Influence of starvation on the critical swimming behaviour of the Senegalese sole (*Solea senegalensis*) and its relationship with RNA/DNA ratios during ontogeny

ANA M. FARIA [1,2], TEJA MUHA [3], ELVIRA MOROTE [4] and
M. ALEXANDRA CHÍCHARO [2]

[1] Eco-Ethology Research Unit, Instituto Superior de Psicologia Aplicada, R. Jardim do Tabaco 34, 1149-041 Lisboa, Portugal.
[2] Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-117 Faro, Portugal.
E-mail: mchichar@ualg.pt
[3] University of Ljubljana, Biotehnical faculty, Janmnikarjeva 101, 1000 Ljubljana, Slovenia.
[4] Institut de Ciències del Mar (CSIC), Passeig Marítim 37-49, Barcelona 08003, Spain.

SUMMARY: Food availability can affect larval survival directly through starvation and indirectly through the effects on larval growth rate, swimming performance and vulnerability to predators. In the present study we evaluate the effects of starvation on growth, nutritional condition and swimming behaviour of the Senegalese sole (*Solea senegalensis*) throughout ontogeny (8 to 14 days after hatching). Biochemical analysis (RNA/DNA ratios) and behavioural experiments (critical swimming speed, U_{crit}) were conducted on larvae reared under 3 feeding treatments: fed ad libitum, deprived of food for 48 hours and deprived of food for 96 hours. Growth was significantly affected by feeding treatment, while only slight decreases in RNA/DNA ratio and swimming performance were registered. Late stage larvae of the three feeding treatments had slower critical speeds than the pre-flexion and flexion stages, which is probably related to the benthic lifestyle acquired by the species at the end of the larval period. These physiological and behavioural changes are in accordance with previous results, which show that flatfish larvae are more resistant to starvation than pelagic species and that they become less active later in development.

Keywords: Senegalese sole, *Solea senegalensis*, feeding treatment, starvation, RNA/DNA ratio, critical swimming speed, U_{crit}, ontogeny.

RESUMEN: INFLUENCIA DEL AYUNO SOBRE EL COMPORTAMIENTO NATATORIO CRÍTICO DEL LENGUADO SENEGALÉS *SOLEA SENEGALENSIS* Y SU RELACIÓN CON EL COCIENTE RNA/DNA DURANTE LA ONTOGENIA. – La disponibilidad de comida puede afectar la supervivencia larvaria directamente a través del ayuno e indirectamente a través de los efectos sobre la tasa de crecimiento larvario, actividad natatoria y vulnerabilidad a los predadores. En el presente estudio evaluamos los efectos del ayuno sobre el crecimiento, condición nutricional y comportamiento natatorio del lenguado senegalés, *Solea senegalensis,* durante la ontogenia (8 a 14 días post-eclosión). Se llevaron a cabo análisis bioquímicos (RNA/DNA) y experimentos de comportamiento (velocidad crítica de natación, U_{crit}) sobre larvas mantenidas bajo 3 condiciones de alimentación: alimentadas (tratamiento *ad libitum*), ayuno a corto plazo (hasta 48 h sin alimentación) y ayuno a largo plazo (hasta 96 h sin alimentación). El crecimiento varió significativamente según el tratamiento alimentario, mientras que el cociente RNA/DNA y la capacidad natatoria mostraron únicamente una ligera disminución. En los tres tratamientos alimentarios los estadios larvarios más avanzados mostraron velocidades críticas más bajas cuando se compararon con las de los estados de preflexión y flexión, lo que está probablemente relacionado con el estilo de vida bentónica adquirido por la especie al final del periodo larvario. Estos cambios fisiológicos y de comportamiento concuerdan con resultados previos que muestran que las larvas de peces planos son más resistentes al ayuno que las de especies pelágicas y se vuelven menos activas con el desarrollo.

Palabras clave: lenguado senegalés, *Solea senegalensis*, tratamiento alimentario, ayuno, cociente RNA:DNA, velocidad de natación crítica, U_{crit}, ontogenia.

INTRODUCTION

Early life stages of marine fishes experience high rates of mortality, with serious implications on species' future recruitment. The two main mortality agents acting upon marine fish larvae are predation and starvation (Bailey and Houde, 1989). These factors are not independent, as starvation leads to a decreased growth rate (Yin and Blaxter, 1986), slower development (Kamler *et al.*, 1990) and changes in behaviour (Sogard and Olla, 1996; Chick and Van den Avyle, 2000). Larvae with a low nutritional status will consequently be smaller, weaker and less developed with regard to sensory and locomotory capacities than well-fed larvae of the same age, thus being more susceptible to predation (but see Billerbeck *et al.*, 2001). For a variety of fish species (herring, hake, cod, flounder, anchovy, striped bass), it has been shown that starved larvae are more susceptible to predation than fed larvae (Bailey, 1984; Neilson *et al.*, 1986; Yin and Blaxter, 1987; Booman *et al.*, 1991). Several studies indicate that high larval condition can be correlated with increased growth rate and enhanced recruitment or juvenile survival under natural conditions (Searcy and Sponaugle, 2001; Sponaugle *et al.*, 2006), but few attempts have been made to correlate larval condition with any behavioural function critical for larval survival, such as swimming behaviour (e.g. Laurence, 1972; Yin and Blaxter, 1987; Chick and Van den Avyle, 2000). Swimming performance is a central determinant of the fitness of fish, determining to a large extent the success of predator avoidance, prey capture and dispersal potential (Reidy *et al.*, 2000; Armsworth, 2001; Plaut, 2001). Larvae in better condition may reveal greater swimming abilities and responsiveness to predators than larvae in poor condition (Chick and Van den Avyle, 2000; Grorud-Colvert and Sponaugle, 2006).

Nucleic acid analysis is an acknowledged practical tool for studying recent overall nutritional condition and growth of larvae and young fish, as well as their responses to environmental variability (e.g. Clemmesen *et al.*, 1997; Chícharo, 1997; Buckley *et al.*, 1999; Caldarone *et al.*, 2003). Specifically, RNA/DNA ratio reflects variations in protein synthesis rates: the quantity of DNA in an animal somatic cell is believed to be normally stable, but the quantity of RNA primarily associated with ribosomes is closely related to the rate of protein synthesis. RNA/DNA ratio has been shown to respond to changes in feeding conditions and growth in periods as short as one to three days in a variety of fish species and is a valid and reliable growth rate estimator (Rooker and Holt, 1996; Buckley *et al.*, 1999; Gwak and Tanaka, 2001).

The present study was undertaken to examine the effect of starvation on growth, nutritional condition and swimming behaviour of laboratory-reared Senegalese sole (*Solea senegalensis*) throughout ontogeny, up to the beginning of metamorphosis, when larvae acquire a benthic life mode. We conducted laboratory experiments assessing the critical swimming speed of larvae reared under three feeding treatments (fed ad libitum, deprived of food for 48 hours and deprived of food for 96 hours) and examined their growth and nutritional condition, measured as RNA/DNA ratio.

MATERIALS AND METHODS

Larvae

Recently hatched larvae of *S. senegalensis*, 4 days after hatching (DAH), were obtained from the Aquaculture Research Station of the National Institute of Biological Resources (INRB-IPIMAR) in Olhão, southern Portugal. Larvae were randomly distributed in five 20-L aquaria, at a density of 100 larvae L^{-1}. Aquaria were filled with filtered seawater, with constant slight aeration and a photoperiod of 12L:12D. Salinity was maintained constant at 37 and the average temperature was 21ºC. Larvae were assigned to three feeding regimes: fed ad libitum, deprived of food for 48 hours and deprived of food for 96 hours. Larvae from the ad libitum treatment were stocked in one aquarium (Aquarium 1, Fig. 1) and fed rotifers from 4 to 9 DAH three times per day and *Artemia* nauplii from 6 to 14 DAH three times per day. Food concentration was maintained at a proper density to guarantee an ad libitum supply of food. The aeration in the tanks was turned off during feeding so that the larvae did not lose extra energy on capturing prey. Larvae were fed at least 1.5 h before experiments were conducted. Larvae from the two starvation treatments were randomly distributed in four aquaria (Fig. 1). Each of the four aquaria provided larvae for both short and long starvation treatments. Aquaria 2 and 3 supplied larvae twice for the short starvation treatment. After larvae had been collected for the long starvation experiments (at 11 and 12 DAH) the remaining larvae in aquaria 2 and 3 were fed once and left starving again for 48 h. Larvae fed ad libitum were tested from 8 DAH to 14 DAH; larvae from the short starvation treatment were tested from 9 DAH to 14 DAH, and larvae from the long starvation treatment were tested from 11 DAH to 14 DAH.

Experimental design

Larvae were carefully removed from the rearing tank using a small container, placed individually in large petri dishes and left undisturbed to allow them to recover from handling (Fuiman and Ottey, 1993). After this period, larvae were transferred to the swimming chamber, one in each lane and allowed to acclimate for 5 min at a minimum flow speed of less than 1 cm s^{-1}. If any behavioural symptoms of stress, such as lying on the bottom or clinging to the sides, were observed after the acclimation period, the individual was removed and replaced by another fish. Water temperatures in the chamber during the study period varied from 21ºC to 23ºC.
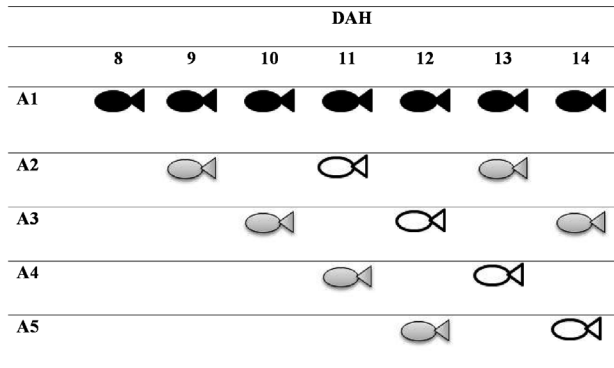
FIG. 1. – Experimental design illustrating which aquarium (A1 to A5) supplied larvae to which feeding treatment and which ages. Black fishes represent larvae from the ad libitum treatment, grey fishes represent larvae from the short starvation treatment, and white fishes represent larvae from the long starvation treatment.

Critical swimming speed

Critical swimming speed (U_{crit}) was measured using a swimming chamber, following the protocols of Stobutzki and Bellwood (1994, 1997). The chamber was made of clear Perspex with 6 parallel swimming lanes, each 30 mm wide, 50 mm high and 180 mm long. A removable lid allowed introduction and removal of fish from the lanes. A strip of black tape on the top of the lid provided fish with a visual reference to maintain position in the flow, and a mesh screen was placed at the upstream and downstream ends of each lane to retain larvae. A section of flow straighteners, 40 mm long, was placed at the upstream end of each lane to minimize turbulence. Previous work demonstrated that at the typical U_{crit}, water velocity was not significantly different between the centre of the lane and 5 mm from the wall (Stobutzki and Bellwood, 1997; Stobutzki, 1998; Fisher et al., 2000). Experimental observations also confirmed that larvae did not show depth preference in the chamber. For details on the swimming chamber characteristics see Faria et al. (2009). Maximum flow speed was 22 cm s^{-1}.

To measure U_{crit}, water velocity was increased by approximately 1 cm s^{-1} every 2 min until the larva was unable to swim against the current for 2 min. Calculation of U_{crit} followed Brett (1964):

$$U_{crit} = U + (t/t_i \cdot U_i);$$

where U is the highest speed a fish was able to maintain, U_i is the velocity increment, t is the time swum in the final velocity increment, and t_i is the time interval for each velocity increment (2 min). After the test, fish were immediately put in liquid nitrogen and then stored at -80ºC for subsequent RNA/DNA analysis. The whole larvae were freeze dried and weighed before the biochemical analysis. This procedure was selected because heads and/or guts are often removed for further age and feeding analysis, and these tissues may influence the overall RNA/DNA ratio (Olivar et al., 2009).

A total of 215 larvae were tested, though the number that provided useful data was lower (n = 161), as each individual did not always provide useful data for all the measured parameters.

Nucleic acid analysis

RNA and DNA were measured with the microplate fluorescent assay (MFA) of Wagner et al. (1998). The MFA assay is a modification of the sequential fluorometric method of Bentle et al. (1981), in which DNA and RNA in a single sample are determined sequentially by the addition of DNase and RNase, using ethidium bromide (EB) as fluorescent dye (Caldarone et al., 2001). Wagner et al. (1998) modified the sequential fluorometric method to the MFA with 96-well microtiter plates by adopting a sarcosyl extraction technique and eliminating the DNase step. The whole larvae were individually homogenized by sonication (3 pulses 50 A during 1 min) with cold sarcosyl extraction buffer. The volume of extraction buffer was 500 μl (0.5%). The samples were then shaken for 1 h at room temperature on a vortex mixer equipped with a multiple-vial head. Next, they were centrifuged (12 000 × g) for 15 min to separate insoluble larvae remains. The samples were diluted 1:10 with Tris buffer to reduce the sarcosyl concentration to 0.05%. In each run, duplicate 50 μl aliquots of supernatants of the samples and duplicates of 0, 0.6, 1.1, 1.7 and 2.3 μg ml^{-1} DNA standard solutions (λ-phagus 0.25 μg μl^{-1} from Roche), 16s-23s E. coli RNA (4 μg μl^{-1}), 0, 3.6, 7.3, 10.9 and 14.6 μg ml^{-1} RNA standard solutions (16s-23s E. coli 4 μg μl^{-1} from Roche) were transferred to Nunclon 96-well, black, round-bottom microplates. The average ratio of DNA and RNA slopes was 5.5±0.8, which can be used to compare RNA/DNA ratio results determined by other protocols (Caldarone et al., 2006). EB solution (30 μl) was added to each well, and the plates were shaken gently at room temperature for 15 min. The EB fluorescence was then scanned on a microplate reader (Biotek synergy HT model SIAFRTD) with 360 nm (excitation) and 590 nm (emission) (first scan- total fluorescence RNA and DNA). Following the first scan, RNase solution (30 μl, 0.12 μg ml^{-1}) was added to each well and the concentration of DNA was calculated directly by the standard curve. The concentration of RNA was determined indirectly by subtraction of DNA fluorescence (second scan) from total fluorescence (first scan).

Statistical analysis

Linear regression analyses were used to determine relationships among size (standard length, SL), age (DAH), RNA/DNA ratio and critical swimming speed (U_{crit}) for each feeding treatment. Normality was tested using the Kolmogorov-Smirnov statistic and plots of residuals, and predicted values were examined. The variables SL, U_{crit} and RNA/DNA ratio were log$_{10}$ transformed to normalize data, but U_{crit} was not nor-

malized by any transformation. Large sample size and the plot of the residuals indicate that analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were robust. To determine whether the slopes of the regressions of size on age differed among the feeding treatments, an ANCOVA was performed with size as the dependent variable, feeding treatment as the fixed factor, and age as the co-variate. Significant effects were further examined by Tukey's HSD multiple comparison tests. To examine the effect of interaction of feeding treatment with ontogeny (pre-flexion, flexion and post-flexion) on mean RNA/DNA ratio and mean U_{crit}, two-way ANOVA were performed after testing for normality and homogeneity of variances. Significant effects were further examined by Tukey's HSD multiple comparison tests. The separate effects of feeding treatment and ontogenetic stage on RNA/DNA ratio and U_{crit} were analyzed by one-way ANOVA. To determine whether the slopes of the regressions of U_{crit} on size differed among the feeding treatments, an ANCOVA was performed with U_{crit} as the dependent variable, feeding treatment as the fixed factor, and size as the co-variate. The relationship between RNA/DNA ratio and U_{crit} was evaluated by regression analysis and an ANCOVA was used to access the effect of feeding treatment on that relationship, with RNA/DNA as the dependent variable, feeding treatment as the fixed factor, and U_{crit} as the co-variate. In all analyses the level of significance was ≤0.05. Notes on ontogenetic progress were made and SL was measured to the nearest 0.01 mm, using the Image J software (version 1.38). Before each photograph was taken, a transparent acetate sheet marked with a millimetre grid was photographed and used as a reference for each measurement in the image analysis software. All statistical tests were conducted using STATISTICA software (Version 6.0).

RESULTS

Effect of starvation on growth and RNA/DNA ratio

Growth in SL of *S. senegalensis* was significantly influenced by feeding treatment ($F_{(2,157)}$ = 29.61, $p<0.0001$). Larvae reared under the long starvation treatment were significantly smaller than larvae reared under the other two treatments (Fig. 2).

RNA and DNA contents of 56 fish of the fed group, 66 fish of the short starvation group and 39 fish of the long starvation group were analyzed (Table 1). The interaction between feeding treatment and ontogeny returned no significant effects ($F_{(4,152)}$ = 0.33, P = 0.86).
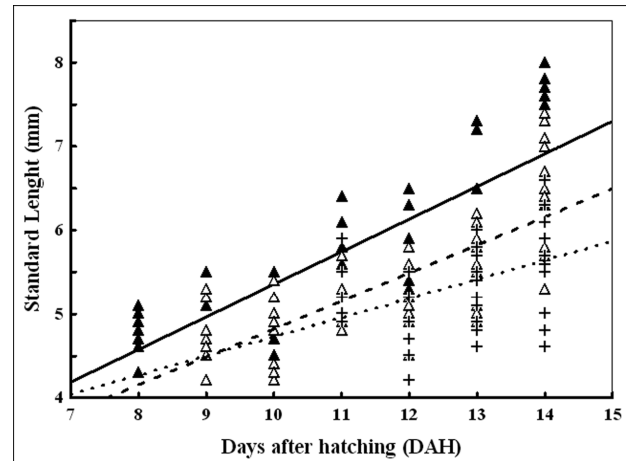

FIG. 2. – Changes in standard length (SL) of fed and starved larvae of *S. senegalensis*. Each symbol represents results for an individual larva. Closed triangles represent fed fish (y = 0.39x + 0.94, r^2 = 0.64); opened triangles represent short starvation fish (y = 0.33x + 0.98, r^2 = 0.56), and cross symbols represent long starvation fish (y = 0.23x + 1.94, r^2 = 0.17). The thick line is the regression line for fed fish; the broken line is the regression line for short starvation fish and the dotted line is the regression line for long starvation fish.

RNA/DNA ratio did not change with ontogeny ($F_{(2,158)}$ = 1.10, P = 0.33), and a long starvation period (96 h) was not enough to significantly decrease nutritional condition ($F_{(2,158)}$ = 2.02, P = 0.13).

Effect of starvation on Critical speed

A total of 161 larvae were tested in critical swimming experiments, of which 56 belonged to the fed group, 66 to the short starvation group and 39 to the long starvation group. Of these, 22 larvae of the fed group, 17 larvae of the short starvation group and 13 larvae of the long term-starvation group did not swim either because they were pre-flexion stages, incapable of swimming against the slowest tested current speed, or post-flexion stages, metamorphosing and settled to the bottom. These larvae were assigned a critical speed of 0 cm s^{-1}.

Critical speed of the fed and short starvation groups revealed a significant relationship with size (fed: $F_{(1,54)}$ = 7.12, P = 0.01; short starvation: $F_{(1,64)}$ = 10.96, P = 0.001), but no significant relationship in larvae of the long starvation treatment was registered ($F_{(1,37)}$ = 0.16, P = 0.69) (Table 2).

The interaction of feeding treatment with ontogenetic stage returned no significant effect on U_{crit} ($F_{(4,152)}$ = 0.59, P = 0.67). Feeding treatment had no influence on U_{crit} ($F_{(2,158)}$ = 0.13, P = 0.88), with performance

TABLE 1. – Number of larvae, age range, mean size (±SE) and mean nucleic acid content of analyzed larvae (±SE) in the three feeding treatments. N = number of larvae, DAH = days after hatching, SL = standard length, W = weight, DW = dry weight, SE = standard error.

	N	DAH	SL (mm)	W (mg)	µg RNA/larva	µg RNA/mg DW	µg DNA/larva	µg DNA/mg DW	RNA/DNA
Ad libitum	56	8-14	5.18±0.13	0.59±0.05	15.54±2.83	36.30 ±6.08	12.58±1.47	27.69±3.23	1.32±0.14
Short starvation	66	9-14	4.86±0.09	0.40±0.03	13.44±2.40	39.45±5.88	11.91±1.15	40.91±4.38	1.56±0.15
Long starvation	39	11-14	4.89±0.10	0.47±0.05	12.43±3.51	39.25±10.54	12.43±0.80	42.53±6.70	0.91±0.19

TABLE 2. – Summary of measurements of critical swimming speed (cm s^{-1}) for *S. senegalensis* in the different feeding treatments: Number (N), size (SL: standard length), age (DAH: days after hatching), relationships between critical speed (U_{crit}: log_{10} transformed) and size (SL: log_{10} transformed SL). Not significant p>0.05.

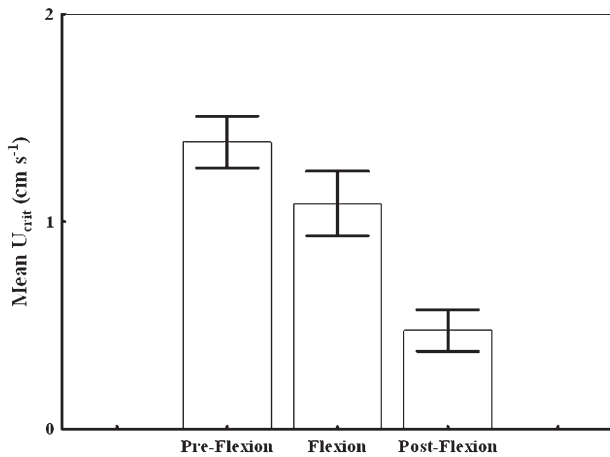|	N	SL (mm)	Age (DAH)	Relationship	R^2	p	Range
Ad libitum	56	3.5-7.5	8-14	y = -0.80x + 0.74	0.12	0.01	0.0-5.0
Short starvation	66	3.7-6.9	9-14	y = -0.91x + 0.81	0.15	0.001	0.0-5.0
Long starvation	39	3.7-6.1	11-14	y = -0.19x + 0.32	0.004	0.69	0.0-3.2

FIG. 3. – Mean U_{crit} (± SE) of *S. senegalensis* larvae in pre-flexion, flexion and post-flexion stages.

ranging from 0 to 5 cm s^{-1} in fed and short starvation larvae, and 0 to 3.2 cm s^{-1} in long starvation larvae. Ontogenetic stage had a significant effect on mean U_{crit} values ($F_{(2,158)}$ = 14.70, P<0.0001). Tuckey-test revealed that post-flexion larvae had a significantly slower swimming performance when compared to earlier developmental stages (Fig. 3). The relationship between RNA/DNA and U_{crit} did not change with ontogenetic stage ($F_{(2,157)}$ = 1.90, P = 0.15), and showed no differences between the feeding groups ($F_{(2,157)}$ = 2.09, p = 0.13) (Fig. 4). Also, the regressions of RNA/DNA
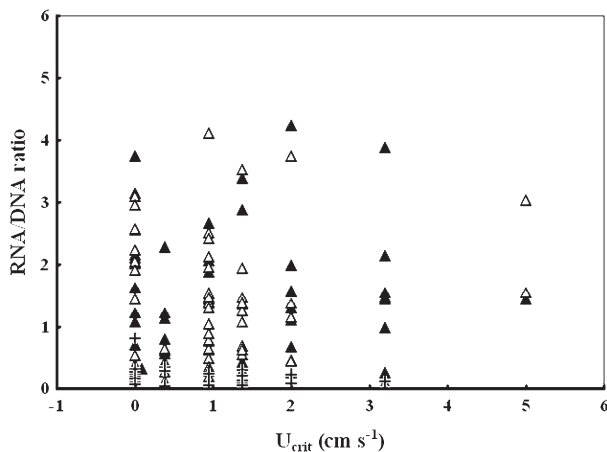

FIG. 4. – Relationship between RNA/DNA ratio and U_{crit} of fed and starved larvae of *S. senegalensis*. Closed triangles represent fed fish, open triangles represent short starvation fish, and cross symbols represent long starvation fish. The x-axis is scaled to start at -1 instead of 0 for a better visualization of the graph.

on U_{crit} were not significant in any feeding treatment (fed: $F_{(1,54)}$ = 1.12, P = 0.29; short starvation: $F_{(1,64)}$ = 3.73, P = 0.06; long starvation: $F_{(1,37)}$ = 2.06, P = 0.16).

DISCUSSION

Effect of starvation on growth and RNA/DNA ratio

Starvation had a significant influence on growth of *S. senegalensis* larvae, with 96 h starvation larvae showing considerably slower growth than fed and 48 h starved larvae. Reduced growth rate caused by starvation will extend the duration of vulnerable ontogenetic stages, and as there are more potential predators for small larvae, starved larvae might suffer a higher cumulative rate of mortality (Cushing, 1975; Shepherd and Cushing, 1980). Survival of starved larvae is then compromised. The results of nutritional condition, measured as RNA/DNA ratio, showed a slight decrease after 96 h of starvation. Latency has been demonstrated to be as short as 1-3 days in some fish species. Clemmesen (1994) reported that fed and starved herring (*Clupea harengus*) larvae (>10 d) could be distinguished only after 3 to 4 days. Other than that, it has been shown that flatfish larvae are more resistant to starvation (Clemmesen, 1987; Yin and Blaxter, 1987) than species with small eggs and yolk-sac larvae, such as *Engraulis mordax* (Lasker *et al.*, 1970), *Paralichtys californicus* and *Hypsopsetta guttulata* (Gadomski and Petersen, 1988), and than species carrying larger yolk reserves, such as *Clupea harengus* (Blaxter and Hempel, 1963) and *Ammodytes americanus* (Buckley *et al.*, 1984).

Richard *et al.* (1991) studied the effect of starvation on RNA/DNA ratio in *Solea solea* larvae and concluded that the time of beginning of starvation was an important factor. Larvae starved from the beginning of exogenous feeding showed a sharp decrease in the RNA/DNA ratio, and could be distinguished from fed larvae after 2 or 3 d of starvation. However, for larvae starved after days 5 and 10, the food deprivation interval must be longer to show a clear effect, and in juveniles starved on day 14 after metamorphosis, the RNA/DNA ratio was not significantly different from that of juveniles fed throughout the experiment (Richard *et al.*, 1991).

Despite studies on how larval condition affects behaviour, in particular swimming performance and escape response (Laurence, 1972; Yin and Blaxter, 1987; Chick and Van den Avyle, 2000; Grorud-Colvert and Sponaugle, 2006), there are no studies on swim-

ming behaviour and nutritional condition measured as RNA/DNA ratio. RNA/DNA ratio has been shown to respond to changes in feeding conditions and growth in periods as short as one to three days in a variety of fish species. It is a valid and reliable growth rate estimator that has been applied in numerous field assessments (e.g. Rooker and Holt, 1996; Buckley *et al.*, 1999; Gwak and Tanaka, 2001; Chícharo *et al.*, 2003) but has never been correlated with any behavioural function. In this study, RNA/DNA ratio did not correlate significantly with critical speed, which may be related to the high resistance to starvation and low swimming performance of *S. senegalensis* larvae. Nevertheless a trend of increase critical speed at high RNA/DNA ratios was observed.

Effect of starvation on Critical speed

Swimming abilities of larval fish are critical behavioural traits since swimming influences the capacity of larvae to find food, escape from predators and control dispersal (Stobutzki and Bellwood, 1994, 1997). In order to determine the potential importance of swimming behaviour, it is fundamental to know how these abilities change during ontogeny (e.g. Leis *et al.*, 2009a,b; Faria *et al.*, 2009). In the present study, a significant decrease in swimming abilities of post-flexion larvae was observed in the three feeding treatments, which implies an ontogeny-related effect. The lack of swimming or reduced swimming of larvae close to settlement is probably related to behavioural changes associated with a benthic lifestyle rather than a decrease in the larvae's ability to swim. These larvae were completing metamorphosis and resembled newly settled fish. Behavioural changes coupled with morphological changes are known to lead to variation in the general metabolism (Bergeron, 1982) and energy expenditure during later development, when flatfish larvae are less active (Blaxter and Staines, 1971). Other studies have also related changes in swimming performance to developmental or ecological transitions (e.g. Dudley *et al.*, 2000; Guan *et al.*, 2008), reporting an improvement in swimming performance until metamorphosis, after which improvements became slower. In disagreement with other studies (Clark *et al.*, 2005; Leis *et al.*, 2006a,b, 2007, 2009a,b; Faria *et al.*, 2009), we found no significant relationship between critical speed and size in any of the feeding treatments. This can be attributed to the small size range of tested larvae, or may be merely indicative of the poor swimming capacities of *S. senegalensis* larvae. Critical swimming speed recorded for Senegalese sole larvae in this study ranged from 0.4 to 5.0 cm s^{-1} (0.2 to 11.9 bl s^{-1}) for the fed and short starvation group, and 0.4 to 3.2 cm s^{-1} (0.6 to 7.3 bl s^{-1}) for the long starvation group. There are no data on critical speed of other Pleuronectiformes, but data available for routine speeds of Soleidae, Pleuronectidae and Paralichthyidae report values of 0.5 to 3 cm s^{-1} (Blaxter, 1986; Miller *et al.*, 1988), which are

close to what we found in this study. These values are in agreement with the typical assumption that temperate fish larvae are poor swimmers. This assumption may be partly due to differences in water temperature. It is well known that temperature influences both the physiology of fish larvae and the physics of the hydrodynamic environment in which larvae are swimming (Fuiman and Batty, 1997). Other than these, variation can result from morphological differences (Stobutzki, 1998; Dudley *et al.*, 2000). Most studies compare temperate clupeiform, gadiform, or pleuronectiform larvae (Blaxter, 1986; Miller *et al.*, 1988) with tropical perciform larvae (Fisher *et al.*, 2000, 2005; Leis and Fisher, 2006). For this reason, comparisons of swimming speed among taxa should take into account phylogeny, methodology, and developmental state.

No food deprivation-induced effects in swimming behaviour were found after a 4-d period of food deprivation, suggesting that there are no condition-related behavioural effects in critical swimming performance after 4 d of starvation. Regarding escape response behaviour, Skajaa and Browman (2007) also concluded that escape response rate of cod larvae deprived of food for 3 d was not affected. In contrast, Yin and Blaxter (1987) found condition-related behavioural changes, with the escape response rate showing a dome-shaped relationship with increasing duration of food deprivation: escape speed in herring (*Clupea harengus*) of different ages and newly hatched cod and flounder (*Platichthys flesus*) initially increased with increasing time of food deprivation, peaked when the larvae were close to the point of no return (when 50% of the larvae will die even when offered food), and subsequently decreased. The escape response rate has also been shown to have a negative relationship with increasing duration of food deprivation. Booman *et al.* (1991) reported a decrease in responsiveness of starved northern anchovy (*Engraulis mordax*) larvae to predatory attacks by adult anchovy. Chick and Van den Avyle (2000) examined the effects of feeding on routine swimming speed and responsiveness to simulated predator attacks of larval striped bass (*Morone saxatilis*) reared under high, medium and low prey treatments and found that larvae reared in the low-prey treatment had slower routine swimming speeds and shorter reactive distances and were less responsive to simulated-predator attacks. The effects of starvation were not evident until larvae had starved for several days. Results then suggest that susceptibility to starvation of fish larvae appears to be species-specific.

Overall, our results indicate that growth was significantly affected by feeding treatment, but only slight decreases in RNA/DNA ratio and swimming performance were observed after 4 days of starvation. These behavioural and physiological changes are in accordance with previous results, which show that flatfish larvae become less active later in development and are more resistant to starvation than pelagic species. In the future it will be imperative to have information on wild larvae in order

to allow inferences of the observed behaviours to their ecology and life history patterns (Leis, 2006).

REFERENCES

Armsworth, P.R. – 2001. Directed Motion in the Sea: Efficient Swimming by Reef Fish Larvae. *J. Theor. Biol.*, 210: 81-91.
Bailey, K.M. – 1984. Comparison of laboratory rates of predation on five species of marine fish larvae by three planktonic invertebrates; effect of larval size on vulnerability. *Mar. Biol.*, 79(Suppl. 3): 303-309.
Bailey, K.M. and E.D. Houde. – 1989. Predation on eggs and larvae of marine fishes and the recruitment problem. *Adv. Mar. Biol.*, 25: 1-83.
Bentle, L.A., S. Dutta and J. Metcoff. – 1981. The sequential enzymatic determination of DNA and RNA. *Anal Biochem.*, 116: 5-16.
Bergeron, J.-P. – 1982. L'aspartate transcarbamylase, indice de croissance des organismes marins: perspectives et Limites. *Publs. Cent. natn. Exploit. Oceans* (CNEXO) (Ser Act. Colloques) 14: 177-19.
Billerbeck, J.M., T.E. Lankford Jr. and D.O. Conover. – 2001. Evolution of intrinsic growth and energy acquisition rates. I. Trade-offs with swimming performance in *Menidia menidia*. *Evolution*, 55(9): 1873-1881.
Blaxter, J.H.S. – 1986. Development of sense organs and behaviour of teleost larvae with special reference to feeding and predator avoidance. *Trans. Am. Fish. Soc.*, 115: 98-115.
Blaxter, J.H.S. and G. Hempel. – 1963. The influence of egg size on herring larvae (*Clupea harengus* L.). *J. Cons. int. Explor Mer*, 28: 211-240.
Blaxter, J.H.S. and M.E. Staines. – 1971. Food searching potential in marine fish larvae. In: D.J. Crisp (ed.), *Proceedings 4th European Marine Biology Symposium*, pp. 467-481. Cambridge University Press, Cambridge.
Booman, C.I., A. Folkvord and J.R. Hunter. – 1991. Responsiveness of starved northern anchovy *Engraulis mordax* larvae to predatory attacks by adult anchovy. *Fish. Bull. (U.S.)*, 89(Suppl. 4): 707-711.
Brett, J.R. – 1964. The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Board. Can.*, 21: 1183-1226.
Buckley, L.J, E. Caldarone and T.-L. Ong. – 1999. RNA–DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. *Hydrobiologia*, 401: 265-277.
Buckley, L.J., S.I. Turner, T.A. Halavik, A.S. Smigielski, S.M. Drew and G.C. Laurence. – 1984. Effects of temperature and food availability on growth, survival and RNA-DNA ratio of larval sand lance (*Ammodytes americanus*). *Mar. Ecol. Prog. Ser.*, 15: 91-97.
Caldarone, E.M., M. Wagner, J.S. Onge-Burnes and L.J. Buckley. – 2001. Protocol and guide for estimating nucleic acids in larval fish using a fluorescence microplate reader. Ref Doc 01-11, Northeast Fisheries Science Center, Woods Hole, MA.
Caldarone, E.M., J.M. St. Onge-Burns and L.J. Buckley. – 2003. Relationship of RNA/DNA ratio and temperature to growth in

larvae of Atlantic cod *Gadus morhua*. *Mar. Ecol. Prog. Ser.*, 262: 229-240.
Caldarone, E.M., C.M. Clemmesen, E. Berdalet, T. Miller, A. Folkvord, G.J. Holt, M.P. Olivar and I.M. Suthers. – 2006. Inter-calibration of four spectro-fluorometric protocols for measuring RNA/DNA ratios in larval and juvenile fish. *Limnol. Oceanogr. Methods,* 4: 153-163.
Chick, J.H. and M.J. Van den Avyle. – 2000. Effects of feeding ration on larval swimming speed and responsiveness to predator attacks: implications for cohort survival. *Can. J. Fish. Aquat. Sci.*, 57: 106-115.
Clark, D.L., J.M. Leis, A.C. Hay and T. Trnski. – 2005. Swimming ontogeny of larvae of four temperate marine fishes. *Mar. Ecol. Prog. Ser.*, 292: 287-300.
Chícharo, M.A. – 1997. Starvation percentages in field caught *Sardina pilchardus* larvae off southern Portugal. *Sci. Mar.* 61 (4): 507-516.
Chicharo, M.A., E. Esteves, A.M.P. Santos, A. dos Santos, A. Peliz and P. Ré. – 2003. Are sardine larvae caught off northern Portugal in winter starving? An approach examining nutritional conditions. *Mar. Ecol. Prog. Ser.*, 257: 303-309.
Clemmesen, C.M. – 1987. Laboratory studies on RNA/DNA ratios of starved and fed herring (*Clupea harengus*) and turbot (*Scophthalmus maximus*) larvae. *J. Cons. int. Explor. Mer*, 43: 122-128.
Clemmesen, C.M. – 1994. The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. *Mar. Biol.*, 118: 377-382.
Clemmesen, C.M., R. Sanchez, C. Wongtschowski. – 1997. A regional comparison of the nutritional condition of SW Atlantic anchovy larvae, *Engraulis anchoita,* based on RNA/DNA contents. *Arch. Fish. Mar. Res.*, 45: 17-43.
Cushing, D.H. – 1975. The natural mortality of the plaice. *J. Cons. Int. Explor. Mer.* 36 (Suppl. 2): 150-157.
Dudley, B., N. Tolimieri, J. Montgomery. – 2000. Swimming ability of the larvae of some reef fishes from New Zealand waters. *Mar. Fresh. Res.* 51: 783-787.
Faria, A., A. Ojanguren, L.A. Fuiman and E.J. Gonçalves. – 2009. Ontogeny of Critical Swimming speed of wild-caught and laboratory-reared larvae of red Drum (*Scieanops ocellatus*). *Mar. Ecol. Prog. Ser.*, 384: 221-230.
Fisher, R., D.R. Bellwood and S.D. Job. – 2000. Development of swimming abilities in reef fish larvae. *Mar. Ecol. Prog. Ser.*, 202: 163-173.
Fisher, R., J.M. Leis, D.L. Clark and S.K. Wilson. – 2005. Critical swimming speeds of late-stage coral reef fish larvae: Variation within species, among species and between locations. *Mar. Biol.*, 147: 1201-1212.
Fuiman, L.A. and D.R. Ottey. – 1993. Temperature effects on spontaneous behaviour of larval and juvenile red drum *Sciaenops ocellatus*, and implications for foraging. *Fish. Bull.*, 91: 23-35.
Fuiman, L.A. and R.S. Batty. – 1997. What a drag it is getting cold: partitioning the physical and physiological effects of temperature on fish swimming. *J. Exp. Biol.*, 200: 1745-1755.
Gadomski, D.M. and J.H. Petersen. – 1988. Effects of food deprivation on the larvae of two flatfishes. *Mar. Ecol. Prog. Ser.*, 44: 103-111.
Grorud-Colvert, K. and S. Sponaugle. – 2006. Influence of condition on behaviour and survival potential of a newly settled coral reef fish, the bluehead wrasse *Thalassoma bifasciatum*. *Mar. Ecol. Prog. Ser.*, 327: 279-288.
Gwak, W.S. and M. Tanaka. – 2001. Developmental changes in RNA/DNA ratios of fed and starved laboratory-reared Japanese flounder larvae and juveniles, and its application to assessment of nutritional condition for wild fish. *J. Fish Biol.*, 59: 902-915.
Kamler, E., M. Szlamiñska, A. Przybyl, B. Barska, M. Jakubas, M. Kuczyñski and K. Raciborsku. – 1990. Developmental response of carp, *Cyprinus carpio*, larvae fed different foods or starved. *Environ. Biol. Fishes*, 29: 303-313.
Lasker, R., H.M. Feder, G.H. Theilacker and R.C. May. – 1970. Feeding, growth, and survival of *Engraulis rnordax* larvae reared in the laboratory. *Mar. Biol.*, 5: 345-353.
Laurence, G. – 1972. Comparative swimming abilities of fed and starved larval largemouth bass (*Micropterus salmoides*). *J. Fish. Biol.*, 4: 73-78.
Leis, J.M. – 2006. Are larvae of demersal fishes plankton or nekton? *Adv. Mar. Biol.*, 51: 59-141.

Leis, J.M. and R. Fisher. – 2006. Swimming speed of settlement-stage reef-fish larvae measured in the laboratory and in the field: A comparison of critical speed and in situ speed. *Proc. 10th Int. Coral Reef Symp.*, Okinawa: 438-445.

Leis, J.M., A.C. Hay and T. Trnski. – 2006a. *In situ* ontogeny of behaviour in pelagic larvae of three temperate, marine, demersal fishes. *Mar. Biol.*, 148: 655-669.

Leis, J.M., A.C. Hay, D.A. Clark, I.S. Chen and K.T. Shao. – 2006b. Behavioural ontogeny in larvae and early juveniles of the giant trevally, *Caranx ignobilis* (Pisces: Carangidae). *Fish. Bull.*, 104: 401-414.

Leis, J.M., A.C. Hay, M.M. Lockett, J.-P. Chen and L.-S. Fang. – 2007. Ontogeny of swimming speed in larvae of pelagic-spawning, tropical, marine fishes. *Mar. Ecol. Prog. Ser.*, 349: 255-267.

Leis, J.M., A.C. Hay and G.J. Howarth. – 2009a. Ontogeny of in situ behaviours relevant to dispersal and population connectivity in larvae of coral-reef fishes. *Mar. Ecol. Prog. Ser.*, 379: 163-179.

Leis, J.M., R.F. Piola, A.C. Hay, C. Wen and K.-P. Kan. – 2009b. Ontogeny of behaviour relevant to dispersal and connectivity in the larvae of two non-reef demersal, tropical fish species. *Mar. Fresh. Res.*, 60: 211-223.

Miller, T.J., L.B. Crowder, J.A. Rice and E.A. Marschall. – 1988. Larval size and recruitment mechanisms in fishes: toward a conceptual framework. *Can. J. Fish. Aquat. Sci*., 45: 1657-1688.

Neilson, J.D., R.I. Perry, P.F. Valerio and K.G. Waiwood. – 1986. Condition of Atlantic cod *Gadus morhua* larvae after the transition to exogenous feeding: morphometrics, buoyancy and predator avoidance. *Mar. Ecol. Prog. Ser.*, 32(2-3): 229-235.

Olivar, M.P., M. Diaz and M.A. Chicharo. – 2009. Tissue effect on RNA/DNA ratios of marine fish larvae. *Sci. Mar.*, 73S1: 171-182.

Plaut, I. – 2001. Critical swimming speed: its ecological relevance. *Comp. Biochem. Physiol. A. Comp. Physiol.*, 131: 41-50.

Reidy, S.P., S.R. Kerr and J.A. Nelson. – 2000. Aerobic and anaerobic swimming performance of individual Atlantic cod. *J. Exp. Biol.*, 203: 347-357.

Richard, P., J.-P. Bergeron, M. Boulhic, R. Galois and J. Person-Le Ruyet. – 1991. Effect of starvation on RNA, DNA and protein content of laboratory-reared larvae and juveniles of *Solea solea*. *Mar. Ecol. Prog. Ser.*, 72: 69-77.

Rooker, J.R. and G.J. Holt. – 1996. Application of RNA/DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). *Mar. Fresh. Res.*, 47: 283-290.

Searcy, S. and S. Sponaugle. – 2001. Selective Mortality during the larval-juvenile transition in two coral reef fishes. *Ecology,* 82: 2452-2470.

Shepherd, J.G. and D.H. Cushing. – 1980. A mechanism for density-dependent survival of larval fish as the basis of a stock–recruitment relationship. *J. Cons. Int. Explor. Mer*., 39(Suppl. 2): 160-167.

Skajaa, K. and H.I. Browman. – 2007. The escape response of food-deprived cod larvae (*Gadus morhua* L.). *J. Exp. Mar. Biol. Ecol.*, 353: 135-144.

Sogard, S.M. and B.L. Olla. – 1996. Food deprivation affects vertical distribution and activity of a marine fish in a thermal gradient: potential energy-conserving mechanism. *Mar. Ecol. Prog. Ser.*, 133: 43-55.

Sponaugle, S., K. Grorud-Colvert and D. Pinkard. – 2006. Temperature-mediated variation in early life history traits and recruitment success of the coral reef fish *Thalassoma bifasciatum* in the Florida Keys. *Mar. Ecol. Prog. Ser.*, 308: 1-15.

Stobutzki, I.C. – 1998. Interspecific variation in sustained swimming ability of late pelagic stage reef fish from two families (Pomacentridae and Chaetodontidae). *Coral Reefs*, 17, 111-119.

Stobutzki, I.C. and D.R. Bellwood. – 1994. An analysis of the sustained swimming abilities of pre- and post-settlement coral reef fishes. *J. Exp. Mar. Biol. Ecol.*, 175: 275-286.

Stobutzki, I.C. and D.R. Bellwood. – 1997. Sustained swimming abilities of the late pelagic stages of coral reef fishes. *Mar. Ecol. Prog. Ser.*, 149: 35-41.

Wagner, M.M., E.G. Durbin and L.J. Buckley. – 1998. RNA/DNA ratios as indicators of nutritional condition in the copepod *Calanus finmarchicus*. *Mar. Ecol. Prog. Ser.*, 162: 173-181.

Yin, M.C. and J.H.S. Blaxter. – 1986. Morphological changes during growth and starvation of larval cod (*Gadus morhua* L.) and flounder (*Platichthys flesus* L.). *J. Exp. Mar. Biol. Ecol.*, 104(1-3): 215-228.

Yin, M.C. and J.H.S. Blaxter. – 1987. Escape speeds of marine fish larvae during early development and starvation. *Mar. Biol.*, 96: 459-468.