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Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory

C. Fernández-Díaz*, M. Yúfera*¶, J. P. Cañavate†, F. J. Moyano‡, F. J. Alarcón‡ and M. Díaz‡

**Instituto de Ciencias Marinas de Andalucía (CSIC), Apartado Oficial E-11510, Puerto Real, Cádiz, Spain; †CICEM El Toruño (Junta de Andalucía), Apartado 16, E-11500, Puerto de Santa María, Cádiz, Spain; and ‡Dept. Biología Aplicada, Escuela Politécnica Superior, Universidad de Almería, E-04120 Almería, Spain*

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The metamorphosis of *Solea senegalensis* was studied in larvae reared at 20° C and fed four different feeding regimes. A, *Artemia* (4 nauplii ml^{-1}); B, *Artemia* (2 nauplii ml^{-1}); C, mixed diet (2 nauplii ml^{-1} and 3 mg ml^{-1} microencapsulated diet); and D, microencapsulated diet (3·7 mg ml^{-1}). Rotifers were also supplied in all cases during the first days of feeding. These feeding regimes supported different growth rates during the pre-metamorphosis period (regime A, G=0·376 day^{-1}; regime B, G=0·253 day^{-1}; regime C, G=0·254 day^{-1}; regime D, G=0·162 day^{-1}). Larvae started metamorphosis 9 days after hatching (DAH) when fed the regime A, 13 DAH with regime B, 11 DAH with regime C and 15 DAH with regime D. A minimum 5·6–5·9 mm L_T was required under all feeding regimes to initiate the metamorphosis. Eye translocation was completed when the larvae reached 8·6–8·7 mm L_T (regimes A, B and C), but only 7·3 mm L_T with regime D. 4·4–6·2 days were required to complete eye migration under the regimes A, B and C, and 18·3 days under the regime D. This transformation is concomitant with changes in body reserves, and with the pattern of some digestive enzymes.

Key words: metamorphosis; larval growth; eye migration; enzymatic activity; *Solea senegalensis*.

INTRODUCTION

Metamorphosis in flatfish is characterized by a dramatic anatomical transformation during development that involves a 90° rotation in body position and the migration of one eye to join the other on an ocular upper side. Such transformation is associated with the settlement of larvae in the substratum and the change from the pelagic to benthic habitat, and consequently it implies important changes in food habits and in digestive physiology (Tanaka *et al.*, 1996). The transformation in flatfish occurs at a wide range of sizes depending on species and environmental circumstances (Policansky, 1982; Osse & van den Boogaart, 1997). Whether size or age are determinant factors in starting the metamorphosis is a question that has been addressed in several studies (Chambers & Leggett, 1987; Amara & Lagardère, 1995; Osse & van den Boogaart, 1997). Besides, the evident ecological implications such a question is also important in laboratory populations in which growth and therefore size of larvae depend strongly on the rearing conditions.

¶Author to whom correspondence should be addressed. Tel.: +34 956 832612; fax: +34 956 834701; email: manuel.yufera@icman.csic.es

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The Senegal sole *Solea senegalensis* Kaup is a flatfish well adapted to temperate waters and exploited in extensive aquaculture production in Spain and Portugal (Drake *et al.*, 1984; Dinis *et al.*, 1999). Due to its high potential interest for aquaculture, the developmental biology has been studied recently (Dinis, 1992; Vázquez *et al.*, 1994; Sarasquete *et al.*, 1996; Martínez *et al.*, 1999; Parra *et al.*, 1999; Ribeiro *et al.*, 1999*a*, *b*; Yúfera *et al.*, 1999*a*). These studies have reported that, under laboratory conditions at temperatures of *c.* 20° C, Senegal sole larvae have a relatively fast development, turning to benthic life between the second and third week after hatching. After this time, acid protease activity appears and increases progressively although acid digestion becomes dominant by the fifth week of life (Martínez *et al.*, 1999), when gastric glands are developed (Ribeiro *et al.*, 1999*a*) and the stomach starts to function. Little research has focused on describing the process of transformation to juvenile stage in this flatfish.

The present study aimed to describe the pattern of metamorphosis in *S. senegalensis* under laboratory conditions. To obtain a better understanding of this process, the time, duration and size at the different stages of transformation have been determined on the basis of eye migration in larvae growing under different feeding regimes and consequently at different rates. The process has been examined in terms of changes in anatomy, energy content and activity of acid digestive enzymes.

MATERIALS AND METHODS

Eggs were obtained from captive broodstock, which spawned naturally. Experiments were replicated by using two batches of eggs from spawn collected on different days. Water temperature during the spawning period ranged from 18 to 20° C. Eggs were incubated at 19° C in 300 l cylinder-conical incubating tanks with gentle aeration and a continuous up-welling water flow of 0·5 l min^{-1}. After hatching, larvae were stocked in 400 l tanks at 20 ± 1° C temperature and 33–35 g l^{-1} salinity. Initial larval density was adjusted to 50 individuals per litre. A continuous illumination of 1500 lx at the water surface was provided. These are the usual methods during the first weeks for intensive rearing of marine larval fish in temperate regions.

Larvae started to feed on rotifers on day 2 after hatching. From day 5, four different feeding regimes were supplied to obtain different larval growth: A, high live prey density, 10 rotifers ml^{-1}+4 *Artemia* nauplii ml^{-1}; B, standard condition, 5 rotifers ml^{-1}+2 *Artemia* nauplii ml^{-1}; C, mixed live and inert diet, 5 rotifers ml^{-1}+2 *Artemia* nauplii ml^{-1} and 3 mg ml^{-1} respectively; D, inert diet, 3·7 mg ml^{-1}. These daily amounts increased with larval age (Table I). Algae (*Nannochloropsis gaditana*) were supplied once a day. Rotifers (*Brachionus plicatilis* strain S-1) and *Artemia* nauplii (San Francisco strain) were also given once a day and their respective rations were adjusted to the desired final density considering the remaining prey from the previous day. The microcapsules were manufactured in the laboratory by interfacial polymerization of the dietary protein. The manufacturing procedure and the diet composition are detailed in Yúfera *et al.* (1999*b*). The inert diet was distributed in six feedings per day with an automatic feeder. The particle concentration in the water ranged between 5 and 15 particles ml^{-1}. All treatments were replicated. From day 6 after hatching, 15% total water volume was exchanged daily and then increased gradually every four days to achieve a 100% renewal at the end of the experiments.

To determine the larval total length (L_T) and the status of metamorphic development, samples of 50–75 larvae were anaesthetized periodically with ethyl-4-aminobenzoate and examined under light stereoscope. Then the larvae were rinsed with distilled water and dried at 85° C to constant weight.

TABLE I. Daily amounts of microalgae (10^6 cells ml^{-1}), rotifers (individual ml^{-1}), *Artemia* nauplii (individual ml^{-1}), microencapsulated diet (MC, in mg dry weight ml^{-1}), given to *Solea senegalensis* larvae under four different feeding regimes in 400 l tanks

Regime	Feed	Larval age (days)				
		2–4	5–9	10–15	16–20	⩾21
A	Algae	0·3	0·3			
	Rotifer	10	10			
	Artemia		4	4	5	6
	Microcapsules					
B	Algae	0·3	0·3			
	Rotifer	5	5			
	Artemia		2	2	2·5	3
	Microcapsules					
C	Algae	0·3	0·3			
	Rotifer	5	5			
	Artemia		2	2	2·5	3
	Microcapsules		3	3·7	4·5	5·2
D	Algae	0·3				
	Rotifer	10				
	Artemia					
	Microcapsules		3·7	7·5	9	10

Specific growth rate (G) was calculated as the slope of the exponential regression of dry weight data on larval age. For each treatment regressions were calculated for the larval period and for the metamorphic period respectively. The day on which eye migration had started in 50% of the larvae was considered as the inflexion point between the two stages. Each regression was calculated using the pooled data of the two replicates. t-test for slope comparison was utilized to compare the growth rate under the different treatments and periods. Larval density in the rearing tanks was estimated on day 2 (first feeding) and days 25, 28 or 37 (end of experiments) by counting larvae in ten randomly chosen 250 ml samples.

To characterize the process, five sub-stages were defined according to the position of the left eye (Fig. 1). These sub-stages were: 0, symmetric larvae with vertical swimming plane; 1, the left-eye starts to migrate toward the dorsal position, until it touches the midline of the dorsal surface: the individuals are not completely symmetrical; 2, the migrating eye can be seen from the right-ocular side up to reaching the midline of the dorsal surface; 3, the individuals change their swimming plane and the eye continues migrating within the ocular side; 4, eye translocation is completed and the orbital arch is clearly visible (cf. Table II). Frequency of sub-stages was calculated using the pooled data of the two replicates. The percentages of larvae that started and completed the metamorphosis were plotted as probit units *v.* either the logarithm of age or size of the larvae in each case. Larval age and the average larval length at which 50% of the population started and at which 50 and 95% of the population finished the transformation was calculated from the corresponding regressions.

Samples for chemical analyses were taken at the beginning of eye migration and toward the end of metamorphosis for each treatment. The larvae were washed with distilled water and then freeze-dried. Carbon (C) and nitrogen (N) content were determined with an Elemental Analyzer model. 1106 (Carlo Erba Science) using cyclohexanone 2·4-dinitrophenylhydrazone as standard; sub-samples weighed *c.* 1 mg.

For the determination of the enzymatic activity, pooled samples of larvae (50–1250 individuals, depending on age and size) were removed from day 9 to day 37 after

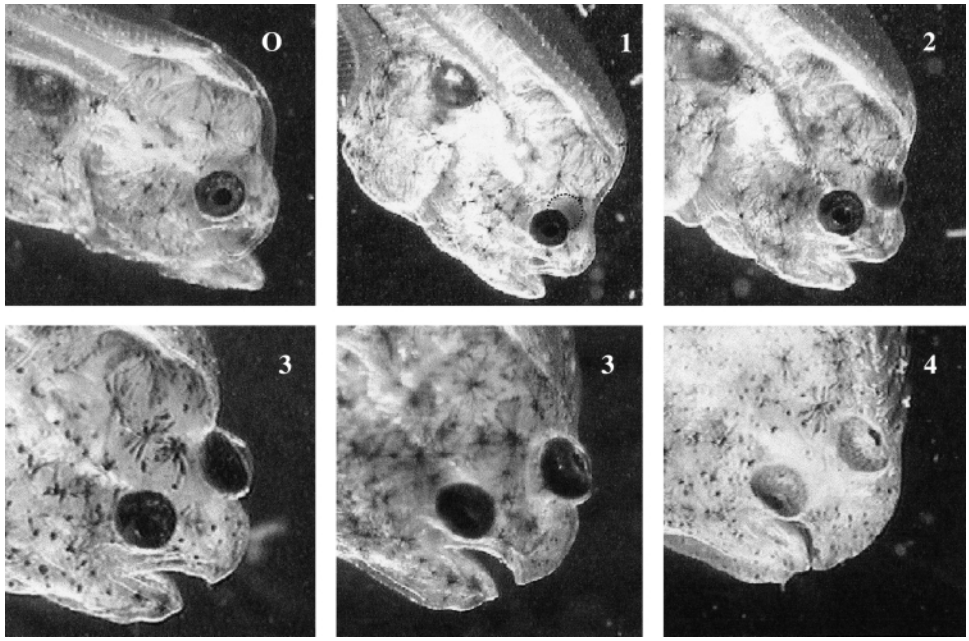

FIG. 1. Eye position in the different stages of metamorphosis for *Solea senegalensis*. See text for explanation.

TABLE II. Nomenclature used in this study and by other authors in defining the different sub-stages during the metamorphosis in flatfish

Solea senegalensis[a]	*Taneiopsetta ocellata*[b]	*Scophthalmus maximus*[c]	*Paralichthys dentatus*[d]
Stage 0	Pre-metamorphic	Stages 1–3	Pre-metamorphic
Stage 1	Early metamorphic	Stage 4a–4c	F-, F
Stage 2	Middle metamorphic	Stage 4d	G, H-
Stage 3	Middle metamorphic	Stage 5a, 5c	H, H+
Stage 4	Late metamorphic	Stage 5d	I

[a]This study; [b]Amaoka, 1970; [c]Al-maghazachi & Gibson, 1984; [d]Keefe & Able, 1993.

hatching, washed with distilled water and freeze-dried before being stored. Sampling was always carried out *c*. 2 h after feeding. Extracts utilized for enzyme assays were obtained after homogenization of sampled larvae (35 mg ml^{-1}) in cold 50 mM Tris-HCl buffer, pH 7·5, followed by centrifugation (13 500 ***g***; 30 min at 4° C). Acid protease activity was measured using the methods detailed by Anson (1938) modified by Díaz *et al*. (1998) using haemoglobin as substrate. One unit of activity was defined as 1 μg of tyrosine released per minute.

RESULTS

The experiment for growth and eye-position determinations ended on day 25 in larvae fed regimes A and C, and on day 28 in larvae fed diet B, when at least 95% of the population had completed metamorphosis. In treatment D, 100% of

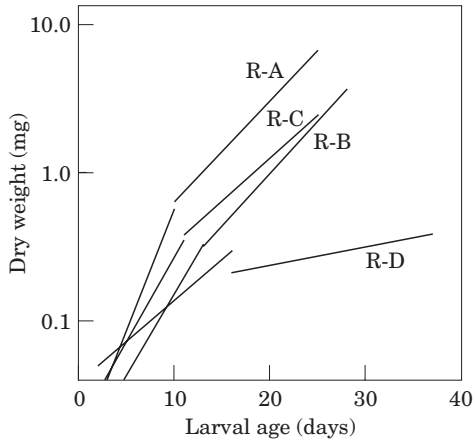

FIG. 2. Growth in dry weight of *Solea senegalensis* during the pre-metamorphic and metamorphic stage under different feeding regimes.

TABLE III. Growth rate of *Solea senegalensis* before and after the beginning of the metamorphosis. In each column, values with the same superscript are not statistically different ($P>0{\cdot}05$)

Feeding regime	Before metamorphosis	After metamorphosis started
A	$0{\cdot}3766^{a}$	$0{\cdot}1567^{a}$
B	$0{\cdot}2527^{b}$	$0{\cdot}1628^{a}$
C	$0{\cdot}2643^{b}$	$0{\cdot}1345^{a}$
D	$0{\cdot}1618^{c}$	$0{\cdot}0260^{b}$

the population had completed the transformation by day 51, but only the data before day 37, when *c.* 80% had metamorphosed, were considered for the calculation of the growth rate (Fig. 2). After this date, the determinations of dry weight in samples of the surviving individuals yielded erratic data. The highest growth rate during the larval phase (from first-feeding to the onset of eye migration) was obtained with regime A, and the lowest rate with regime D (Table III). Afterwards, specific growth rates decreased significantly (*t* test, $P<0{\cdot}05$ in all cases) and were similar in all treatments with the presence of live prey (regimes A, B and C), ranging between 0·135 and 0·163 day^{-1} ($P>0{\cdot}05$). The larvae fed on inert diet (D) showed little growth at this stage ($G=0{\cdot}026$ day^{-1}). The survival of larvae at the end of experiments was >70% in regimes A, B and C. In the treatment D, the survival was 30% at day 25 and 5% at day 37.

Eye migration started between 8 and 12 days after hatching (DHA) depending on the feeding regimes (Fig. 3). Two days later, >50% of the population had initiated the transformation in all cases. Larvae fed on regime D also started eye migration in a similar time and way. Nevertheless, about 30% of the population did not show a regular pattern in starting the transformation. This feature can be observed throughout the different steps of the transformation (Fig. 3). The

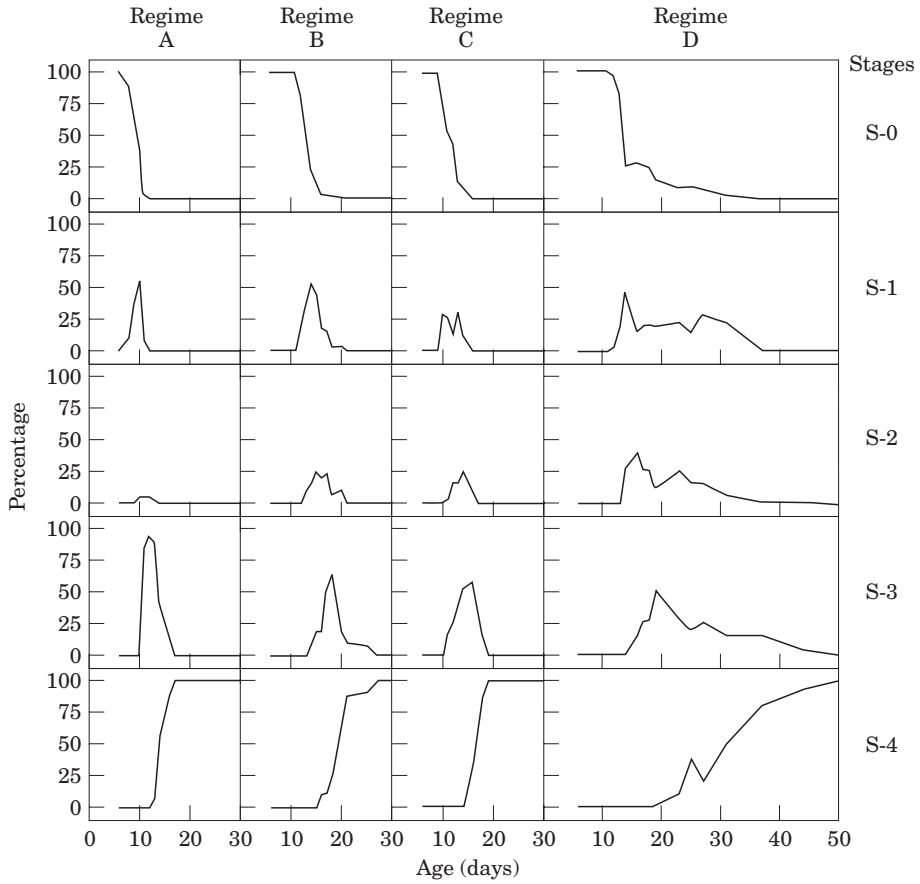

FIG. 3. Frequency histograms of the different metamorphosis stages in *Solea senegalensis* in relation to larval age under different feeding regimes.

age at the end of the process, considering when both 50 and 95% of the population completed metamorphosis, was similar under regimes A, B and C, but practically twice as great under the regime D (Table IV). Due to the irregular pattern in regime D, the age calculated from probits is slightly lower than that observed in Fig. 3.

Eye migration (S1) started at *c.* 4·5–5·0 mm L_T (Fig. 4). Total length calculated from probits when 50% of individuals started eye migration ranged from 5·6 to 5·9 mm (Table IV). The left eye reached the midline of the dorsal surface and the larvae changed the swimming plane (S3) at 6·0–7·0 mm L_T, and completed eye migration (S4) at >8·0 mm L_T, except for the larvae fed regime D which completed metamorphosis at >6·5 mm L_T. Total length when 50% of the population attained metamorphosis was *c.* 8·6 mm, but only 7·3 mm in treatment D. Likewise, total length when practically all individuals (95% population) completed the metamorphosis was *c.* 9·3–9·6 mm but only 7·0 mm in treatment D.

The C : N ratio dropped significantly throughout the process under the four treatments (ANOVA, $P>0{\cdot}05$) (Fig. 5). At the beginning of metamorphosis, the

TABLE IV. Age, total length, and dry weight in *Solea senegalensis* at the beginning (50% of the population starting) and at the end (50 and 95% of the population ending) of metamorphosis. Length and age values have been calculated from probits. Values between parentheses indicate the 95% CL. Dry weight values have been calculated from length values of probits using the length-weight regressions

Feeding regime	A	B	C	D
50% starting				
Age (days)	9·40 (9·19–9·57)	12·50 (12·03–12·88)	11·18 (10·75–11·53)	15·17 (13·10–16·23)
Total length (mm)	5·90 (5·56–6·10)	5·67 (5·57–5·76)	5·64 (5·54–5·74)	5·58 (5·46–5·68)
Dry weight (μg)	0·28	0·26	0·24	0·17
50% ending				
Age (days)	13·81 (13·56–14·03)	18·69 (18·36–19·4)	16·48 (16·25–16·67)	27·69 (25·38–29·40)
Total length (mm)	8·67 (8·54–8·77)	8·58 (8·44–8·68)	8·55 (8·46–8·63)	7·26 (7·06–7·38)
Dry weight (μg)	0·93	0·98	0·86	0·41
95% ending				
Age (days)	16·37 (15·94–16·96)	22·38 (21·58–23·62)	18·56 (18·56–19·22)	43·91 (41·14–48·38)
Total length (mm)	9·57 (9·43–9·78)	9·34 (9·20–9·57)	9·36 (9·21–9·58)	7·90 (7·77–8·13)
Dry weight (μg)	1·27	1·28	1·14	0·55

C : N ratio ranged from 4·6 to 5·6. At the end of the metamorphosis the C : N ratio ranged between 4·2 and 4·8. Lower values for each stage were always obtained in larvae under treatment D.

Specific activity of acid protease in whole larval tissue was maximal at the beginning of metamorphosis. The highest values were reached between 10 and 13 DHA for larvae fed in the presence of live prey (regimes A, B and C) and by 19 DHA in larvae fed in the absence of live prey from day 5 (regime D). Afterwards, this activity decreased sharply to a minimum by the end of the transformation, between 19 and 20 DHA under the regimes, A, B and C and by 25 DHA under the regime D, and them increased again (Fig. 6). This pattern, which corresponded with eye migration, was similar under the different feeding regimes, but it was characterized by a 5 to 6 days' delay in the case of larvae fed only on microcapsules.

DISCUSSION

The different feeding regimes supported different growth rates during the larval stage. Although diet quality assessment was not the aim, it is noteworthy that larvae fed on live prey showed excellent growth and survival, while larvae fed on regime D grew and survived below what would be expected for this species under laboratory conditions (Yúfera *et al.*, 1999*a*), indicating a nutritional

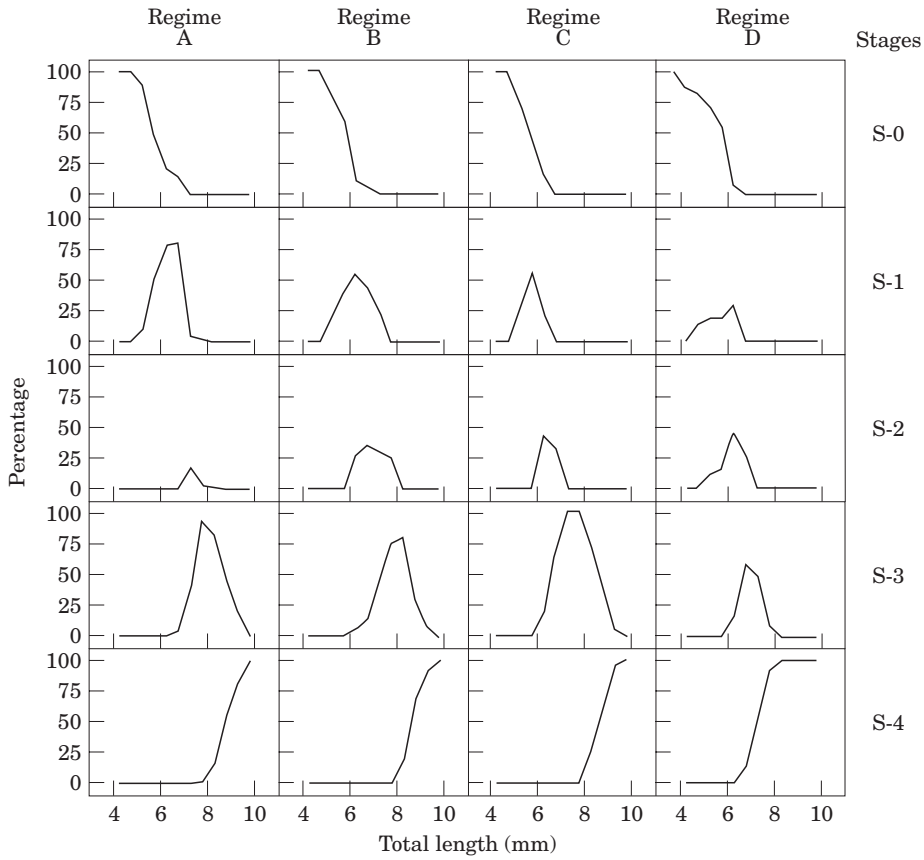

FIG. 4. Frequency histograms of the different metamorphosis stages in *Solea senegalensis* in relation to larval total length under different feeding regimes.

deficiency. The larvae that grew faster began their transformation earlier. Once metamorphosis started, the growth rates decreased significantly although still remained relatively high. More interesting is the fact that during the transformation the growth rates were quite similar in those populations fed on live prey and probably without nutritional deficiency (treatments A, B and C).

Although the age at transformation in *S. senegalensis* varied from day 9 to day 15 (age coefficient of variation (CV) among treatments=0·201; considering when 50% of individuals start the transformation), the larval length varied only in a narrow range (length CV=0·024). This finding has been reported for the closely-related species *Solea solea* (L.) (Amara & Lagardère, 1995) and other flatfish such as *Plathichtys stellatus* (Pallas) (Policansky, 1982) and *Pseudopleuronectes americanus* (Walbaum) (Chambers & Leggett, 1987). All these studies indicate that larval length is relatively constant and less variable than age at the onset of transformation. In spite of the equality in length at the onset of metamorphosis, the average larval dry weight varied in the different treatments at the beginning of the transformation (Fig. 1). Nevertheless, a better approximation of individual weight at a given developmental stage has been calculated from length values obtained from probits using the length-weight

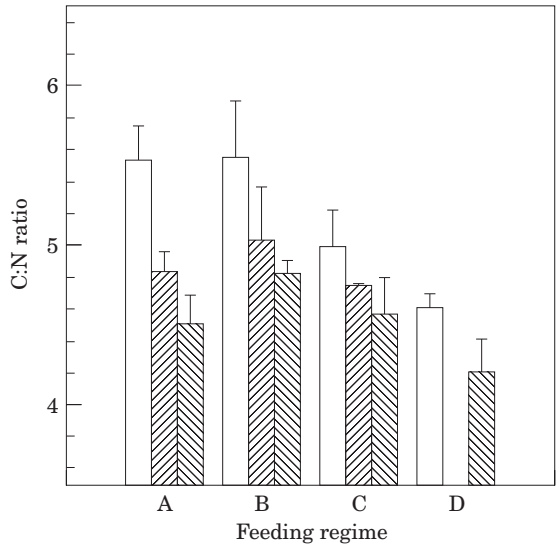

FIG. 5. Changes in C : N ratio in *Solea senegalensis* from the beginning to the end of metamorphosis under different feeding regimes. □, 50% starting; ▨, 50% ending; ▧, 95% ending.

regressions of each corresponding treatment (Table IV). In this case, the differences in dry weight are relatively small, except in treatment D but higher than in total length (dry weight CV = 0·202). Such differences in the condition index of larvae suggest that length reflects the state of ontogeny better than weight.

Transformation from pre- to post-metamorphic stage in flatfish usually takes a variable period, depending on the species and environmental circumstances (Osse & van den Boogaardt, 1997). In the present study, the time required to complete the transformation under the different feeding regimes was similar (4·4–6·2 days), except for regime D, which required 12·5 days. This figure is concordant with similar growth rates exhibited under those regimes. Metamorphosis in *S. senegalensis* started when larvae reached a minimum length of 4·5–5·0 mm, the size and shape which allows eye translocation. As reported by Osse & van den Boogaardt (1997), flattening of the body with a strong positive allometry of body depth, head size and mouth gape, is required to start the transformation.

The observed depletion in the C : N ratio during transformation suggests that larvae are catabolizing carbon-rich compounds, as lipids and carbohydrates. Energy content of the body tissues in *S. senegalensis* increased during the pre-metamorphic period and decreased from the start of metamorphosis (Yúfera *et al.*, 1999*a*). An accumulation of lipid reserves during the pre-metamorphic stage, and used during metamorphosis has been observed in other species (Youson, 1988; Kao *et al.*, 1997; Pfeiler, 1999). Christensen & Korsgaard (1999) also observed a decrease in the C : N ratio in *Pleuronectes platessa* L. at the end of metamorphosis. Metamorphosing lavae of *S. senegalensis*, as in *S. solea* (Lagardère *et al.*, 1999), continue eating actively, suggesting that the matter and energy required for the transformation does not originate exclusively from body reserves. The level of the C : N ratio at the beginning of eye migration under

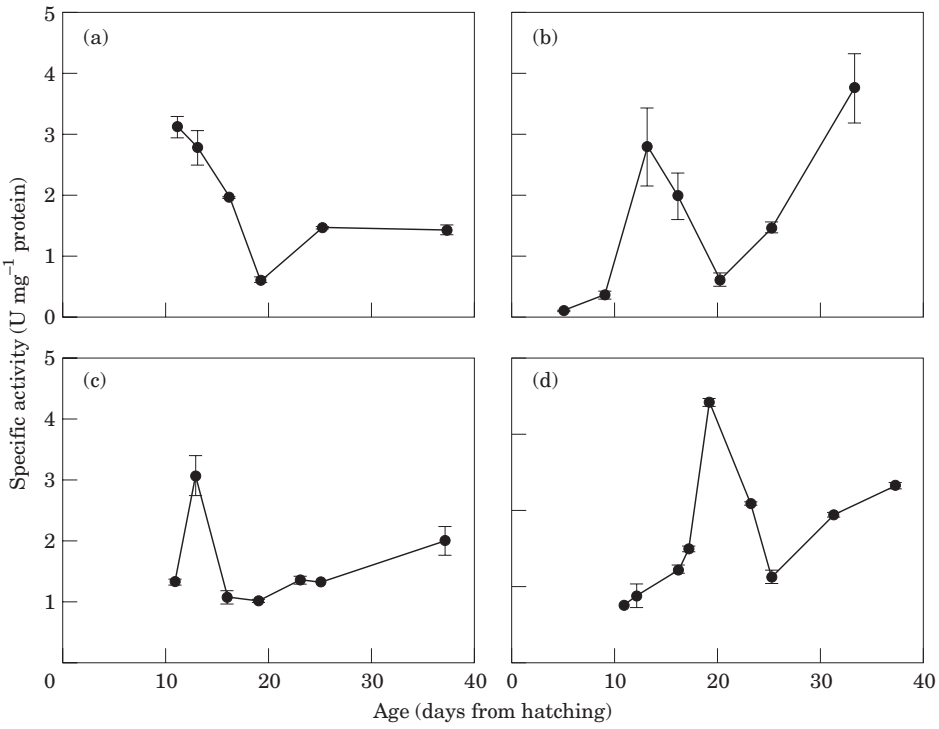

FIG. 6. Changes in acid protease activity (U mg^{-1} protein) in *Solea senegalensis* under different feeding regimes (a) A; (b) B; (c) C; (d) D.

regime D was too low (4·6), and similar to that showed in individuals that completed the transformation in the other treatments. However, the C : N ratio in larvae fed inert food decreased to 4·2 at the end of the transformation, when there was high mortality. This suggests that although the individuals are able to start the metamorphosis under these feeding conditions, there were not enough reserves for anatomic transformation, somatic growth and maintenance activity.

The pattern of protease activity increasing initially during the transformation was reported by Martínez *et al.* (1999). This first peak of activity may not represent a true digestive ability, and is probably associated with the presence of cathepsines related to the mobilization of protein in body tissues. The aspect most relevant to the present study is that this first increase occurred approximately during the eye translocation under the different feeding regimes. A further gradual increase in acid pepsin-like proteases was determined in all cases when most of the population completed eye migration and exhibited benthic life. This agrees with Segner *et al.* (1994) for turbot and it would be related to the development of stomach and gastric glands which in Senegal sole are fully developed 7–10 days after the acquisition of benthic life, under good feeding conditions and at temperatures of 16–19° C (Ribeiro *et al.*, 1999*a*).

In the present study, only total length was individually measured in larvae, while dry weight was determined on pooled samples with several larvae, or extrapolated from probits or length-weight regressions. Therefore, length frequency distribution histograms gave the best reliable reflection of population

variation. Individual variation during early development in larval fish is an aspect that merits special attention. This variation becomes more evident when a factor limits development, such as insufficient feeding. This seems to be the case with larvae grown under the regime D, which exhibited a wider age and size range at the end of metamorphosis. Furthermore, the irregular increase of larvae attaining the final metamorphic stage is probably due, at least in part, to the progressive mortality of those larvae unable to complete the eye migration.

In summary, the status of development determined by the eye position is a good measure of metamorphic transformation in *Solea*, although some events still occur after the eye has reached its final position, such as cranial ossification (Brewster, 1987) and the completion of gut development (Ribeiro *et al*., 1999*a*). The morphological changes occur in parallel with the behavioural and physiological changes and independent of the time required to reach a given developmental level. Individuals change their swimming plane and move to the bottom during the transition from stage 2 to stage 3, starting benthic life. This transformation is concomitant with changes in body reserves, as well as in the pattern of enzymatic activity although the timing depends on environmental conditions, and specifically in the present study on food availability and its nutritional value.

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