Vol. 559: 131–145, 2016
doi: 10.3354/meps11881

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published November 9

Effect of temperature on the growth, survival, development and foraging behaviour of *Sardina pilchardus* larvae

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ABSTRACT: The effect of water temperature on the growth, survival, development and foraging behaviour of European sardine *Sardina pilchardus* larvae was examined in the laboratory. First, the capability of early sardine larvae to cope with starvation was assessed at temperatures from 10 to 22°C. Second, we examined under ad libitum feeding conditions and across the range of temperatures experienced by sardines during spawning along the Atlanto-Iberian coast (13–17°C) the ontogenetic changes in growth, survival and foraging behaviour of sardine larvae. Unfed larvae had similar maximum survival times (11–12 d post hatching, dph) from 13 to 15°C, but the survival time was significantly shorter at the coldest and warmest temperatures tested. The survival of exogenously feeding larvae increased with temperature, but younger endogenously feeding larvae had higher survival at colder temperatures. The cumulative mortality after 25 dph, however, was similar at the 3 temperatures. Not only larval growth rate increased with increasing temperature, but ontogenetic development also occurred sooner and at smaller sizes. Notochord flexion, which is a developmental milestone for fish, occurred 10 d earlier at 17 rather than at 13°C. The time spent swimming and the foraging behaviour (orientations to prey, feeding strikes and successful capture) significantly increased throughout the ontogeny and with temperature. This study highlights how even modest changes in spawning temperature can lead to large changes in the survival and growth of larval sardine. This study also reveals some of the mechanisms whereby inter-annual and seasonal variability in temperature can have significant ecological impacts at the population level.

KEY WORDS: *Sardina pilchardus* · Growth rate · Pelagic fish · Mortality · Foraging behaviour

INTRODUCTION

Temperature influences metabolism and growth in poikilotherms (Clarke & Johnston 1999) and is considered a key factor shaping the ecophysiology and life history strategy of fish (Pörtner & Peck 2010). Temperature plays a particularly important role during the early life of marine fish by influencing rates of metabolism, growth and mortality of marine fish larvae (Houde 1989, Blaxter 1991). The ability of a species, a population or an individual to persist over a range of temperatures is limited by the thermal adap-

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tive capacity of biochemical, cellular and organismal processes which constrains the geographic distribution of stocks and influences inter-annual variability in survival (Jordaan & Kling 2003) and also by the severity of climate impacts (Rijnsdorp et al. 2009). Even within the limits of thermal tolerance, subtle changes in temperature experienced by early larvae cannot only influence survival by altering physiological rates and behaviour but can also influence the performance of survivors later in life (Johnston et al. 2001, Koumoundouros et al. 2009, Moyano et al. 2016). Discriminating the relative contribution of genetic versus environmental factors to the variation in larval performance is necessary if we hope to provide robust predictions of the effects of climate change on fish stocks (Jordaan & Kling 2003, Dahlke et al. 2016).

The European sardine *Sardina pilchardus* is distributed along the eastern North Atlantic coast, from Iceland and the North Sea to Senegal and the Mediterranean Sea (Whitehead et al. 1985). One of the main areas of sardine spawning in the northeast Atlantic is the Iberian Peninsula, where sardine larvae often dominate the ichthyoplankton community (Garrido et al. 2009). Due to persistently low recruitment since 2006, the size of the Iberian sardine stock has substantially decreased, reaching historical minimum values in 2012–2013 (ICES 2014). The decrease in recruitment of European sardines is thought to be directly related to increasing water temperature observed in recent years in the Western Iberian Upwelling Ecosystem (ranging from 0.02 to 0.03°C yr^{-1} since 1985) (Relvas et al. 2009). Field surveys in that region report the highest occurrence of spawning at 14 to 15°C with little or no spawning at water temperatures colder than 12°C and warmer than 16°C (Stratoudakis et al. 2007, Peck et al. 2013). The size-at-maturation is generally smaller in sardines from warmer waters and, likely for this reason, length-at-maturation has decreased and the spawning period has increased in western Iberian waters during the last 2 decades (Silva et al. 2006). In order to understand the modifications in sardine spawning and development, it is essential to know the underlying physiological and behavioural responses of individual larvae at different temperatures.

Laboratory studies can serve to simplify some of the interactions of inherently complex natural systems by removing factors that confound temperature effects, such as food limitation (Jordaan & Kling 2003). To our best knowledge, there are no previous laboratory studies examining ontogenetic changes and/or the effect of temperature on survival, growth and behaviour of exogenously feeding European sardine, with all previous studies restricted to either embryos or unfed larvae (Blaxter 1969, Miranda et al. 1990, Bernal et al. 2008). The present study investigated the effect of temperature on the growth, survival and foraging behaviour of European sardine larvae. First, the capacity of larvae to withstand starvation as a function of temperature was assessed. Second, the effect of temperature on growth, mortality and foraging behaviour was studied for larvae reared with excess food across at a range of temperatures encompassing the vast majority (95%) of those associated with sardine spawning in Atlanto-Iberian waters (Coombs et al. 2006). This study examines the effect of temperature on growth, mortality and foraging behaviour of European sardine larvae under controlled laboratory conditions, and our results are discussed in relation to patterns derived from the field.

MATERIALS AND METHODS

Larval rearing and growth experiments

Sardine larvae were hatched from eggs spawned by broodstock European sardines *Sardina pilchardus* maintained in a 15 000 l cylindrical tank at the Oceanário de Lisboa. Fish were originally captured in 2009 and 2010 by purse seine in coastal waters off Peniche (western Portugal). Adult fish started spawning naturally after adjusting the temperature (15°C) to natural conditions and increasing the light regime (16 h light:8 h dark) to the maximum number of daylight hours occurring in western Iberia. The eggs were collected daily in the morning from the broodstock using 500 µm mesh egg collector bags placed in the skimmers of the tank. At that time, most eggs were found halfway through egg development (Stage V, Gamulin & Hure 1955). A total of 2000 viable eggs was placed into 5 l glass beakers containing gently aerated water from the broodstock tank. Over the next 1 to 2 h, the temperature was slowly adjusted to 17, 15 or 13°C. Finally, eggs were gently transferred to 30 l cylindrical tanks filled with seawater of salinity 35 at their rearing temperature. Eggs were incubated using a 16 h light:8 h dark light regime and gentle aeration to mix the water and maintain a high oxygen concentration. Surface light levels were kept at 55 to 58 µmol s^{-1} m^{-2} (Philips Master TL-D Super 80 58W fluorescent lamp).

Growth experiments consisted of transferring batches of ca. 2000 sardine eggs to the above-mentioned 30 l tanks and rearing the embryos and larvae either unfed or fed a mixed diet of dinoflagel-

lates *Gymnodinium* sp., rotifers *Brachionus* sp. and nauplii, copepodites and adults of the copepod *Paracartia grani*. Details of the feeding conditions, which translate into saturated feeding and growth rates of sardine larvae, can be found in Silva et al. (2014) and Caldeira et al. (2014). Each morning, after checking the food concentration in the tanks, 20 to 30 % of the tank water was renewed by syphoning the bottom of the tank, and new food was added to obtain the pre-established food concentration. Every other day pH, ammonia and oxygen were measured (Hanna Instruments 9828) to confirm that high water quality was maintained throughout the experiment. In the starvation experiment, 5 different temperatures were tested (10, 13, 15, 17, and 22°C ± 0.4°C). Experiments using fed larvae were conducted at 13, 15 and 17°C, which correspond to the coldest, peak and warmest temperatures, respectively, measured during peak spawning by sardines in Iberian waters (Coombs et al. 2006).

Throughout the experiment, groups of larvae were randomly sampled from experimental tanks and rapidly anesthetized with MS-222 to determine their size (total length, TL) under a stereomicroscope (± 0.05 mm accuracy) and posteriorly preserved in formaldehyde. When embryos were found to be in an advanced developmental stage (Stage X; Gamulin & Hure 1955), tanks were inspected frequently to determine the time when 50 % of the larvae hatched. Larval size was determined at the time when ~50 % of the larvae had hatched. Given that at the temperatures tested the hatching period can last up to 10 h (Bernal et al. 2008), larval size-at-hatch would be temperature-dependently overestimated, and for that reason larval size at the day of hatching was not formally compared between temperatures. Larvae were sampled from each tank each day until death in the case of unfed larvae. In the case of fed larvae, samples were taken until 50 d post-hatching (dph) at 15 and 17°C and at 25 dph at 13°C, where technical problems did not allow measurements beyond that age due to accidental loss of larvae. Mean larval TL for fed larvae was determined from measurements made on 20 (at hatching), ≥10 (3 to 15 dph) and 5–10 (≥16 dph) individuals. Measurements of sardine larvae were made at 0, 3, and 5 dph and then every 5 d until the end of the experiment. Every day, the bottom of each tank was syphoned, and dead larvae were counted to determine age-specific survival rates.

Sardine larvae preserved in formaldehyde were digitally photographed under a stereoscope (Leica S8 APO, zoom 8:1 with a Canon EOS SLR550 camera), and the development of important ontogenetic traits of fish larvae, such as fin formation and notochord flexion, were registered for larvae reared at each of the 3 temperatures.

Foraging behaviour

The behaviour of fed larvae was observed at 3 temperatures (13, 15 and 17°C). Every day and for each temperature treatment, randomly selected larvae (≥20 observations) were individually followed and their behaviour registered for a 1 min interval (focal-animal technique, Martin & Bateson 1993). During observations, water mixing was stopped by removing the aeration so that individual larvae could be followed in the absence of turbulence which may affect larval behaviour. The time spent swimming by the larvae during the 1 min observation period (locomotory model action pattern), as an indicator of foraging effort, was logged. The foraging MAPs recorded, adapted from Barlow (1968), included the occurrence of orientations (also termed fixations or s-shape positions when detecting a prey) and the frequency of lunges or attacks on prey; given that not all orientations resulted in successful attacks, the percentage of complete feeding sequences with respect to the total number of orientations was also estimated. At 15 and 17°C, behavioural observations were made until the larvae were 50 and 25 dph at 13°C.

Data analysis

For fed larvae from 3 to 25 dph at each of the 3 temperature treatments, growth rate was assessed from the exponential fit of TL (in mm) and age data using the equation:

$$\mathrm{TL} = L_0 \mathrm{e}^{kt} \quad (1)$$

where L_0 is length at 3 dph, k is the instantaneous growth rate and t is age in dph. In the case of larvae reared until 50 dph at 15 and 17°C, data were adjusted from 3 to 50 dph to a Laird-Gompertz growth curve by an iterative nonlinear regression routine:

$$\mathrm{TL} = L_0 \mathrm{e}^{\left(\frac{A_0}{\alpha}\right)\left(1-\mathrm{e}^{-\alpha t}\right)} \quad (2)$$

where A_0 is the growth rate at time 0, and α is the rate of exponential decay.

To compare the relationship between size and age between larvae reared at different temperatures from age 3 to age 25 dph (common range in ages between the 3 temperatures), first generalized linear models (GLMs) with an identity link were used to as-

sess if the interactions of age and temperature were significant. When the interaction term was significant, pairwise comparisons of slopes were conducted using an ANCOVA model. When the differences between the slopes were significant, the regression was repeated excluding the interaction term. When the differences between the slopes were not significant, a new regression model excluding the interaction term was fitted, and both regression models (with and without the interaction model) were compared using ANOVA, and then the most parsimonious model was selected. Growth rates of 3 to 50 dph larvae reared at 15 and 17°C and fitted to Laird-Gompertz growth curves were compared by using the method to compare non-linear models described in Chen et al. (1992).

Cumulative survival rates of sardine larvae reared with excess food were calculated from Day 0 to 25 dph. Comparison of the survival rates of larvae reared with different temperatures was conducted using the Logrank (Mantel cox) *z*-test. Cumulative survival rates were considered different when $p < 0.05$.

GLMs were used to test the significance of the interaction between age and temperature on the time spent swimming, the number of orientations, number of attacks on prey and the percentage of successful attacks on prey for pre-flexion sardine larvae (≤25 dph for larvae reared at 13 and 15°C and ≤20 dph for larvae reared at 17°C). When the interaction term was significant, pairwise comparisons of slopes were conducted using an ANCOVA model. When the differences between the slopes were significant, the regressions were repeated excluding the interaction term. When the differences between the slopes were not significant, a new regression model excluding the interaction term was fitted and both regression models (with and without the interaction model) were compared using ANOVA, and then the most parsimonious model was selected.

Foraging efficiency (FE) was estimated as

$$FE = K \times 100 / C \quad (3)$$

where K is the growth rate of sardine larvae and C is the capture rate (here estimated as the number of attacks on prey) at each of the 3 temperatures. Both K and C were expressed in carbon units (µg C) by using available conversions of larvae length to carbon content and prey mean length of prey (*Paracartia grani*) to carbon content (Caldeira et al. 2014). Statistical analyses were performed using Matlab 8.1 (R2013a) from Mathworks (mortality data) and the open source software R version 2.9.2 of R Development Core Team (growth and behavioural data).

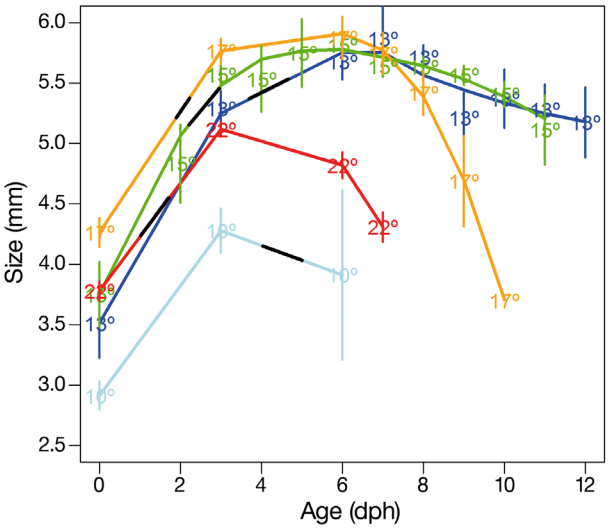

Fig. 1. Mean size-at-age (total length ± SE, mm) versus age (d post-hatching, dph) of unfed European sardine larvae at 5 different temperatures. Black lines represent the interval at which the yolk sac was exhausted and eyes were pigmented. Mean size-at-age at age 0 corresponds to the mean size when 50% of the larvae have hatched in the tank, not the precise size-at-hatch

RESULTS

Larval growth under starvation

Sardine larvae hatched sooner at higher temperatures, and the time between egg collection to 50% hatch ranged from ca. 72 to 30 h at incubation temperatures of 10 and 22°C, respectively. The mean (±SD) TL at the time of 50% hatch was 2.9 ± 0.3, 3.6 ± 0.3, 3.8 ± 0.7, 4.3 ± 0.3 mm and 3.8 ± 0.2 mm at 10, 13, 15, 17 and 22°C, respectively. Time until the yolk-sac absorption and the larvae started exogenous feeding was 2.5 d at 22°C and 4.5 d at 10°C.

The growth of endogenously feeding (yolk sac) larvae increased with increasing temperature except at the lowest (10°C) and highest (22°C) temperatures. At 10°C, larval length-at-age remained fairly constant until death and only increased from 0 to 3 dph at 22°C, sharply decreasing afterwards. At the other 3 temperatures tested, larval size increased until 6–7 dph and then either abruptly dropped (17°C) or steadily declined (13° and 15°C) (Fig. 1). Maximum duration of survival of unfed larvae was 6, 12, 11, 12 and 7 dph at 10, 13, 15, 17 and 22°C, respectively (Fig. 1).

Growth and survival of feeding larvae

The batches of sardine larvae used in feeding trials hatched approximately 60, 48 and 30 h after the em-

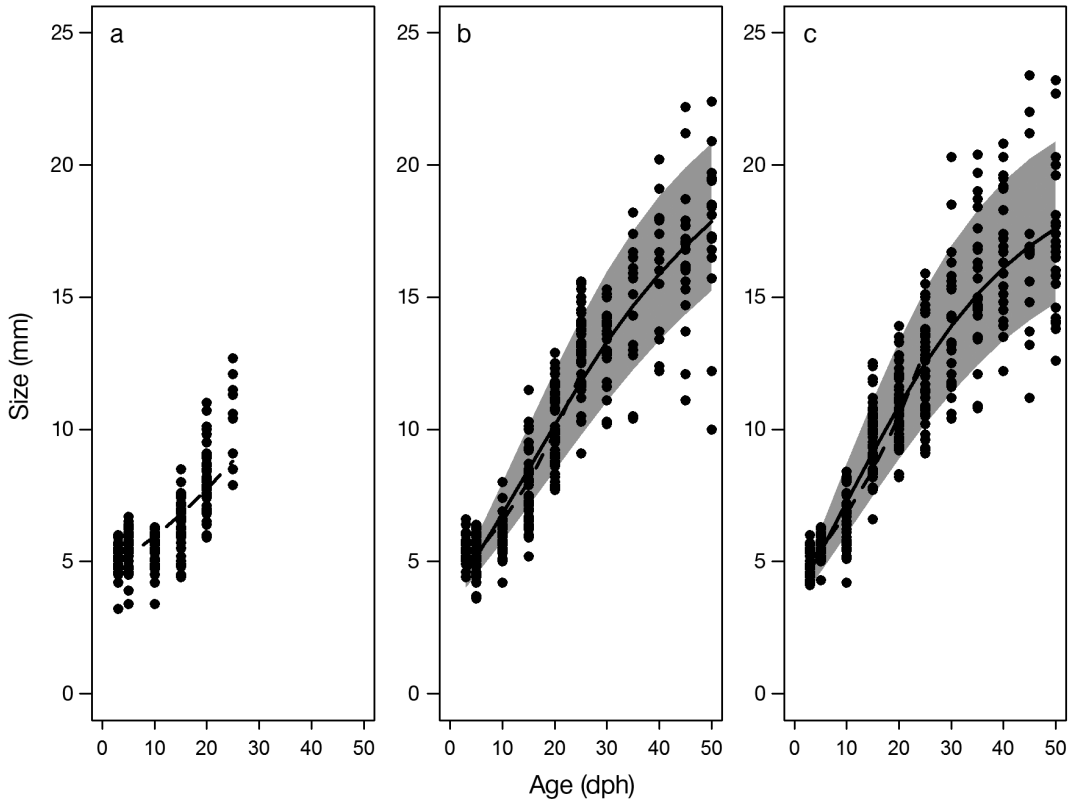

Fig. 2. Total length (mm) versus age (d post-hatching, dph) of fed European sardine larvae at 3 different temperatures: (a) 13°C, (b) 15°C, (c) 17°C. Exponential growth curve fitted for the period from 3 to 25 dph for all temperatures (dashed line). Grey area represents the 95% confidence interval of the Laird-Gompertz curve fitted to the period from 3 to 50 dph for larvae reared at 15 and 17°C

bryos were collected from the adult spawning tank, at 13, 15 and 17°C, respectively. There was a significant increase in size (TL) with age with increasing temperature (Fig. 2, Tables 1–3). The GLM model showed a significant effect of temperature on growth for larvae growing from 3 to 25 dph at 13, 15 and 17°C (Akaike's information criterion [AIC] = 2694.8; $p < 0.0001$). The exponential increase in larval size from 3 to 25 dph (slope) was similar for larvae reared at 17 and 15°C and higher than that of larvae reared at 13°C (Tables 1 & 2). The ANOVA model compar-

Table 1. Results of ANCOVA models analysing the relationship between larval size (total length, mm) and age (d post hatching, dph) for *Sardina pilchardus* larvae reared from 3 to 25 dph at 13,15 and 17°C

Temp. (°C)	df	p-value		*F*	ρ	R^2
		Intercept	Slope			
13 and 15	1	<0.001	0.001	941	<0.001	0.81
13 and 17	1	0.023	<0.001	1033	<0.001	0.82
15 and 17	1	0.005	0.714	1710	<0.001	0.88

Table 2. Exponential regression of the relationship between larval size (total length, mm) and age (d post hatching, dph) for *Sardina pilchardus* larvae reared at 13, 15 and 17°C from 3 to 25 dph

Period	Temp. (°C)	Regression equation	No. of ind.	Confidence interval (95% confidence bounds)		Goodness of fit	
				Slope	Intercept	Adjusted R^2	One-tailed probability (p-value)
3–25 dph	13	$y = 4.12e^{0.0258age}$	257	0.0231–0.0286	4.44–4.77	0.58	<0.001
	15	$y = 4.12e^{0.0416age}$	291	0.0395–0.0437	4.16±4.43	0.84	<0.001
	17	$y = 4.32e^{0.0433age}$	292	0.0412–0.0453	4.28–4.55	0.85	<0.001

Table 3. Relationship between larval size (total length, mm) and age (d post hatching, dph) for *Sardina pilchardus* larvae reared at 15 and 17°C from 3 to 50 dph adjusted to a Laird-Gompertz growth curve. *t*: age in dph; L_0: length at 3 dph; α and r: coefficient of the Laird-Gompertz growth curve (Eq. 2)

Period	Temp. (°C)	Regression equation	No. of ind.	Confidence interval (95% confidence bounds) L_0	α	r
3–50 dph	15	$TL = 3.68e^{\left(\frac{0.074}{0.041}\right)(1-e^{-0.041t})}$	386	3.52–4.02	0.064–0.086	0.035–0.047
	17	$TL = 3.86e^{\left(\frac{0.081}{0.048}\right)(1-e^{-0.048t})}$	403	3.30–4.03	0.074–0.101	0.044–0.058

ing the regressions with and without the interaction term of age and temperature for larvae reared at 15 and 17°C from 3 to 25 dph showed that removing the interaction did not significantly affect the fit of the model (F = 0.13; p = 0.71), suggesting that exposure temperature did not significantly alter the growth rate of larvae for this age interval. The regression of larval size depending of larval age and temperature without an interaction term for larvae reared at 15 and 17°C from 3 to 25 dph showed that the intercept was significantly different for larvae reared at the 2 temperatures (F = 24.77; p < 0.0001); therefore, size-at-age was significantly higher for larvae reared at 17°C.

Growth rates of larvae reared from 3 to 50 dph at 15 and 17°C were significantly different (F = 5.76; p > 0.0001), and larval growth rate was higher for larvae reared at the higher temperature (Fig. 2, Table 3). Cumulative survival rates were significantly different between 13 and 15°C (z = 4.75, p < 0.001), between 13 and 17°C (z = 5.26, p < 0.001) and between 15 and 17°C (z = 2.04, p = 0.041 < 0.05) (Fig. 3). Mortality during the endogenous feeding stage increased with increasing temperature, but when exogenous feeding was the only feeding mode used by the larvae (>5 dph), daily mortality was significantly higher at lower temperatures. From 12 dph (for 15 and 17°C) and 15 dph (for 13°C) onwards, daily mortality was extremely low when compared to the first days after hatch (Fig. 3).

The development was faster at warmer temperatures. In general, fin formation occurred earlier and at smaller sizes with increasing temperature (Table 4). At 10 dph, the pectoral and caudal fins were first observed at 10 dph for larvae reared at 13 and 15°C and at 5 dph for larvae reared at 17°C. The beginning of notochord flexion was observed at 10, 15 and 20 dph, for larvae reared at 13, 15 and 17°C, respectively, and the complete flexion occurred at 25 and 20 dph for larvae reared at 15 and 17°C, respectively. Not only did notochord flexion occur earlier in time for higher temperatures, it also occurred at smaller sizes. At 13°C, notochord flexion was not complete by the end of the observations (at 25 dph). Development of the caudal fin was complete at 40 dph (16.2 mm TL) and 30 dph (13.7 mm TL) for larvae reared at 15 and 17°C, respectively.

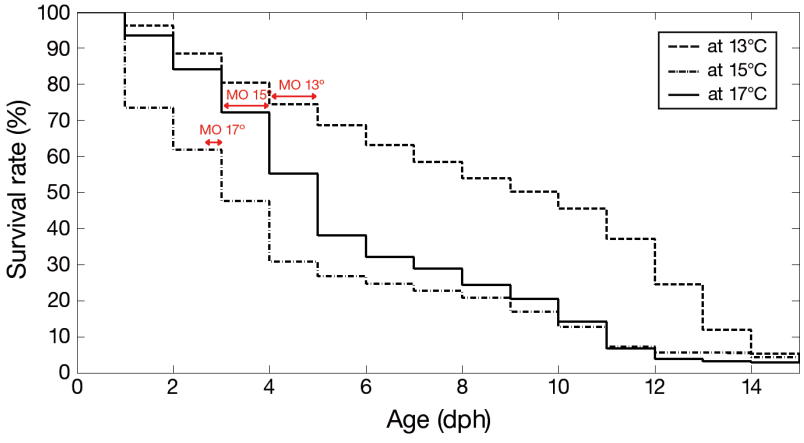

Fig. 3. Cumulative survival rate of *Sardina pilchardus* larvae reared with high concentration diet and under 3 different temperatures using the Kaplan-Meier method. Survival data not shown after 15 d post-hatching (dph) as subsequent changes were negligible (<3%). Red arrows represent the time period when the mouth opens (MO) and exogenous feeding begins

Locomotory behaviour and foraging of well-fed larvae

At 13, 15 and 17°C, 1 to 2 dph sardine larvae spent most of their time inactive, at the surface or near the bottom of the tank. Older larvae started swimming horizontally and vertically, spending most of the time in the upper half of the tank until 25 dph and near the bottom half of the tank at ages >25 dph. Prior to an age of 25 dph, larvae paused frequently to search for prey while from approximately 25 dph onwards, larvae

Table 4. Timing of the main morphological events occurring during larval development of sardine *Sardina pilchardus* at 13, 15 and 17°C. Pigmentation refers to the appearance of many small scattered melanophores in the dorsal region extending from the head to the tail, with one ventral caudal melanophore. Pectoral fins refer to the time when the insertion of the pectoral fins become oblique. Caudal fin complete refers to the time when the caudal fin has the complete number of fin rays. dph: d post hatching; –: no data

Temp. (°C)	Age (dph)	Mean size ± SD (mm)
Hatching day, no pigmentation		
13	0	3.6 ± 0.64
15	0	3.8 ± 0.27
17	0	4.1 ± 0.34
Pigmentation, yolk sac, mouth open		
13	4/5	5.5 ± 0.63
15	3/4	5.4 ± 0.41
17	3	5.0 ± 0.49
Pectoral fins and beginning of caudal fin formation		
13	10	5.5 ± 0.60
15	10	5.9 ± 0.60
17	5	5.5 ± 0.41
Beginning of notochord flexion		
13	20	8.1 ± 1.32
15	15	7.7 ± 1.29
17	10	6.3 ± 0.92
Beginning of dorsal fin development		
13	20	8.1 ± 1.32
15	20	10.0 ± 1.41
17	15	9.7 ± 1.26
Notochord flexion complete		
13	–	–
15	25	13.1 ± 1.52
17	20	10.8 ± 1.25
Dorsal fin complete		
13	–	–
15	30	13.2 ± 1.25
17	25	12.1 ± 1.82
Beginning of anal fin development		
13	–	–
15	35	14.5 ± 2.29
17	25	12.1 ± 1.82
Caudal fin complete		
13	–	–
15	40	16.2 ± 2.49
17	30	13.7 ± 2.44

started searching for prey while swimming. Time spent swimming by pre-flexion sardine larvae increased significantly with age, and its interaction with temperature was also significant (GLM AIC = 6995; $p < 0.0001$) (Tables 5 & 6, Fig. 4). The rate of increase in time spent swimming was higher for larvae reared at 17°C when compared to those reared at 13 and 15°C. The ANOVA testing for differences between the regression models with and without the interaction term of temperature and age for larvae reared at 13 and 15°C were not significant ($F = 0.846$; $p = 0.357$), suggesting that the rate of increase in swimming with pre-flexion larvae age was similar for larvae reared at these 2 temperatures. However, the intercept was significantly different between the two ($F = 393$; $p < 0.0001$; $R^2 = 0.39$); therefore, the time spent swimming at age was higher at 15°C.

At the start of exogenous feeding (3 to 5 dph), foraging activity was low; the (mean ± SD) frequency of orientation was 0.19 ± 0.54, 0.79 ± 1.36, and 1.75 ± 1.99 prey min^{-1} for larvae reared at 13, 15 and 17°C, respectively. The frequency of attacks (lunges) for first-feeding larvae was 0.09 ± 0.31, 0.40 ± 0.95 and 0.81 ± 1.24 attacks min^{-1} at 13, 15 and 17°C, respectively (Fig. 4). Foraging activity significantly increased with increasing age for pre-flexion larvae, and temperature significantly affected the slope of the increase in the frequency of larval orientations or fixations to prey (AIC = 8573; $p < 0.0001$), attacks on prey (AIC = 6995; $p < 0.0001$) and percentage of successful attacks on prey (AIC = 16776; $p < 0.0001$). The slope of the relationship between these 3 foraging behaviours with age increased with increasing temperature, with 13 < 15 < 17°C, except for the slope of the percentage of successful attacks to prey, which was significantly higher at 15 than at 17ºC (Tables 5 & 6).

After flexion and dorsal fin began to develop (25 dph for 13 and 15°C, 20 dph for 17°C, Table 4), time spent swimming was significantly different according to the temperatures with which larvae were reared (ANOVA, $F = 8.2$; $p = 0.0005$) and was particularly high at 17°C and similar between 13 and 15°C (p-values of Tukey-test pairwise comparisons of 0.57, 0.001 and 0.02 for 13–15, 13–17 and 15–17°C, respectively). Orientations towards prey as well as attacks on prey were also significantly different between temperatures during the flexion stage (ANOVA tests: $F = 12.1$ and 4.6; $p < 0.0001$ and 0.012, for comparisons of orientations and attacks, respectively). Orientations to prey at this stage were lower at 13°C and similar between 15 and 17°C (p-values of Tukey-test pairwise comparisons of 0.001, <0.0001 and 0.238 for 13–15, 13–17 and 15–17°C, respectively). Attacks on prey were also lower at 13°C and similar between 15 and 17°C (p-values of Tukey-test pairwise comparisons of 0.002, 0.015 and 0.990 for 13–15, 13–17 and 15–17°C, respectively). The successful attacks on prey were not significantly different between the 3 temperatures (ANOVA; $F = 0.99$; $p = 0.376$).

Larvae reached 50 % of their maximal value of time spent swimming at an age of 5.3 and 14.7 dph at 17 and 15°C, respectively (Fig. 4, Table 7). At approximately 15 and 20 dph for 17 and 15°C, respectively, sardine larvae spend all the observational time swimming, presenting a constant swimming behaviour.

Table 5. Results of the linear regression analyses ($y = ax + b$) of the effect of age (d post hatching, dph) on the time spent swimming (SWI,%), frequency of orientations (Orient.), frequency of attacks and frequency of successful attacks on prey of *Sardina pilchardus* larvae during 3 developmental stages: pre-flexion (from 3 to 25 dph at 13 and 15°C and from 3 to 20 at 17°C) and post-flexion (25 to 50 dph at 15°C and 20 to 50 dph at 17°C) and after caudal fin formation (40 dph at 15°C and 50 dph at 17°C)

Developmental stage	Relation	Temp. (°C)	*a* (±SE)	*b* (±SE)	p	R^2
Pre-flexion	SWI vs. Age	13	0.029±0.002	0.15±0.024	<0.001	0.30
		15	0.027±0.001	0.30±0.020	<0.001	0.37
		17	0.038±0.01	0.31±0.001	<0.001	0.47
	Orient. vs. Age	13	0.09±0.011	0.28±0.159	<0.001	0.11
		15	0.28±0.017	−0.67±0.277	<0.001	0.27
		17	0.50±0.034	−0.26±0.417	<0.001	0.32
	Attacks vs. Age	13	0.02±0.005	0.12±0.069	<0.001	0.05
		15	0.17±0.013	−0.77±0.207	<0.001	0.20
		17	0.24±0.034	−0.29±0.417	<0.001	0.27
	Success attacks vs. Age	13	0.58±0.235	14.15±3.217	0.01	0.01
		15	1.73±0.013	10.15±0.207	<0.001	0.11
		17	1.34±0.283	24.72±3.445	<0.001	0.05
Post-flexion	SWI vs. Age	15	0.002±0.0004	0.88±0.015	<0.001	0.06
		17	$-0.0001 \pm 9 \times 10^{-5}$	0.99±0.0032	0.196	0.001
	Orient. vs. Age	15	0.35±0.031	−1.20±1.194	<0.001	0.16
		17	0.08±0.038	12.66±1.319	0.033	0.01
	Attacks vs. Age	15	0.36±0.028	−5.07±1.051	<0.001	0.204
		17	0.12±0.033	6.63±1.154	<0.001	0.023
	Success attacks vs. Age	15	1.30±0.124	14.83±4.682	<0.001	0.145
		17	0.54±0.033	46.61±1.154	<0.001	0.03
Caudal fin formation	SWI vs. Age	15	−0.0001±0.006	1.00±0.316	0.09	0.01
		17	0.0002±0.012	0.98±0.513	0.302	0.0001
	Orient. vs. Age	15	0.35±0.145	−1.67±6.637	0.014	0.021
		17	0.001±0.079	16.14±3.160	0.982	−0.002
	Attacks vs. Age	15	0.326±0.133	−3.67±6.115	0.015	0.020
		17	−0.06±0.069	14.77±2.774	0.352	−0.003
	Success attacks vs. Age	15	0.91±0.447	31.85±21.843	0.056	0.011
		17	0.06±0.246	66.71±9.807	0.782	−0.002

Table 6. Results of ANCOVA models analysing the relationship between foraging behaviour (time spent swimming [SWI,%], frequency of orientations, frequency of attacks and frequency of successful attacks on prey) and Age (d post hatching, dph) of *Sardina pilchardus* larvae during 3 developmental stages: pre-flexion (from 3 to 25 dph at 13 and 15°C and from 3 to 20 dph at 17°C) and post-flexion (25 to 50 dph at 15°C and 20 to 50 dph at 17°C) and after caudal fin formation (40 dph at 15°C and 50 dph at 17°C). MAPS: modal action patterns

Developmental stage	MAPS	Temperatures (°C)	df	p-value		*F*	ρ	R^2
				Intercept	Slope			
Pre-flexion	SWI	13 and 15	1	<0.001	0.358	262	<0.001	0.39
		13 and 17	1	<0.001	<0.001	270	<0.001	0.44
		15 and 17	1	<0.001	<0.001	270	<0.001	0.42
	Orientations	13 and 15	1	0.004	<0.001	190	<0.001	0.31
		13 and 17	1	0.193	<0.001	270	<0.001	0.44
		15 and 17	1	0.390	<0.001	183	<0.001	0.33
	Attacks	13 and 15	1	<0.001	<0.001	140	<0.001	0.25
		13 and 17	1	0.047	<0.001	229	<0.001	0.41
		15 and 17	1	0.130	0.007	115	<0.001	0.23
	Success	13 and 15	1	0.366	<0.001	44	<0.001	0.09
		13 and 17	1	0.026	0.042	34	<0.001	0.09
		15 and 17	1	0.262	0.001	36	<0.001	0.09
Post-flexion	SWI	15 and 17	1	<0.001	<0.001	26	<0.001	0.06
	Orientations	15 and 17	1	<0.001	<0.001	59	<0.001	0.13
	Attacks	15 and 17	1	<0.001	<0.001	70	<0.001	0.15
	Success	15 and 17	1	<0.001	<0.001	42	<0.001	0.09
Caudal fin formation	SWI	15 and 17	1	0.205	0.310	5	<0.001	0.02
	Orientations	15 and 17	1	0.027	0.047	3	0.017	0.01
	Attacks	15 and 17	1	0.010	0.014	3	0.033	0.01
	Success	15 and 17	1	0.175	0.136	2	0.058	0.007

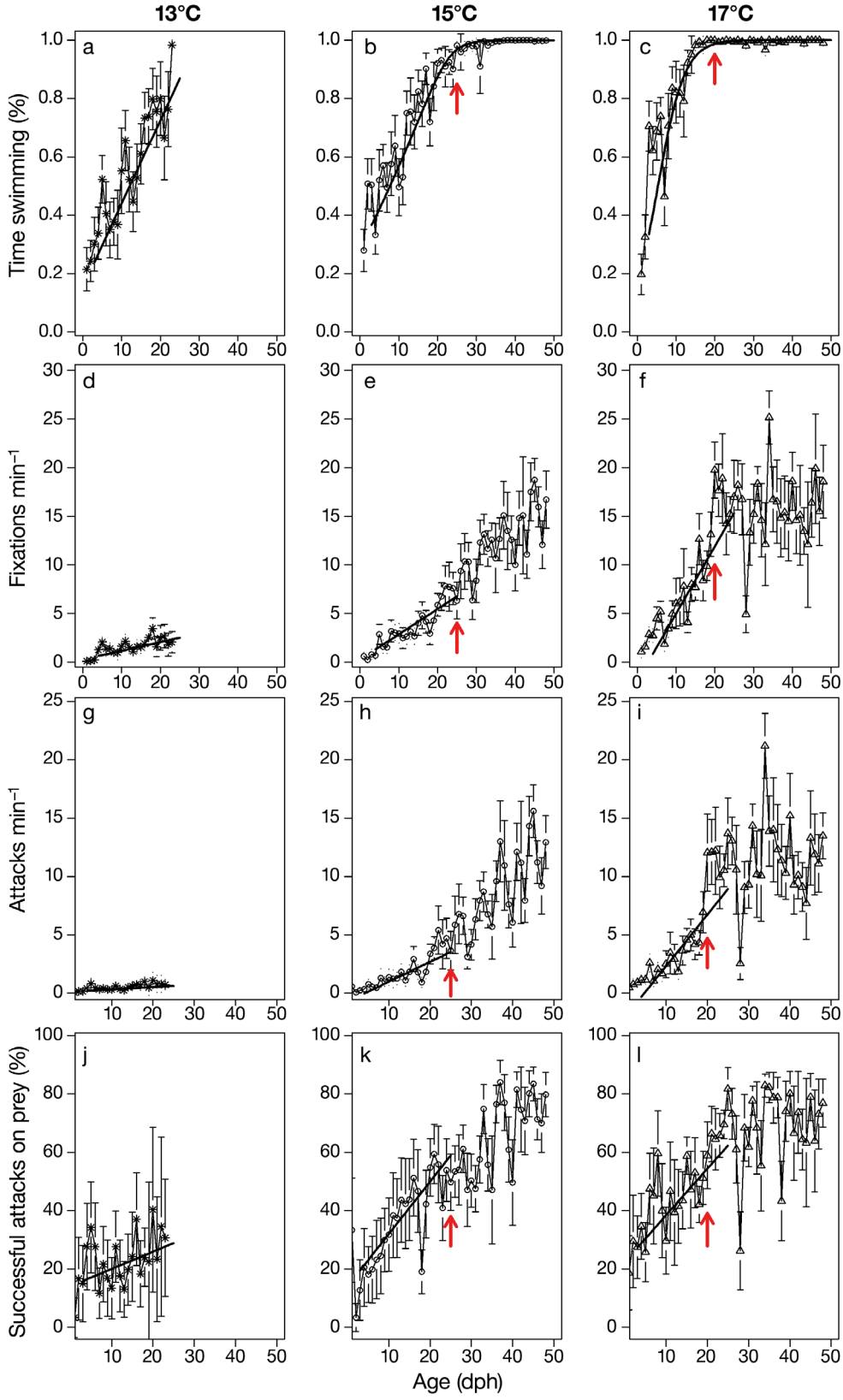

Fig. 4. Mean ± 0.95 confidence interval bars of the (a,b,c) time spent swimming, (d,e,f) frequency of fixations or orientations, (g,h,i) frequency of lunges during a 60 s period of observation throughout larval ontogeny of *Sardina pilchardus* and (j,k,l) percentage of successful attacks on prey of larvae reared with high prey concentrations and at 3 different temperatures: 13, 15 and 17°C. Red arrows represent the timing when notochord flexion is complete. Parameters of the linear and logistic regressions are provided in Tables 5 & 7

Table 7. Results of the Richard's logistic regression of the effect of temperature and age (d post hatch, dph) on the percentage of time spent swimming by *Sardina pilchardus* larvae from 3 to 50 dph at 15 and 17°C

Parameter	15°C	17°C
Bottom asymptote	−1.1	0
Top asymptote	1	1
Inflexion point at (*x*,*y*)	14.7, 0.7	5.3, 0.5
Goodness of fit	0.90	0.93
SE	0.47	0.42
50% activity	7.7	5.3

During the post-flexion stage, foraging parameters were higher for larvae reared at 17°C when compared to those reared at 15°C (Tables 5 & 6). Time spent swimming did not increase significantly for larvae reared at 17°C after flexion, contrary to those reared at 15°C, for which there was a slight but significant increase (Table 5). Orientations and attacks on prey as well as successful attacks on prey increased with age for larvae reared at 15 and 17°C during the post-flexion stage and were significantly higher at 17°C (Tables 5 & 6), and at the temperature when the flexion of the notochord was complete corresponded to a sharp increase in foraging ability of the larvae reared at 17°C, contrasting to larvae reared at 15°C, for which the increase in foraging was less abrupt.

After the caudal fin formation (30 and 40 dph for larvae reared at 17 and 15°C, respectively), time spent swimming did not increase significantly for larvae reared at 15 and 17°C as it was near to maximum. All foraging parameters were significantly higher at 17°C when compared to those of larvae reared at 15°C after caudal fin formation. Moreover, foraging parameters such as orientations and attack on prey significantly increased with age for larvae reared at 15°C, contrary to larvae reared at 17°C, for which these rates did not increase with age after the caudal fin formation (Tables 5 & 6). The percentage of successful attacks on prey did not increase with age after the caudal fin formation for larvae reared at 15 and 17°C (Fig. 4, Tables 5 & 6).

DISCUSSION

Despite the ecological and commercial importance of sardines and anchovies in the world's oceans, controlled laboratory studies on the growth, feeding and mortality of any life stage of these fishes are rare (Peck et al. 2013). This study is the first to examine the effect of temperature on the growth, mortality and foraging behaviour of European sardine larvae reared from hatch through first-feeding and well into the exogenous feeding period. Our results highlight the controlling role that temperature plays on larval development, growth and feeding performance.

After fertilizing and incubating eggs at the same temperature, the yolk-sac period of sardine larvae described in Blaxter (1969) and Miranda et al. (1990) was 2 to 5 d between 20 and 10°C, respectively, which agrees with the observations in the present study. Although the rate of embryonic development increased with increasing temperature, the survival of unfed larvae was highest within a more narrow range of temperatures typically occurring during the spawning period of sardine in Iberian Atlantic waters. Time needed from hatch until dead from starvation was slightly lower in the present study than reported for larvae collected from northwestern Iberia (Miranda et al. 1990) and slightly longer than reported for larvae collected from the English Channel (Blaxter 1969). Part of this variation can be the result of physiological adaptations of marine fish embryos to prevailing local conditions for populations living in different latitudes, as shown by Tarifeño et al. (2008) for Peruvian anchovies.

The growth pattern of unfed sardine larvae was similar to that described by Blaxter (1969). Larvae increased in length with age until the yolk reserves were exhausted, when substantial decreases in length subsequently occurred. This decrease in mean length was particularly marked at higher temperatures. At 22°C, sardine larvae growth was practically null immediately after the endogenous feeding period, and larvae died sooner than at 13 to 17°C, probably as the result of a sharp increase in metabolic demands with the increase in temperature. For the 13–17°C range, similar to that experienced by sardines spawning off Iberia, there were no relevant differences in the endurance to starvation of the larvae, although the decline in larval mean size for unfed larvae was steeper at 17°C. It appears, then, that the point of no return (when larvae will die regardless of whether prey becomes available) is overall quite similar within this temperature range (Pepin 1991).

Daily mortality was very high for the first weeks of life and then significantly decreased at 12 dph for larvae reared at 15 and 17°C and at 15 dph for larvae reared at 13°C. This decrease can be related to the beginning of the flexion of the notochord, when larvae became more efficient swimmers and foragers. There were significant differences in the daily mortality of fed larvae reared at 13, 15 and 17°C, and the

patterns observed depended on the life stage. During the endogenous feeding period, daily mortality was higher for larvae reared at higher temperatures, but in exogenously feeding larvae, daily mortality was higher for larvae reared at the lowest temperature. Interestingly, total cumulative mortality was similar between the 3 temperatures at the end of the experimental period (25 dph). Similar results were reported for the larvae of Atlantic cod *Gadus morhua* (von Herbing Hunt et al. 1996), suggesting that, within a range of temperatures near the centre of the tolerance range, there is little effect of temperature on cumulative mortality. According to the authors, intrinsic factors may be the predominant agents of mortality prior to yolk-absorption, but this shifts to extrinsic (environmental) factors as the fish begin to feed. Increased mortality during the egg and yolk-sac stage is known to occur at higher temperatures (Houde 1989). Alternatively, increased larval mortality at lower temperatures after the endogenous feeding could be related to the decreased foraging and growth potential at sub-optimally cold temperatures, and consequently, larvae have the smallest size-at-age (Pepin 1991).

Within the range 13–17°C, the growth of well-fed sardine larvae increased with increasing temperature, which is in accordance with previous studies demonstrating that, within the optimal temperature range for a species, growth and development rates increase at warmer temperatures (Pepin 1991). Growth rates of small pelagic fish larvae were described to have a domed-shaped relationship with sea surface temperature (SST) (Takasuka et al. 2007), increasing up until a maximum after which they decrease with increasing temperature. According to the present results, the inflexion point when growth starts to decrease with increasing temperature for sardine larvae (upper thermal tolerance limit) is >17°C. However, the upper thermal tolerance point for egg development was not tested. Eggs were spawned and fertilized in the adult tank at 15°C and were collected and transferred to different test temperatures halfway through egg development. Since the thermal tolerance of eggs tends to be lower than that of larvae (Pepin 1991, Jordaan & Kling 2003), the thermal limits of sardine egg development from fertilization is likely to have a narrower range than that shown here for the larvae. In a previous study, the broodstock fish used in the present study naturally produced eggs at 18 to 19°C, and those eggs and larvae were reared at this (relatively warm) temperature. Despite using the same feeding protocol, no larvae survived beyond the first week, and many larvae displayed skeletal deformities, suggesting that these temperatures represent the upper lethal temperature for Iberian sardine larvae (S. Garrido unpubl. data). Thermal tolerance is expected to increase as fish grow up to the juvenile and adult stages. A recent work conducted in the Mediterranean Sea (Schismenou et al. 2016) has found that larvae occurred in areas of SST ranging from 13 to 17°C, and a linear and positive relationship of sardine larvae growth rate was found with temperature. In contrast, sardine juveniles developed in a wider range of temperatures (12–27°C) and had a dome-shaped growth response, with an optimum at around 24°C.

Temperature-specific growth rates of small pelagic fish larvae can be quite different depending upon the ecosystem/region, year and/or study (Peck et al. 2013). Optimal temperature for sardine spawning off the east Atlantic (Iberian) waters is from approximately 13 to 17°C, with warmer waters being used at lower latitudes (off northwest Africa, 15–20°C) and colder waters being utilized at higher latitudes (British Islands, 10–17°C) (Coombs et al. 2006). It is likely that there are local adaptations of sardine populations to prevalent environmental conditions and that populations living at higher latitudes are more tolerant to lower temperatures compared to those living at lower latitudes areas. Sundby (2000) observed that Atlantic cod stocks from the colder areas within the species distribution range tended to have increased recruitment in warmer than average years, whereas those from warmer areas within the range had increased recruitment in cooler than average years. The authors hypothesize that the causal effect of temperature on the growth and recruitment of North Atlantic cod populations can partly be the result of a direct effect by changes in physiological processes of cod and partly an indirect effect through trophic transfer and availability of prey correlated to temperature. Nevertheless, the range of temperatures that promotes high rates of larval survival and strong recruitment might be much narrower than could be inferred from the geographic extent of the species (Jordaan & Kling 2003).

Several lines of evidence from the laboratory, such as the midpoint of the zone of thermal tolerance (Bernal et al. 2008), showed that sardine eggs do not hatch at 10°C, and results mentioned above showed that larvae hatching from eggs reared at >18°C did not survive beyond the first week (S. Garrido unpubl. data). Evidence from the field, such as the median temperature experienced in spawning areas (Coombs et al. 2006) and the tendency for recruitment to be maximized at intermediate tem-

peratures (S. Garrido et al. unpubl.), suggest that the optimal temperature for Atlanto-Iberian sardine during early life would be around 15°C. In our experiments, however, early sardine larvae had high growth capacity at temperatures >15°C. This apparent mismatch between temperatures of spawning and of maximum larval growth has also been described for cod (Jordaan & Kling 2003), where peak spawning occurs at 5–7°C, but larvae are able to grow and survive at >15°C. Since standard and active metabolic rates, and consequently prey requirements, of ectotherms are positively related to temperature, constrains in the food supply will eventually reduce the temperature at which growth performance is maximized (Brett 1979, Jobling 1997, Sundby 2000, Jordaan & Kling 2003). When facing restricted feeding conditions, larvae will die faster at higher temperatures (Laurel & Blood 2011). Thus, in the wild, fish growth tends to be maximized at temperatures a few degrees lower than those at which food consumption is maximal (e.g. Jobling 1993). Furthermore, other researchers have suggested that having maximum field abundance at temperatures slightly colder than optimal temperature for growth is a form of metabolic insurance (Thomas et al. 2012), since thermal windows for performance are shifted to the right, making critical (intolerably warm) temperatures often only a few degrees above optimum growth temperatures (Pörtner & Peck 2010, Boersma et al. 2016). For these reasons, it is not surprising that European sardine larvae tend to occur most frequently in field samples collected in slightly colder waters (Coombs et al. 2006) than their laboratory maximum (17°C, when larvae grew more than those reared at 15°C and had similar cumulative mortalities, as opposed to those reared at 18°C, as mentioned above). In our experiments, larvae were exposed to optimal feeding conditions that may be rarely encountered in the sea. Furthermore, seawater temperature often covaries with food availability (Rankin & Sponaugle 2011); therefore, the indirect effect of temperature on availability of prey may be just as important as the direct physiological effect of temperature on the foraging and growth capacity of fish (Loeng 1989). In regions where temperatures are higher than in the Ibero-Atlantic area, such as the Mediterranean Sea, food availability probably plays a pivotal role in determining larval survival. In the Mediterranean Sea, sardine spawns preferentially at 12–14°C, but spawning also occurs up to 19°C (Palomera et al. 2007). For larvae developing in that area under warmer conditions, growth was described to be higher when compared to the western Iberia, and larval condition was strongly dependent of high food availability (Catalán et al. 2006).

Elevated temperatures can accelerate the timing of ontogenetic development more than the rate of growth (Fuiman et al. 1998). Not only did larvae grow faster at higher temperatures, but ontogenetic development also occurred sooner and at smaller sizes. The timing of morphological development of sardine larvae varied inversely with temperature, but as observed by Fuiman et al. (1998), not every event was equally accelerated by temperature. In general, fin formation occurred earlier and at smaller sizes with increasing temperature, and this was translated into higher foraging abilities at age for larvae growing in higher temperatures. The time spent swimming and the foraging behaviour of sardine larvae increased with age/size. The increase in the frequency of prey capture with age/size is a consequence of larger larvae swimming faster (Silva et al. 2014) and being able to capture prey within a wider size range (Caldeira et al. 2014). By being able to select larger prey, larger larvae maximize the net rate of energy gain, because, all other things being equal, larger prey yield more energy per unit effort. The swimming activity and the foraging performance of sardine larvae both increased with increasing temperature (from 13 to 17°C), and the effect of temperature was more marked on prey pursuit and prey attack frequencies. Larvae spend all their time swimming (at least during daylight hours) when notochord flexion begins and dorsal fin starts to form (Fig. 4, Table 4). The ontogenetic increase in the swimming ability of sardine larvae matches the timing of notochord flexion (Silva et al. 2014), coinciding with the beginning of the caudal fin formation and development of the swim bladder (Santos et al. 2007). This ontogenetic event also marks the time when foraging abilities (number of orientations and attacks on prey and percent success in capturing prey) sharply increased, which was more abrupt for larvae reared at 17°C than for larvae reared at 15°C. After the caudal fin formation, there was no increase in larval swimming and foraging with age for larvae reared at 17°C, supporting the idea that this is an important developmental milestone for fish, marking a clear separation of higher abilities (Gibb et al. 2006, Somarakis & Nikolioudakis 2010, Kopf et al. 2014). These morphological changes occurred approximately 10 d earlier at 17°C rather than at 13°C. Earlier improvement of swimming performance for larvae growing at higher temperatures is not only important for the capacity to conduct diel vertical migration influencing advective

transport patterns but also for the ability to successfully forage and escape predators, coinciding with the timing when fish start the schooling behaviour (Somarakis & Nikolioudakis 2010). At this stage, larvae are described to switch from an anguilliform to a subcarangiform swimming style (Batty 1984), and this corresponds to the time when substantial endurance swimming is developed (Clark et al. 2005). Moreover, temperature can affect the phenotype of the larvae under development, altering the relative timing of development of the different tissues and organs (such as muscles and bones) during very early ontogeny (Johnston et al. 2001, Koumoundouros et al. 2009 and references within). This has been demonstrated in other clupeids such as Atlantic herring (Moyano et al. 2016) as well as in European sardine, where larvae growing in higher temperatures had a higher recruitment of muscle fibres compared to conspecifics given similar feeding conditions at lower temperatures (Catalán et al. 2004). The temperature experienced during the embryonic and early larval stages, therefore, can have profound consequences for the survival of larvae and later stages (Koumoundouros et al. 2009).

In contrast to the sardine larvae studied here, Hunter (1972) reported no effect of age on the percentage of successful attacks (average 52%) in the larvae of northern anchovy *Engraulis mordax*. Moreover, through an age of 30 dph, those larvae displayed a mean ± SD of 1.75 ± 0.20 feeding strikes min^{-1} at 17–18°C (Hunter 1972), which is similar to what was observed in the present study at 13°C (1.4 ± 1.8) but markedly lower than feeding strike frequencies observed at temperatures of 15°C (3.7 ± 5.45 min^{-1}) or 17°C (7.1 ± 6.75 min^{-1}), which suggests a higher foraging activity for European sardine larvae.

The substantial enhancement of foraging at warmer temperatures might not necessarily result in enhanced larval growth, since metabolic costs also increase with increased swimming activity, which could potentially offset any gains due to increased feeding success (Checkley 1984, Kiørboe et al. 1987). It is worth noting, however, that the different components of each foraging event have different expenditures of energy, e.g. swimming speeds during attack result in specific metabolic rates that are twice those in pursuit bursts (Hunt von Herbing et al. 1996). Therefore, increased temperature may have translated into only marginally higher energy expenditures and only slight reductions in growth efficiency, because pursuit success also increased with increasing temperature.

The response to temperature of different biological processes is not necessarily similar. The temperatures at which ingestion rate, growth rate and conversion efficiency are maximized can be different (Jobling 1993, 1997). Larvae experiencing lower temperatures will have longer stage durations which lengthens the period of time when they are most vulnerable to predators, whereas larvae at warmer temperatures will have to ingest more prey per unit growth, and these higher costs decrease the time needed for individuals to reach the point-of-no-return in poor prey fields (Pepin 1991). At the same time, larvae experiencing warmer temperatures may experience higher potential predation mortality through both increased encounter rates with predators and reduced energy available for predator evasion (Litvak & Leggett 1992, Lankford et al. 2001, Jordaan & Kling 2003). A recent modelling study suggested that benefits of faster growth at warmer temperature can be offset by increases in predator feeding rates (Akimova et al. 2016). As a result of these associated trade-offs, the temperature at which maximum daily growth is realized (Otterlei et al. 1999, Steinarsson & Björnsson 1999) may not be the optimal temperature for survival. Additionally, density-dependent effects may impede the benefits of increased growth rates by increasing competition for food in the wild, as described for cod (Holt & Jørgensen 2014).

The survival of sardine larvae and their potential for recruitment will depend on the environmental conditions and how these conditions affect the growth and mortality rates of the eggs and larvae. Temperature has been involved as a major factor in both year-to-year fluctuations and long-term trends in fish populations (e.g. Loeng 1989). In our experiments with well-fed sardine larvae, growth rate increased with increasing temperature. This higher growth was associated with large increases in foraging activity, therefore implying that sardine larvae will depend on high prey concentrations in order to survive at high temperatures. Sardines have an extensive distribution range from the waters off Ireland and the UK to northwestern Africa, experiencing a relatively broad temperature range. It is likely that the selective pressures acting to limit productivity at the higher latitudinal limit of distribution are more likely related to temperatures falling below tolerance thresholds of the species, whereas food availability probably plays a pivotal role influencing the upper energetically affordable tolerance limit of temperatures at the lower latitudinal limit. Therefore, future studies examining the influence of temperature on

the growth and survival of sardine larvae should take into account the combined influence of temperature and food availability, particularly if projections of global warming are associated with changes in plankton productivity.

Acknowledgements. This work was supported by Fundação para a Ciência e Tecnologia (FCT) as part of the project VITAL (Vital rates of pelagic fish larvae PTDC/MAR/111304/2009). Project MODELA (PTDC/MAR/098643/2008) partially supported this work. S.G. was partially supported by FCT through 2 post-doctoral fellowships (SFRH/BPD/38332/2007 and SFRH/BPD/105419/2014). The microalgae *Rhodomonas baltica* was provided by the Assemble Program (grant 227799) funded under the European Community – Research Infrastructure Action under the FP7 'Capacities'. E.S. was funded by project TOPCOP (CTM2011-23480). Thanks are due to A. Teodósio (CCMAR, Univ. Algarve) and all the team of the Oceanário de Lisboa for their collaboration as team of project VITAL. This work contributes to project UID/Multi/04326/2013 from the Portuguese Foundation for Science and Technology (FCT).

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Editorial responsibility: Stylianos Somarakis, Heraklion, Greece

Submitted: January 5, 2016; Accepted: August 25, 2016
Proofs received from author(s): October 7, 2016