Natural carbon stable isotope ratios as indicators of the relative contribution of live and inert diets to growth in larval Senegalese sole (*Solea senegalensis*).

Julián Gamboa-Delgado[1], José Pedro Cañavate[2], Ricardo Zerolo[2] and Lewis Le Vay[1].

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School of Ocean Sciences

College of Natural Sciences

Bangor University

Menai Bridge, Anglesey, UK

LL59 5AB

Tel +44 (0) 1248 351151

Fax +44 (0) 1248 716367

osp216@bangor.ac.uk

2

CIFPA "El Toruño", IFAPA

Junta de Andalucía

Apartado 16, 11500

Puerto de Santa María

Cádiz, Spain

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Abstract

The relative contributions of live *Artemia* metanauplii and an inert diet for growth of Senegalese sole larvae and postlarvae were assessed through the analysis of carbon stable isotopes ratios ($\delta^{13}C$) in both diets and whole larval tissue. Larvae were reared on four dietary regimes: 100% live prey (rotifers and *Artemia*), 100% inert formulated diet and two co-feeding regimes of 70:30 and 30:70 ratios of *Artemia* and inert diet, respectively. Larvae from the live food regime and both co-feeding regimes showed a steep increase in $\delta^{13}C$ from 10 days after hatching (DAH) as a result of the onset and continuation of *Artemia* consumption. From 12 DAH fish larvae from all the regimes showed significant isotopic differences as their $\delta^{13}C$ increased to final asymptotic values of -15.1, -15.6 and -16.3 ‰ in the live food, 70:30 and 30:70 regimes, respectively. Carbon turnover rates in larvae from both live food and co-feeding regimes were relatively high (0.071 to 0.116 d^{-1}) but more than 90% of the observed change in fish tissue isotopic values was accounted for by the retention of carbon in new tissue growth. A two-source, one-isotope mixing model was applied to estimate the nutritional contribution of *Artemia* and inert diet to postlarvae growth in the co-feeding regimes. At 23 DAH, the relative contribution of live and inert diets to tissue growth in larvae was respectively, 87 and 12% for the 70:30 co-feeding regime and 73 and 27% for the 30:70 co-feeding regime. At 17 DAH, the estimated proportion of tissue carbon derived from the inert diet was higher at 23 and 38% for the 70:30 and 30:70 regimes, respectively. The results suggest that co-feeding regimes in *S. senegalensis* larvae may be adjusted to meet ontogenetic changes in the capacity for larvae to utilise inert diets. The contrasting levels of carbon isotope discrimination between diet and tissue in larvae reared on either 100% live feed or 100% inert diet

indicate relatively poor utilization of nutrients from the inert diet. The use of isotopic discrimination factors as potential indicators of the digestive physiological performance of a consuming organism in regards to its diet is discussed.

Keywords: Stable isotopes; nutrient assimilation; carbon turnover; *Solea senegalensis*

1. Introduction

The Senegalese sole is one of several potential candidates that might contribute to diversification of farmed species of marine fish in Europe, due to its characteristics of high growth rate, tolerance to a range of salinities and high commercial value. Although various biological aspects of the larval, postlarval and juvenile stages of this species have been studied over the last 15 years (e.g. Parra et al., 1999; Ribeiro et al., 1999; Morais et al., 2004; Conceição et al., 2007), further information is still needed with regard to nutrition and physiology during its early life stage. The larval development of *S. senegalensis* is characterized by rapid growth followed by an early metamorphosis (20-22 DAH) and transition from the planktonic to benthic stages. In common with the majority of larval rearing techniques for marine fish (Teshima et al., 2000), hatchery production of sole larvae is still highly dependant on live feeds such as rotifers and *Artemia,* while weaning of post-settlement juveniles to formulated feeds can be relatively difficult due to their selective feeding behaviour and slow acceptance of inert diets. In rearing larvae of many marine fish species, varying proportions of inert diets may be used in co-feeding regimes to partially replace live feeds (Appelbaum, 1989; Rosenlund et al., 1997; Kolkovski and Tandler, 2000), which are expensive and frequently variable in nutritional quality. This feeding strategy is designed to overcome the inability of the undeveloped digestive system of early larval stages to break down inert food particles, by taking advantage of the increased digestion and assimilation of inert diets in the presence of live food within the gut (Walford et al., 1991; Kolkovski et al., 1997). Co-feeding strategies have potential economic benefits through the improvement of the nutritional status of the larvae as well as pre-conditioning them for better acceptance of inert weaning feeds at an earlier

stage (Cahu and Zambonino-Infante, 1995; Rosenlund et al., 1997). The degree to which live foods for marine fish larvae can be replaced with inert diets has been steadily increasingly as a result of the development of new microdiets and delivery methods (Fletcher et al., 2007; Langdon et al., 2007). In sole larvae, a number of studies have reported the use of different inert diets as partial substitutes for live feed (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2002; Chang et al., 2006; Palazzi et al., 2006). However, the extent to which inert diets contribute directly to sole larval growth has not been determined.

The majority of techniques employed to determine assimilation of nutrients are indirect methods that are difficult to apply in marine animals at the very early life stages and often require some manipulation of dietary ingredients, such as incorporation of labelled components using radio- or stable isotopes (Tonheim et al., 2004; Verschoor et al., 2005). The use of stable isotopes provides a safe alternative to the use of radio-isotopes and represents a straightforward technique to directly trace the flow and incorporation of carbon and nitrogen from unadulterated inert diets and live prey into the target organism (Schlechtriem et al., 2004; Jomori et al., 2008), providing an integrated nutritional history of organic matter assimilation and retention (Yokoyama et al., 2002). The use of this approach relies on the assumptions that isotope values in tissues of consuming organisms reflect those of their diet and that tissue isotope signature may change over time due to dietary variations (Fry, 2006). The application of mixing models allows estimation of the proportion of nutrients derived from different food sources retained in the consuming organism. Carbon accounts for approximately 50% of the dry weight of most life forms; hence the consuming organism-diet relationship in terms of carbon stable isotope composition can be used to

identify dietary components contributing to growth both, in pond culture (Schroeder, 1983; Nunes et al., 1997) and in larval rearing systems (Schlechtreim et al., 2004). Comparisons of different ratios of carbon stable isotopes signatures ($\delta^{13}C/^{12}C$, $\delta^{13}C$ hereafter in the text) in available natural foods and supplied feeds have been useful in gaining insight into the flow of organic matter in aquatic environments and into the consuming organisms (Schroeder, 1983; Fry, 1991) which in turn, closely resemble their diet $\delta^{13}C$ values (DeNiro and Epstein, 1978). After a consuming organism has reached isotopic equilibrium with its diet, a difference in isotope values between diet and consuming organism is frequently observed (Fry and Arnold, 1982). This isotopic fractionation is the result of heavier isotopes being preferentially selected at the molecular level in the organism tissues during the sum of metabolic reactions, hence producing an isotope discrimination factor, $\delta^{13}C_{body\text{-}diet}$.

The present study experimentally estimates the relative contribution of carbon from *Artemia* and from a routinely used commercial inert feed to tissue growth in larvae of *Solea senegalensis*, through the analysis of naturally occurring carbon stable isotope ratios in different feeding items and in the consuming organism whole bodies. A second objective was to assess the carbon turnover rate in larval tissue and to explore the potential of using the isotope discrimination factor as an indicator of diet performance in nutritional studies.

2. Material and methods

2.1 Supply of larvae and rearing system

Larval rearing was conducted at CIFPA "El Toruño" aquaculture laboratory, Cadiz, Spain. *Solea senegalensis* eggs were flow-collected from a spawning tank in which 39 wild-caught breeders were kept under ambient light and temperature conditions. A batch of 130,000 eggs was incubated in a 300 l conical-bottom tank with 50% water exchange h^{-1} using seawater pumped from a channel connecting to the Bay of Cadiz and passed through sand and cartridge filters (10 and 3 μm). Temperature, dissolved oxygen and salinity in the incubation tank were maintained at 21.5 °C, 7.4 mg l^{-1} and 33 g l^{-1}, respectively. Two days after hatching (DAH), larvae were transferred into 300 l rearing tanks and stocked at an initial density of 50 individuals l^{-1}. The larval tanks were filled to a volume of 100 l with filtered seawater and moderate aeration was provided. No water exchange was made in the larval tanks until 6 DAH. During the larval rearing period water quality conditions were as follows: salinity 33.7 ± 1.2 g l^{-1}, dissolved oxygen 6.8 ± 0.9 mg l^{-1}, total NH_3-N 0.15 ± 0.07 mg l^{-1} and temperature 21.1 ± 1.3 °C. A light:dark photoperiod was set as 16:8 h with mean light intensity of 303 lux. Salinity, temperature and dissolved oxygen were determined with a WTW 340i multi-parameter instrument and light intensity with a Lutron LX-102 light meter. The ammonium concentration in seawater was monitored using the phenol-nitroprussiate method.

2.2 Live and inert diets

Isotope mixing models generate enhanced outputs describing assimilation of dietary components when the different nutritional sources show contrasting isotope values. The estimation of carbon turnover rate is also improved if this condition holds. Therefore, diets were selected with the aim of providing a relatively wide range of $\delta^{13}C$ values. To

achieve this, the carbon stable isotope values of different *Artemia* strains, dry diets and enrichment products were determined before the larval culture experiment. *Isochrysis galbana* (T-ISO strain) was mass produced in 50 l cylindrical vessels using a nutrient mixture of sodium nitrate (7.6 mol l^{-1}), ferric chloride (0.05 mol l^{-1}), monosodium phosphate (0.8 mol l^{-1}), trace metals, EDTA and vitamins B1 and B12. Guillard's F/2 nutrient medium was used to scale up stock cultures. In order to avoid an unwanted influence on the $\delta^{13}C$ of the microalgal cells, CO_2 was not supplemented in the aeration. The rotifer *Brachionus plicatilis*, strain-S, was mass cultured on baker's yeast and later enriched for 18 h with the same pre-cultured *I. galbana*. *Artemia* cysts (*Artemia franciscana,* Vinh-Chau strain, Vietnam) were hatched under standard conditions. Twenty four h after hatching, metanauplii were also enriched with *I. galbana* for a further 24 h. In order to adjust the amount of live and inert diet proportions fed in the different feeding regimes, the dry weight of enriched rotifers and enriched *Artemia* metanauplii were estimated by concentrating the individuals in dense homogenous small volumes of known density. Five replicate samples were rinsed in distilled water, dried for 24 h at 60 °C and weighed to the nearest 10 µg. The inert diet AgloNorse (EWOS, Norway) had a protein content of 59% and was fed to larvae and postlarvae in particles sizes of 300-500 and 400-700 µm, respectively. The moisture content of the inert diet (10%) was also taken into account in establishing the feeding regimes on a dry weight basis.

2.3 Experimental design

Four different feeding regimes with differing levels of live food replacement were randomly assigned to larvae in triplicate tanks. All 12 tanks received a similar low

concentration of *I. galbana* and enriched rotifers from 2 to 6 DAH (Table 1). The positive control live food regime (LF) consisted of rotifers and *Artemia* from 2 to 9 DAH and only *Artemia* from 10 DAH onwards. Two co-feeding regimes were provided. The first (70LF:30ID) comprised 70% live food and 30% inert diet on a dry weight basis as described by Cañavate and Fernandez-Diaz (1999). Treatment 30LF:70ID comprised the opposite live food/inert diet proportions, 30% live food and 70% inert diet. A negative control regime was used and it consisted of only inert diet from 7 DAH onwards. Rotifers and microalgae were added to the tanks in a single, morning dose. Artemia metanauplii was supplied in two equal daily rations, a first ration was added at 09:00 while a second ration was kept at 4° C and delivered at 17:00. Inert feed was supplied three times a day at 09:00, 13:00 and 17:00.

2.4 Sampling and carbon stable isotope analysis

Microalgae, rotifer and *Artemia* samples were centrifuged or sieved, rinsed with distilled water and frozen at -80 °C until pre-analysis treatment. Throughout the larval rearing period, every 24 to 48 h, samples of 5 to 30 pooled larvae (depending on larval age/weight and treatment) were collected from each replicate tank for stable isotope analysis. Sampling of fish was done before the first feeding and were starved for 24 h and eventually killed by freezing. Total length and metamorphosis stage were estimated. Samples were then oven dried (65 °C for 24-48 h), weighed and kept in a desiccator. Nine hundred to 1100 µg samples of ground dry fish tissue and diets were packed into tin cups (D1008, Elemental Microanalysis Ltd, UK) to be analyzed for carbon and nitrogen content and carbon stable isotope ratios using a PDZ Europa Scientific Roboprep elemental analyzer coupled to a PDZ Europa Hydra 20/20 stable

isotope ratio mass spectrometer (Crewe, UK) at the University of California, Davis. Carbon stable isotope ratios are expressed in delta (δ) notation, which is defined as parts per thousand (‰) deviations from Pee Dee belemnite, the standard reference material (Eq. 1). Every 12 measurements, two validation samples were run and the $\delta^{13}C$ standard deviation from these reference samples was 0.1 ‰. We use the term "discrimination factor" following Cerling and Harris (1999) and Pearson et al. (2003) to describe changes in isotope values between a consuming organism (whole body or specific tissue) and its diet.

$$\delta^{13}C = (R_{sample}/R_{standard} - 1) \cdot 1000 \quad (1)$$

where $R = {}^{13}C/{}^{12}C$

2.5 Estimation of nutrient assimilation

The assimilated and retained proportions from the different feeding items were estimated using a two-source one-isotope mixing model (Phillips and Gregg, 2001). The model takes into account the stable isotope value variability of both, the sources (live and inert diets) and the isotope mixture (fish tissue). One of the model assumptions is that the consuming organism is in isotopic equilibrium with its diet. After a constant isotopic difference between diet and fish was reached, isotope values were corrected for discrimination factors before introduction into the mixing model. This was done by introducing into the model the isotopic profiles of both, fish fed only inert and live diets (positive and negative controls), which set up two different trophic levels instead of having introduced only the isotopic value of their respective diets.

Isotopic profiles of fish from the co-feeding regimes were also introduced into the model to estimate the assimilation proportions and their 95% confidence intervals. Carbon contents in the inert diet and enriched *Artemia* metanauplii were slightly, but statistically different (Table 2); therefore, in order to calculate the total amount of carbon contributed by each feeding source, the following equation (Fry, 2006) was used:

$$f_{total1} = f_1 \cdot W_2/(f_1 \cdot W_2 + f_2 \cdot W_1) \text{ and } f_{total2} = 1\text{-}f_{total1} \quad (2)$$

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

$$f_1 = (\delta^{13}C_{fish\ sample} - \delta^{13}C_{source2})/(\delta^{13}C_{source1} - \delta^{13}C_{source2}) \text{ and } f_2 = 1\text{-}f_1$$

W_1 and W_2 represent the percent carbon content in each of the two sources.

The amount of carbon relative to the amount of nitrogen present (C:N ratio) in fish at different larval ages was also estimated as an indicator of changes occurring as endogenous and exogenous nutrients were being used up by the larvae.

2.6 Changes in larval tissue isotope composition during development and estimation of carbon turnover rate

Stable isotope value shifts observed in animal tissues following a change in diet may be due to growth and/or metabolic turnover. In order to separate these factors and to obtain an estimate of the carbon turnover rate and its effect on isotopic change, the isotope

values in the tissues of fish following the transition in diet from rotifers to *Artemia* and to the co-feeding regimes, were fitted to an exponential model (Eq. 3, Hesslein et al., 1993) that integrates growth and isotope value shift over time.

$$R_{sample} = R_n + (R_o - R_n)e^{-(k+m).t} \quad (3)$$

Where R_{sample} is the fish tissue isotope value at time *t*, R_o is the isotope value of the fish tissue in equilibrium with the initial diet, R_n is the isotope value reached when the animal is in equilibrium with a new diet. *m* is the metabolic turnover rate and it was derived from the equation and calculated using iterative non-linear regression. An estimate of the growth (constant *k*) is required for the model and this was obtained from an exponential growth equation (Eq. 4).

$$W = W_o \cdot e^{(k \cdot t)} \quad (4)$$

Where W is the final dry weight, W_o is the initial weight, *t* is the time (d) and k is the growth rate expressed per day (d^{-1}). In this approach, Hesslein's model coefficients *k* and *m* indicate the magnitude of the isotopic rate of change in relation to growth and metabolic turnover, respectively, and can also provide an estimation of the time necessary for half of the body tissue to reach isotopic equilibrium after consuming the new diet (half time, t_{50}):

$$t_{50} = \ln 2/(m+k) \quad (5)$$

2.7 Statistical analysis.

Data are expressed as mean ± standard deviations. Carbon content in inert and live diets, and discrimination factors were compared by means of Student's *t*-tests. Differences between $\delta^{13}C$ values, dry weights, percentage metamorphosis and survival were analyzed by one way ANOVA, after variance homogeneity was verified by Levene's test. Tukey's pairwise comparisons were used to detect treatments that differed significantly from each other. Parameters in Equation 3 were estimated by iterative non-linear regression. All the tests were done using SPSS 12.0 software (SPSS Inc.) at a significance level of $P< 0.05$.

3. Results

3.1 Stable isotopes values in live and inert diets

Carbon stable isotope values determined in a range of larval food items are shown in Table 2. *I. galbana* grown in vessels where only pumped air was used showed a ^{13}C enrichment substantially higher than microalgae cells produced with supplementary CO_2. Changes in isotope values in 24-h starved and T-ISO-enriched *Artemia* metanauplii were minimal as compared to post-hatched nauplii. There was a significant difference (6.6 ‰) in $\delta^{13}C$ between the enriched *Artemia* metanauplii when compared to the inert diet value. This isotopic divergence was sufficient to allow assessment of the contribution of carbon from both sources using the mixing model.

3.2 Larval growth and survival

More than 90% of the fish larvae in the LF regime had completed metamorphosis by 23 DAH. Fish in this treatment had a mean final dry weight of 598 ± 46 μg which was significantly higher than the weight of fish in the other feeding regimes at the end of the experiment (Fig. 1). There were no significant differences between treatments LF and 70LF:30ID in regards to total length, metamorphosis and survival by 23 DAH (Table 3). Larvae in the ID regime exhibited very low survival and at 18 DAH all remaining fish in this treatment were collected for further analysis.

3.3 Isotopic changes in larval tissue during development, in relation to dietary treatment

Recently-hatched larvae (0 DAH) had a $\delta^{13}C$ value of -19.4 which increased significantly to -18.6 on the following 4 days as yolk reserves were utilised. This was also indicated by a decline in C:N ratio in larval tissue during the same time period (Fig. 4). From 4 DAH, a decrease in $\delta^{13}C$ was observed, corresponding to the start of exogenous feeding. Over the same time period, the C:N ratio steadily increased until metamorphosis, though this trend was variable in relation to the different feeding regimes. Isotopic values for larvae from the dietary treatments were differentiated at 7 DAH, with the $\delta^{13}C$ values in fish fed only inert diet becoming significantly lower as compared to the other three regimes. Larvae from the LF regime and both co-feeding regimes showed a steep increase in $\delta^{13}C$ enrichment after 10 DAH as a result of the onset and continuation of *Artemia* consumption. At 12 DAH larvae from all the regimes showed significant isotopic differences as fish values from the LF and 70LF/30ID regimes kept increasing toward the enriched *Artemia* isotopic value (Fig 3). The enrichment observed in treatments LF, 70LF:30ID and 30LF:70ID started to

stabilize from 17 DAH onwards at isotopic asymptotic values of -15.1, -15.6 and -16.3 ‰, respectively. Most fish had metamorphosed to the benthic stage by this period.

3.4 Relative contribution of live prey and inert diet to tissue growth

The individual mean dry weight of enriched rotifers and enriched *Artemia* metanauplii was 0.226 ± 0.013 µg and 2.23 ± 0.27 µg, respectively. During the 23-day rearing period the LF regime received 23.4 g of *Artemia* metanauplii (on a dw basis). The 70LF:30ID regime was fed 15.7 g of *Artemia* and 6.2 g of inert diet, respectively representing 71.8 and 28.2 % of total rations offered. The 30LF:70ID regime received 7.0 g of *Artemia* and 12.7 g of inert diet, representing 35.7 and 64.3 % of total rations respectively. In contrast, the ID regime received only 14.6 g of inert diet as a result of feeding rations being adjusted according to observed survival.

At 23 DAH, the estimated proportions of larval tissue derived from *Artemia* and inert diet, and their 95% confidence intervals, were 0.88 (0.85-0.90) and 0.12 (0.09-0.15) respectively, for the 70LF:30ID regime and 0.73 (0.69-0.77) and 0.27 (0.23-0.31) for the 30LF:70ID regime (Table 4). By comparison, in younger larvae at 17 DAH, the estimated proportion of tissue carbon derived from the inert diet was 0.23 (0.19-0.28) and 0.38 (0.22-0.54) for the 70LF:30ID and 30LF:70ID regimes, respectively.

3.5 Carbon turnover and isotopic discrimination

Fish larvae were not supplied with rotifers after 10 DAH, when feeding regime LF was shifted to *Artemia* and co-feeding regimes 70LF:30ID and 30LF:70ID were changed to

combinations of *Artemia* and inert diet. Over the 13 days after these diet shifts, linearization of the trend lines (fitted Eq. (3) in Fig. 5) for predicted tissue $\delta^{13}C$ values and subsequent slope comparisons, showed significant differences in the rate of isotopic change detected in fish from the three feeding regimes. The estimated time (half life) for the carbon from the new feeding items to be incorporated in new body tissue was 3.5 ± 0.6 days for fish in the 30LF:70ID regime as compared to 2.5 ± 0.2 and 2.7 ± 0.5 days in the LF and 70LF:30ID regimes, respectively. The high growth rate observed in the fish from the three regimes (fitted Eq. (4) in Fig. 1) resulted in an increase in biomass of over 700% on a dry weight basis, over a period of 13 days. Even though the turnover rates for carbon were also relatively high (*m* values ranging from 0.071 to 0.116 d^{-1}, Fig. 5), the rapid addition of biomass and the predicted values from the model suggest that the change in the fish isotopic values was mainly accountable (>90%) to the retention of carbon from the *Artemia* and inert diet in new tissue growth. The observed isotopic discrimination values between the fish tissue and their diets in isotopic equilibrium were +0.8 ± 0.14 ‰ for the LF regime and +2.3 ± 0.07 ‰ for the ID regime (Fig. 3).

4. Discussion

Solea senegalensis larvae go through a relatively rapid morphological development in which a shift from the pelagic to the benthic stage occurs between 15 to 20 DAH (Fernández-Díaz et al., 2001). During this period, rapid ontogeny of digestive capacity takes place as the digestive tract elongates and the liver and pancreas increase their size and enzyme production (Ribeiro et al., 1999). Yolk reserve consumption is positively correlated with a decrease in larval dry weight; once the yolk has been depleted and

exogenous feeding starts, the trend is reversed and a high growth rate follows. Feeding regimes used in fish larviculture make use of a relatively low number of diet types that usually include microalgae, rotifers, *Artemia* and artificial feed. Mixing models used to ascertain nutrient assimilation generate narrower confidence intervals when fewer feeding items are present in the system, giving information about the proportions of nutrients being assimilated from different sources and that are in consequence, nutritionally important. An implicit assumption of the model is that the consuming organism is in isotopic equilibrium with its diet (Fry and Arnold, 1982); therefore, the application of stable isotopes to estimate assimilation or trophic position is limited when the target animals go through different metamorphic and/or trophic stages, consequently preventing an isotopic equilibrium to be reached. A number of studies have reported that an increase in body weight from 3 to 4-fold is required for the consuming organism to reach an isotopic equilibrium with its diet (Fry and Arnold, 1982; Vander Zanden et al., 1998). The time period that is required to allow sufficient integration of dietary isotopes to reflect a diet depends on the metabolic rate of the tissue examined and the growth rate of the organism (Hobson and Clark, 1992; Hesslein et al., 1993). In the present study, larvae from the feeding regimes LF, 70LF:30ID and 30LF:70ID increased their weight 7 to 11-fold from 10 DAH (41-49 ug dw) when fish started to receive *Artemia* and/or artificial feed and until 23 DAH (322-598 ug dw). This high growth rate and the use of only two feed sources during the second half of the larviculture, suggests that changes in tissue isotopic values were primarily due to the addition of newly-synthesized tissue.

The small size of marine fish larvae makes it difficult to isolate specific organs or tissues in order to analyze their isotope values differentially. In the present experiment,

only pools of whole, 24-h starved animals were analyzed. This minimized the metabolic routing effect that occurs when different tissues take up dietary nutrients at different incorporation rates, consequently affecting the apparent diet carbon contributions measured in different tissues or organs (Phillips and Koch, 2002; Podlesak and McWilliams, 2006). The observed increase in $\delta^{13}C$ from 0 to 5 DAH suggests that lipid tissue, usually less enriched in ^{13}C in relation to other tissues (Peterson and Fry, 1987; Hobson and Welch, 1995) was actively being used during the endogenous trophic stage of the larvae, causing isotopic enrichment as vitelline sources were being depleted. The C:N ratio sharply decreased during this period, consistently indicating a catabolism of lipid reserves. Even though the fish larvae were supplied with live rotifers from 2 DAH to ensure live feed availability during the unsynchronized onset of the exogenous feeding, it appears that the actual consumption started between 3 to 4 DAH as indicated by a sharp change of trend in the fish tissue $\delta^{13}C$ towards the rotifer isotope value (-22.6) at 5 DAH. Parra et al. (1999) observed that a peak in oxygen uptake in *S. senegalensis* larvae occurred around 4 DAH which is possibly correlated to the onset of exogenous feeding and a higher swimming activity. In the present study, on this same period, the C:N ratio started to increase as a consequence of assimilation and retention of exogenous nutrients.

Supplying inert feeds to early stage larvae has positive effects in the weaning period. Cañavate and Fernández-Díaz (1999) observed that *S. senegalensis* are more willing to accept inert particles at earlier larval stages than later in development. Similarly, Barnabe and Guissi (1994) noted that European sea bass larvae seem to accept artificial feed at specific periods of their development, and concluded that this is probably associated with ontogenetic changes in behaviour and digestive physiology. In the

present study, results from the isotope mixing model suggest that in the co-feeding regimes the estimated proportion of retained carbon from the inert diet was higher at earlier than later larval stages, indicating also an increasing utilization of *Artemia* as larvae further developed. This latter tendency was also observed in the 30LF:70ID regime, where the *Artemia* metanauplii supply was restricted. Early sole larvae (3 DAH) have an average mouth opening size of 403 μm (R. Zerolo, personal communication); therefore, older larvae (7 DAH) might have been able to consume both feeding items easily. Inert feed consumption was verified by microscopic observations; nevertheless, fish in the latter regime showed a strong isotopic deviation towards the *Artemia* value. This observation suggests that (1) even with a low amount of available live feed in the rearing tanks, the metanauplii were selectively ingested and better assimilated, as might be expected for larval marine fish offered live prey and (2) a proportion of the inert diet was not consumed during the earlier larval stages as the inert diet sinks to the bottom and is less available to the pre-metamorphic larvae. Estimates of relative contribution of the diets at 17 DAH should be considered with caution as isotopic equilibrium between diet and fish tissues had not been fully reached at this stage.

Organic tissue often does not reflect the gross isotopic composition of the diet. Instead, it reflects the isotopic composition of the constituents of the diet from which the tissue was biosynthesized (Gannes et al., 1997). *Artemia* metanauplii show a high digestibility when consumed by fish larvae; Morais et al. (2004) demonstrated that when *S. senegalensis* larvae feeds on *Artemia*, the amino acids and protein of the latter show a digestibility range from 77 to 83%. When compared to other marine fish larvae, *S. senegalensis* shows a high capacity for digesting live prey even during the early stages

(Conceição et al., 2007). Fish larvae in the ID regime exhibited low survival and growth rate, as is frequently observed when marine fish larvae are exclusively fed on inert diets (Kelly et al., 2000; Teshima et al., 2000). The $\delta^{13}C$ shift observed in fish from all treatments, except in regime ID, equilibrated after 17 DAH, when more than 50% of the fish larvae in treatments LF and 70LF:30ID had metamorphosed to the benthic stage. This stage has been characterized as a period of low metabolic activity and decreased feed consumption in *S. senegalensis* and it is possibly related to an energy saving strategy (Parra and Yufera, 2001).

Isotopic discrimination occurs because organisms have an affinity to incorporate heavy isotopes rather than the lighter and more common isotopes. This is the result of heavy isotopes concentrating in molecules were the bond strengths are greatest, the effect being amplified in every chemical and enzymatic transformation (Peterson and Fry, 1987). There is gathering evidence indicating that isotopic discrimination is species- and tissue-specific (DeNiro and Epstein, 1981, Tiezen et al., 1983; Yokoyama et al., 2005; Stenroth et al., 2006). Therefore, the frequently assumed carbon isotopic discrimination range from 0 to 1 ‰ might not be applicable to all cases. Considerable variance in the reported values demonstrate that careful validation of assumptions about discrimination are critical for interpreting stable isotope data from field and laboratory studies (Crawley et al., 2007). McCutchan et al. (2003) reported $\delta^{13}C$ discrimination values of +0.5 ±1.3 ‰ in aquatic and terrestrial animals. Vander Zanden and Rasmussen (2001) accounted a mean value of +0.47 ± 1.2 ‰ in an aquatic food web; discrimination factors were lower for herbivores than for carnivores. In the present study isotopic discrimination values of +0.8 ± 0.14 and +2.3 ± 0.07 ‰ were observed when sole larvae fed only on *Artemia* or on inert diet, respectively. High discrimination

factors are possibly related to a lack, or deficiency of specific dietary nutrients necessary for a particular life stage; any or both of these conditions may increase the feeding rates as a strategy to cope up with a nutrient deficit. This causes further metabolic cycling of non-essential nutrients and increases the discrimination factor between animal tissue and diet (Martínez del Rio and Wolf, 2005). For example, in regards to protein metabolism, Roth and Hobson (2000) support the hypothesis that nitrogen stable isotope discrimination factors between consuming organism and diet ($\delta^{15}N_{body\text{-}diet}$) will decrease as the biological value of the dietary protein increases. Such premise has been recently supported by Robbins et al. (2005) through an analysis of published data in which a negative correlation ($r^2 = 0.72$) was determined between the $\delta^{15}N_{body\text{-}diet}$ in mammals and birds, and the biological value of their dietary proteins. In the present case, the higher discrimination value in larvae fed the formulated inert diet supports the assumption of nutrient scarcity through either inadequate assimilation or an unbalanced nutrient profile.

Newly-assimilated nutrients are used to synthesize organic tissue and/or to replace degraded molecules, resulting in growth and tissue replacement, respectively. Turnover rates are important for determining how quickly the isotopic signature of an animal's diet is incorporated into its tissues (Pearson et al., 2003). Tissues of fast growing animals exhibit faster turnover rates and a shorter half-life of carbon and nitrogen in the tissue than slow growing animals (MacAvoy et al., 2005), and this may be reflected in turnover at different life stages. For example, the carbon turnover rates observed in the present study (0.071 to 0.116 d^{-1}) were higher than those reported for juvenile *Salmo salar* (0.008 - 0.022 d^{-1}, Jardine et al., 2004) and considerably higher than values for carbon turnover in adult *Coregonus nasus* (0.0018 d^{-1}, Hesslein et al., 1993). Bosley et

al. (2002) reported a carbon half life of 2.2 ± 0.3 d for recently metamorphosed winter flounder, reared at 18 °C following a dietary shift from rotifers to *Artemia*. In the present study, larvae under a similar diet regime (LF) showed a carbon half life of 2.5 ± 0.2 d at an average water temperature of 21.1 °C. As isotopic differences increase between initial animal tissue and diet, resolution of turnover rates tends to improve (Fry and Arnold, 1982). In the present study, predicted tissue $\delta^{13}C$ values fitted well to the observed values (r^2 = 0.83 to 0.93), indicating that sufficient dietary difference in natural $\delta^{13}C$ can be found in foods commonly used for marine fish larvae to make assessment of growth and metabolic replacement rates an effective useful approach to studying the nutritional physiology of organisms under specific feeding regimes and controlled rearing conditions.

Most fish larviculture facilities are still highly dependant on live feed to produce good quality postlarvae and juveniles. Even though inert feeds are being increasingly used as part of co-feeding regimes, higher replacement ratios have resulted in low growth and survival (Teshima et al., 2000). The poor performance of artificial diets has been related to their inability to meet specific nutritional requirements of the larvae (Fletcher et al., 2007) and to the inadequate incorporation of nutrients by the fish due to poor ingestion, digestion and/or assimilation (Chang et al., 2006). In a recent study, Jomori et al. (2008) determined the stable carbon and nitrogen isotopes profiles in live and inert diets and in pacu fish larvae in order to identify periods in which larvae started using nutrients from an inert diet after being weaned from *Artemia* nauplii. In this context, the assessment of the natural abundances of different stable isotopes naturally present in larval and postlarval stages and in their diets can yield relevant information to (1) elucidate the origin and fate of nutrients that contribute to growth and (2) to

define periods at which the larvae are physiologically better prepared to ingest and assimilate nutrients from live and inert feeds. Verschoor et al. (2005) have pointed out the high sensitivity of the use of stable isotopes in the estimation of ingestion and assimilation rates as low as 2.5 ng of carbon ind^{-1} h^{-1} in zooplanktonic organisms, which highlights the application potential of these techniques in the study of larval and postlarval nutrition. Further controlled laboratory-feeding experiments are required to determine the effect of new ingredients and diets of known isotopic profile on the metabolism and growth of marine larvae. Additional comparative laboratory experiments might indicate if the degree of isotopic discrimination is consistent and correlated to specific characteristics of nutritionally complete and deficient diets so as to be used as an indicator of the relative nutritional value of new aquaculture ingredients and feeds.

Conclusion

The assessment of the natural abundances of carbon stable isotopes allowed determining the differential contributions of live and inert feed during the growth of Senegalese sole larvae to postlarval stage. This study also confirms that the inert feed used in co-feeding regimes is physiologically better utilized during specific growth stages, indicating that co-feeding regimes can be optimized when they are designed considering periods and peaks of maximal ingestion and assimilation. In the feeding schedules of many marine larval rearing operations, there are much less trophic elements than in the larval natural environment; therefore, the estimation of isotopic profiles in dietary items and consuming organisms, as well as their integration into mixing models proves less complex, consequently generating valuable information that

might further assist in the evaluation of new diets. As observed in the present study and reported in other manuscripts, different discrimination factors in regards to diets of different nutritional composition, are potential indicators of the digestive physiological performance of an organism.

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Table 1

Summary of four experimental feeding regimes used in the larval culture of *Solea senegalensis*

Days after Hatching	T-ISO (cells $ul^{-1}d^{-1}$)	Rotifers (ind $ml^{-1}d^{-1}$)	Artemia (ind $ml^{-1}d^{-1}$)	Inert diet (mg $l^{-1}d^{-1}$)
Live food				
2-6	100	10	-	-
7-9	100	15	1.8	-
10-12	-	-	4.5	-
13-17	-	-	6.5	-
18-23	-	-	7.5	-
70LF–30ID				
2-6	100	10	-	-
7-9	100	15	1	1.5
10-12	-	-	3	3
13-17	-	-	4	5
18-23	-	-	5	5
30LF–70ID				
2-6	100	10	-	-
7-9	100	9.7	0.7	5.1
10-12	-	-	0.7	7.8
13-17	-	-	1.4	9.3
18-23	-	-	2.2	9.3
Inert diet*				
2-6	100	10	-	-
7-9	-	-	-	8.4
10-12	-	-	-	9.4
13-17	-	-	-	13.4
18-23	-	-	-	13.4

*Fish collected 18 DAH

Table 2

Carbon content and carbon stable isotopes ratios ($\delta^{13}C/^{12}C$) in *Solea senegalensis* postlarvae and different larval diets. Means ± SD of 3 to 6 samples

Organism/feeding item	C (mg g^{-1})	$\delta^{13}C/^{12}C$
Baker's yeast	449 ± 16	-23.2 ± 0.0
Inert diet (AgloNorse)	464 ± 4	-22.4 ± 0.1
Isochrysis galbana cells		
Air only	567 ± 15	-21.0 ± 0.0
Air + CO_2 injected	583 ± 12	-43.3 ± 0.1
Rotifers *Brachionus plicatilis*		
Mass cultured on yeast	434 ± 20	-23.9 ± 0.1
Enriched on T-ISO (4 generations)	451 ± 8	-22.2 ± 0.0
Enriched and after 16 h in rearing tanks	471 ± 14	-21.4 ±0.1
Artemia, Vinh-Chau strain		
Posthatched nauplii	500 ± 31	-15.7 ± 0.1
24 h starved metanauplii	441 ± 7	-15.3 ± 0.3
24 h enriched metanauplii (T-ISO)	418 ± 9	-15.6 ± 0.2
Enriched and after 16 h in rearing tanks	437 ± 12	-15.8 ± 0.1
Solea senegalensis larvae		
Recently hatched larvae	487 ± 0	-19.4 ± 0.0
23 days after hatching:		
Regime LF	425 ± 30	-15.1 ± 0.3
Regime 70LF/30IF	433 ± 15	-15.6 ± 0.2
Regime 30LF/70IF	448 ± 30	-16.3 ± 0.2
Regime IF (18 DAH)	448 ± 18	-20.1 ± 0.1

Table 3

Survival, individual dry weight, total length and percentage of *Solea senegalensis* larvae completing metamorphosis at 23 DAH under different feeding regimes (means ± SD)

Feeding regime	Survival (%)	Dry weight (µg)	Total length (mm)	Metamorphosis (%)	Carcass C:N ratio
Live food	55.2 ± 4.3[a]	598 ± 46[a]	6.85 ± 1.66[a]	93 ± 9.7[a]	3.67
70LF/30ID	48.2 ± 3.1[a]	489 ± 41[b]	6.66 ± 1.28[a]	82 ± 6.9[a]	3.72
30LF/70ID	30.7 ± 1.7[b]	322 ± 32[c]	5.46 ± 1.0[b]	53 ± 5.4[b]	4.03
Inert diet*	13.1 ± 1.8[c]	50 ± 16[d]	3.85 ± 0.69[c]	2 ± 0.1[c]	3.61

* Fish in the inert diet regime were collected 18 DAH.
Different superscripts indicate significant differences at $P<0.05$.

Table 4

Estimated percentage contribution of live and inert feeds to tissue growth in *Solea senegalensis* larvae and postlarvae grown under two different co-feeding regimes

Time	70LF/30ID*			30LF/70ID*		
	min.	mean	max.	min.	mean	max.
17 DAH						
Artemia	72.2	76.6	80.9	45.6	61.6	77.7
Inert diet	19.0	23.4	27.7	22.3	38.4	54.4
20 DAH						
Artemia	70.0	82.2	94.3	61.3	67.3	73.3
Inert diet	5.7	17.8	30.0	26.7	32.7	38.7
23 DAH						
Artemia	84.8	87.6	90.4	69.1	72.9	76.7
Inert diet	9.6	12.4	15.2	23.3	27.1	30.9

* Regime 70LF/30ID had a proportion of 70% live food and 30% inert diet on a dry weight basis. Treatment 30LF/70ID had the opposite live food/inert diet proportion.

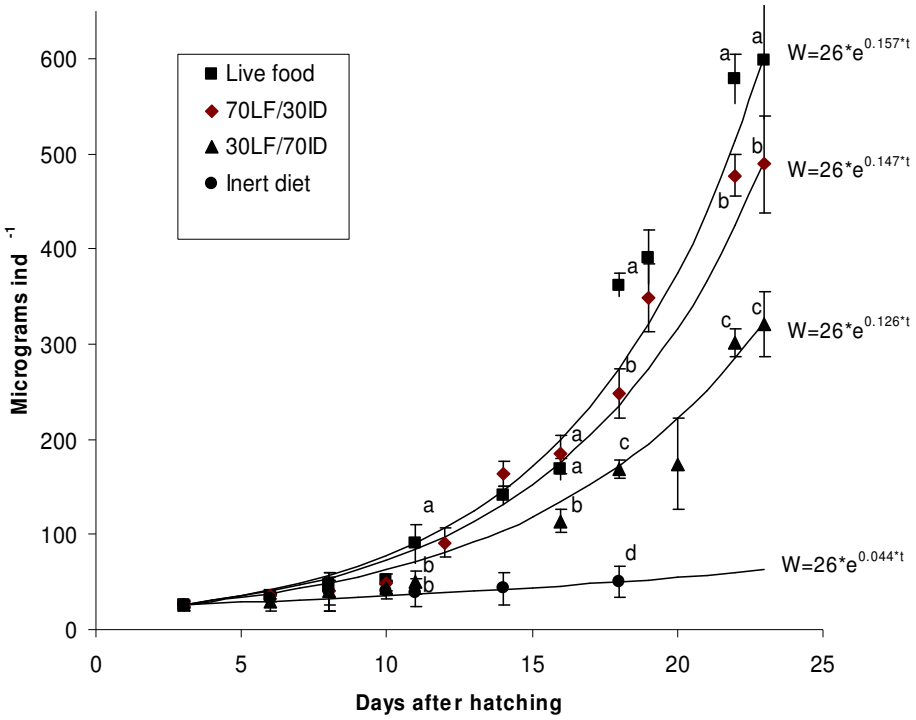

Fig.1. *Solea senegalensis* larval growth under different feeding regimes. Experimental growth curves were fitted to dry weight data. Mean of 15 samples, vertical bars indicate standard deviations. Different subscripts denote statistical differences.

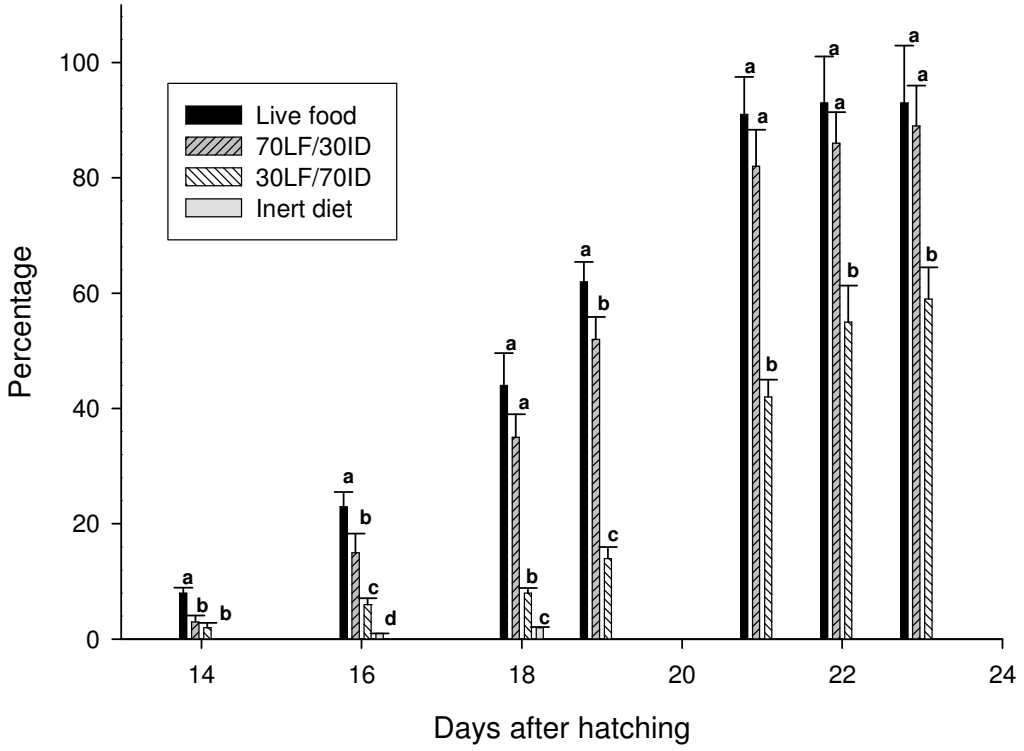

Fig. 2. Percentage (± SD) of *Solea senegalensis* postlarvae reaching benthic stage in different feeding regimes. Different superscripts indicate significant differences between treatments ($P<0.05$).

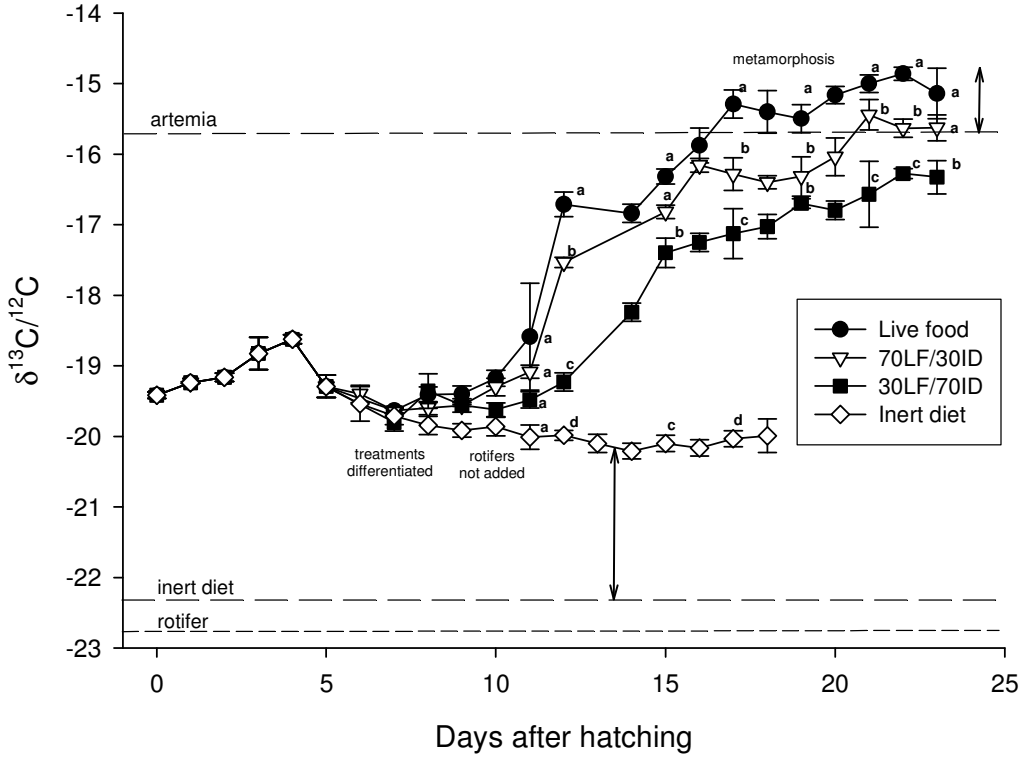

Fig. 3. Changes in carbon stable isotope ratios in whole tissue of *Solea senegalensis* larvae raised on different feeding regimes. Means of 6-9 samples ± standard deviations. Verticals arrows refer to the degree of isotopic discrimination between diet and the consuming organism (LF and ID) after equilibrium. Different subscripts indicate differences between treatments on that particular day.

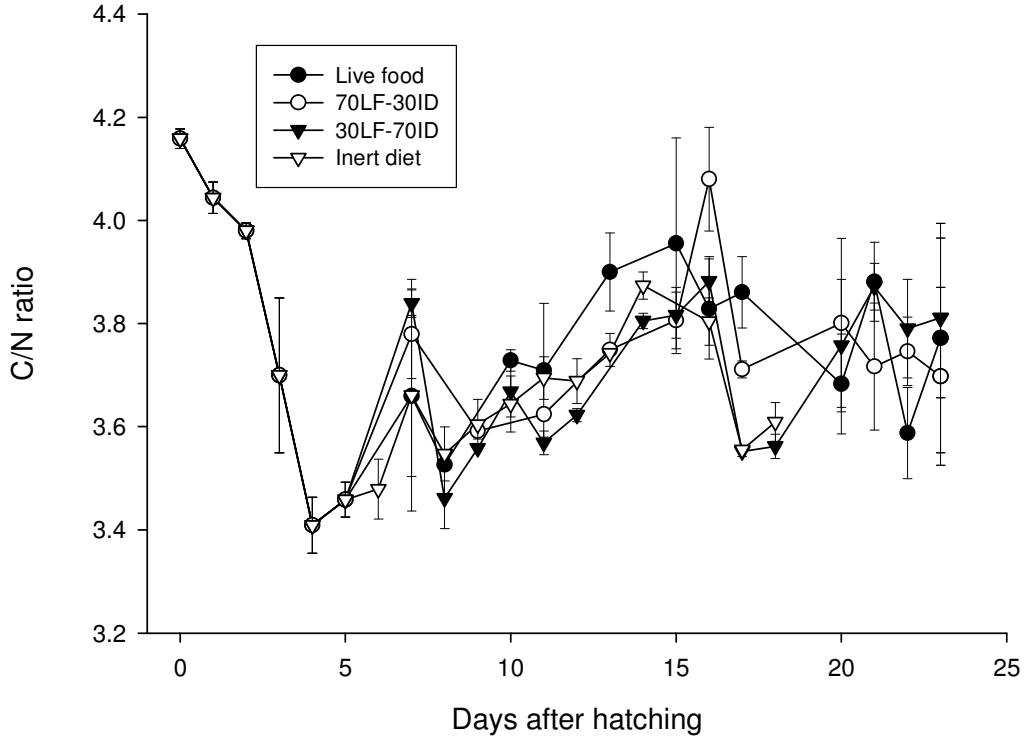

Fig. 4. Changes in carbon/nitrogen ratio in *Solea senegalensis* larvae and postlarvae raised on four different feeding regimes. Mean of 6-9 samples ± SD indicated by vertical bars.

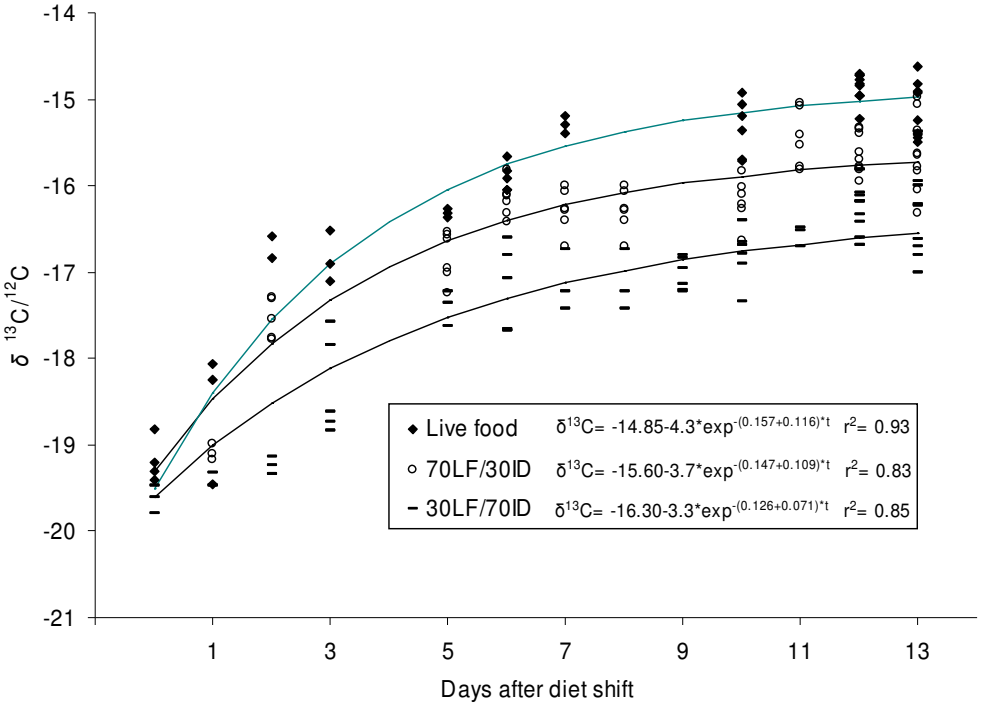

Fig. 5. Carbon stable isotope value changes after a dietary shift 10 DAH from rotifers to *Artemia* metanauplii and two co-feeding regimes using different *Artemia*/inert diet proportions. Predicted values from the Hesslein's model were fitted to observed data using non-linear iterative regression.