-graded and distributed into 15, 2-l capacity round glass flasks at an initial density of 150 larvae l^{-1}. Flasks were individually fitted with glass tubes to keep gentle aeration and circulation. The flask array was set in a water-bath tray and there was no water exchange until larvae reached M_3; thereafter, partial and total water exchanges were carried out daily. Seawater used for water replacement was taken from a recirculation system and was treated through mechanical filters (50 and 5 μm), fluidized bed biofilters, foam fractionator and an 8-unit ultraviolet lamp array.

2.3. Feeding regimes and sampling

Five different larval feeding regimes were designed to supply similar amounts of carbon by estimating the individual dry weight of recently hatched *Artemia* nauplii (mean dry

weight 1.56 μg) and after correcting for the moisture content present in the inert diets (9.4%). Preliminary analysis indicated that carbon contents in *Artemia* nauplii and inert diets were similar (48.2 ±0.6 and 47.7 ±1.1%, respectively; P= 0.855). Feeding regimes consisted in 100% *Artemia* nauplii (100A) (Vinh-Chau strain, Vietnam), 100% inert diet (100I) at two particle sizes [Frippak 2CD (30-90 μm) and 3CD (80-150 μm), INVE Technologies, Dendermonde, Belgium] and 3 co-feeding regimes in which *Artemia* was replaced with inert diets at 25, 50 and 75% (75A/25I, 50A/50I and 25A/75I, respectively) of the daily carbon supply as indicated in Table 1. Microalgae supply was stopped before distributing the animals in the experimental array, so that from M_1 to postlarval stage 5 (PL_5) animals received only *Artemia* and/or inert diet. Shrimps were fed 4 times per day at 08:00, 12:00, 16:00 and 20:00 h. Each of the first three feeding doses supplied 23% of the daily ration and the last supplied 31% of the amount of feed offered per day. Recently-hatched *Artemia* nauplii were kept at 4ºC to minimize utilisation of energy reserves prior to be fed to the shrimp. Inert diet doses were thoroughly suspended in sea water before addition into the experimental flasks using a 5 ml pipette. Feeding rations were progressively adjusted on a daily basis in relation to the experimental feeding regimes, observed survival and number of sampled animals. Samples of 10 to 30 shrimp larvae or PL were randomly collected every 24 h from each replicate flask and were pooled for stable isotope analysis. In order to minimize the amount of food present in the gut, sampled animals were starved for 5 h in filtered sea water, as it has been estimated that complete gut evacuation time in this species is approximately 3 h (Beseres et al., 2006), the time period being considerably smaller for larval and postlarval stages (Villamar and Langdon, 1993). Animals were killed by freezing and the larval sub-stages immediately determined. Individual larval mean dry weight was estimated to the nearest 10 μg after drying samples of pooled animals at 60 °C until constant weight.

Table 1 Experimental larval feeding regimes used to estimate nutrient contributions from inert diet and *Artemia* nauplii co-fed to *Litopenaeus vannamei* mysis and early postlarvae. Estimated total dietary carbon supply (mg C d^{-1}) from both sources is indicated in parenthesis.

Stage/Feeding item	100A	75A-25I	50A-50I	25A-75I	100I
$Zoea_{1-3}$					
C. gracilis (cel ul^{-1})	130-150	130-150	130-150	130-150	130-150
M_1					
Artemia (ind ml^{-1})	4.0(*6.0*)	3.0(*4.5*)	2.0(*3.0*)	1.0(*1.5*)	-
Inert diet [a] (mg l^{-1})	-	2.0(*1.7*)	4.0(*3.4*)	6.0(*5.1*)	8.0(*6.9*)
M_2					
Artemia	6.0(*9.0*)	4.5(*6.8*)	3.0(*4.5*)	1.5(*2.3*)	-
Inert diet [a]	-	3.0(*2.6*)	6.0(*5.1*)	9.0(*7.7*)	12.0(*10.3*)
M_3					
Artemia	8.0(*12.0*)	6.0(*9.0*)	4.0(*6.0*)	2.0(*3.0*)	-
Inert diet [a]	-	4.0(3.4)	8.0(*6.9*)	12.0(*10.3*)	16.0(*13.7*)
PL_{1-3}					
Artemia	10.0(*15.0*)	7.5(*11.3*)	5.0(*7.5*)	2.5(*3.8*)	-
Inert diet [b]	-	5.0(*4.3*)	10.0(*8.6*)	14.0(*12.0*)	18.0(*15.4*)
PL_{4-5}					
Artemia	11.0(*16.5*)	8.5(*12.8*)	5.5(*8.3*)	2.7(*4.1*)	-
Inert diet [b]	-	5.5(*4.7*)	11.0(*9.4*)	15.4(*13.2*)	19.8(*17.0*)

[a] Frippak 2CD
[b] Frippak 3CD

Shrimp biomass was expressed as µg of carbon, as indicated by their dry weight and elemental composition of samples from respective treatments and times. Samples of microalgal cells, *Artemia* nauplii and shrimp at different stages and sub-stages (fertilized eggs, larvae and postlarvae) were centrifuged or sieved, rinsed with distilled water and kept at -80 °C until isotopic analysis.

2.4. Stable isotope analysis and estimation of nutrient contribution

Pre-analysis sample preparation and analytical methods for determination of carbon and nitrogen contents, carbon isotopic ratios ($\delta^{13}C$) and procedures to estimate proportional nutrient contribution from inert diets and *Artemia* using a two-source, one-isotope mixing model (Phillips and Gregg, 2001) are described in Gamboa-Delgado et al. (2008). Carbon isotope discrimination factors ($\Delta^{13}C$) were estimated as the difference between $\delta^{13}C$ mean values of shrimps and the $\delta^{13}C$ of their respective diets after reaching isotopic equilibrium. Positive and negative isotopic control values used to correct for $\Delta^{13}C$ in the mixing model were taken from the isotopic differences between diets and shrimps fed exclusively *Artemia* or inert diets, respectively. Estimated nutrient incorporation values from two sources are expressed as means and their truncated confidence intervals.

2.5. Estimation of carbon turnover rates

In order to obtain an estimate of the metabolic carbon turnover rate, changes in $\delta^{13}C$ values in shrimp following a dietary shift from *C. gracilis* to the different experimental feeding regimes were fitted to the following exponential model (Hesslein et al., 1993) in order to integrate growth and isotope value shift over time:

$$C_{sample} = C_n + (C_o - C_n)e^{-(k+m)t} \quad (1)$$

Where C_{sample} is the $\delta^{13}C$ value in shrimp tissue at time t, C_o is the $\delta^{13}C$ value in shrimp tissue in equilibrium with the initial diet, C_n is the $\delta^{13}C$ value reached when shrimps are in equilibrium with a new diet. Parameters k and m represent growth (d^{-1}) and metabolic tissue turnover rate (d^{-1}), respectively. The former was estimated as $k = log$(final weight/initial weight)/time(days). m was derived from Equation 1 and calculated using

iterative non-linear regression to find the best fit. In this model, coefficients k and m indicate the magnitude of the isotopic rate of change in relation to growth and metabolic turnover, respectively, providing also an indicator of the time necessary for half of the body tissue to reach isotopic equilibrium after consuming a new diet (half time, t_{50}):

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

In order to allow comparison with other studies, parameters k, m and t_{50} were estimated on a dry weight basis and not in regards to carbon weight. The C:N ratio of shrimp eggs and different larval stages was also estimated at different times to associate C:N changes with isotopic shifts and also as an indicator of nutritional variations occurring as endogenous and exogenous nutrients were utilized.

2.6. Statistical analysis

Carbon contents and $\delta^{13}C$ values of inert diets and *Artemia* nauplii were compared by means of Student's t-tests. Dietary isotopic effects on tissue values at different times, mean dry larval carbon weights, percentage metamorphosis and survival 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 goodness of fit tests were applied to determine statistical differences in the expected (carbon proportions in the co-feeding regimes) and observed estimated proportions of dietary carbon incorporated in shrimp tissue. Parameters m and k in Eq. (1) were estimated by iterative non-linear regression. All tests were done using SPSS 12.0 software (SPSS Inc.) at a significance level of $P<0.05$.

3. Results

3.1. Larval growth and carbon turnover rates

Shrimps in the co-feeding regimes exhibited significantly higher survival than those fed either only *Artemia* or inert diet (P<0.001) (Table 2). Larvae fed 100% inert diet exhibited low survival and only 4% of the surviving animals reached PL_1. Animals reared on co-feeding regimes 100A, 75A/25I, 50A/50I metamorphosed to PL_1 stage faster. At the end of the experiment there were no significant differences in growth between treatments (except in animals raised under feeding regime 100I) due to high variability (Fig. 1). Over the study period (9 d) shrimp had increased their weight (μg C) by between 800 and 1100%, except those fed inert diet alone for which the weight increase was only 64%. Table 3 shows the estimated half time (t_{50}) for carbon replacement in shrimp tissue. The low growth rate observed in shrimps fed only inert diets produced a mean carbon t_{50} of 2.5 ±0.3 d, which was significantly higher (P<0.001) than t_{50} observed in the other treatments (1.2 ±0.1 to 1.6 ±0.2 d). No significant differences were observed between treatments in regards to metabolic turnover rates (m) due to high variability (P= 0.148), while growth rate (k) was significantly lower only in animals fed 100% inert diet (P<0.001). The C:N ratio of fertilized eggs decreased very fast after hatching and stabilized through the naupliar stages, although during the zoea stages the ratio further decreased influenced by the microalgae. There was an increase after M_1, when experimental feeding regimes were diversified (Fig. 2).

Table 2 Survival, metamorphosis and mean individual final weight (µg C) of *Litopenaeus vannamei* reared on five different feeding regimes from M_1 to early postlarval stage (n= 15, means ±SD).

Feeding regime	Survival (%)	Final weight (µg C)	Metamorphosis to PL_1 (%)	Weight increase (%)
100A	69.2 ± 9.6^{c}	101 ± 6^{a}	100 ± 0^{a}	1118
75A/25I	99.3 ± 1.2^{a}	82 ± 11^{a}	93 ± 7^{a}	918
50A/50I	89.4 ± 6.9^{b}	86 ± 18^{a}	97 ± 5^{a}	951
25A/75I	87.3 ± 8.0^{b}	72 ± 14^{a}	43 ± 8^{b}	799
100I	35.1 ± 6.2^{d}	14 ± 3^{b}	4 ± 3^{c}	64

Mysis 1 initial dry weight = 9 ±3 ug C
Different superscripts indicate significant differences at $P<0.05$.

Table 3 Growth rates (k), estimated carbon metabolic turnover rates (m) and estimated carbon half time (t_{50}) in tissue of shrimp *Litopenaeus vannamei* reared from mysis 1 to postlarval stage on feeding regimes having different levels of *Artemia* replacement.

Feeding regime	k (d $^{-1}$)	m (d $^{-1}$)	t_{50} (d)	$\Delta^{13}C$ (‰)
100A	0.239 ± 0.005^{a}	0.261 ± 0.103^{a}	1.4 ± 0.2^{a}	1.3
75A/25I	0.218 ± 0.014^{a}	0.381 ± 0.178^{a}	1.2 ± 0.2^{a}	-
50A/50I	0.225 ± 0.037^{a}	0.334 ± 0.095^{a}	1.2 ± 0.1^{a}	-
25A/75I	0.204 ± 0.024^{a}	0.239 ± 0.063^{a}	1.6 ± 0.2^{a}	-
100I	0.040 ± 0.019^{b}	0.241 ± 0.033^{a}	2.5 ± 0.3^{b}	4.1

Different superscripts indicate significant differences at $P<0.05$.

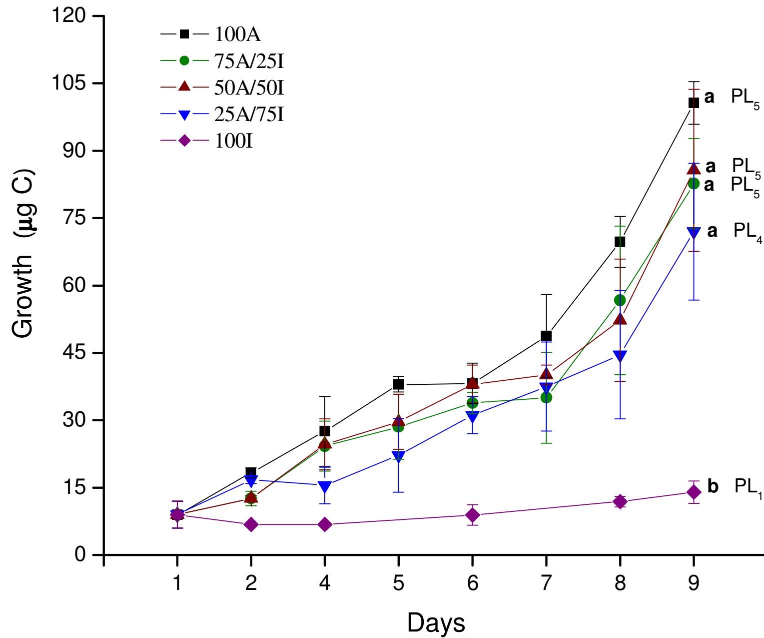

Fig. 1 Growth (µg C) of *L. vannamei* from mysis 1 to early postlarvae fed five different feeding regimes having varying levels of inert diet replacing *Artemia* nauplii. Final metamorphosis stages at the end of the experiment are indicated. Mean of 15 samples, vertical bars indicate SD and different subscripts denote statistical differences ($P<0.05$).

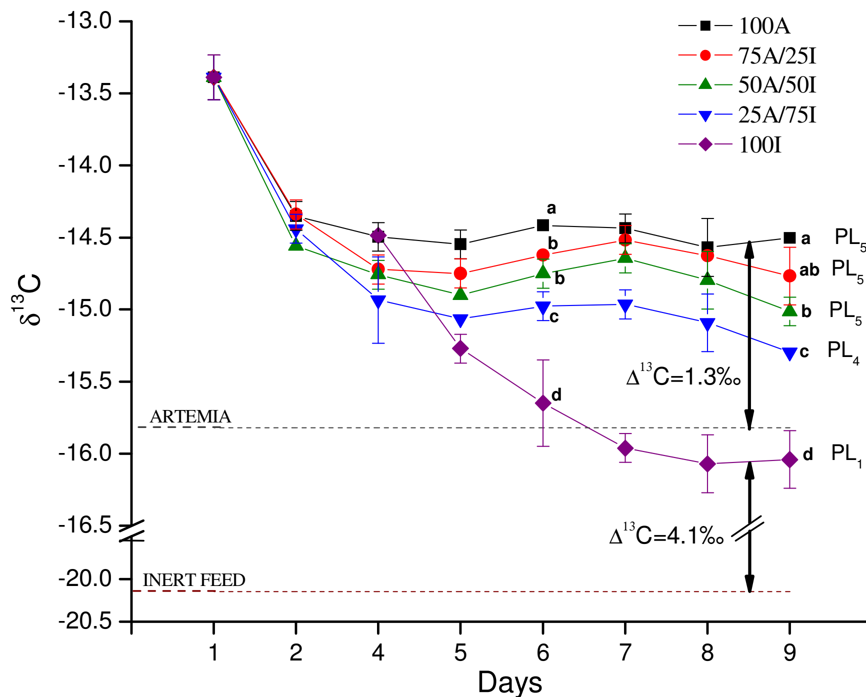

Fig. 2 Changes in δ^{13}C values and C:N ratios in *L. vannamei* tissue during larval development from egg to postlarvae 1. Larvae were fed only on *C. gracilis* (zoea stages) and *Artemia* nauplii (mysis stages). Mean of 3-5 samples of pooled animals ±SD indicated by vertical bars. Horizontal dotted lines represent δ^{13}C values of live foods.

3.2. Influence of diet on $\delta^{13}C$ values and shrimp-diet carbon isotopic discrimination

Artemia nauplii and both inert diets had $\delta^{13}C$ mean values of -15.8 ±0.1 and -20.2 ±0.1‰, respectively, compared to -14.9 ±0.1‰ for *C. gracilis* used to feed the zoea stages. The $\delta^{13}C$ value of fertilized shrimp eggs was -19.7‰ and rapidly increased to -17.9‰ over the naupliar stages (Fig. 2); thereafter, the isotopic value of the larval tissue for the zoea stages (Z_3 = -14.9‰) quickly reflected that of the algal diet, although $\delta^{13}C$ changes indicate that isotopic equilibrium was not reached.

After metamorphosis to M_1 and immediately before supplying the experimental dietary treatments, $\delta^{13}C$ values had increased to -13.4‰ (Fig. 2). 24 h after the start of the experiment, shrimp isotopic values were significantly influenced by their corresponding feeding regimes (Fig. 3). Results indicate that shrimp reached isotopic equilibrium with their respective diets by day 5 (PL_1), with the exception of shrimps fed exclusively on the inert diet, which grew more slowly and had a greater difference in initial $\delta^{13}C$ value between tissue and diet, and so did not reach isotopic equilibrium until day 7. At stages PL_4 and PL_5, the $\delta^{13}C$ of shrimps fed the three co-feeding regimes showed a slight deviation towards the value of the inert diet. Postlarval shrimp fed only on *Artemia* nauplii exhibited carbon discrimination factors ($\Delta^{13}C$ = +1.3‰) that were significantly smaller that those observed in shrimp fed only inert diets ($\Delta^{13}C$ = +4.1‰) (Fig. 3).

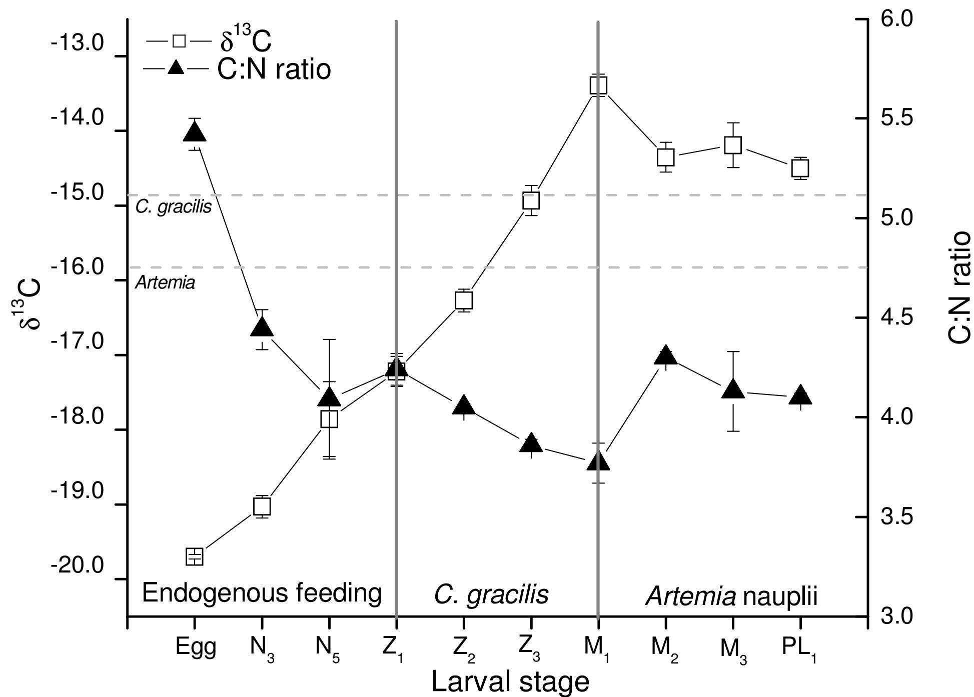

Fig. 3 δ^{13}C changes in tissue of *L. vannamei* mysis and postlarvae after a dietary shift from *C. gracilis* to five experimental feeding regimes having different levels of *Artemia* nauplii replacement by inert diet. Vertical arrows refer to Δ^{13}C between diet (100A and 100I) and shrimp after reaching isotopic equilibrium. Superscripts indicate isotopic differences between treatments (P<0.05) and horizontal dotted lines denote δ^{13}C values of both food sources. Postlarval stage on final day is indicated. Means of 3–6 samples of 10-30 pooled animals ±SD.

3.3. Relative contribution of *Artemia* *and inert diet to larval and postlarval growth*

δ^{13}C values in both food sources were significantly different (P<0.001) and shrimp quickly reached isotopic equilibrium with their respective diets, thus allowing use of the isotopic mixing model to estimate the relative nutrient contributions from *Artemia* and inert diet. Carbon contents in *Artemia* nauplii and inert diets were similar; therefore, no corrections were applied to estimate carbon contributions from the two sources (Fry, 2006; Gamboa-Delgado et al., 2008). Results from the isotope mixing model indicate that observed carbon contributions from *Artemia* nauplii were significantly higher (P<0.001)

than expected contributions indicated by the carbon proportions established in the three co-feeding regimes (Tables 1 and 4), while nutritional contributions from the inert diet were lower than expected. From M_3 onwards, isotopic values of animals fed the different regimes, except those fed only on inert diet, showed similar trends over time (Fig. 3), thus suggesting that estimated nutrient contributions were very similar from M_3 to PL_3 within respective dietary treatments. Over this period, results from the mixing model indicate that *Artemia* nauplii and inert diet contributions in the 50A/50I regime ranged from 77 to 86% and from 13 to 24%, respectively. At PL_4, results indicated that shrimps fed on the 50A/50I regime, had incorporated 87% of tissue carbon from *Artemia* nauplii and 13% from the inert diet, the latter value increased to 27% on PL_5 (Table 4).

Table 4 Estimated nutritional contributions (%) of *Artemia* nauplii and inert diets to growth of *L. vannamei* mysis (M) and postlarvae (PL) raised under three different co-feeding regimes and as indicated by dietary and tissue $\delta^{13}C$ values integrated into an isotope mixing model (n = 10-30 pooled animals).

Stage/Food	75A/25I [a]			50A/50I			25A/75I		
	min.	mean	max.	min.	mean	max.	min.	mean	max.
M_3 [b]									
Artemia	69.0	85.4	100	67.1	83.2	99.3	55.8	72.1	88.5
Inert diet	0.0	14.6	31.0	0.7	16.8	32.9	11.5	27.9	44.2
PL_1									
Artemia	69.3	86.3	100	59.5	76.5	93.4	49.1	66.0	82.9
Inert diet	0.0	13.7	30.7	6.6	23.5	40.5	17.1	34.0	50.9
PL_2									
Artemia	71.7	87.3	100	61.4	78.0	94.8	50.7	66.3	81.8
Inert diet	0.0	12.7	28.3	5.2	22.0	38.6	18.2	33.7	49.3
PL_3									
Artemia	77.8	94.7	100	69.3	86.3	100	48.5	65.4	82.2
Inert diet	0.0	5.3	22.2	0.0	13.7	30.7	17.8	34.6	51.5
PL_4									
Artemia	79.3	96.7	100	69.4	86.7	100	48.1	65.3	82.5
Inert diet	0.0	3.3	20.7	0.0	13.3	30.6	17.5	34.7	51.9
PL_5									
Artemia	70.0	87.3	100	55.4	72.7	89.9	34.8	52.0	69.2
Inert diet	0.0	12.7	30.0	10.1	27.3	44.6	30.8	48.0	65.2

[a] Feeding regime 75A/25I consisted of 75% *Artemia* nauplii and 25% inert diet on a carbon-weight basis. Treatment 25A/75I had opposite percentages. In all cases, estimated carbon incorporations were significantly different to those expected in Table 1.
[b] Nutritional contributions from stages M_3 to PL_2 were calculated using the $\Delta^{13}C$ values of $PL_{3\text{-}5}$ fed only inert diet.

4. Discussion

4.1. Larval growth and survival

The present study evaluated the proportional nutritional contributions of *Artemia* and inert diet to the growth of shrimp larvae from M_1 to PL_5 by assessing the $\delta^{13}C$ values in both dietary sources and animal tissue. During the study period, shrimp increased their carbon weight from 8 to 11-fold in all treatments except those fed only on inert diet. It has been shown that increases in body weight from 3 to 4-fold are necessary for observing isotopic equilibriums between penaeid shrimp and constant diets (Fry and Arnold, 1982; Al-Maslamani, 2006; Gamboa-Delgado and Le Vay, 2009). The higher survival observed in co-fed shrimp mysis and PL as compared to animals fed only *Artemia* is in agreement with studies reporting nutritional benefits when complementing live feed with inert diets and/or using additives to manipulate the nutritional composition of live feed, which improves the overall response of shrimp with respect to live food alone (Léger et al., 1985; Coutteau et al., 1997; Gallardo et al., 2002; Calderon et al., 2004). Brito et al. (2004) also reported higher growth in *L. vannamei* and *L. setiferus* fed co-feeding regimes as compared to regimes consisting only in *Artemia* without microalgae. Some studies have reported good survival and complete larval development to PL_1 when shrimp are fed only on microalgae (Kuban et al., 1985; Rodriguez et al., 1994; Naranjo et al., 1999). In the present study, and in order to prevent an isotopic influence from the microalgae on the $\delta^{13}C$ values of the animals and also on nutrient incorporation values indicated by the isotopic mixing model, *C. gracilis* was added only during the zoea sub-stages. Mysis shrimp fed only inert diets showed lower survival and growth than values observed in the other treatments. Although slow growth was foreseen in this treatment, low survival was unexpected as previous studies have reported high survival in shrimp larvae fed inert diets only (Jones et al., 1979; Kurmaly et al., 1989; D'Abramo et al., 2006). On the other hand, co-feeding *Artemia* with

the inert diet significantly improved survival compared to use of *Artemia* alone and even at up to 75% *Artemia* replacement, while the 25 and 50% replacement levels supported growth equivalent to the use of *Artemia* alone.

4.2. Influence of diet on isotope value changes and carbon turnover rate

Shrimp eggs showed very negative (isotopically depleted) $\delta^{13}C$ values, most likely due to their high lipid content and possibly also due to effects of the shrimp broodstock diet and metabolism. Lipids are depleted in $\delta^{13}C$ (DeNiro and Episten, 1978) and as a result, the fast consumption of the isotopically-depleted yolk reserves over the naupliar stages was reflected in a steep, near-linear, increase in $\delta^{13}C$ values that persisted through the zoea stages when shrimp larvae were fed microalgae. Isotopic equilibrium was finally reached following consumption of *Artemia* nauplii. After the experimental dietary treatments were differentiated, shifts in $\delta^{13}C$ values of shrimp fed on the different feeding regimes (except those fed on inert diet) stabilized quickly in the mysis and postlarval stages. Rapid isotopic changes are due to both, high growth and metabolic turnover rates, in the present study indicated by parameters from the exponential model (Hesslein et al., 1993). Fry and Arnold (1982) also observed fast $\delta^{13}C$ turnover in *P. aztecus* shrimp postlarvae fed *Artemia*, which led to short carbon metabolic half time in tissue (4 d). Although the high metabolic turnover rates observed in the present study were not statistically different, their integration into equation (2) with the growth rates, indicated significantly higher carbon t_{50} in animals fed 100% inert feed (2.5 ±0.3 d). The faster growing animals in the other feeding treatments exhibited carbon t_{50} ranging from 1.2 to 1.6 d. The carbon t_{50} in tissue decreases as a function of high growth rates as a result of isotopic dilution, which affects the tissue isotopic values determined by the previous diet; therefore the carbon t_{50} can be used as an indicator of diet performance. The C:N ratio of fertilized eggs decreased very

fast after hatching, indicating fast consumption of lipid reserves during the naupliar stages. The decreasing trend slowed down through the zoea stages when larvae first-fed on microalgae. An increase in C:N ratio after M_1 was associated to the intake of *Artemia* and indicated growing lipid reserves. Observed changes in C:N ratio followed the opposite trend of $\delta^{13}C$ values since the catabolism of isotopically depleted lipids increased the $\delta^{13}C$ values in larval tissue.

4.3. The relative contribution of Artemia and inert diet to larval tissue growth

Decapod larvae employ either filter feeding or raptorial feeding methods depending upon their trophic level. Zoea stages of penaeids are herbivorous and employ filter feeding but shift to raptorial feeding during the later mysis stages (Jones et al., 1997). Under conditions of high food availability, penaeid larvae show high ingestion rates and rapid turnover in the gut (Kurmaly et al., 1989a), while a high digestive enzyme content enables them to rapidly extract the more digestible components from the food items with a relatively low overall assimilation efficiency but high net energy gain (Le Vay et al., 2001). Results from the present study indicate that carbon contributions from *Artemia* to the growth of mysis and early PL was significantly higher than the expected values established by the proportions of feed provided in the co-feeding regimes. The higher incorporation of nutrients from *Artemia* nauplii might be partially attributed to their higher digestibility in penaeid larvae and PL as compared to inert diets. In the present study, estimated inert diet contribution to growth from M_3 to PL_5 ranged from 3 to 15% in the feeding regime 75A/25I, increased to 13 to 27% in the 50A/50I regime and finally, in regime 25A/75I, contributed from 28 to 48% of the incorporated carbon in shrimp tissue. Dietary isotopic influences from both food sources on shrimp tissue and data integration into the isotopic mixing model thus indicate that significantly higher than expected

proportions of nutrients supporting larval growth originated from *Artemia* nauplii in the co-feeding regimes. Villamar and Langdon (1993) point out that excessive leaching losses, sub-optimal nutrient composition and poor digestibility of inert diets have prevented complete replacement of live food organisms. In caridean larvae, Kumlu and Jones (1995a) observed that animals having guts full of artificial diets showed poor growth and survival, suggesting that these larvae cannot digest or possibly assimilate enough artificial diet to supply growth requirements due to low levels of digestive enzymes during early stages. In penaeid mysis larvae, previous studies have observed increased digestive protease activity in response to inert diets, which has been attributed to low dietary protein availability (Le Vay et al., 1993). Interestingly, in the present study, those shrimp fed the 25% *Artemia* replacement regime demonstrated much greater retention of dietary carbon from the inert diet than those fed that diet alone. This may have resulted from greater ingestion and/or assimilation of the inert diet in the presence of *Artemia*, combined with the higher growth rate in the co-feeding treatment. Also, the higher survival observed in shrimp raised on co-feeding regimes, as compared to shrimp fed only *Artemia*, indicates that the inert diet supplied essential nutrients not found in *Artemia* nauplii, while the lower growth and lower carbon incorporation in shrimps fed regimes consisting in 75 and 100% of inert feed confirmed results from previous studies that showed that inert diets were less digestible than *Artemia* (Léger et al., 1986; Le Vay et al., 2003). In the present study, *Artemia* was supplied as nauplii added in an unenriched form and without microalgae, therefore suggesting that, in the co-feeding regimes, the inert feed may have supplied nutrients that were otherwise scarce or not found in *Artemia* nauplii (for example *n*-3 unsaturated fatty acids) thus promoting higher survival. DHA levels in *Artemia franciscana* nauplii are generally very low or undetectable (Barclay and Zeller, 1996; Han et al., 2001), as is the case for the *Artemia* strain used in the present study (Nghia et al.,

2007). In contrast, the fatty acid profile of the inert diet used in the present experiment, shows significantly higher contents of DHA (Liu et al., 2007) than those reported for *Artemia*. Results from the present study indicate that replacing 50% of *Artemia* (on a dry weight basis) with inert diets in co-feeding regimes represents a good strategy as growth, survival and metamorphosis rates were similar to those observed at 25% *Artemia* replacement. $\delta^{13}C$ values of PL_4 and PL_5 fed the three co-feeding regimes showed a slight deviation towards the value of the inert diet, suggesting a higher ingestion and/or assimilation efficiency as the postlarval stages progressed. The low growth observed in larvae fed only on inert diet was reflected in a longer period of time to reach isotopic equilibrium, therefore contributions of nutrients at stages M_3, PL_1 and PL_2 were estimated using $\Delta^{13}C$ values for PL_3 to PL_5 fed only inert diet. Assumptions associated with the use of isotope mixing models (see review by Martinez del Rio et al., 2009) to assess nutritional contributions from dietary sources were met in the present study. These include the similar elemental (carbon) composition in the food sources, quantified discrimination factors and avoidance of tissue isotopic routing by using whole animals for tissue analysis. Assimilation efficiency of the dietary sources was not assessed in this study, but they are likely to be different since *Artemia* nauplii are known to be more digestible in penaeid larvae and PL when compared to inert diets (Léger et al., 1986; Le Vay et al., 2003). This difference might imply that in the present study, nutritional contributions from *Artemia* nauplii might in fact have been slightly higher than indicated by the isotopic mixing model.

4.4. Shrimp – diet isotopic discrimination

Different carbon discrimination factors ($\Delta^{13}C$) between organisms and diets have been linked to different factors such as the C:N ratio and lipid content of the diets (DeNiro and

Episten, 1978; Fantle et al., 1999; Post et al., 2007), dietary protein:energy ratio and amino acid profile (Gaye-Siessegger et al., 2004; O'Brien et al., 2005). In the present study, shrimps fed only either *Artemia* nauplii or inert diet, showed contrasting carbon discrimination factors ($\Delta^{13}C$ = +1.3 and +4.1‰, respectively). These observations are similar to those reported by Al-Maslamani (2006), who also observed different $\Delta^{13}C$ values in *P. semisulcatus* fed only *Artemia* (+1.6‰) and various inert diets (-1.3 to +6.8‰). $\Delta^{13}C$ values between shrimp and *Artemia* observed in the present study are very similar to those reported by Fry and Arnold (1982) in *P. aztecus* PL fed on *Artemia* ($\Delta^{13}C$= +1.0 to +1.1‰), while Dittel et al. (1997) also observed contrasting $\Delta^{13}C$ values when *L. vannamei* PL were fed zooplankton (+0.4‰) or detritus (+6.0‰). Similar observations have been reported in fish larvae fed only *Artemia* ($\Delta^{13}C$= +0.8‰) or inert diets ($\Delta^{13}C$= 2.3‰) and it has been suggested that such differences are related to a lack or deficiency of specific dietary nutrients (Martínez del Rio and Wolf, 2005; Gamboa-Delgado et al., 2008). Other studies have supported this premise by indicating that the amino acid profile of the diets might account for differences in $\Delta^{13}C$ values. For example, $\delta^{13}C$ analysis of individual amino acids in juvenile *Callinectes sapidus* has revealed that crabs fractionate the carbon isotopes in essential and non-essential amino acids differently (Fantle et al., 1999).

4.5. The use of stable isotopes in larval nutrition

Carbon and nitrogen stable isotopes have been successfully used to assess digestion and assimilation in fish larvae fed on live food (Schlechtriem et al., 2005), to determine incorporation of individual dietary sources co-fed to fish larvae and postlarvae (Gamboa-Delgado et al., 2008) and to identify periods when larval fish are physiologically better suited to incorporate specific nutrients (Jomori et al., 2008). Assessing nutrient

contribution in crustacean larvae using stable isotopes is difficult due to rapid ontogeny through larval stages and trophic levels, which frequently prevent the organisms form reaching isotopic equilibrium. However, as observed in the present study, the very fast growth of penaeid larvae and PL (fed on 100, 75 and 50% of *Artemia* nauplii) achieved isotopic equilibrium with the diets in as little as 5 d. The use of isotopic mixing models is limited when the dietary sources have overlapping isotopic profiles. Evaluation of nutrient incorporation thus requires previous knowledge (or estimation) of the diet isotopic values in order to ensure enough resolution on the isotopic changes occurring in tissue over time as a result of dietary intake. Although the use of live feeds represents clear advantages in the culture of marine larvae such as high digestibility, availability in the water column and suitability for nutritional enrichment, they are expensive to culture and provide a vector for the introduction of pathogenic micro-organisms into larval culture tanks (Southgate and Partridge, 1998), therefore, there are continuous efforts in developing and improving inert diets that can be used at higher replacement levels. In this regard, the use of stable isotopes provides an additional useful tool in assessing nutrient incorporation from experimental diets and also in designing improved feeding regimes. The resolution of such nutritional studies in larval and postlarval organisms will be enhanced through the application of compound-specific stable isotope analysis, which has allowed tracing individual fatty acids (Evans et al., 2003) and essential and non-essential amino acids (O'Brien et al., 2003, 2005; McCullagh et al., 2008) in invertebrates and fish. Further experiments might indicate if different isotopic discrimination factors between consuming organisms and diets are consistently associated to different dietary characteristics.

5. Conclusion

The present study demonstrated the use of stable isotopes to estimate nutritional carbon contributions in shrimp larvae and postlarvae co-fed inert diet and *Artemia*. Shrimp mysis and early postlarvae incorporated higher than expected amounts of nutrients from live feed compared to an inert diet in three different co-feeding regimes. It is suggested that the co fed inert diets supplied nutrients not found in unenriched *Artemia* nauplii, thus promoting higher survival than shrimp fed only on *Artemia*. Results indicate lower than expected carbon contributions from the inert diets to tissue growth, possibly due to lower digestibility. Incorporation of nutrients from the inert diet increased as postlarvae developed. Assessment of carbon stable isotopes at natural abundance levels in the dietary items and consuming organisms allows estimating nutrient allocation form different sources, providing that the latter have contrasting isotope values among them and in relation to the consumer’s isotopic value before inclusion of the new diet. The possibility of manipulating the isotopic values of different larval dietary items increases the resolution of such studies. For example, through addition of different culture media, the carbon and nitrogen isotope values in microalgal cells can be easily manipulated and in turn, these can be fed to *Artemia* and rotifers to the same end.

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