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Effects of temperature, salinity and feeding frequency on growth and mortality of twaite shad (*Alosa fallax*) larvae

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ABSTRACT

Key-words: *clupeidae, anadromous, twaite shad, stocking, rearing*

Available knowledge on the ecological requirements of the twaite shad *Alosa fallax* larvae is limited, restricting the development of effective management and conservation measures for this anadromous clupeid in European rivers. In this study, the effects of water temperature, salinity and feeding frequency on *A. fallax* larval growth and mortality were evaluated. For a period of ten days after the onset of exogenous feeding, *A. fallax* larvae exhibited higher survival rates when subjected to salinities of 2.5 $g{\cdot}L^{-1}$ and 5 $g{\cdot}L^{-1}$, from trials conducted at 0 $g{\cdot}L^{-1}$, 2.5 $g{\cdot}L^{-1}$, 5 $g{\cdot}L^{-1}$, 10 $g{\cdot}L^{-1}$, 15 $g{\cdot}L^{-1}$, 20 $g{\cdot}L^{-1}$. Higher food availability resulted in higher larval growth and survival rates during this period. Water temperature effects on larvae growth and survival was evaluated for a period of three months after hatching. *Alosa fallax* larvae exhibited higher growth and survival rates when subjected to temperatures of 24 °C and 28 °C, in contrast to trials conducted at 20 °C. These results are compared to other *Alosa* spp. and considerations on conservation measures are discussed in light of the results.

RÉSUMÉ

Effets de la température, la salinité et la fréquence d'alimentation sur la croissance et la mortalité des larves d'alose feinte (*Alosa fallax*)

Mots-clés : *clupeidae, anadrome, conservation, empoissonnement, élevage*

Les connaissances disponibles sur les exigences écologiques des larves d'alose feinte *Alosa fallax* sont faibles, limitant le développement des mesures de conservation et de gestion efficace pour ce clupéidé anadrome des rivières européennes. Dans cette étude, les effets de la température de l'eau, de la salinité et de la fréquence d'alimentation sur la croissance et la mortalité des larves d'*A. fallax* ont été évalués. Pendant une période de dix jours après le début de l'alimentation exogène, les larves d'*A. fallax* ont montré des taux de survie plus élevés lorsqu'elles sont soumises à des salinités de 2,5 $g{\cdot}L^{-1}$ et 5 $g{\cdot}L^{-1}$, à partir d'essais menés à 0 $g{\cdot}L^{-1}$, 2,5 $g{\cdot}L^{-1}$, 5 $g{\cdot}L^{-1}$, 10 $g{\cdot}L^{-1}$, 15 $g{\cdot}L^{-1}$, 20 $g{\cdot}L^{-1}$. Une plus grande disponibilité des aliments a entraîné une croissance des larves et des taux de survie plus

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élevés au cours de cette période. Les effets de la température de l'eau sur la croissance des larves et la survie ont été évalués pour une période de trois mois après l'éclosion. Les larves d'*Alosa fallax* montrent une croissance plus élevée et des taux de survie meilleurs lorsqu'elles sont soumises à des températures de 24 °C et 28 °C, contrairement aux essais réalisés à 20 °C. Ces résultats sont comparés à ceux d'autres *Alosa* spp. et les considérations sur les mesures de conservation sont discutées à la lumière des résultats.

INTRODUCTION

The twaite shad *Alosa fallax* (Lacépède, 1803) is an anadromous clupeid widespread along the Northeastern Atlantic coast, from Morocco to the Baltic Sea, and throughout the Mediterranean Sea (Quignard and Douchement, 1991). This species is in decline in most European streams (Aprahamian *et al.*, 2003), eventhough some twaite shad populations have recovered in several estuaries (Lopéz *et al*., 2007; Magath and Thiel, 2013). Despite recent twaite shad populations increases human impacts remain unsolved, such as limited stream connectivity, narrowing of spawning grounds and general modification of river hydrology (Collares-Pereira *et al.*, 2000). The combined effect of these impacts imposes significant pressures on the freshwater phases of anadromous fish life cycles, ultimately compromising recruitment (McDowall, 1992; McDowall, 1999). In addition, deterioration of water quality and overexploitation may further worsen the condition of weakened stocks (Maes *et al.*, 2008).

Given the current population trends of anadromous fish throughout the northern hemisphere (Limburg and Waldman, 2009), the necessity for, and implementation of mitigation measures directed towards the conservation of *Alosa* species are becoming increasingly common (Waldman and Limburg, 2003; Greene *et al.*, 2009). As so, understanding the environmental factors that mediate larval fitness is of prime importance to identify and modulate the ecological constraints underlying anadromous species decline (Jonsson *et al.*, 1999). An increasing need to optimize larvae rearing also arises from the improvement of fish condition required to maximize effective recruitment when stocking programs are implemented (Fushimi, 2010; Brown and Day, 2002; Hendricks, 2003). Despite extensive literature on the effects of the environmental factors conditioning *Alosa* spp. larval development (Esteves, 2011) little information is specifically available for *A. fallax* (Esteves and Andrade, 2012). Water temperature, salinity and prey availability were recognized as important factors for twaite shad larvae abundance (Esteves, 2011), however no effects on larvae growth and mortality were studied.

The current study aimed to evaluate the effects of water temperature, salinity and feeding frequency on *Alosa fallax* larval growth and mortality under controlled conditions. Firstly, it was evaluated the influence of salinity and feeding frequency on larval growth and mortality after first feeding. Secondly, it was compared growth and mortality rates under different temperature treatments. The findings presented here, provide baseline information required to develop adequate measures for the successful management and conservation, such as captive rearing of twaite shad larvae and juveniles.

MATERIALS AND METHODS

Two sets of experiments were performed. The first experiment evaluated the combined effects of salinity and feeding frequency on larval growth and mortality for a short period after the onset of exogenous feeding. The second experiment assessed the effects of water temperature on growth and mortality from the embryo to the juvenile stage.

Twaite shad eggs were obtained from five females, fertilized by a dry method with sperm collected from five males, all captured while spawning in early June 2011 in the Guadiana river, Portugal (Lat: 37°40′52.45"N, Long: 7°39′43.25"W). An hour after collection, the eggs

were placed in cylindrical-conical incubators under laboratorial conditions, exposed to the natural diel photoperiod. Fertilization rate was estimated 24 h after collection from a sample of approximately 7000 eggs.

For the first experiment, a batch of eggs was maintained in a cylindrical-conical incubator until hatching. Larvae were then transferred to a general rearing aquarium kept at a temperature of 22 °C (±1 °C) and salinity of 0 $g{\cdot}L^{-1}$. From this aquarium, ten larvae were distributed to each of the experimental aquaria (n = 36, 1 L) upon the onset of exogenous feeding (3rd day after hatching). Testing of salinity (0 $g{\cdot}L^{-1}$, 2.5 $g{\cdot}L^{-1}$, 5 $g{\cdot}L^{-1}$, 10 $g{\cdot}L^{-1}$, 15 $g{\cdot}L^{-1}$, 20 $g{\cdot}L^{-1}$) and feeding frequency (once a day, 1 × day^{-1}, and three times a day, 3 × day^{-1}) was performed with three replicates per combined treatment. Desired salinities were obtained by dissolution of sea salt, larvae were fed *ad libitum* with freshly-hatched *Artemia fransciscana* nauplii and water temperature was controlled at 24 °C (±1 °C). Larvae were maintained under these conditions from the onset of exogenous feeding until the 10th day after the start of the experiment. Mortality was registered daily for each replicate and total length (L_T ± 0.1 mm) of the surviving larvae was measured on the 10th day. Measurements took place under a microscope with an incorporated micrometric scale.

In the second experiment, approximately 1000 eggs were transferred to an incubator jar installed inside each rearing aquarium (n = 12, 100 L). This process took up to six hours past egg fertilization. Water temperature in the aquaria was controlled at 20 °C, 24 °C and 28 °C (±1 °C) in groups of four replicates per temperature treatment. After hatching, salinity was gradually raised from 0 $g{\cdot}L^{-1}$ to 4 $g{\cdot}L^{-1}$ (1 $g{\cdot}L^{-1}{\cdot}day^{-1}$) for all replicates. Larvae were initially fed three times a day (morning, noon, evening) with approximately 10^6 freshly-hatched *Artemia franciscana* nauplii per aquarium. Weaning began at the 45th day after hatching with 0.5 mm pellets (Skretting Gemma Wean Diamond) and was extended until the 75th day. From the 75th day onwards larvae were fed solely on this formulated diet, six times a day. During the first month post hatching (Period I), 20 larvae were measured (L_T ± 0.1 mm) from each replicate every other day. For the second and third months post hatching (Period II), 20 larvae were measured per replicate every two weeks. Larvae were anesthetized prior to measuring in a 0.5 $mL{\cdot}L^{-1}$ solution of clove oil in order to reduce stress and improve measurement accuracy.

> DATA ANALYSIS

For the first experiment, L_T attained at the 10th day after first feeding was compared using non-parametric statistics, as data did present neither a normal distribution nor homogeneity of variances, even after $\log_{10}$ transformation (Zar, 1999). In each feeding frequency treatment, a Kruskal-Wallis test was performed to compare differences between sizes (L_T) among salinity treatments, and was followed by a Dunn test as post hoc comparison (Zar, 1999). Mann-Whitney tests were run to compare differences between sizes (L_T) between the two feeding frequencies in each salinity treatment (Zar, 1999). Mean treatment mortality rates were calculated as the number of surviving larvae relatively to the initial number of larvae in each aquarium. Differences between mortality rates among salinity treatments for each feeding frequency were accessed through survival curves comparison using the logrank test, a modification of Gehan-Wilcoxon test. Analysis was performed using "survival" R-package (function = survdiff, rho = 1) (R-project).

For the second experiment, linear regressions were determined for each temperature treatment between attained L_T and time (days after fertilization) for each of the two growth periods (Period I – first month; Period II – second and third months), considering the replicate data together. Linear regressions within each period were compared across temperature treatments by means of an ANCOVA. Normality and homoscedasticity were tested, using Shapiro-Wilk and Levene tests respectively (Zar, 1999). Mean mortality rates were determined for each temperature treatment at the end of the experiment by determining the number of surviving larvae relatively to the estimated number of hatched larvae (number of eggs × fertilization rate). Differences between observed mortality rates were accessed using a χ^2 test. All data and statistical analysis were performed using R software (R-project).

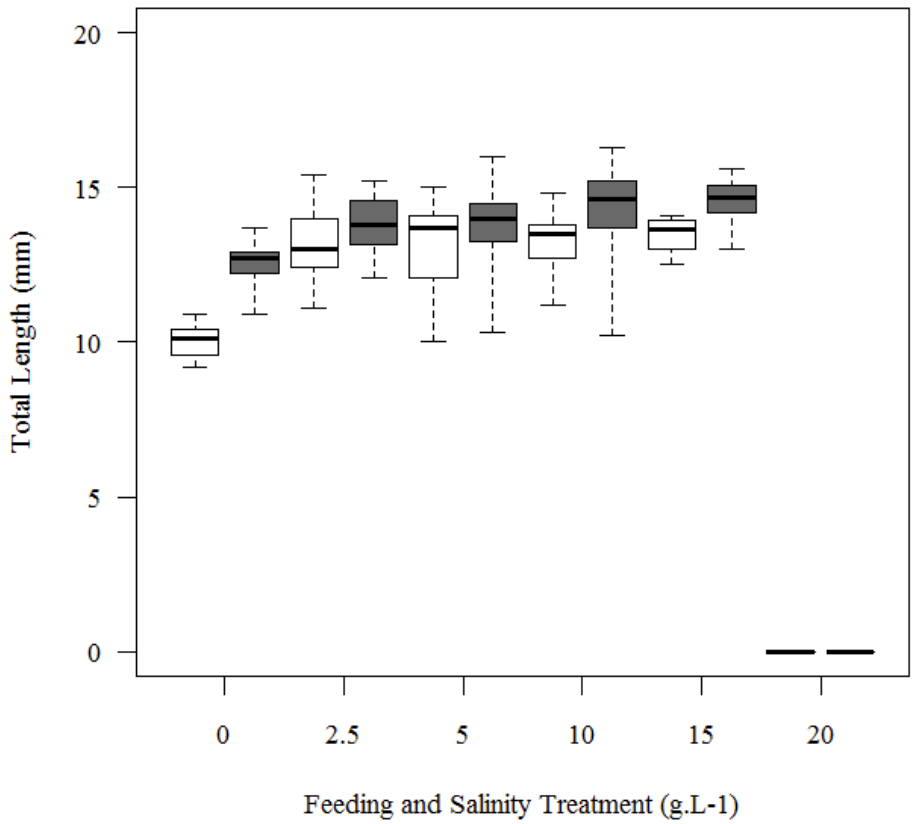

Figure 1
Box-plots with mean total length (L_T, mm) attained by Alosa fallax larvae at the 10th day after first feeding when fed once a day (white) and three times a day (grey) per salinity treatments; box represent the upper and lower quartiles and lines represent minimum and maximum values; 20 g·L^{-1} treatment not represented due to high mortality rates.

RESULTS

Larval total length varied significantly according to salinity in both feeding tests: 1 × day^{-1} (Kruskal-Wallis test, $\chi^2 = 40.771$, $P < 0.001$, *d.f.* 4) and 3 × day^{-1} (Kruskal-Wallis test, $\chi^2 = 28.909$, $P < 0.001$, *d.f.* 4) (Figure 1). Larvae fed 1 × day^{-1} at 0 g·L^{-1} were significantly smaller than those subjected to 2.5 g·L^{-1}, 5 g·L^{-1}, 10 g·L^{-1} (Dunn test, $Q > 3.291$, $P < 0.01$, *d.f.* 5) and 15 g·L^{-1} (Dunn test, $Q > 2.807$, $P < 0.05$, *d.f.* 5). Larvae fed 3 × day^{-1} at 0 g·L^{-1} were also significantly smaller than those kept at 2.5 g·L^{-1}, 10 g·L^{-1}, 15 g·L^{-1} (Dunn test, $Q > 3.291$, $P < 0.01$, *d.f.* 5) and 5 g·L^{-1} (Dunn test, $Q > 2.807$, $P < 0.05$, *d.f.* 5). Fish larvae fed less frequently presented significantly smaller mean L_T at all salinity treatments (Mann-Whitney test, $P < 0.05$) except for 5 g·L^{-1} (Mann-Whitney test, $U = 200.5$, $P > 0.05$).

Mortality was found to differ significantly among salinity treatments for both feeding frequencies (1 × day^{-1}, $\chi^2 = 220$, $P < 0.001$, *d.f.* 5; 3 × day^{-1}, $\chi^2 = 227$, $P < 0.001$, *d.f.* 5). Mean mortality rates generally increased with salinity regardless of feeding frequency, the exception being larvae kept at 0 g·L^{-1} that presented higher mortality rates (≈40.0%) than those kept at 2.5 g·L^{-1} (≈10.0%) and at 5 g·L^{-1} (≈30.0%, Figure 2). A salinity of 20 g·L^{-1} was lethal (100.0%) in a couple of days after exposition. Higher feeding frequency lead to lower mortality rates particularly at 15 g·L^{-1} (Figure 2).

The three different temperature treatments resulted in significantly different growth rates in both Period I and Period II (Table I, Figure 3). During Period I, larvae subjected to 20 °C exhibited lowered growth rates in comparison to those exhibited by larvae subjected to 24 °C (ANCOVA, $F = 4047$, $P < 0.001$, *d.f.* 1, 1161) and 28 °C (ANCOVA, $F = 4620$, $P < 0.001$, *d.f.* 1, 1229). In Period II, this pattern was similar with larvae reared at 20 °C exhibiting lower

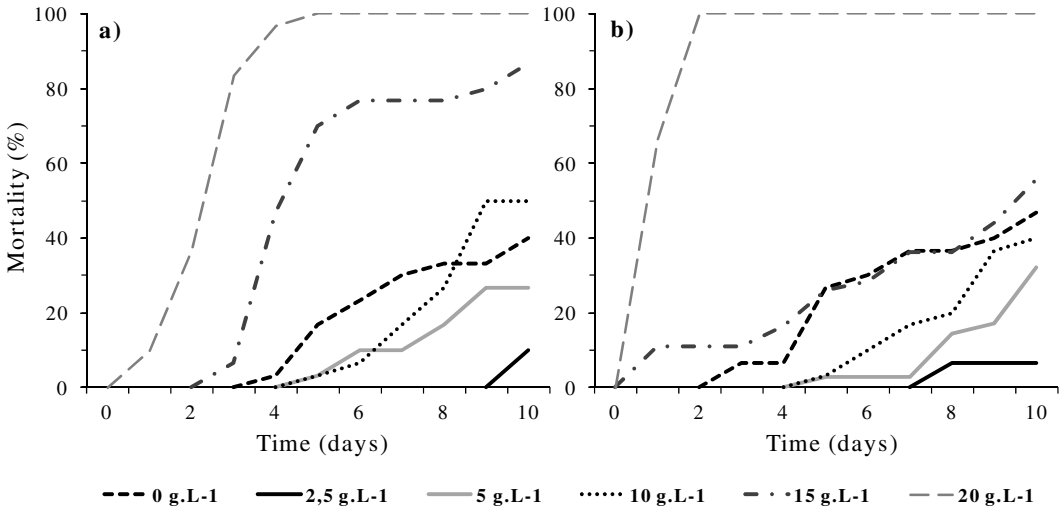

Figure 2
Mean cumulative mortality (%) for the two different feeding frequencies treatments (a – once a day; b – three times a day) per salinity treatments: 0 g·L^{-1} – black dashed line; 2.5 g·L^{-1} – black solid line; 5 g·L^{-1} – grey solid line; 10 g·L^{-1} – black dotted line; 15 g·L^{-1} – black dotted-dashed line; 20 g·L^{-1} – grey dashed line.

Table I
Growth rates determined from linear relationship between Total Length (L_T, mm) and Time (T, days) for the three temperature treatments in the two different periods: $L_T = a + bT$, where "b" is the slope and "a" is the intercept; R^2 is the explained variation; values of the T-test (t) and associated p-values (P).

Period	Temperature	*a*	*b*	R^2	*T*	*P*
I	20 °C	7.06	0.20	0.68	32.02	0.00
	24 °C	6.79	0.39	0.87	67.19	0.00
	28 °C	6.55	0.40	0.88	70.60	0.00
II	20 °C	12.46	0.12	0.23	5.73	0.00
	24 °C	12.23	0.20	0.45	15.61	0.00
	28 °C	12.09	0.18	0.44	15.81	0.00

growth rates than larvae reared at 24 °C (ANCOVA, $F = 279$, $P < 0.001$, *d.f.* 1, 415) and 28 °C (ANCOVA, $F = 282$, $P < 0.001$, *d.f.* 1, 431). Growth rates between treatments at 24 °C and 28 °C were significantly different for Period I (ANCOVA, $F = 9475$, $P < 0.001$, *d.f.* 1, 1434) and Period II (ANCOVA, $F = 492$, $P < 0.001$, *d.f.* 1, 622) (Table I). There was a reduction on growth during Period II relatively to Period I (Figure 3, Table I). Mean mortality observed at the end of the experiment was significantly different among temperature treatments ($\chi^2 = 415.822$, $P < 0.001$, *d.f.* 2), being similar for individuals kept at 24 °C (71.2% ± 11.0%) and 28 °C (72.6% ± 6.7%) while much higher at 20 °C (98.3% ± 0.6%).

DISCUSSION

The results obtained with the present experimental work provide the first insight on the growth and mortality responses of larval *A. fallax* when subjected to distinct temperature, salinity and feeding frequency treatments under laboratorial conditions. Overall, these results were in accordance with available information for other North American (*e.g.* Leach and Houde, 1999; Jia *et al.*, 2009) and European *Alosa* species (*e.g.* Aprahamian and Aprahamian, 2001; Bardonnet and Jatteau, 2008). Young *A. fallax* larvae were able to withstand environments with salinities ranging from 0 g·L^{-1} up to 15 g·L^{-1}, thriving at 2.5 g·L^{-1} while being unable to survive at 20 g·L^{-1}. Food availability improved growth and survival of *A. fallax* larvae, while lower water temperatures (20 °C) led to the reduction of both growth and survival.

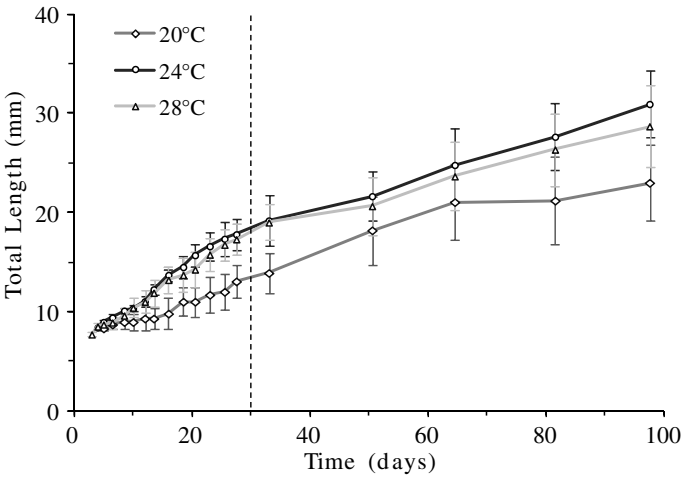

Figure 3
Mean total length (L_T, mm) attained by Alosa fallax larvae along the 90 days experiment for each treatment temperature (20 °C – diamond, dark grey line; 24 °C – circle, black line; 28 °C – triangle, light grey line; bars represent standard deviations); vertical dashed line represents the beginning of Period II.

Alosa fallax mainly spawns in tidal freshwater, where both eggs and young larvae may passively drift to oligohaline and mesohaline environments (Caswell and Aprahamian, 2001; Esteves and Andrade, 2008). During the downstream drift, larvae face an increasing salinity gradient that can exert a significant environmental pressure when functional osmoregulation is not yet fully developed (Limburg and Ross, 1995; Bardonnet and Jatteau, 2008). In fact, young *Alosa* spp. larvae are known to be unable to osmoregulate at sea salinity (Zydlewski and McCormick, 1997; Leguen *et al.*, 2007) and herein a salinity of 20 g·L^{-1} was lethal to young *A. fallax* larvae in a couple of days after exposition. Conversely, the lowest mortality rates for young *A. fallax* larvae were observed at oligohaline environments (2.5 g·L^{-1} and 5 g·L^{-1}) supporting the hypothesis that these environments are more suitable to this species. The reduced osmotic pressure difference between the larvae and its environment might enable a higher investment in development rather than osmoregulation, increasing larvae condition and survival (Limburg and Ross, 1995). The higher *Alosa* spp. larvae abundances observed by Esteves and Andrade (2008) in the oligohaline and mesohaline sections of the Guadiana estuary support these results. However, *A. fallax* larvae presented clear differences in salinities optimum relatively to other *Alosa* spp., with *A. alosa* and *A. sapidissima* larvae optimum closer to 10 g·L^{-1} (Limburg and Ross, 1995; Bardonnet and Jatteau, 2008). The observed salinity optima differences for larvae growth and survival could result from species specific physiologic development. However, distinct methodological approaches were used between the current study and others previously mentioned. For instance, Bardonnet and Jatteau (2008) analyzed different larval stages and had a different experimental set regarding exposure time and salinity ranges.

Food availability and quality are recognized as utmost important aspects to improve fish larvae growth and survival (Houde, 2008; Fushimi, 2010). Concordantly, in the current study, increasing feeding frequency enhanced both growth and survival of *A. fallax* larvae. The observed differences in mortality between feeding treatments were greater at a higher salinity (15 g·L^{-1}). When subjected to this condition, a higher feeding frequency greatly improved young *A. fallax* larvae survival, providing indirect evidence of the high energetic requirements of osmoregulation, and the dependence of fish on a sure exogenous energy intake during early life (Wiggins *et al.*, 1985; Johnson and Dropkin, 1995). Due to *Artemia* spp. nauplii limited survivability in freshwater (Lavens and Sorgeloos, 1996), at 0 g·L^{-1} larval growth was hampered by low prey availability (live nauplii) when in comparison to higher salinity treatments.

Alosa spp. larvae fish growth is intrinsically dependent on temperature as it is a determining factor of poikilothermic metabolic rates (Aprahamian and Aprahamian, 2001). In fact, twaite

shad larvae growth rates seemed to increase with increasing water temperature (Figure 3), however some of the observed growth rate differences (24 °C and 28 °C) are not biologically meaningfull. The results seem to suggest that *A. fallax* larvae have a thermal optimum (≈24 °C) above the most commonly experienced temperatures during the spawning season in the Guadiana and other rivers (Gerkens and Thiel, 2001; Esteves and Andrade, 2008). As the spawning season progresses (from March to June) later larvae cohorts may benefit from increasing water temperature. From RNA/DNA ratios, Esteves *et al.* (2009) found that metabolic activity is potentiated by the higher temperatures to which *A. fallax* larvae are exposed later in the spawning season, which also potentiate general ecosystem productivity providing larvae with a higher abundance of resources (Crecco and Savoy, 1985). In contrast, Esteves and Andrade (2012) observed that the highest growth rates for *A. fallax* larvae occurred early in the spawning season, suggesting that maybe only the fastest growing larvae survive during this period. According to Perez and Munch (2010), faster growing larvae tend to present higher individual fitness given that developed fish are able to swim, feed and avoid predation more efficiently. These aspects should be further investigated in the light of the results here presented for the population of the Guadiana river. Considering that this river shelters the southernmost Atlantic populations of the European *Alosa* spp. (Quignard and Douchement, 1991), it would be of particularly interest to assess possible thermal optima shifts relatively to Northern Atlantic populations (Houde, 1989; Arendt, 1997).

The effects of salinity, temperature and feeding on *A. fallax* larvae growth and mortality are unlikely to be shaped by major methodological shortcomings. Despite the high mortality registered during larval development relatively to other *ex situ* studies (*e.g.* Howey, 1985), clearly different responses to the tested factors were observed among treatments. Some methodological limitations such as timing of weaning and food type probably led to increased mortality, but these effects were constant among treatments. Smaller pellet sizes and delayed weaning should be considered in future research studies aiming for higher larvae survivability. Additionally, comparing lengths between deceased and live fish should provide an insight on the hypothesis that faster growth would result in increased survivability (Houde, 2002). In the current study, only young larvae tested for their responses to different salinity and food availability treatments and future studies should be extended to comprise older larval stages (*e.g.* Leguen *et al.*, 2007). Nonetheless, the effects of these two factors are likely to most strongly influence larvae growth and mortality at an early age, when the osmotic and digestive systems are still developing (Lavens and Sorgeloos, 1996; Zydlewski and McCormick, 1997).

The early life stages are considered as the most critical in fish development, when environmental conditions have greater influence on larval growth and survival (Blaxter, 1974; Houde, 1987). Anadromous *Alosa* spp. larvae and juvenile stages are bound to occur in upper estuaries, in tidal freshwater and brackish waters (Esteves, 2011). However, these environments are subjected to high anthropogenic intervention and, in the Guadiana catchment, the increasing water demand for human consumption has been exacerbating the impacts on the estuarine system (Collares-Pereira *et al.*, 2000; Costa *et al.*, 2001). The recent human derived changes have altered water thermal and haline regimens and consequently the estuary production and food availability patterns as a result of river flow regulation (Morais *et al.*, 2009). These changes in water parameters could have significant effects on anadromous species effective recruitment as suggested by Esteves *et al.* (2009) and supported by current results. To improve the condition of nursery habitats, ecological flows should be implemented in order to achieve a balance between leading environmental factors, such as temperature and salinity, and enhance *A. fallax* recruitment (Chícharo *et al.*, 2006). These ecological flows should enhance twaite shad recruitment, by increasing water temperature (≈24 °C) and extending mesohaline section of the estuary (≈5–18 $g \cdot L^{-1}$), and consequently increasing larvae growth rates and reduce fish mortality. The results here presented come as a much needed complement to the ecological knowledge on this species, providing also some baseline information that might be useful for future rearing programs as potential measures for the conservation of this threatened anadromous species (Philippart, 1995; Sarrazin and Barbault, 1996).

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