Effect of different diets on proteolytic enzyme activity, trypsinogen gene expression and dietary carbon assimilation in Senegalese sole (*Solea senegalensis*) larvae

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Abstract

The effect of diet on larval growth, anionic trypsinogen gene expression (*ssetryp1*), and trypsin and chymotrypsin activities was assessed in *Solea senegalensis*. Changes in larval carbon stable isotope ($\delta^{13}C$) composition were used to estimate carbon assimilation. Diets were supplied for 20 d to fish held in larval rearing tanks and consisted of live rotifers, *Artemia* nauplii, rotifers followed by *Artemia*, rotifers co-fed with inert diet and inert diet alone. Growth was significantly faster in larvae fed only *Artemia* and those fed rotifers and *Artemia* (k= 0.381-0.387 d^{-1}). Trypsin and chymotrypsin activities increased from 3-4 days after hatching (DAH) in all dietary treatments, while *ssetryp1* transcripts increased at 4-5 DAH only in larvae fed live prey. *ssetryp1* gene expression was activated later in larvae fed only *Artemia* and this corresponded with *Artemia* $\delta^{13}C$ values being reflected in larval tissue. Larval $\delta^{13}C$ values also indicated greater selection and/or assimilation of rotifers in relation to the inert diet. Results demonstrate that during early larval development of sole, diet modulates *ssetryp1* gene expression. The rapid and intense response to diets that promoted different growth and survival suggests the suitability of this biomarker as a nutritional status indicator in early sole larvae.

Keywords: *Solea senegalensis*, larval nutrition, enzyme activity, trypsinogen, gene expression, stable isotopes, assimilation

1. Introduction

Among the different species viable for marine aquaculture production, the Senegalese sole (*Solea senegalensis*) represents an ideal candidate to further diversify production in Europe. Even though efficient larval rearing and weaning strategies have been developed for this species in recent years (Cañavate and Fernández-Diaz, 1999; Engrola et al, 2007; 2009a;

2009b), more information is required on the developmental changes in the processes of food ingestion, digestion and assimilation. This is of special relevance to the goal of significant replacement of live feed by inert diets, which can be designed to satisfy specific nutritional requirements. Artificial diets are usually associated with reduced growth, malformations and higher stress susceptibility in feeding larvae due to low diet acceptance, combined with poor ingestion, digestion and assimilation, and lack of essential micronutrients (Koven et al., 2001; Cahu et al., 2003). 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; Le Vay and Gamboa-Delgado, 2010). Changes in the isotopic composition of organisms generally results from dietary shifts (Hesslein et al., 1993; Carleton and Martínez del Rio, 2010) and the rate of isotopic incorporation depends on tissue growth and metabolic turnover (Fisk et al., 2009). Carbon is present in all organic molecules hence carbon turnover can be considered as an indicator of total tissue turnover (MacAvoy et al., 2005).

Trypsin is a key proteolytic enzyme influencing the digestive capacity of fish larvae (Pedersen et al., 1987; Moyano et al., 1996; Perez-Casanova et al., 2006). It is synthesized in the pancreas as a pre-proenzyme and stored in secretory granules as trypsinogen, the proenzyme form. In the intestine, enterokinase removes the N-terminal activation peptide converting it in its active form, trypsin. In turn, the resulting trypsins themselves activate all other pancreatic digestive zymogens including more trypsinogens (Chen et al., 2003). In *S. senegalensis*, four trypsinogen types have been identified: anionic trypsinogen (*ssetryp1*), cationic trypsinogen (*ssetryp2*) and 2 psychrophilic trypsinogens (*ssetryp3* and *ssetrypY*) (Manchado et al., 2008). *ssetryp1* was the most abundant, with expression greatly activated early in the first week of larval life. This expression pattern is well-correlated with a total

trypsin activity peak at 9 days after hatching (DAH) (Ribeiro et al., 1999). Moreover, trypsin enzymatic activity has been proposed as a good indicator of nutritional status in young sole (Engrola et al., 2007). Information on expression patterns and enzymatic activities under different feeding regimes is crucial to optimize feeding regimes and aquaculture protocols in this species. The present study aimed to evaluate the effects of commonly-used diets that are known to support different growth rates in early sole larvae, so that effects of diet on trypsinogen gene expression, trypsin and chymotrypsin activity and nutrient assimilation could be investigated.

2. Material and methods

2.1. Senegalese sole and rearing system

The experiment was conducted at IFAPA "El Toruño" aquaculture centre (El Puerto de Santa María, Cádiz, Spain). Wild captive broodstock fish were kept under ambient light and temperature (16 – 22 °C) conditions. Eggs from a collective spawn were incubated in a 200 l conical-bottom tank having a water exchange of 50% h^{-1}. Recently posthatched larvae were sampled every 24 h in order to monitor their initial $\delta^{13}C$ values, which were maternally defined and considered as isotopic baseline points before supplying the experimental feeding regimes. As soon as larvae reached 2 DAH they were transferred into 300 l-capacity rearing tanks at an initial fish density of 50 ind l^{-1}. Rearing tanks were double-walled to maintain a narrow water temperature range. If necessary, spaces between walls were synchronically flooded with thermostatically-controlled water pumped from a chiller. During the experimental period, seawater was maintained under the following mean conditions: temperature 21.1 ±0.8 °C, dissolved oxygen 7.4 ±1.0 mg l^{-1} and salinity 33 ±1.2 g l^{-1}. A light:dark photoperiod was set as 16:8 h with mean light intensity of 300 lux. Moderate bubbling and water circulation were provided by diffused air pumped through a ring-shaped

tube attached to the tank bottom. In order to minimize the handling, no water exchange was practised until larvae reached 6 DAH. During the larval experiment, the ammonium concentration in seawater was monitored using the phenol-nitroprussiate method and over this period total NH_3–N remained below 0.15±0.07 mg l^{-1}.

2.2. Experimental feeding regimes

Five different dietary regimes that are commonly used in rearing *S. senegalensis* larvae (Cañavate and Fernández-Diaz, 1999; Engrola et al., 2007, 2009b) (Table 1) and that are known to support varying growth rates were randomly assigned to duplicate tanks holding a mean number of 15,000 fish larvae each. Feeding regimes receiving a single food type from 3 to 10 DAH were fed exclusively rotifers (Rotifers group), *Artemia* (Artemia group) or inert diet (Inert diet group). A co-feeding regime consisted of rotifers and inert diet supplied on a 50:50 ratio estimated on a dry weight basis and was also supplied from 3 to 10 DAH (Co-feeding group). In a fifth regime, rotifers were added from 3 to 5 DAH followed by *Artemia* from 6 to 10 DAH (Rotifers/Artemia group). An unfed treatment was used as a negative control. All feeding regimes received 100 cells ul^{-1} of *Isochrysis galbana* (Tahitian strain, T-ISO) from 3 to 10 DAH to condition the water. From 11 to 20 DAH all fish were fed only *Artemia* metanauplii enriched for 24 h with T-ISO. Estimation of carbon turnover rates in consumers is enhanced when their respective dietary sources exhibit contrasting isotope values in regards to the consumer's tissues, therefore feeding items were selected in order to provide a relatively wide range of isotopic values. Thus, $\delta^{13}C$ values of rotifers, two *Artemia* strains, inert diet and microalgae were determined before the larval culture (Table 2). In order to estimate turnover rates at the postlarval stage, the isotopic profiles of fish fed only inert diet or *Artemia* were further manipulated by feeding them two different types of *Artemia* (Vinh Chau, Viet Nam or INVE-32 strain) which are known to have differing $\delta^{13}C$

Table 1. Experimental larval diets used to assess trypsin and chymotrypsin activity, trypsinogen gene expression and carbon isotopic changes in whole larval tissue of *Solea senegalensis*. T-ISO= *Isochrysis galbana*, Tahitian strain.

Days after Hatching	T-ISO (cells $\mu l^{-1}d^{-1}$)	Rotifers (ind $ml^{-1}d^{-1}$)	*Artemia* (ind $ml^{-1}d^{-1}$)	Inert diet (mg $l^{-1}d^{-1}$)
Rotifers				
3-5	100	20	-	-
6-10	100	20	-	-
11-14	-	-	6.6 (I)[a]	-
15-20	-	-	8.5 (I)	-
Rotifers/*Artemia*				
3-5	100	20	-	-
6-10	100	-	3.3 (I)	-
11-14	-	-	6.6 (I)	-
15-20	-	-	8.5 (I)	-
Artemia				
3-5	100	-	0.3 (I)	-
6-10	100	-	3.3 (I)	-
11-14	-	-	6.6 (I)	-
15-20	-	-	8.5 (VN)[b]	-
Co-feeding				
3-5	100	10	-	8.3
6-10	100	10	-	9.4
11-14	-	-	6.6 (I)	-
15-20	-	-	8.5 (I)	-
Inert diet				
3-5	100	-	-	16.6
6-10	100	-	-	19.0
11-14	-	-	6.6 (VN)	-
15-20	-	-	8.5 (I)	-
Starved				
3-5	100	-	-	-
6-10	100	-	-	-
11-14	-	-	-	-
15-20	-	-	-	-

[a] I = *Artemia* INVE-32 strain

[b] VN = *Artemia* Viet Nam strain

values, from 11 DAH and 15 DAH (Table 1). In order to facilitate its capture and ingestion, *Artemia* supplied before 7 DAH was offered as nauplii and as enriched metanauplii from 7 to 20 DAH. *Isochrysis galbana*, rotifers *Brachionus plicatilis* and *Artemia* nauplii were mass produced and enriched as described in Gamboa-Delgado et al. (2008). Daily rations of live and inert diets provided in the different feeding regimes were adjusted in order to supply equivalent amounts of dry matter which were calculated based on modifications of the feeding protocol presented by Cañavate and Fernández-Diaz (1999). The mean dry weight of enriched rotifers (0.22 μg) and *Artemia* metanauplii (2.23 μg) were estimated as described in Gamboa-Delgado et al. (2008). The inert diet AgloNorse (EWOS, Norway; 59% crude protein, 20% crude fat, 5% carbohydrates and 12% ash; manufacturer's data) was fed to larvae in particles sizes ranging from 300 to 500 μm. The moisture content in inert feed (10%) was considered in establishing the feeding regimes on a dry weight basis. *Artemia* metanauplii and rotifers were supplied in two daily rations; a first ration was added at 09:00 h while a second ration was kept at 4 °C and delivered at 17:00 h. Inert feed rations were thoroughly suspended in seawater and supplied in three daily doses at 09:00, 13:00 and 17:00 h. The intermittent supply of this diet and the mild aeration in the rearing tanks allowed keeping a constant presence of inert feed particles in suspension, so that food availability was comparable to live prey treatments. Before the first daily feeding, the inner surfaces of the tanks were scrubbed and simultaneously siphoned out to remove uneaten feed and debris. For isotopic analysis, fish larvae (10 to 20 animals, according to dry weight required for analytical procedures) were randomly sampled every 24 h over the first 6 DAH and every 2 to 3 d thereafter. From 3 DAH, animals were allowed to starve for 12 h in order to minimize food presence in the gut which might have affected the $\delta^{13}C$ values. Larvae were killed by immersion in water/ice slurry, the total length measured and the metamorphosis rate determined according to Fernández-Diaz et al. (2001). Larvae in

samples were counted, pooled and oven dried (24 h, 60 ºC) to estimate mean dry weight and stored at -80 ºC until pre-treatment for isotopic analysis.

Table 2. Carbon contents and stable isotopes ratios ($\delta^{13}C$) in different larval feeds and whole body tissue of *Solea senegalensis* reared on feeding regimes consisting of live and/or inert feed. Means ± SD of 3 to 4 samples.

Organism/feeding item	C (mg g^{-1})	$\delta^{13}C$ (‰)
Isochrysis galbana cells		
Air + CO_2 injected	548 ± 35	-57.9 ± 0.3
Rotifers *Brachionus plicatilis*		
Mass cultured on yeast	439 ± 34	-24.7 ± 0.2
Enriched on T-ISO (18 h)	484 ± 23	-36.4 ± 0.3
Artemia, Viet Nam strain		
Posthatched nauplii	514 ± 14	-16.0 ± 0.1
Metanauplii (Enriched on T-ISO 24 h)	523 ± 35	-18.5 ± 0.3
Artemia, INVE-32 strain		
Posthatched nauplii	534 ± 25	-19.9 ± 0.1
Metanauplii (Enriched on T-ISO 24 h)	520 ± 23	-23.3 ± 0.2
Baker's yeast	453 ± 3	-24.5 ± 0.1
Inert diet (AgloNorse)	528 ± 23	-23.3 ± 0.1
Solea senegalensis		
Recently hatched larvae	526 ± 9	-19.4 ± 0.0
10 days after hatching and fed on:		
Rotifers	505 ± 18	-27.1 ± 0.2
Artemia	473 ± 6	-23.5 ± 0.5
Inert diet	478 ± 40	-20.1 ± 0.1
Co-fed Rotifers-Inert diet	483 ± 10	-24.8 ± 0.0
Rotifers and *Artemia*	490 ± 21	-25.4 ± 0.2

2.3. Gene expression analysis

A previous study of our group demonstrated that anionic trypsinogen *sstryp1* expression was significantly modified during the early larval development (3 to 8 DAH) of sole (Manchado

et al., 2008). Hence, to evaluate a possible effect of feeding regimes on *ssetryp1* expression, transcripts levels were quantified during this critical period. For gene expression analysis, 500 to 1,300 larvae (depending on individual weight) were collected daily from 3 to 8 DAH from each replicate tank and pooled. Samples were washed with DEPC water, frozen in liquid nitrogen and stored at -80 ºC until use. Three subsamples were taken from each sample of pooled animals. Homogenization, RNA isolation, cDNA synthesis and real-time PCR analyses were conducted as reported in Manchado et al. (2008). Primers used in this study were Ssetryp1·1 (F) and Ssetryp1·2 (R) for the target gene (anionic trypsinogen, *ssetryp1)* and SseGAPDH231·1 and SseGAPDH231·2 for the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, *gapdh-2*) (Manchado et al., 2007a, 2007b; Infante et al., 2008; Manchado et al., 2008). Relative mRNA expression for *ssetryp1* was determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).Values from fish sampled 3 DAH and prior to first feeding were used as calibrators.

2.4. Digestive enzyme analysis

For enzymatic determination, the assays were performed on whole bodies of larvae. From 3 to 8 DAH and before the first daily feeding ration, 100 to 300 fish larvae (depending on individual weight) were collected from each replicate tank and pooled. Three subsamples were homogenised in 5 volumes (w/v) of ice-cold distilled water, followed by centrifugation (16,000 g, 30 min, 4 ºC). The supernatant was used for assays of trypsin, chymotrypsin and protein content. Trypsin activity was measured with a fluorimetric assay using Z-L-arginine (CBZ-L Arg-MCA, Sigma C8022) as substrate, following Bolasina et al. (2006). Chymotrypsin activity was measured with a fluorimetric assay using N-Succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (CBS, Sigma S9761) as described in Moyano et al. (2005). Determination methods were adapted to using a microplate reader. Enzyme specific

activities were expressed in units expressing percentage increase of emission min^{-1} per mg of protein at 30 ºC (mU mg $protein^{-1}$). The concentration of soluble protein in extracts was determined by the Bradford method (Bradford, 1976) adapted to microplate reading and using bovine serum albumin as a standard.

2.5. Stable isotope analysis and nutrient turnover rates

Sample methods and protocols to determine total carbon and nitrogen contents and $\delta^{13}C$ values, as well as procedures to estimate proportional nutrient contributions from inert diet and rotifers in the co-feeding regime (using a two-source, one-isotope mixing model; Phillips and Gregg, 2001) were conducted as described in Gamboa-Delgado et al. (2008). An exponential model describing isotopic change (Hesslein et al., 1993) was used to estimate and compare the degree at which observed isotopic changes in fish following a change in diet were due to growth and/or metabolic turnover, and also to compare different carbon turnover rates observed in whole tissue of fish fed on different regimes. The model integrates growth (k) and isotope shifts over time and provides an estimate of the elemental turnover rates (m) and their effect on isotopic change. Iterative non-linear regression was used to estimate the proportion of isotopic change in tissue caused by metabolic turnover (m). The time necessary for half of the body tissue to reach isotopic equilibrium or to be replaced after consuming the new diet (half time, t_{50}) was also estimated from the parameters in the exponential model (MacAvoy et al., 2005). The C:N ratio of fish at different larval stages was estimated in order to associate C:N and isotopic changes with nutritional variations occurring as endogenous and exogenous nutrients were utilized.

2.6. Statistical analysis

Data are expressed as mean ± standard deviations. The treatment-specific growth rate constant (k) was estimated by fitting an exponential growth model to the observed wet weights. In order to verify isotopic differences (required by the isotopic mixing model) and similar elemental content in rotifers and inert diet used in the co-feeding regime, carbon concentration and isotope values of both sources were compared by Student's t-tests. Differences in $\delta^{13}C$ values, dry weights, metamorphosis and survival between fish from different feeding treatments were analyzed by one-way ANOVA. Survival rates were arcsin transformed. Normal distribution and variance homogeneity were verified by Levene's and Lilliefors tests, respectively. Tukey's pairwise comparisons were used to detect treatments that differed significantly from each other. All the tests were done using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) at a significance level of $p<0.05$.

3. Results

3.1. Larval growth and survival

At the end of the experiment, there were significant differences among treatments in mean final dry weight, survival and metamorphosis but not in total length (Figs. 1 and 2, Table 3). Fish from the Rotifers and Rotifers/Artemia groups showed higher dry mean weights at the end of the experiment. Higher survival was observed in both latter groups and in the Co-feeding group. Significantly lower survival and mean dry weight were observed in fish from the Artemia group and the Inert diet group (Table 3). Observed growth rates (k) were significantly higher in fish fed only *Artemia* and fish fed rotifers followed by *Artemia* (k= 0.381-0.387 d^{-1}) from 3 to 10 DAH. Fish fed inert diet showed lower growth rate (0.071 d^{-1}); however, compensatory growth was observed in this latter group after the dietary shift to enriched *Artemia* metanauplii from 11 to 20 DAH (0.290 d^{-1}) (Table 4).

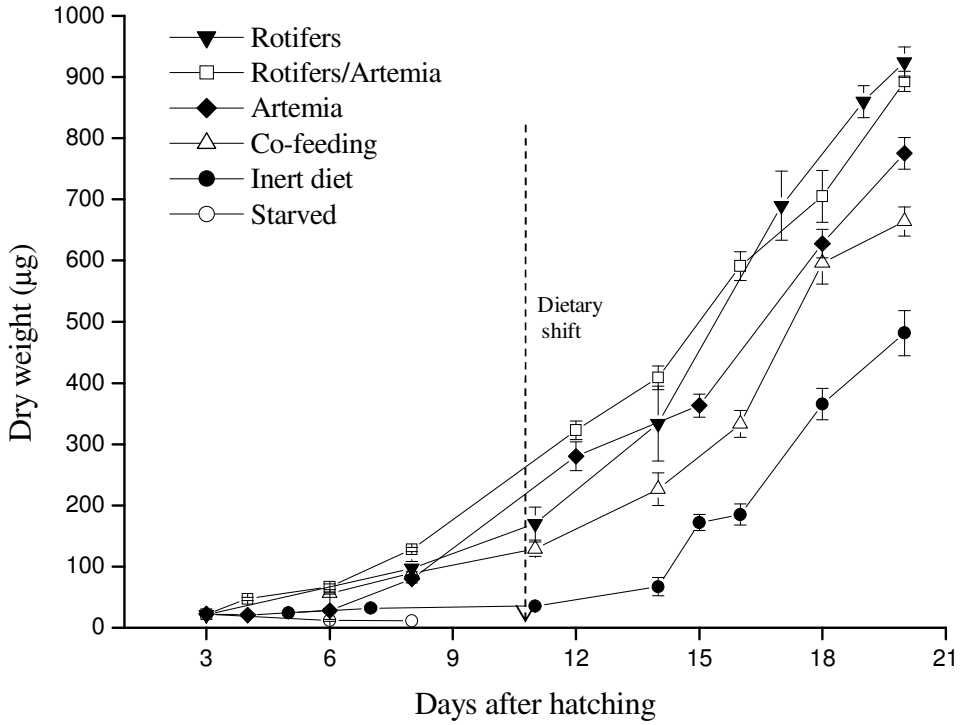

Fig. 1. Larval growth of *Solea senegalensis* reared on different diets supplied from 3 to 10 DAH followed by a dietary shift to enriched *Artemia* metanauplii from 11 to 20 DAH (except treatment Rotifers-Artemia, 6 DAH). Mean of 15 samples, vertical bars indicate standard deviations.

Table 3. Survival, individual dry weight, total length and percentage of *Solea senegalensis* larvae completing metamorphosis 20 DAH after being reared on different feeding regimes (mean ± SD, n = 10-20)

Feeding regime (3 - 10 DAH)	Survival (%)	Dry weight (µg)	Total length (mm)	Metamorphosis to 20 DAH (%)
Rotifers	76.1 ± 6.1^{a}	924 ± 25^{a}	8.4 ± 1.6^{a}	100 ± 0^{a}
Rotifers/*Artemia*	85.3 ± 5.9^{a}	893 ± 16^{a}	8.0 ± 1.5^{a}	95 ± 3^{b}
Artemia	64.3 ± 1.0^{b}	775 ± 35^{b}	7.2 ± 1.4^{a}	95 ± 4^{b}
Co-feeding	79.5 ± 7.9^{a}	664 ± 41^{c}	6.9 ± 1.3^{a}	98 ± 2^{ab}
Inert Diet	65.6 ± 1.3^{b}	481 ± 37^{d}	7.0 ± 1.6^{a}	50 ± 8^{c}

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

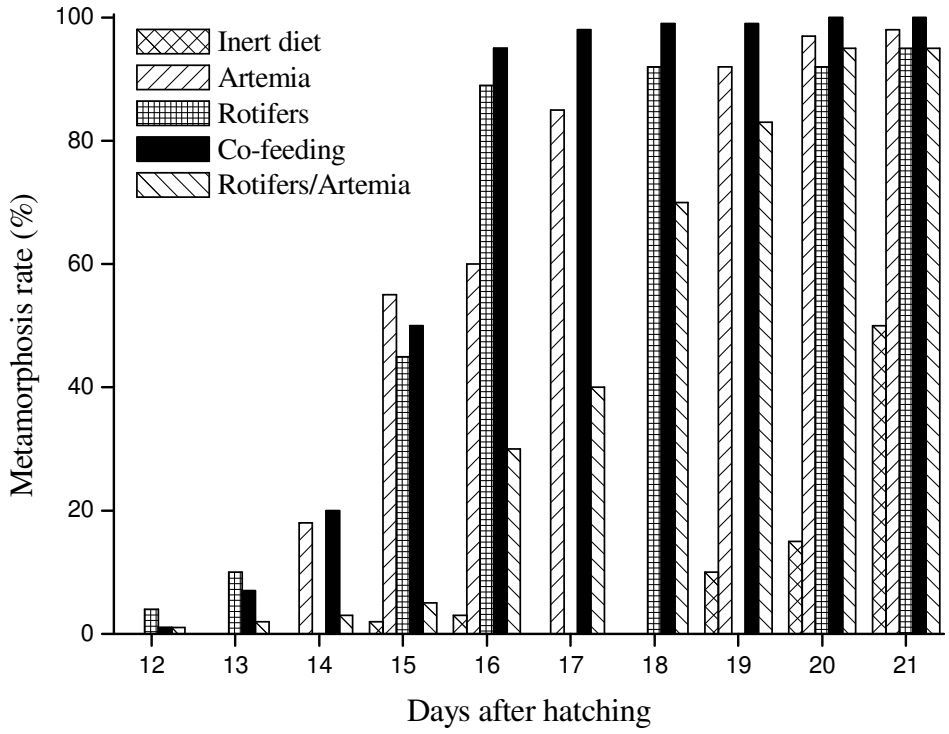

Fig. 2. Percentage (±SD) of *Solea senegalensis* postlarvae reaching benthic stage after being reared on different diets consisting of live and/or inert feed.

3.2. Expression of ssetryp1 under different feeding regimes

mRNAs peaked at 4 DAH in those regimes involving rotifers as prey (Rotifers, Rotifers/Artemia and Co-feeding groups, Fig. 3) although fold induction was lower in fish from the Co-feeding group (5.3-fold). Thereafter, mRNAs decreased progressively until recovering the steady-state levels. The expression profile was quite similar in fish from the Artemia group, although the peak occurred slightly later at 5 DAH. No peak was observed in animals fed inert diet only, although mRNAs increased progressively until 8 DAH (Fig. 3).

3.3. Trypsin and chymotrypsin activities

Trypsin specific activity increased from 3 to 4 DAH in all feeding regimes studied. Moreover, enzymatic activity peaked at 6 DAH in larvae fed only *Artemia* (~6-fold). Trypsin activity in larvae fed inert diet increased progressively until it reached approximately 500 mU mg protein^{-1} at 8 DAH (Fig. 3). In contrast, larvae from feeding regimes including rotifers (Rotifers, Rotifers/Artemia and Co-feeding groups) showed trypsin activity that either remained stable or increased slightly from 4 to 8 DAH (100-330 mU mg protein^{-1}). Chymotrypsin activity profiles followed similar trends as those observed for trypsin, although activities were significantly higher in fish from all feeding regimes except those fed inert diet. Chymotrypsin activity in fish fed rotifers followed by *Artemia* decreased significantly from 4 to 8 DAH.

3.4. Influence of diet on fish tissue C:N ratio and stable isotope values

Immediately after hatching, the C:N ratio decreased steeply as lipid reserves were consumed over the first 3-4 DAH (Fig. 4). On 4 and 5 DAH, tissues of unfed fish and those in the Artemia group reached significantly lower C:N ratios. Most values remained statistically unchanged from 4 to 8 DAH. Thereafter, ratios increased progressively until stabilization near or at metamorphosis (15 DAH). δ^{13}C values in fish tissue were quickly influenced by their respective dietary regimes (Fig. 5a). From 3 DAH onwards, isotopic changes were very fast in fish fed on rotifers, *Artemia*, rotifers followed by *Artemia* and co-fed inert diets and rotifers. However, larvae fed only *Artemia* showed a delay in isotopic influence on tissue as compared to the other treatments. Carbon isotopic shifts were not obvious between fish and inert diet because δ^{13}C values in the latter were similar to those observed in recently hatched fish.

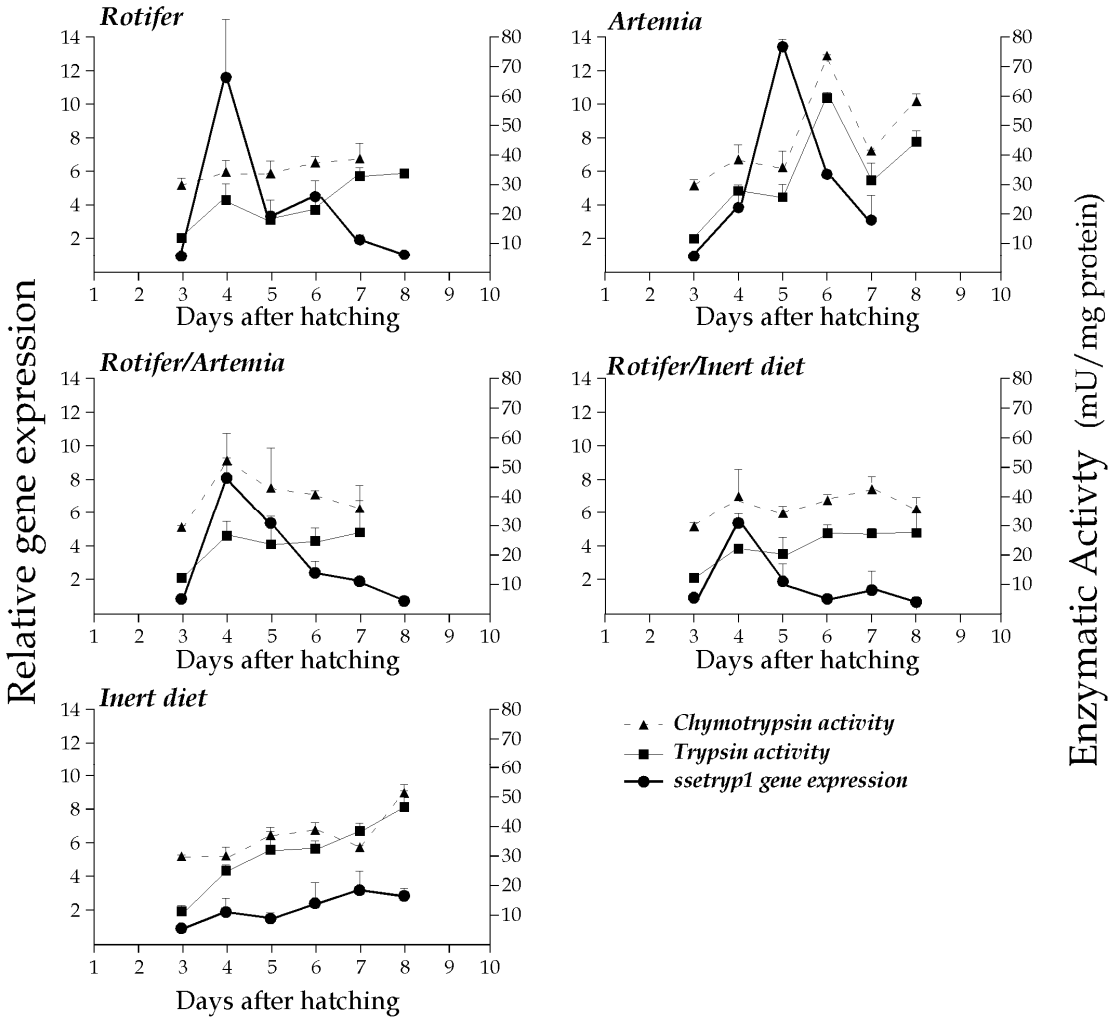

Fig. 3. Specific activity of trypsin and chymotrypsin (mU mg protein^{-1}, right axis) in whole *Solea senegalensis* larvae fed different feeding regimes (means ± SD, n = 3) and relative *ssetryp1* expression (left axis). Data were expressed as the mean fold change from the calibrator group (3 DAH).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

Further changes in $\delta^{13}C$ values influenced by the dietary shifts to *Artemia* (6 DAH in the Rotifers/Artemia group and 11 DAH in all other treatments) were also clearly observed as steep isotopic changes in fish tissue (Fig. 5b). $\delta^{13}C$ values in diet and fish from the Co-feeding group allowed for estimating the contribution of each dietary source to tissue growth. Results from the isotopic mixing model indicated that by 10 DAH, from 84 to 96%

of the incorporated dietary carbon in tissue was derived from rotifers and only 4 to 14% was provided by the inert diet.

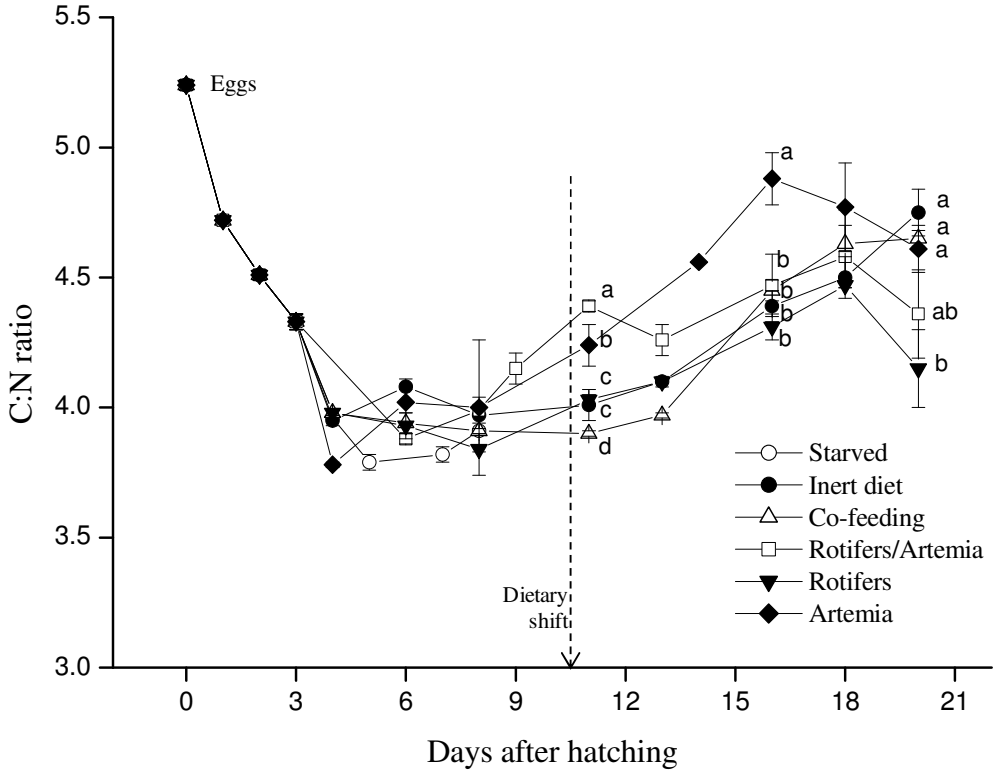

Fig. 4. Changes in carbon/nitrogen ratio in whole tissue of *Solea senegalensis* larvae fed different diets. Vertical arrow indicates a dietary shift to enriched *Artemia* metanauplii from 11 to 20 DAH. Mean of 2-3 pooled samples ± SD indicated by vertical bars.

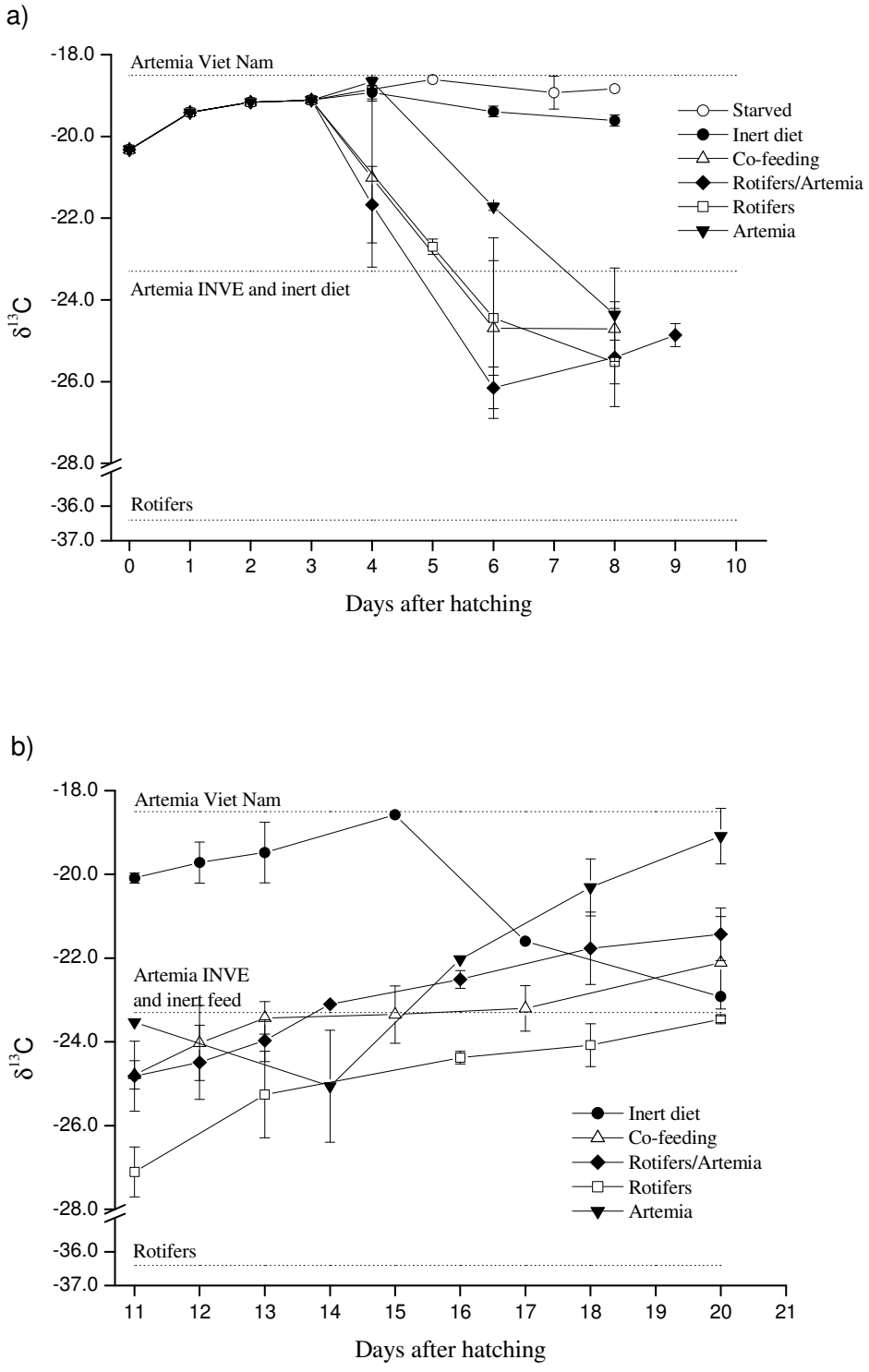

Fig. 5. Changes in carbon isotope values (‰) in body tissue of *Solea senegalensis* reared on five different diets from 0 to 10 DAH (a) and from 11 to 20 DAH (b). Diets were shifted to enriched *Artemia* metanauplii from 11 to 20 DAH, except treatment Rotifers-Artemia (6 DAH). Mean of 15 samples, vertical bars indicate standard deviations.

3.5. Carbon turnover rates

Carbon turnover rates and residency half time in tissue (t_{50}) at two different larval ages (before and after the dietary shift to *Artemia*) were estimated from parameters in the exponential model of isotopic change (Table 4). Results indicate that in general, carbon t_{50} in tissue of early larval stages was very short and ranged from 1.3 to 1.5 d, except for fish larvae reared only on inert feed (4.4 d). Values increased in all dietary regimes after fish further developed and received *Artemia* metanauplii (2.4 to 4.0 d). Estimated carbon turnover rates (m) exhibited high variability mostly due to varying degrees of fit of the predicted isotopic values on the rapidly changing observed values, therefore ANOVA analysis failed to detect significant differences.

Table 4. Growth rates (k*), estimated carbon turnover rates (m**) and half times in tissue (t_{50}) of *Solea senegalensis* at two different larval ages (3-10 and 11-20 DAH) reared under different diets.

Diet	Age (DAH)	k (d^{-1})	m (d^{-1})	t_{50} (d)	R^2
Rotifers	3-10	0.291 ±0.045^{b}	0.173 ±0.096^{a}	1.5	82
Rot-Art	3-10	0.387 ±0.001^{a}	0.145±0.890^{a}	1.3	67
Artemia	3-10	0.381 ±0.007^{a}	0.154±0.750^{a}	1.3	77
Co-feeding	3-10	0.254 ±0.026^{b}	0.218±0.168^{a}	1.5	79
Inert diet	3-10	0.071 ±0.008^{c}	0.088±0.054^{a}	4.4	45
Starved	3-8	-0.028 ±0.015^{d}	-	-	-
Rotifers	11-20	0.180 ±0.056^{a}	0.042±0.019^{a}	3.1	91
Rot-Art	11-20	0.112 ±0.004^{b}	0.062±0.019^{a}	4.0	84
Artemia	11-20	0.101 ±0.013^{b}	0.122±0.430^{a}	3.1	85
Co-feeding	11-20	0.188 ±0.002^{a}	0.032±0.032^{a}	3.2	85
Inert diet	11-20	0.290 ±0.005^{c}	nd	nd	nd

Different superscripts indicate significant differences at $p<0.05$ within age groups.
* k =log(final weight/initial weight)/time(days).
** Parameter m was estimated from the model proposed by Hesslein et al. (1993) using iterative non-linear regression.

4. Discussion

4.1. Dietary effects on larval growth and C:N ratio

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

weaning (68 DAH) in sole (Engrola et al., 2009b). Possible explanations for improved larval performance and increased nutrient assimilation observed when live and dry diets are co-fed are the contribution of digestive enzymes from the live food organisms and an increased supply of more suitable nutrients (Kolkovski et al., 1993; Rosenlund et al., 1997). Moreover, modulation of trypsinogen gene expression by feeding regime is another factor to be taken into account as described below.

4.2. Effect of feeding regime on trypsinogen gene expression and enzymatic activities

In the present study, trypsin and chymotrypsin activities exhibited quite similar profiles within each diet. Trypsin activity did not reflect changes observed in the *ssetryp1* gene expression except in fish from the Artemia group. This difference between mRNA expression profiles and enzymatic activity has been previously reported in other species suggesting a post-transcriptional (Gawlicka and Horn, 2006) or even translational (Péres et al., 1998; Wang et al., 2006) regulation of trypsin activity. However, some considerations should be taken into account: *i*) Senegalese sole possess at least six cDNAs encoding trypsinogens (Manchado et al., 2008). They can be classified into three groups (I or anionic, II or cationic and III or psychrophilic) with distinct catalytic properties (Asgeirsson et al., 1989; Ahsan and Watabe, 2001; Gudmundsdottir and Palsdottir, 2005) and substrate preferences (Fletcher et al., 1987). This implies that trypsin activity does not represent *ssetryp1* necessarily; *ii*) Diet composition, as well as type and abundance of prey can modulate trypsin activity. Some studies have reported that live prey can contribute up to 8-10 % of trypsin activity (Gawlicka et al., 2000; Perez-Casanova et al., 2006). Furthermore, Pedersen and Hjelmeland (1988) showed evidence of trypsin activity being retained in the gut of herring fed on varying amounts of live food; *iii*) Compound diets might contain trypsin inhibitors modulating activity (Ribeiro et al., 2002). These findings illustrate that

numerous factors can modulate trypsin activity, hence explaining observed differences between expression and activity profiles in the present and other studies. Nevertheless, post-transcriptional regulation cannot be discarded and deserves to be investigated.

Serine proteases are key digestive enzymes accounting for up to 75–80 % of the total protease activity (Moyano et al., 1996) and trypsin and chymotrypsin represent the main proteases associated with food conversion efficiency, growth rate and nutritional condition in larvae (Lemieux et al., 1999; Nolting et al., 1999; Applebaum and Holt, 2003; Perez-Casanova et al., 2006). However, enzyme activity in fish larvae is age-dependent and modulated by diet composition (Cahu and Zambonino-Infante, 2001). Varying responses in terms of proteolytic activity has been observed when fish larvae are fed diets having different C:N ratios and an increasing amount of prey in the gut stimulates the total proteolytic activity (Lauff and Hofer, 1984). A better knowledge of serine protease regulation at the transcriptional and protein level is crucial to advance in the design of formulated diets that can replace live feeds during larval culture. In *S. senegalensis*, the anionic trypsinogen *ssetryp1* is the most abundant expressed trypsinogen, exhibiting a bell-shape expression profile during development (from 2 to 9 DAH) (Manchado et al., 2008). In this study, we have quantified the expression of *ssetryp1* in larvae fed on five distinct feeding regimes during the first critical 10 days of life. Our results indicate that transcription of *ssetryp1* is modulated during early larval development by diet. Larvae fed on live preys increased *ssetryp1* transcripts rapidly at 4 and 5 DAH to recover the steady-state levels progressively. The delay in the peak observed in larvae from the Artemia group, together with the delayed, dietary-influenced shift in tissue $\delta^{13}C$ values, could be easily explained by the higher prey size of the nauplii (530 μm) and a major difficulty for its capture in these early stages as explained above. In contrast, no peak was observed in larvae fed inert diet,

and mRNAs increased continuously as larvae grew. Additionally, a lower induction was determined in larvae in the Co-feeding group and integration of their $\delta^{13}C$ values in the isotopic mixing model indicated that even though only from 4 to 14% of carbon incorporated into tissue was derived from the inert diet, the ingestion of the latter elicited a different response in the expression of *ssetryp1*. Previous results have demonstrated that food ingestion and diet composition seem to be key factors modulating trypsinogen gene expression. A fast induction of anionic trypsinogen transcription after feed ingestion (peaking at 12 h after re-feeding) has been reported in *Seriola quinqueradiata* juveniles (Murashita et al., 2007). Moreover, larvae of *Pelteobagrus fulvidraco* and *Dicentrarchus labrax* fed on *Artemia* showed lower mRNA levels of anionic trypsinogen than those fed compound diets (Péres et al., 1998; Wang et al., 2006). However, in these studies, expression was quantified later, at 7 or 10 days after supplying the diets. All these indicate that the anionic trypsinogen is regulated at transcriptional level and that its expression can be used to monitor at molecular level the mechanisms that determine the digestive physiology in *S. senegalensis* larvae.

4.3. Effect of diet on fish tissue isotopic composition and carbon turnover rates

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 (Kaufman et al., 2008). In the present experiment the observed high growth rates suggest that $\delta^{13}C$ changes in fish tissue were primarily due to addition of newly-synthesized tissue. Isotopic incorporation is rapid in fast growing ectotherms (Martínez del Rio et al., 2008) and in fish, growth seems to account for most of the observed isotopic change (Suzuki et al., 2005). In the present study, isotopic changes observed in fast-growing early sole larvae were also mainly accounted for by high growth rates rather than metabolic turnover rate. In

fact, isotopic values of food sources were quickly reflected in the whole bodies of fish as indicated by $\delta^{13}C$ values and t_{50} estimations. Even though the growth rates (k) observed in the present experiment were statistically different between dietary treatments and fish age/size, significant differences were not detected in carbon metabolic turnover rates due to the high variability of the data. Results also indicate short half times (t_{50}) of dietary carbon in tissues of early larvae fed live diets (1.3 to 1.5 d). These results are consistent with those reported by Bosley et al. (2002) and Gamboa-Delgado et al. (2008) after they estimated nutrient turnover using stable isotopes and reported an average carbon t_{50} of 2.3 d in tissues of marine flat-fish larvae. Carbon turnover rates and t_{50} values (4.4 d) of fish fed only inert diet should be considered cautiously as isotopic values in these fish were similar to those in recently hatched larvae, hence changing isotopic values did not showed an exponential trend and predicted values fitted poorly (r^2= 45). However, previous experiments performed on the same species in our laboratory have indicated relatively high t_{50} values (3.5 d ±0.6) when larval sole is reared on co-feeding regimes having high proportions (70% on a dw basis) of inert diet (Gamboa-Delgado et al., 2008). In the present experiment, a general increase in carbon t_{50} was observed as fish further developed and after all feeding regimes were shifted to enriched *Artemia*. These increases are likely correlated to the slower growth rate characteristic of the relatively inactive benthic stage. From a nutritional perspective, the faster the animal isotopic composition resembles the dietary isotope values, the greater is the nutrient retention from the diet (Buchheister and Latour, 2010; German and Miles, 2010); therefore, turnover rates and tissue t_{50} values reflect the influence of a specific diet and its components on the nutritional physiology of a target species.

In conclusion, growth and metamorphosis in *S. senegalensis* larvae are greatly affected by diet. Larvae fed on rotifers and rotifers followed by *Artemia* showed optimal values for

growth, metamorphosis completion and survival. $\delta^{13}C$ values indicated that rotifers and inert diet co-fed in similar dry weight proportions supplied significantly different levels of dietary carbon to the growth of fish larval tissue. The lower incorporation of carbon from the inert diet suggests a strong selection of live food and a low digestibility and/or assimilation of nutrients from the former. Fish larvae fed only *Artemia* showed reduced growth and survival, most likely due to less efficient prey capture and low availability of specific nutrients. *Ssetryp1* gene expression (but not trypsin) was modulated by feeding regimes showing a clear relationship with growth and survival. Dietary treatments supporting optimal growth and survival increased expression levels rapidly, whereas no activation was detected in larvae fed inert diet. Moreover, larvae co-fed on rotifers and inert feed showed lower *ssetryp1* transcripts than larvae fed live preys, while those fed on *Artemia* increased mRNAs one day later. Together, these results suggest that quantification of *ssetryp1* transcripts could be used as a biomarker for evaluation of digestive capacity and growth in early life stages of sole during the planktonic and benthic phases. Analysis of digestive enzymes also provides valuable information on nutrient utilization over time and under different nutritional conditions, while stable isotope analysis represents an additional nutritional tool to assess nutrient incorporation from different dietary sources and to estimate elemental turnover rates in tissue.

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