Ontogeny of swimming behaviour in sardine *Sardina pilchardus* larvae and effect of larval nutritional condition on critical speed

L. Silva[1], A. M. Faria[2], M. A. Teodósio[3], S. Garrido[4],*

[1]University of Algarve, Campus de Gambelas, 8000 Faro, Portugal

[2]Eco-Ethology Research Unit, Instituto Superior de Psicologia Aplicada, R. Jardim do Tabaco 34, 1149-041 Lisbon, Portugal

[3]CCMAR, University of Algarve, Campus de Gambelas, 8000 Faro, Portugal

[4]Centro de Oceanografia, Laboratório Marítimo da Guia, Faculdade de Ciências, Universidade de Lisboa, Avenida Nossa Senhora do Cabo, n°939, 2750-374 Cascais, Portugal

ABSTRACT: The ontogeny of swimming behaviour in sardine *Sardina pilchardus* larvae was studied, from hatching to 75 days post-hatch (dph), by measuring the critical swimming speed (U_{crit}) and observing locomotory behaviour. In addition, the effect of larval nutritional condition on U_{crit} at the onset of their swimming abilities (20 to 25 dph) was evaluated by rearing larvae under 4 different feeding treatments. Diets consisted of different concentrations of dinoflagellates, rotifers and the copepod *Acartia grani,* and a wild plankton assemblage. Recently hatched larvae were mostly inactive, but from 2 dph onwards larvae started to swim freely in the rearing tank, and time spent swimming increased throughout ontogeny. Larvae younger than 20 dph (i.e. <7.90 mm TL) could not swim for the entire adjustment period at the minimum current speed, but thereafter U_{crit} increased significantly with larval age and length, reaching a maximum of 9.47 cm s^{-1} at 19.10 mm TL and 55 dph. Growth, survival and the nutritional condition of sardine larvae, assessed by the RNA residual index, were significantly higher for larvae reared with the high-concentration diet, contrary to the other derived nucleic acids indices (RNA/DNA and DNA/DW), which showed no differences between diets. Despite differences in the survival and growth rates of sardine larvae, U_{crit} at the onset of swimming did not differ significantly among diets, but was significantly related to larval nutritional condition as assessed by the RNA residual index. Overall, our results show that early larval stages of sardines have poor swimming ability and probably rely on food patches in the wild to survive; however, close to metamorphosis (especially from 45 dph onwards), larvae spend most of the time swimming and are capable of resisting the mean current speeds of their natural environment, which may strongly enhance chances for survival.

KEY WORDS: Ontogeny · Critical swimming speed · U_{crit} · *Sardina pilchardus* · Nucleic acid derived indices · RNA/DNA · Foraging

INTRODUCTION

Small pelagic fish species are not only important in the world's fisheries, but also in the ecological processes of marine systems, as they play a significant role in connecting lower and upper trophic levels (Cury et al. 2000). Fisheries management is often confounded by high variability in population size that occurs annually for small pelagic fish stocks. This variability is proposed to be related to the high mortality rates during the early life stages, which ultimately influence recruitment (Blaxter & Hunter 1982, Houde 1987). Starvation and predation are recognized as the 2 main agents of mortality during the

larval stage (Bailey & Houde 1989). These factors are not independent, and it has been shown for a variety of fish species (i.e. herring, hake, cod, flounder and anchovy) that starved larvae are more vulnerable to predation than fed larvae (Bailey 1984, Neilson et al. 1986, Booman et al. 1991, Takasuka et al. 2003).

While several studies indicate that high nutritional condition of fish larvae can be correlated with high growth rates and enhanced recruitment or juvenile survival under natural conditions (Searcy & Sponaugle 2001, Bergenius et al. 2002, McCormick & Hoey 2004, Sponaugle et al. 2006), few attempts have been made to correlate larval condition with behavioural functions critical for larval survival, such as swimming behaviour (Laurence 1972, Chick & Van Den Avyle 2000). Swimming plays an important role in larval survival, with the potential to influence an individual's ability to capture prey (Peck & Hufnagl 2012), avoid predators (Reidy et al. 2000), control dispersal patterns (Stobutzki & Bellwood 1997) and, ultimately, the levels of self-recruitment in some marine populations (Sponaugle et al. 2002). Although larvae can only be considered 'effective swimmers' when swimming faster than mean ambient current speed (Leis & Stobutzki 1999), horizontal swimming speeds lower than 'effective speeds' can strongly influence dispersal trajectories if swimming direction is normal to the current direction (Leis 2006).

Larvae in good condition will most likely have greater swimming abilities and responsiveness to predation than larvae in poor condition (Chick & Van Den Avyle 2000, Grorud-Colvert & Sponaugle 2006). Larval condition may be estimated by a variety of morphometric, biochemical, histological or otolith growth indices. The RNA/DNA ratio is one of the most commonly used biochemical indices in the study of nutritional and larval growth (Chícharo 1998, Buckley et al. 2004, 2008, Chícharo & Chícharo 2008). It is based on the assumption that the DNA concentration remains constant in a somatic cell even during starvation and the amount of RNA varies in proportion to protein synthesis (Chícharo 1998), meaning that nutritional condition increases with increases in RNA/DNA. Other nucleic acid-derived indices have been proposed, such as the DNA/dry weight index (DNA/DW); which is also sensitive to nutritional status since cell weight decreases while DNA concentration maintains constant during a reduction in nutritional condition (Bergeron 1997). The DNA/DW index increases when condition decreases, since more cells are present for the same weight of tissue of starved larvae. Several studies advocate caution in the use of these last 2 indices due to their

dependency on larval size, and advise the removal of this allometric effect by instead using a residual-based index from RNA content and an independently determined variable such as standard length or dry weight (RNA residual index) (Suthers et al. 1996, Chícharo et al. 1998).

European sardine *Sardina pilchardus* (Walbaum, 1792) represents one of the most important fisheries in the NE Atlantic. Peak spawning occurs from November through April in the coastal Iberian upwelling ecosystem (Figueiredo & Santos 1989, Ré et al. 1990, Santos et al. 2004). Larvae of this species are exposed to favourable feeding conditions owing to upwelling and high primary production, but are also exposed to offshore transport, which can result in high mortality rates (Bailey 1981, Parrish et al. 1981, Mann & Lazier 1991, Santos et al. 2007). One possible mechanism of avoiding dispersal is to perform vertical migrations. Santos et al. (2006) showed that the highest concentration of sardine larvae (4 to 22 mm total length, TL) off western Iberia occur between 10 and 25 m depth during the day, whereas during the night larvae tend to occupy the upper layers (5 to 10 m), due to their ability to perform vertical migrations. These authors suggest that the relationship between sardine larval survival and oceanographic processes (conditions for transport and dispersion) is the major factor impacting the recruitment variability of this species. However, little is known about the horizontal swimming capacity of sardine larvae, how these abilities change throughout ontogeny and how they may contribute to retention in favourable areas. Critical swimming speed (U_{crit}) has been widely used to study the horizontal swimming speed of larvae in relation to a variety of ecological, biological, and environmental factors (Green & Fisher 2004), and it is also important to recognize the potential effect of swimming behaviour on larval dispersal patterns (Stobutzki & Bellwood 1994, Plaut 2001, Leis et al. 2009a,b).

The aims of this study are (1) to describe the ontogeny of the swimming abilities (measured by U_{crit}) and locomotory behaviour of sardine *S. pilchardus* larvae from hatch to 75 days post hatch (dph) in optimal temperature (15°C), salinity (35) and food density conditions, and relate the variation in swimming abilities throughout ontogeny with the morphological development of the larvae; and (2) to ascertain if a variation in larval nutritional condition (as a consequence of larval growth with different diet types and concentrations) affects the onset of the critical swimming abilities of larvae.

MATERIALS AND METHODS

Sardine spawning and experimental design

Sardine eggs were obtained from adult fish captured by purse-seine fishery in June 2009 and July 2010 off the western Iberian Peninsula, and maintained in captivity at the Lisbon Aquarium, Oceanário de Lisboa, in a large cylindrical tank (15 000 l). Fish spawning was naturally induced by increasing food concentration and manipulating photoperiod and temperature. Each morning, egg collectors placed in the overflows of the adult fish tank were inspected and, when present, eggs were collected and counted. When the number of eggs exceeded 1000, they were transferred to rearing tanks.

To address the first objective of this study (i.e. to describe the ontogeny of the swimming abilities of sardine larvae in optimal environmental conditions), larvae were reared under optimal conditions of temperature (15°C), salinity (35), which correspond to natural environmental conditions present when sardine spawning peaks off western Iberia (Bernal et al. 2007), and food density (corresponding to Diet C, described below). Larval growth and morphological development, locomotory behaviour and U_{crit} were determined from hatch until the age corresponding to maximum survival in the rearing tanks (see protocols described below).

To address the second objective of this study (i.e. to investigate the effect of larval nutritional condition on the onset of larval swimming abilities), larvae were reared under 4 different diet types and concentrations in optimal temperature (15°C) and salinity (35) conditions. Their growth rate, nucleic-acid derived indices of nutritional condition and U_{crit} were determined and compared between feeding treatments, restricting this comparison to the common ages when all treatments had live larvae.

Feeding treatments simulated low (Diet A), medium (Diet B), and high prey concentrations (Diet C), and one treatment (Diet D) used wild plankton as prey (Tables 1 & 2). Prey were introduced into the rearing tanks, and included preferred prey items and sizes for this species in natural conditions (Morote et al. 2010). The dinoflagellate *Gymnodinium* sp., the rotifer *Brachionus plicatilis* and the calanoid copepod *Acartia grani* were reared and maintained at the Lisbon Aquarium (Oceanário de Lisboa). Wild plankton

was collected in estuarine waters near the Lisbon Aquarium using a WP2 net with a mesh size of 55 µm. The microalgae *Nannochloropsis* spp. was added daily to all rearing tanks (green water technique) to feed rotifers and copepods and increase contrast inside the tanks. In order to keep microalgae in suspension, tanks were supplied with gentle air bubbling, to avoid damage to the larvae (Scura & Jerde 1977). Each morning, after checking for the food concentration in the tanks, 20 to 30 % of the tank water was renewed and new food added to obtain the pre-established concentrations of each treatment. Stock cultures of copepods were fed daily with *Rhodomonas* spp. while rotifers were fed daily with an excess of *Nannochloropsis* spp. Experiments were conducted in cylindrical tanks of 30 l, except for Diet D, which was conducted in a 50 l cylindrical tank. A larger tank volume was used in this case to maintain the water quality similar to the other experiments,

Table 1. Prey types and concentrations in the 4 different feeding treatments (Diets A, B, C, D, corresponding to low, medium and high concentration, and wild diet, respectively) used to rear *Sardina pilchardus* larvae. Larval age (dph) refers to the day(s) when each prey type was introduced into the experimental tanks. Composition of wild plankton (Diet D) is described in Table 2

| Prey item | Concentration (no. prey ml⁻¹) | | | | Larval age |
	Diet A	Diet B	Diet C	Diet D	(dph)
Gymnodinium sp.	1500	1500	1500	1500	0
Rotifers	5	5	25	0	3
Wild plankton (±SE)	0	0	0	1.32 ± 0.65	≥0, daily
Acartia grani (nauplii)	1	2	5	0	≥4, daily
Acartia grani (copepodites)	1	2	2	0	≥20, daily
Acartia grani (adults)	1	2	2	0	≥30, daily

Table 2. Prey types and concentrations (±SE) of Diet D, based on wild plankton

Prey type	No. prey ml⁻¹
Copepod eggs	0.03 ± 0.00
Copepod nauplii	0.93 ± 0.49
Calanoida copepodits	0.1 ± 0.08
Cyclopoida copepodits	0.03 ± 0.03
Harpacticoida copepodits	0.02 ± 0.02
Unidentified copepods	0.01 ± 0.02
Cirripedia nauplii	0.01 ± 0.02
Evadne spp.	0.04 ± 0.05
Tintinnids	0.06 ± 0.03
Gastropoda	0.01 ± 0.01
Apendicularia	0.03 ± 0.03
Bivalvia veliger	0.01 ± 0.02
Crustacean eggs	0.01 ± 0.02
Unidentified crustaceans	0.01 ± 0.02
Total prey	1.32 ± 0.65

since natural food could increase the contamination of the tanks.

In order to determine growth rate, swimming ability, and morphometric and nutritional condition of the larvae, samples were collected throughout the ontogeny for each replicate of the 4 feeding treatments (Table 3). Each sampling day, 2 to 6 larvae were tested for their swimming ability (described below), after which larvae were immediately measured (TL, mm) and preserved in liquid nitrogen for posterior determination of the nutritional condition. Additional larvae from all feeding treatments (3 to 5 ind.) were sacrificed each sampling day and preserved in formaldehyde to study morphological development throughout ontogeny (described below).

Growth rates and morphological development

TL was measured *in vivo* for larvae reared on each of the 4 diet treatments, and growth rates for each experimental tank were fitted to exponential growth curves using the equation:

$$TL = l_0\, e^{kt} \tag{1}$$

where l_0 is length at hatching, k is the instantaneous growth rate and t is age (dph). Maximum survival rates were determined as the day the last larva was alive.

Photographs of sardine larvae preserved in formaldehyde were taken under a stereoscope (Leica S8 APO, zoom 8:1) coupled with a digital camera (Canon EOS SLR550), and morphometric measurements were taken using Image J software (version 1.46). Morphometric measurements encompassed the development of important characteristics of fish larvae, such as diameter and yolk-sac area, area of oil globule, eye diameter, height behind the pectoral fin, and body height above anus. Other morphological changes throughout ontogeny were also noted, such as fin formation and notochord flexion.

Nutritional condition

Nutritional condition was assessed by the following nucleic acid-derived indices: sRD (standardized RNA/DNA ratios), DNA/DW and RNA residual indices. Nucleic acid analysis was carried out for sardine larvae grown at the 4 feeding treatments. The procedures used to quantify nucleic acids in individual fish larvae are outlined in Esteves et al. (2000) and Caldarone et al. (2001). Due to tissue effects on RNA and DNA concentration, whole larvae were analysed (Olivar et al. 2009). The protocol involves mechanical and chemical homogenization of tissues and subsequent fluorescence-photometric measurements using ethidium bromide (EB) as a specific nucleic acid fluorochrome dye. Fluorescence was measured on a microplate reader (Biotek synergy HT model SIAFRTD) using an excitation wavelength of 365 nm and an emission wavelength of 590 nm. Endogenous fluorescence (before EB addition) from the first set of samples for each tissue was measured and found to be negligible, so it was not considered in the calculations of nucleic acid concentrations. Total fluorescence was first read, and then samples were incubated with ribonuclease A (Type-II A) at 37°C for 30 min, and then cooled to room temperature before reading. The fluorescence due to total RNA, mainly ribosomal, was calculated as the difference between total fluorescence (RNA and DNA) and the fluorescence measured after ribonuclease treatment, which is assumed to be due to DNA. Concentrations were determined by running standard curves of DNA-EB and RNA-EB every day with known concentrations of λ-phagus DNA (0.25 µg µl^{-1}) and 16S–23S *E. coli* RNA (4 µg µl^{-1}) (Roche), in the appropriate range of values. The average ratio of DNA and RNA slopes (mean ± SE) was 2.84 ± 0.20. The RNA/DNA ratios were standardized (sRD) using this information and the reference slope ratio of 2.4, according to Caldarone et al. (2006). The residual based index from RNA content and an independently determined variable (in this case TL), was used to remove

Table 3. Sampling procedure for *Sardina pilchardus* larvae grown under 4 different feeding treatments with medium egg concentration; number of experimental days; and sampling intervals for swimming experiments, locomotory behaviour observations, length and morphology measures

	Diet A	Diet B	Diet C	Diet D
Replicates	4	2	2	2
Egg concentration (no. ml^{-1})	0.07	0.09	0.06	0.1
End experiment (d)	30	50	75	25
Swimming	Every 5 d since hatch			
Locomotory behaviour	–	–	Every 2 d	–
Length	Every 3 d (0–10 dph), every 5 d after 10 dph			
Morphology	Every 3 d (0–10 dph), every 5 d after 10 dph			

the allometric size effect with an overall, simple regression of ln(RNA + l) on ln(TL) (Suthers et al. 1996, Chícharo et al. 1998).

Locomotory behavioural observations

Larval behavioural observations were conducted for larvae reared on the high concentration diet (Diet C). The focal animal technique (Martin & Bateson 1993) was used to observe a randomly selected larva for a 1 min interval. From 0 to 58 dph, 5 larvae were observed; from 60 to 66 dph, 3 larvae; and from 68 to 75 dph, 2 larvae (Table 3). Observations were conducted solely on larvae of high food concentration that had similar growth rates to that of wild larvae. Behaviours were categorized into modal action patterns (MAPs), adapted from Barlow (1968) (Table 4). During observations, aeration was stopped in order to follow the larva and to avoid the influence of turbulence on larval behaviour. Six MAPs, grouped into 2 classes (locomotory and foraging), were recorded. Foraging behaviours were recorded as frequency of occurrence, whereas locomotory behaviours were recorded as time variables. Successful attacks were recorded when a fixation lead to a lunge.

Critical swimming speed

A swimming chamber, following the design of Stobutzki & Bellwood (1994), was used to measure U_{crit} of sardine larvae. The chamber was made of clear Perspex containing 6 parallel swimming lanes,

Table 4. Definition of modal action patterns (MAPs; after Barlow 1968) recorded for sardine *Sardina pilchardus* larvae fed the highest concentration diet throughout ontogeny

MAP	Definition
Locomotory	
Swimming	Forward movement of the larva through the water column
Pause	Larva is motionless and stationary in the water column
Vertical position	Larva is in pause and in vertical position in the water column
Foraging	
Orientation	The head is directed towards a prey item
Fixate	The larva is stationary and bends its body into an 'S' shape position
Lunge	The larva moves towards the prey item

each 30 mm wide, 50 mm high and 180 mm long. A removable lid allowed placing and removing larvae from the lanes. A mesh was placed at both ends of each lane in order to retain larvae in the chamber. Flow straighteners (40 mm long) at the upstream and downstream of each lane minimized turbulence. Previous studies have shown that water velocity is not significantly different between the centre of the lane and in the 5 mm closest to the wall (Stobutzki & Bellwood 1997, Stobutzki 1998, Fisher et al. 2000). A submersible pump moved water from a 50 l tank to the swimming chamber in a closed-flow system. A valve at the upstream end of the swimming chamber controlled water velocity. A protractor mounted on the valve handle calibrated flow rates in swimming lanes based on handle angle. For the different angles, the time taken for the outlet water to fill a 5 l container was recorded and divided by the cross-sectional area and number of lanes. The procedure was repeated 3 times for a specific valve angle, and the average from the 3 trials was considered the flow velocity.

In each sampling day, 2 to 6 larvae were tested per treatment. Larvae were carefully removed from the rearing tanks and transferred to the swimming chamber. Due to their small size, larvae were tested one at a time to maintain accurate measurements for each individual. Larvae were maintained for 5 min at a flow speed of 1.5 cm s^{-1}; individuals that displayed symptoms of stress after this adjustment period were removed and replaced. Water temperature (15°C) and salinity (35) matched the conditions in the rearing tank. After the adjustment period, water velocity was increased by ~1.2 cm s^{-1} every 2 min until the larva was unable to swim against the flow for 2 min. U_{crit} was calculated following Brett (1964):

$$U_{crit} = U + (t / t_i \times U_i) \tag{2}$$

where U is the penultimate speed a fish was able to maintain; U_i is the velocity increment (1.2 cm s^{-1}); t is the time swum in the final velocity increment; and t_i is the time interval for each velocity increment (2 min).

The Reynolds number (Re) (Webb & Weihs 1986), a common measurement tool to characterize different hydrodynamic conditions, was calculated to determine whether larvae were swimming in viscous (Re < 300) or inertial (Re > 1000) conditions (Leis 2006), using the formula:

$$Re = U_{crit} \times TL / v \tag{3}$$

where v is the kinematic viscosity of seawater (viscosity of seawater at 15°C = 1.20×10^{-6} m^2 s^{-1}).

Data analysis

ANCOVA was used to assess if the regression between size and age were comparable between larvae reared on different diets. For this ANCOVA model, the logarithm of larval TL depending on larval age (continuous variable) and diet (factorial variable design with 4 factors corresponding to the 4 diets) was performed. As larval maximum survival differed between diets, this analysis was performed only for the period common to all diets (< 26 dph). ANCOVA was also used to assess if the regression between U_{crit} and size (or age) were comparable between larvae reared on different diets.

The increase of larval fixations on prey with larval age was adapted to Ivlev's equation (Ivlev 1961):

$$F = F_{\max} \times (1 - e^{-a \times age}) \qquad (4)$$

where $F_{\max}$ is the maximum fixation rate, a is a constant describing the rate at which fixation approaches the maximum rate, and age is larval age in dph. The same equation was fitted to the increase in lunges with age:

$$L = L_{\max} \times (1 - e^{-b \times age}) \qquad (5)$$

where $L_{\max}$ is the maximum lunge rate and b is a constant describing the rate at which lunges approach the maximum rate. In both cases, fitting was done on non-transformed data using an iterative non-linear regression routine.

An index of condition was derived from the residuals of an overall, simple regression of ln(RNA + l) on ln(TL), according to Suthers et al. (1996). The effect of diet on the nutritional condition was also tested using a linear model. Finally, a linear model was used to investigate the effect of nutritional status of the larvae on U_{crit}, depending on larval nutritional condition. These tests were also restricted to the period common to all diets (< 26 dph).

The 'R' software (version 2.15.1) was used for all statistical analyses and graphics.

RESULTS

Growth rate and morphological development

The timing of morphological development of sardine larvae was similar between larvae reared on the 4 different diets. Approximately 72 h after spawning, sardine larvae hatched with a mean (±SE) length of 4.05 ± 0.37 mm, a yolk sac of 0.49 ± 0.06 mm² and no pigmentation. During ontogeny, eye diameter in-

creased by 7.13 μm d⁻¹, to a maximum of 737.50 μm at 70 dph. Height behind the pectoral fin and body height above anus also increased with age, at a rate of 15.10 and 13.09 μm d⁻¹, respectively. Eye pigmentation started at 3 dph, coincident with the mouth opening. At 10 dph, the pectoral fins were observed and the caudal fin begun to form. The beginning of notochord flexion was observed at 1 dph, and its complete flexion at 25 dph. At 20 dph larval caudal fin began to form and was complete at 40 dph. The anal fin was observed for the first time at 35 dph (Table 5).

Maximum survival varied significantly for larvae reared under the 4 different feeding treatments, ranging from 25 d for larvae reared with Diet D to 75 d for larvae reared under the high concentration diet (Diet C). The maximum survival of larvae reared on Diets B and A was 50 and 30 d, respectively. Larval growth rates varied according to the feeding treatment, ranging from 0.49 mm d⁻¹ (Diet B) to 0.24 mm d⁻¹ (Diet D) (Fig. 1, Table 1). The comparison of larval growth rates between different diets for the common ages (the first 25 dph) showed that there were significant differences of size according to age and diet type (ANCOVA, $F = 467.3$, $p < 0.001$, $n = 332$). In particular, during this period, larval growth rates were not statistically different between Diets C and B concentration diets (growth rates of 0.35 and 0.38 mm d⁻¹, respectively), but were significantly higher than growth rates observed for Diet A (0.27 mm d⁻¹) and for Diet D (0.24 mm d⁻¹), which contained a low concentration of different prey types (Table 2).

Table 5. Timing of the main morphological events occurring during sardine *Sardina pilchardus* larvae development

| Age (dph)	Total length (mean ± SE, mm)	Morphological characteristic
0	4.05 ± 0.37	Hatching day, no pigmentation, yolk sac
3	5.40 ± 0.17	Eye pigmentation, yolk sac, mouth open
10	6.00 ± 0.44	Pectoral fins and beginning of caudal fin formation
15	7.53 ± 1.22	Beginning of notochord flexion
20	10.28 ± 1.34	Beginning of dorsal fin development
25	12.00 ± 2.01	Notochord flexion complete
30	13.47 ± 1.55	Dorsal fin complete
35	14.43 ± 3.14	Beginning of anal fin development
40	16.00 ± 3.22	Caudal fin complete
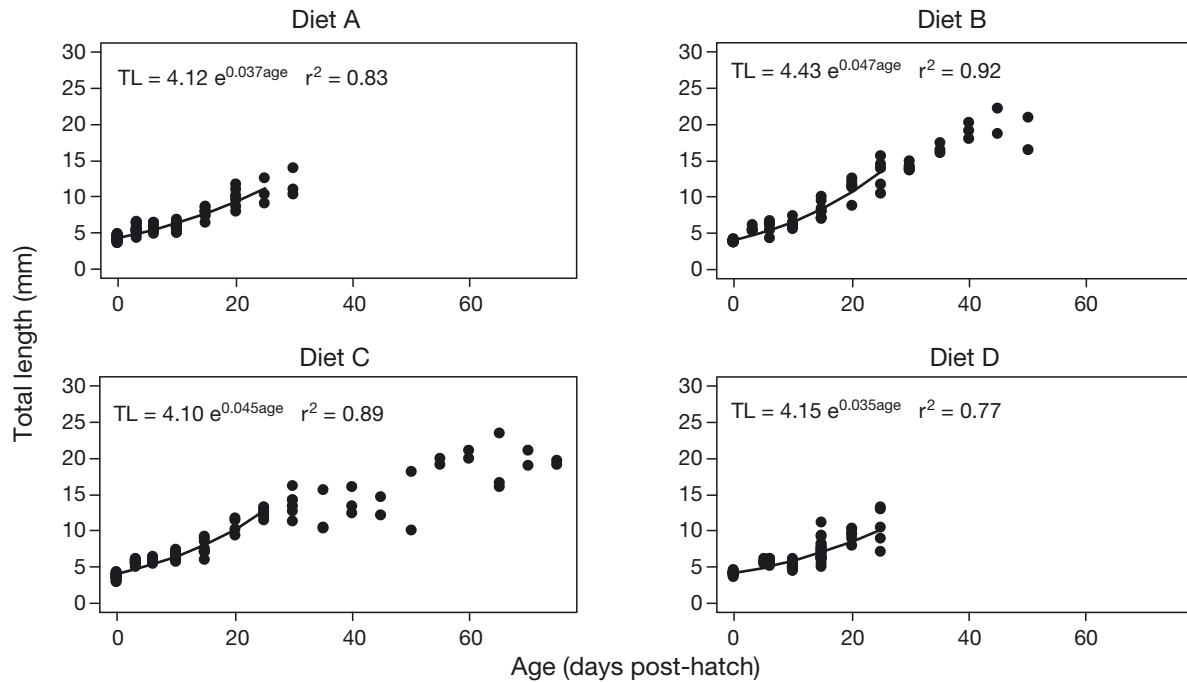
Fig. 1. Larval length vs. age for the entire experimental duration for sardine *Sardina pilchardus* larvae reared under 4 different diets. Equations were fitted for the common ages (0 to 25 dph)

Nutritional condition

For the first 25 dph, regression models testing the dependence of larval nutritional condition (assessed by the sRD, DNA/DW and the RNA residual index) on larval length and diet type showed that size did not influence sRD (ANCOVA, $F = 1.65$, $p = 0.41$, $n = 59$) and the RNA residual index (ANCOVA, $F = 5.84$, $p = 0.60$, $n = 59$), whereas the DNA/DW significantly decreased with larval age (ANCOVA, $F = 13.44$, $p < 0.005$, $n = 59$). The nucleic acid content of sardine larvae grown with Diet D was lower in terms of µg RNA mg^{-1} DW and higher in terms of µg DNA mg^{-1} DW than larvae grown under the other diet regimes (Table 6). The RNA residual index was higher for larvae reared on Diet C when compared to larvae reared under the other feeding regimes (ANCOVA, $F = 7.67$, $p < 0.005$, $n = 59$). Larval condition was not significantly different between diets when estimated using the sRD (ANCOVA, $F = 2.06$, $p = 0.11$, $n = 59$) and the DNA/DW (ANCOVA, $F = 0.61$, $p = 0.6$, $n = 59$) indices.

Locomotory behavioural observations

Locomotory behavioural observations were performed for larvae reared on Diet C. During the first 2 dph, sardine larvae spent most of their time inactive, initially at the surface (0 dph), and then close to the bottom of the tank (1 dph). After this period, larvae started to swim up and down in the water column.

Table 6. Mean (±SE) concentrations and ratios of nucleic acids of sardine *Sardina pilchardus* larvae reared under different diets across all ages sampled. Number of larvae (n), age groups (days post hatch, dph), length (total length, TL), mean nucleic acid content of analyzed larvae (RNA/DW), DNA/dry weight index (DNA/DW), standardized RNA/DNA (sRD) and residuals for RNA (resDNA) are shown separately for the 4 diets

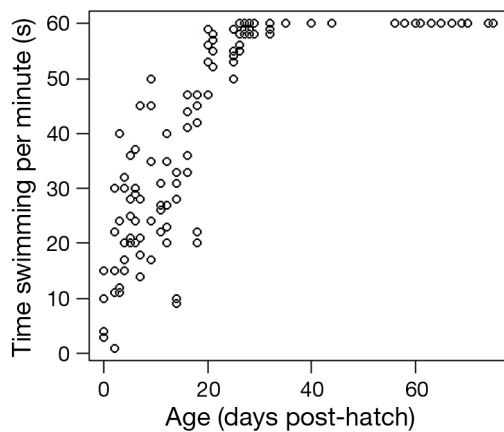| Diet	n	Age (dph)	TL (mm)	RNA/DW	DNA/DW	sRD	resRNA
A	17	0–30	10.49 ± 1.63	8.59 ± 17.56	15.68 ± 18.49	0.39 ± 0.31	−0.18 ± 0.29
B	33	0–50	14.94 ± 3.31	4.98 ± 7.28	10.12 ± 12.14	0.42 ± 0.30	0.10 ± 0.50
C	34	0–75	14.74 ± 3.72	9.51 ± 18.83	7.17 ± 8.80	0.38 ± 0.24	0.19 ± 0.51
D	12	0–25	9.96 ± 1.85	3.12 ± 6.42	17.88 ± 29.05	0.22 ± 0.12	−0.23 ± 0.35

Fig. 2. Time spent swimming by sardine *Sardina pilchardus* larvae during a 60 s period of observation throughout larval ontogeny for larvae reared with high prey concentrations (Diet C)

Fig. 4. Critical swimming speed (U_{crit}) of sardine *Sardina pilchardus* larvae throughout ontogeny for larvae from all feeding treatments. Each symbol represents the U_{crit} for an individual larva

The first 15 dph were characterized by pause–travel behaviour, with larvae spending most of the time (60%) pausing (Fig. 2). Time spent swimming increased with larval age, and from 26 dph onwards larvae shifted to constant locomotory behaviour and spent all the observation time swimming. Foraging behaviour was first noticed when larvae started exogenous feeding at 4 to 5 dph; larval fixation and attacks (lunges) increased with age, with an increase of 0.25 fixations d^{-1} and 0.08 lunges d^{-1} (Fig. 3). The sequence of orientation, fixation and attack on prey that characterizes foraging behaviour was not always successful, given that not all fixations resulted in an attack on the prey (Fig. 3). Lunges occurred at a rate
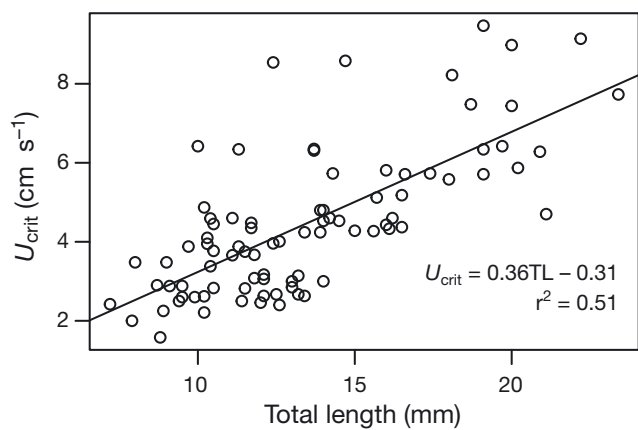
of less than 2 attacks min^{-1} at 25 dph, with a percentage of complete feeding sequences of 25% for this age. At 60 dph, lunges increased to approximately 5 attacks min^{-1}, with 74% being successful, complete sequences (Fig. 3).

Critical swimming speed

A total of 372 larvae were tested to evaluate U_{crit}; 94 from Diet C, 68 from Diet B, 120 from Diet A and 90 from Diet D. Larvae <20 dph could not swim at the minimum current speed for the entire adjustment period and exhibited bursting and gliding, unable to counteract the flow. These were excluded from subsequent analyses. U_{crit} were determined for 86 larvae that swum at the lowest tested current speed. U_{crit} values ranged from 1.58 to 9.47 cm s^{-1}, over a range of lengths from 7.90 (20 dph) to 23.40 (55 dph) mm TL, corresponding to 1.80 to 6.89 body lengths s^{-1} (BL s^{-1}) (Fig. 4, Table 7). The maximum U_{crit} recorded (9.47 cm s^{-1}) was from a larva 55 dph with 19.10 mm TL and reared on Diet C. U_{crit} increased with larval length for every diet, although the variability in swimming ability at a given larval age/length was high. The rate of increase in swimming speed for each 1 mm increase in TL ranged from 0.38 to 0.49 cm s^{-1}; with TL explain-
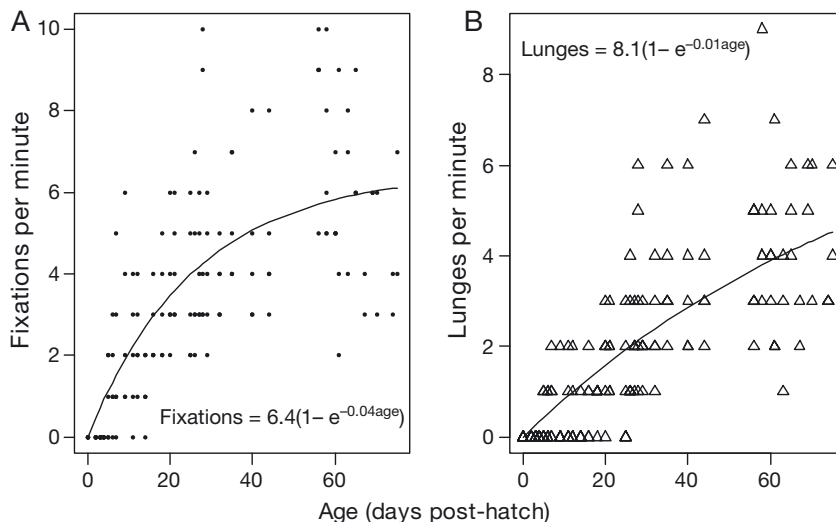

Fig. 3. Frequency of (A) fixations and (B) lunges on prey items by sardine *Sardina pilchardus* larvae throughout ontogeny for larvae reared on high prey concentrations (Diet C)

Table 7. Results of regression analyses on the effects of sardine *Sardina pilchardus* larvae length (total length, TL) and age (dph) on critical swimming speed (U_{crit}). Range and means ($\pm$SE) of U_{crit} and relative swimming speed (body lengths per second, BL s^{-1}) as well as maximum survival are also shown. p < 0.001 for all regressions

| Diet	Variables	Linear equation	R^2	U_{crit} (cm s^{-1}) Range	Mean ± SE	Relative swimming speed (BL s^{-1}) Range	Mean ± SE	Survival (dph)
A	U_{crit} vs. TL	$0.45x - 2.26$	0.70	2.00–4.80	3.08 ± 0.87	2.53 – 4.13	3.18 ± 0.56	30
	U_{crit} vs. age	$0.11x - 0.38$	0.64					
B	U_{crit} vs. TL	$0.47x - 2.60$	0.88	1.58–9.14	4.50 ± 1.60	1.80 – 4.64	3.08 ± 0.70	50
	U_{crit} vs. age	$0.17x - 0.83$	0.83					
C	U_{crit} vs. TL	$0.49x - 2.42$	0.80	2.21–9.47	4.60 ± 2.16	1.90 – 6.89	3.26 ± 1.29	75
	U_{crit} vs. age	$0.12x - 0.40$	0.78					
D	U_{crit} vs. TL	$0.38x - 1.93$	0.47	2.25–4.87	3.38 ± 0.76	2.02 – 4.77	3.36 ± 0.87	25
	U_{crit} vs. age	$0.10x - 0.55$	0.39					
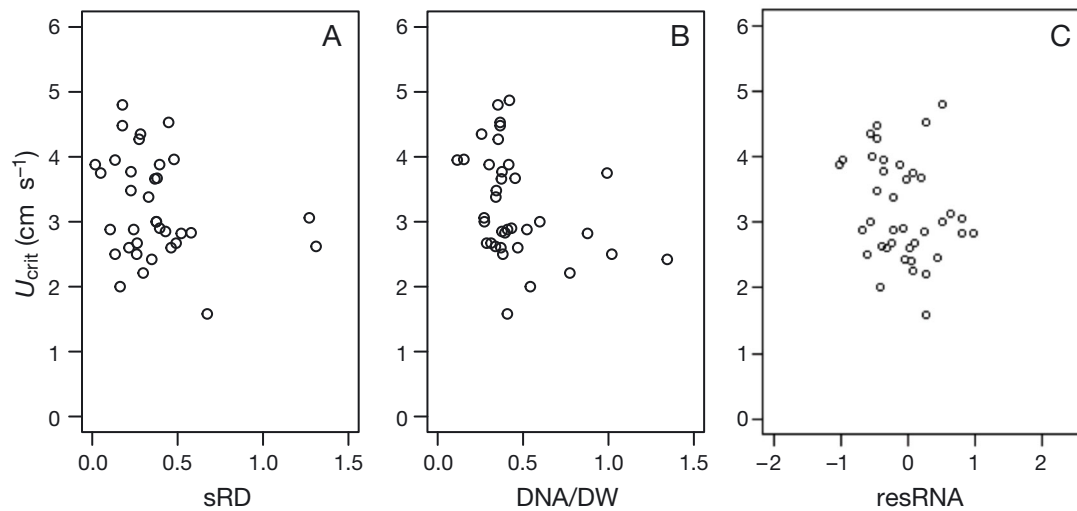
Fig. 5. Relationship between critical swimming speed (U_{crit}) and nutritional condition of sardine *Sardina pilchardus* larvae at 20 to 25 days post-hatch (dph), assessed by the (A) standardized index RNA/DNA (sRD), (B) DNA/DW index and (C) RNA residual index (resRNA). An increase in RNA/DNA and RNA residual index corresponds to better nutritional condition, while a decrease in DNA/DW corresponds to better nutritional condition

ing 79% of the variation in larval swimming speed. Length was also a better predictor of U_{crit} than age (Table 7). Larval swimming speeds were also investigated at this age range as a function of length and diet. Larval length at these ages influenced U_{crit} (ANCOVA, $F = 8.54$, p = 0.005, n = 40) but no significant differences were found between diets (ANCOVA, $F = 1.59$, p = 1.59, n = 40). On the other hand, the regression models testing the dependence of U_{crit} on larva length and the 3 indices of larval nutritional condition showed that U_{crit} was related to larval condition when assessed using the RNA residual index (ANCOVA, $F = 5.79$, p = 0.02, n = 38), but not the sRD (ANCOVA, $F = 5.36$, p = 0.06, n = 34) or the DNA/DW (ANCOVA, $F = 0.59$, p = 0.44, n = 35) (Fig. 5).

Re of sardine larvae in these experiments ranged from 20.89 to 746.81. Most larvae (81%) had a Re < 300, and would therefore be swimming in a largely viscous hydrodynamic environment when swimming at their U_{crit}. Only larvae larger than 17.31 ± 4.09 mm TL had a Re > 300, and would be swimming in an environment where viscous and inertial forces dominate. The smallest larva that was able to swim in this intermediate environment was 11.30 mm TL. No larvae swum in an inertial environment.

DISCUSSION

The early development of swimming abilities in fish larvae is critical for survival, dispersion and transport

(Leis 2006). Data on swimming performance of cold temperate fish larvae, particularly small pelagic species, are limited. Here we describe, for the first time, the ontogeny of the swimming abilities of sardine larvae.

Swimming performance improved significantly with larval age and length, similar to other species (e.g. Clark et al. 2005, Leis et al. 2007, 2009a,b, 2012, Guan et al. 2008, Faria et al. 2009, 2011), reaching a maximum of 9.47 cm s^{-1} at 19.10 mm TL and 55 dph. However, variability in the swimming ability of sardine larvae of the same age was high. Accordingly, length was a better predictor of swimming performance than age, which can be explained by the wide range of larval lengths observed at a given age. This is probably a consequence of slow growers being able to survive under laboratory conditions in the absence of predators, resulting in a high inter-individual variability of sardine larvae growth within each feeding treatment. The overlap between slow growers reared in better conditions and fast growers reared in poorer conditions makes it difficult to obtain significant differences between treatments. This can also explain why, at the onset of larval swimming abilities (20 to 25 dph), U_{crit} was only dependent on larval length and was not significantly different between larvae reared on different diets.

Sardine larvae began to resist currents of 1.5 cm s^{-1} at 20 dph (8.33 ± 0.97 mm TL), coinciding with the beginning of notochord flexion and caudal fin formation. At this age, larvae have already developed a swim bladder (Santos et al. 2007), which is necessary for buoying control and consequently for vertical migration behaviour (Somarakis & Nikolioudakis 2010). At approximately the same time, an increase in body height above anus and height behind the pectoral fin was also observed, coincident with the development of fins, body musculature and increased ossification of the skeleton. These ontogenetic changes result in gradually improved swimming performances as the larvae develop.

Most studies on the critical swimming speed of fish larvae have been conducted on perciform tropical species, and more recently on temperate species. Compared to other tropical species (1.80 to 64 cm s^{-1}; Fisher et al. 2000, Leis et al. 2007, 2009a,b) and even temperate species (1 to 27.30 cm s^{-1}; Clark et al. 2005, Faria et al. 2009, 2011), sardine larvae swimming abilities are lower, although maximum speed overlaps at the lower ends of the range described for other species. In terms of relative swimming speed (BL s^{-1}), sardine larvae have lower values (1.80 to 6.89 BL s^{-1}) than those reported for perciform tropical

reef fish (0.30 to 31.10 BL s^{-1}; Leis et al. 2007, 2009a,b) and some temperate species (2 to 20 BL s^{-1}; Clark et al. 2005, Faria et al. 2009, 2011). However, relative speeds are similar to values reported for non-perciform cold-water species (Gadiforms and Scorpaeniforms; Guan et al. 2008).

Taxonomy is an obvious factor that needs to be considered when making comparisons of swimming performances (Leis 2010), due to highly different body morphologies and muscle mass among taxa (Fisher & Hogan 2007). In addition to morphology, temperature also affects swimming performance, since higher temperatures allow more efficient functioning of fish muscle cells (Hunt von Herbing 2002). Moreover, temperature is also linked to viscosity effects on fish larval motion (Fuiman & Batty 1997, Hunt von Herbing 2002). The viscosity of water increases with decreasing temperature, which means that larvae require either higher speed or greater size to reach a given Re (Leis 2006). Larvae swim best in inertial environments (Re > 1000) than in a viscous ambient (Re < 300) (Leis 2006, Leis et al. 2012). In this study, most sardine larvae (81%) swam in a viscous environment, and only 19% (>40 dph) swam in an environment where both viscous and inertial forces dominated. The fact that most larvae swam in a viscous environment might help in explaining the lower speeds in comparison to other species. To our knowledge, there is only one study that has investigated the critical swimming behaviour of clupeiforms (Fisher et al. 2005). These authors studied U_{crit} of late-stage larvae of 2 tropical clupeiforms (28 to 30°C) and recorded an average speed of 32 cm s^{-1} for *Jenkinsia* spp. (average TL: 37.70 mm) and 12.70 cm s^{-1} for *Spratelloids* sp. (average TL: 30.80 mm). These are significantly better swimming performances than sardines, although larval size and temperature may in part be responsible for this difference. Differences in performance may also be attributable to different methodologies. There is no standard protocol in the literature for conducting U_{crit} experiments (Kolok 1999), and aspects such as velocity, increment size and interval length vary among studies. However, Hogan et al. (2007) concluded that U_{crit} of fish lavae is relatively robust to variations in methodology. Moreover, the interspecific comparisons addressed here are from studies where the U_{crit} methodology was quite similar.

Despite being poor swimmers compared to most perciform species of temperate and tropical environments, the swimming abilities of sardine larvae described here likely influence their distribution in the wild. Sardine spawning in Portugal peaks be-

tween November and April in upwelling areas (Figueiredo & Santos 1989, Ré et al. 1990, Santos et al. 2004) and occurs along the continental shelf, with temperature ranging from 12 to 17°C (Bernal et al. 2007). Off western Iberia, sardine larvae are distributed in the upper 20 to 25 m of the water column (Santos et al. 2006). In this area, current speeds of 4 to 10 cm s^{-1} are frequent (Vitorino & Coelho 1998, Santos et al. 2004, 2006, Zuo et al. 2007, dos Santos et al. 2008), which are lower than the maximum swimming speeds obtained here for late-stage sardine larvae (9.47 cm s^{-1}). It is unlikely that larvae in the wild swim at these U_{crit} values long enough to influence their transport (Leis 2006); rather, they probably combine vertical (Santos et al. 2006) and horizontal swimming to control their dispersion or to prevent advection offshore.

As expected, locomotory behaviour, and consequently foraging activity, improved with ontogeny. Early stages spent more time in pause (57% in pause at 5 dph and 33% at 15 dph), which is probably associated with a 'saltatory' foraging strategy, where searching for prey occurs while pausing between swimming events (Browman & O'Brien 1992a,b). As larvae developed, the time spent swimming increased, and larvae adopted a cruise strategy (from 26 dph onward), in which, according to Munk & Kiørboe (1985), the search for prey occurred during active swimming. This change in foraging behaviour was also observed in another clupeioid, the northern anchovy *Engraulis mordax* (Hunter 1972), reared at the same temperature (15°C).

Although sardine larvae hatched with similar total length (4.05 ± 0.06 mm TL) to that found in their natural environment off the Portuguese coast (Santos et al. 2007), most morphological events (such as the beginning of notochord flexion, and the formation of dorsal and anal fins) occurred at smaller sizes when compared to wild larvae (Santos et al. 2007), which could either be related to the lower growth rates observed in the present study or to differences in water temperature. Larval growth rates obtained in this study ranged from 0.24 (Diet D) to 0.49 (Diet C) mm d^{-1} for a size range of 2.90 to 23.40 mm TL, which are lower than the mean growth rates estimated for wild sardine larvae caught in the Bay of Biscay (0.70 mm d^{-1}; Diaz et al. 2011), and similar to those recorded in Portuguese estuaries (0.41 to 0.57 mm d^{-1}; Ré 1983, 0.29 to 0.42 mm d^{-1}; Chícharo et al. 2012) at similar water temperatures (14 to 15°C). Growth rates obtained in the laboratory are generally lower than those estimated for larvae growing in the wild, as observed for the European anchovy

Engraulis encrasicolus (Garrido et al. 2012) and Atlantic herring *Clupea harengus* (Checkley 1984). This is likely a consequence of the absence of predation under laboratory conditions, in contrast to the wild where slow growers may be removed by predation (Takasuka et al. 2003).

Growth rates of sardine larvae varied significantly with prey concentration. Larvae reared with high food concentrations (Diets B and C) had higher growth rates than those fed with Diet A (<2 prey ml^{-1}), even when a highly diversified diet of wild prey (Diet D) was used. These low growth rates were similar to those obtained by Blaxter (1969), using low concentrations (<1 prey ml^{-1}) of wild plankton. In both cases, the low growth rates and maximum survival of sardine larvae using wild plankton are likely related to the low concentration used when compared to reared plankton. On the other hand, there was no difference in growth rates using 2 and 5 nauplii ml^{-1} (Diets B and C), which is probably a consequence of both being optimal feeding levels for the larvae. The fact that these high prey concentrations are needed to guarantee similar growth rates to those observed in the wild suggests that sardine larvae require high plankton densities to survive. This probably means that larvae need to stay on food patches and avoid advection offshore to less favourable environments, which depends on the development of important survival skills such as swimming abilities.

Mean sRD (0.37 ± 0.27 SE) was significantly lower than the average ratio (1.36) observed for wild larvae growing in the same temperature off the Iberian Peninsula (Chícharo et al. 2012, Santos et al. 2006). Although the lower nutritional condition of sardine larvae in the present study might be related to the absence of predators (which may remove larvae in poor condition in the wild; Pepin 1990), we cannot sustain that all laboratory-reared larvae are in poor nutritional condition, because the Fulton's condition index of the larvae reared with the high prey concentration diet (Moyano et al. 2014) was similar to that of fast-growing sardine larvae in the wild (Fulton's somatic condition factor was 0.1 to 0.4 for 10 to 30 dph larvae; Laiz-Carrión et al. 2011). We also cannot argue that the lack of a relationship of diet concentration with larval condition is due to the RNA/DNA ratio being a poor condition proxy, since the RNA/DNA ratio has not been calibrated to growth rates in the larvae of this species. Our findings of low sRD, despite the high values of growth rates, suggests that more work is needed to calibrate sRD for use as a growth proxy in European sardine larvae growing in relatively warm (≥15°C) waters.

The condition of sardine larvae estimated by sRD and the DNA/DW was not significantly different between diets. Since there is a size effect on the nutritional condition of the larvae, the RNA residual index was used to eliminate the influence of length on the results (Suthers et al. 1996, Chícharo et al. 1998). The RNA residual index revealed significant differences in larval condition between the highest food concentration diet and the other feeding treatments. Contrary to the other indices, the RNA residual index was also related to sardine larvae U_{crit}, revealing that larval condition has a significant effect on its swimming abilities. This is especially relevant during the larval phase, which is characterized by a dynamic development of new capabilities and fast improvements and a period of constant ecological challenges, such as obtaining food, avoiding predation and finding suitable habitat (Houde 1987, Fuiman 2002).

The present study provides a new insight into the behaviour of sardine larvae, suggesting that the swimming abilities of the later stages may actively contribute to larval retention in food patches, which in turn could allow larvae to achieve high growth rates and better nutritional condition, thus enhancing chances of survival. Future steps must include other measures of swimming performance (such as sustained swimming speeds) and comparisons to wild larvae, since behaviour may differ between wild and laboratory-reared larvae.

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