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The role of salinity in shaping the early life stages of *Aphanius fasciatus*

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ABSTRACT

Salinity is a key environmental factor shaping the physiology, reproduction, and survival of estuarine and coastal fish species. *Aphanius fasciatus*, a euryhaline killifish inhabiting Mediterranean transitional water ecosystems, is listed in Annex II of the European Habitat Directive due to its ecological significance. Although highly tolerant to salinity fluctuations, its presence in oligohaline waters is limited. This study investigates the effects of salinity on *A. fasciatus* reproductive and early developmental stages by assessing (1) egg deposition rates, (2) hatching success across a salinity gradient, (3) egg mortality across a salinity gradient, and (4) larval survival probabilities. Results revealed that egg mortality is statistically higher and fry survival rate statistically lower in oligohaline conditions. Our findings contribute to understand how salinity influences reproductive success and early-stage survival, providing valuable insights into the species' ecological resilience. This knowledge is essential for conservation strategies in transitional waters, where salinity fluctuations are intensified by climate change. The discussion supports the use of ecological systems modelling as a valuable tool for studying and managing complex systems, such as transitional aquatic environments.

1. Introduction

Salinity is a major environmental factor influencing aquatic organisms' physiology, reproduction, and survival (Alkhamis et al., 2023; Arafat et al., 2023; Nielsen et al., 2003; Pistole et al., 2008). Variations in salinity can induce significant changes in growth rates (Liu et al., 2024), reproductive success, and overall fitness, potentially affecting population dynamics (Altavilla et al., 2025; Dawood et al., 2023; Vlahos et al., 2023). With the ongoing rise in global temperatures, climate change exacerbates salinity fluctuations through increased evaporation and altered precipitation patterns (Pfleiderer et al., 2019).

Even though environmental instability may drive epigenetic modifications facilitating phenotypic plasticity in the long term (Abdelnour et al., 2024), rapid shifts in salinity regimes may compromise organismal fitness, population abundance, and stability, ultimately affecting ecosystem resilience (DeYoe et al., 2023; Scapin et al., 2019). This is particularly relevant in coastal and transitional water ecosystems, which are naturally exposed to high variability in abiotic conditions, further intensified by climate-driven factors such as sea-level rise and extreme rainfall events (de Azevedo et al., 2023; Adams et al., 2023; Palmer et al., 2011).

Although many species inhabiting these environments exhibit euryhaline adaptations, their ability to tolerate fluctuations does not necessarily imply immunity to acute environmental stressors (Altavilla et al., 2025), particularly during early life stages. Salinity shapes early developmental processes, influencing egg fertilisation, yolk sac absorption, and larval growth (Bœuf and Payan, 2001). Despite this, estuaries and coastal lagoons often provide favourable conditions for the reproduction and early development of many fish species due to abundant food resources, sheltered habitats essential for larvae/juveniles survival, and suitable salinity gradients. Several studies have demonstrated that intermediate salinity levels can enhance growth rates, likely due to reduced energy costs associated with osmoregulation [see Bœuf and Payan (2001) and cited literature].

Understanding the effects of salinity on survival and growth is particularly relevant for euryhaline species inhabiting transitional waters, where salinity naturally fluctuates (Dubey et al., 2016). Among these, the Mediterranean killifish *Aphanius fasciatus* (Valenciennes, 1821) has gained attention not only for its adaptability to coastal lagoons and salt marshes but also for its potential as an ecological indicator species (Altavilla et al., 2025; Facca et al., 2020), a sentinel species (Lionetto et al., 2023), and as a biological control agent for mosquito

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larvae (*Aedes caspius*, *A. detritus*) in brackish water environments (Veronesi et al., 2023). While this species exhibits a high salinity tolerance, its reproductive success might not be uniform across the entire salinity range, and its presence in oligohaline waters is notably limited. To date, the absence of *Aphanius fasciatus* in oligohaline environments is not attributed to abiotic factors such as salinity but it is primarily linked to competition with the invasive species *Gambusia holbrooki*, which thrives in low-salinity habitats and exhibits both aggressive behaviour and trophic dominance over native species (Rincón et al., 2002).

In line with this, a recent study by Altavilla et al. (2025) demonstrated that *A. fasciatus* successfully courts and spawns across a broad salinity spectrum (5, 15, 30, and 45 PSS), with no significant differences observed in courtship behaviour or egg deposition rates. However, hatching success, larval survival, and growth in oligohaline environments remain unexplored variables.

In the context of climate change, marinization due to sea level rise contrastsw with extreme rainfall events and water dulcification, underlining the need to fill this knowledge gap.

To fully understand the ecological dynamics of *Aphanius fasciatus* under salinity fluctuations, this research investigates 1) the effect of salinity on eggs deposition rates, 2) eggs mortality across a salinity range 3) hatching success from oligohaline to hypersaline condition, and 4) larval survival probabilities under the salinity levels tested. We hypothesise that while spawning may occur across a wide salinity range, early developmental success might be compromised in extreme salinity conditions.

2. Material and methods

2.1. The studied species

The Mediterranean killifish *Aphanius fasciatus* is a small cyprinodont species commonly found in coastal lagoons and river mouths across the Mediterranean region (Bianco, 1995). It is classified as an estuarine resident fish and is listed in Annex II of the European Habitat Directive (92/43/CEE) as a species of community interest, requiring the establishment of special conservation areas (Cavraro et al., 2011; Franco et al., 2006; Franzoi et al., 2010). Although *A. fasciatus* demonstrates a broad tolerance to varying physico-chemical conditions, it shows a preference for brackish and hyperhaline environments influenced by tidal fluctuations (Cavraro et al., 2014; Kessabi et al., 2010; Leonardos and Sinis, 1998; Triantafyllidis et al., 2007). Environmental factors play a crucial role in shaping its life history traits and influence the investment in secondary sexual characteristics (Cavraro et al., 2013a). Existing literature provides preliminary insights into the reproductive behaviour of *A. fasciatus*, suggesting a polygynandrous mating system characterised by strong sexual selection and intense male-male competition (Altavilla et al., 2024; Cavraro et al., 2013b; Marconato, 1982; Grech and Schembri, 1993; Malavasi et al., 2010). Furthermore, individuals engage in reproductive behaviours from an early stage of life, displaying the full mating repertoire regardless of body size (personal communication).

2.2. Experimental design and procedure

Aphanius fasciatus individuals were collected in a marginal habitat of the Venice lagoon, naturally subjected to salinity fluctuations due to rainfall events and tides. Physico-chemical parameters at the sampling site were measured in situ using a multiparameter probe. During the reproductive period, recognisable by the males' intensified colouration and the appearance of the secondary sexual trait such as a black band on the caudal fin (Altavilla et al., 2024), salinity ranged from 28 to 31 pss. From June to September, water temperature ranged from 26 to 30 °C reaching its peak in August. Fish were collected using a passive capture method (baited fish traps) to prevent physical injuries and excessive stress. Individuals were placed in buckets equipped with airstones and transported to the Ca' Foscari University Zoology Laboratory within 15 min. Once in the laboratory, fish were sorted by sex and housed in eight 140 L aquariums at 30 pss, which corresponded to the salinity measured at the sampling location. Each aquarium contained 16 males and 12 females, selecting only females with a prominently swollen belly and males with pronounced breeding colouration to ensure mating rituals. Fish were housed following the method proposed by Altavilla et al. (2025), separating females from males using a glass divider with small perforations that allowed water and pheromone circulation within the aquarium system, while still preventing tactile interaction. This system maximises the likelihood of getting fish ready to mate due to the high pheromone levels in the water. Soft green turf (breeding patch) was added to each aquarium to recreate an ideal spawning surface.

Fish were acclimatised in the housing tanks for 24 h at 30 pss (control), and then salinity was adjusted over 48 h by adding either freshwater or hypersaline water incrementally to reach the target conditions in two aquariums for each treatment: 5 pss, 15 pss, 30 pss, and 45 pss. The photoperiod regime followed the natural cycle for the latitude and time of year (July 2023, 45°28′41.7″N 12°15′21.0″E), and fish were fed twice a day ad libitum (Prodac® Biogran Small BS250). After the 72h of acclimation period, the glass septa were removed to allow male–female interaction, and the fish were allowed to freely interact and reproduce for two days. After this period, all mature individuals were moved to different housing tanks and acclimatised to the sampling site's salinity and water temperature before being released at the collection location following the Italian national legislation criteria, which implements EU Directive 2010/63/EU on the protection of animals used for scientific purposes (Legislative Decree No. 26, 2014).

The breeding patches were checked twice a day for two days, and the collected eggs were placed in 50 mL beakers, partially covered to minimise water evaporation. A maximum of 20 eggs were transferred into each beaker and monitored until hatching, resulting in a total of 35 beakers for the 5 pss treatment, 65 for 15 pss, 60 for 30 pss, and 35 for 45 pss. Each beaker was considered as one replicate. Beakers were filled with the aquarium's water where spawning occurred, and laboratory temperature was set at 28 °C, which corresponded to the average temperature recorded during the sampling period. Hatching occurred between the 10th and the 14th day after spawning. Eggs that failed to hatch within 20 days from spawning and showed no signs of embryonic development, as well as those that turned white, were considered non-viable (Janhunen et al., 2023). Once hatched, *A. fasciatus* juveniles were moved to the housing tanks at the corresponding salinity, where they were fed ad libitum with specific small-grain food and monitored monthly for 200 days. All the procedures described above were followed while minimising direct contact with the eggs or individuals involved.

2.3. Data analysis

Statistical differences in the total number of spawned eggs across salinity treatments were not investigated since this variable has already been addressed in a recent study using more targeted methodologies and statistically robust approaches (Altavilla et al., 2025). The effect of salinity on reproductive performance and individual survival was investigated by considering the following variables: number of eggs laid (EL), number of dead eggs (DE), percentage of hatched eggs (HP), and fry survival rate (SR) (Table 1). The collected data were analysed within the R statistical environment (v. 3.6.0; R Core Team, 2019; www.r-project.org). To assess differences across salinity level, a linear mixed-effects model (lmer) was fitted using the nlme package (Pinheiro et al., 2022), including the random effect of the beaker in which the eggs hatched. When the assumption of homogeneity of variances was violated (Levene's test, $p < 0.05$), a heterogeneous variance structure (varIdent) was included in the mixed model to allow residual variances to differ across salinity levels (pss). This approach allowed each treatment group to have its own residual variance, thereby controlling for

Table 1
Analysed variables with corresponding label and description.

Analysed variable	Label	Description
Number of eggs laid	EL	Total number of spawned eggs in each treatment in 48 h
Number of dead eggs	DE	Total number of dead or unhatched eggs for each treatment
Percentage of hatched eggs	HP	(Total number of hatched eggs/Total number of spawned egg) * 100
Fry survival rate	SR	Fry condition (alive = 1, dead = 0) monitored monthly for 200 days
Day since deposition		Number of days elapsed since the spawning date

heteroscedasticity and improving the reliability of parameter estimates. Fry survival rate was measured by means of a Generalised Linear Mixed-Effects Regression (GLMER) with binomial distribution included in the lme4 R package (Table 2). To visualise the survival rate, fry survival probability was calculated from the fitted GLMER using the predict (.) function, and the survival probability curves was plotted.

3. Results

A total of 762 eggs were collected, with 5 and 45 pss groups showing lower number of spawned eggs (respectively 19.4 % and 17.6 % of the total) while 15 and 30 pss providing 63 % of the total collected eggs (Fig. 1).

Eggs mortality increases over time in all treatments with the 15 pss groups showing the best performance (Fig. 2a and b) and 5/45 pss treatment experiencing higher mortality rate, respectively 27 % and 32.1 % of the total spawned eggs. Significant statistical differences were found among groups in egg mortality (Linear Mixed Effects Model, **m1**, $P < 0.05$) where 15 pss treatment showed significant less mortality (12.1 %) compared to the other groups (Table 3). Mortality reached 21.2 % in the control group (30 pss).

Hatching percentage dropped considerably after 10 days from deposition in 5, 15, and 30 pss treatments, while remained almost constant at 45 pss treatment (Fig. 3b). Statistical differences were found among groups with 15 pss treatments showing significant higher hatching percentage (averagely 81 %), as shown in Fig. 3a and Table 4 (Linear Mixed Effects Model, **m2**, $P < 0.05$). Again, 5 pss treatment showed the lower performance (62 %) while hatching percentage reached 65 % in the control group and 67 % in the 45 pss treatment. The interaction term between salinity and days since deposition was not statistically significant in both DE and HP models,.

After 200 days, only 25 fry survived out of 425. The Generalised Linear Mixed-Effects Regression revealed a significant negative effect of time on fry survival rate ($P < 0.001$), and a statistically significant higher mortality at 5 pss compared to the other treatments (5–15: $P < 0.001$; 5–30: $P < 0.001$; 5–45: $P < 0.01$). The control group (30 pss) exhibited the best performance with highest overall survival rate. As shown in Fig. 4, survival probability declines markedly in the early larval stages across all salinity treatments, confirming the sensitivity of this period. However, 15, 30 and 45 pss groups' survival probabilities dropped approximately after 50 days post hatching, while 5 pss group exhibited a much earlier decline within the first few days after hatching.

Table 2
List of the models fitted with the corresponding random term and weighted argument (DE = Number of dead eggs HP= Percentage of hatched eggs, SR= Fry survival rate, PSS= Salinity).

Model label	Model Type	Model structure	Random Term	Weights Argument
m1	lmer	DE ~ PSS * day since deposition	Housing beaker	PSS
m2	lmer	HP ~ PSS * day since deposition	Housing beaker	PSS
m3	glmer	SR ~ PSS * day since deposition	Spawning date	

4. Discussion

The results of this study confirm that *A. fasciatus* is an euryhaline species capable of surviving, reproducing, and growing in a wide range of salinity conditions. Previous studies on *A. fasciatus* have demonstrated that this species is capable of displaying the full courtship repertoire and maintaining consistent spawning performance across different salinity conditions (Altavilla et al., 2025), as also observed in other studies on congeneric or ecologically equivalent species (Oltra and Todoli, 2000; Perschbacher et al., 1990). However, the presence of the species along the Italian coast seems to be confined to the coastal environment, and just a few cases of *Aphanius* populations inhabiting inland waters are reported. To the best of our knowledge, the unique stable populations in inland waters are reported in the Imera River in Sicily (Lo Duca and Marrone, 2009). In the Venice Lagoon environment, *A. fasciatus* appears to be confined to marginal habitats where, although salinity may fluctuate due to heavy rainfall or exceptional high tide events, salinity generally ranges between 15 and 35 pss. The species has not been recorded in lagoon areas with salinity levels below this range (Cavraro et al., 2014, 2017).

Up to date, the absence of *A. fasciatus* in oligohaline environments has been chiefly attributed to biotic interaction, such as competition with the alien species *Gambusia holbrooki* (Monti et al., 2021; Valdesalici et al., 2015), but the influence of salinity in the first life stage on the species was never investigated. Previous studies on *Fundulus grandis*, the ecological equivalent of *A. fasciatus* in the American salt marshes, revealed that low salinity affects egg fertilisation with implication on the first life stage of the population, consequently driving habitat colonisation (Ramee and Allen, 2016). In light of this, our results present novel findings of *A. fasciatus* reproductive output and offspring viability that could provide a new ecological perspective on the species' spatial distribution, explaining its absence in freshwater and in transitional water environments with salinity around 5 pss.

It has been demonstrated that hyposaline conditions might increase egg size due to osmotic pressure compromising embryonic development (Holliday and Blaxter, 1960). In the present study, egg diameter has not been measured to reduce egg manipulation, but it could be an interesting topic for future studies on the species. In species like *Fundulus heteroclitus* and *Dicentrarchus labrax*, similar patterns of increased larval mortality and reduced growth have been observed under low salinity conditions, further supporting the hypothesis that early developmental stages are particularly vulnerable to osmotic challenges (Morgan and Iwama, 1991; Brown et al., 2011). On the other side, hypersaline conditions might cause dehydration, affecting embryo vitality and hatching success (Rhody et al., 2010). The present study aligns with these findings, suggesting that despite the adult *A. fasciatus* resilience to salinity fluctuations, early life stages experience critical physiological constraints that likely shape habitat suitability and distribution. The lower reproductive success found at 5 and 45 pss can be linked also to the incomplete development of osmoregulatory organs during early life stages since larvae must rely on epidermal ionocytes for osmoregulation that are less efficient than gill (El-Leithy et al., 2019; Hiroi and McCormick, 2012; Varsamos et al., 2005; Guh et al., 2015).

All salinity treatments showed a general increase in egg mortality around day 8, with a particularly marked rise at 30 pss. The higher number of dead eggs observed in the 30 pss treatment may be explained by the fact that this group initially contained a considerably higher number of deposited eggs compared to the 5 and 45 pss groups. A higher initial egg count naturally increases the absolute number of non-viable eggs due to physiological attrition. Notably, this increase in mortality was not observed in the 15 pss treatment, despite a comparable initial egg count.

Despite *A. fasciatus* resiliency, the 15 pss treatments performed best

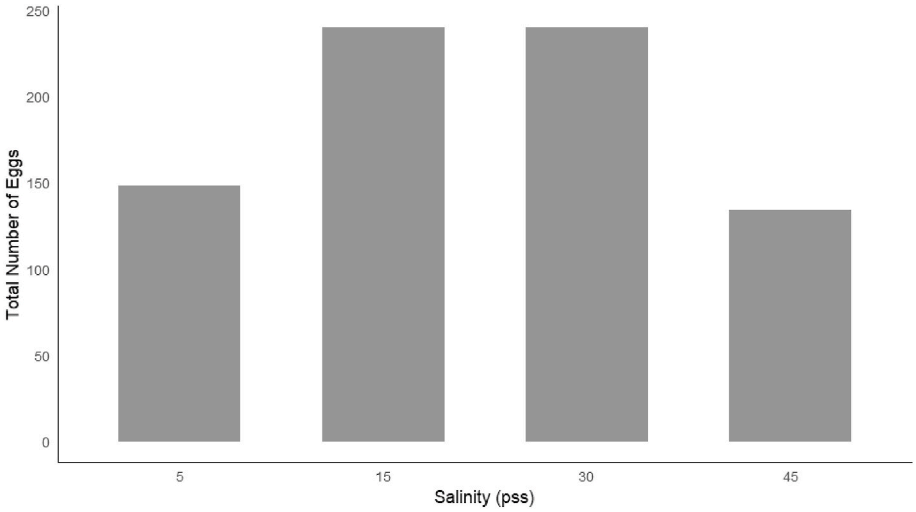

Fig. 1. Total number of laid eggs (EL) in the four analysed groups (5, 15, 30 and 45 pss).

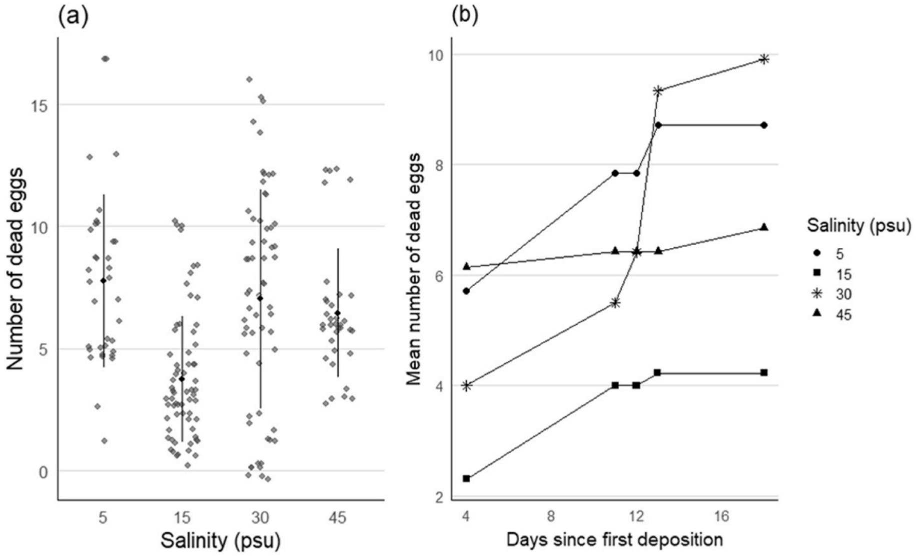

Fig. 2. (a) number of dead eggs, mean number of dead eggs and standard deviation in the 4 analysed groups (5, 15, 30 and 45 pss). Each dot represents a single replicate (becker). Black points indicate group means, and vertical bars represent standard deviations. (b) mean number of dead eggs from the spawning date (0) to 15 days after deposition at the four salinity treatments (5, 15, 30 and 45 pss).

Table 3
Results of the post hoc comparision between groups of the Linear Mixed Effects Model m1. Estimate value and P value are showed. Significant P values are highlighted in bold.

Treatments contrast (pss)	Estimate	P-value
5–15	3.554	**<0.001**
5–30	0.430	>0.05
5–45	1.314	>0.05
15–30	−3.124	**<0.001**
15–45	−2.240	**<0.01**
30–45	0.884	>0.05

in all the analysed variables, highlighting the optimal salinity window that minimises egg mortality, maximising hatching success. This result is consistent with Bœuf and Payan (2001), who showed how intermediate salinity maximises fish growth in euryhaline species. In agreement with the aforementioned findings, the fry predicted probability of survival dropped considerably in the 5 pss group in the early life stage, leading to zero soon after, probably explaining the absence of the studied species in oligohaline environments. 45 pss treatment also showed lower survival probability, even if not statistically significant, emphasising the importance of maintaining intermediate salinity habitats, which may serve as crucial nursery grounds.

Considering the complexity of the lagoon ecosystems and the variability driven by climate change, we highlight the need for analytical tools capable of integrating such findings into a broader framework. An important future direction in this context lies in applying ecological systems modelling as developed in the works of H.T. Odum and E.P. Odum. This approach allows for a visual and dynamic representation of ecosystem functioning through diagrams that include energy sources, transformations, storage, feedback, and losses. Applying this methodology to the case of resident species such as *A. fasciatus* would enable the construction of a dynamic model of reproduction and larval survival as a function of salinity, integrating variables such as temperature, predation, competition (e.g., with *Gambusia holbrooki*), and hydrological flows. Several pioneering studies have demonstrated how energy systems modelling can offer deep insights into the functioning of coastal and estuarine systems, especially when salinity is a key ecological driver

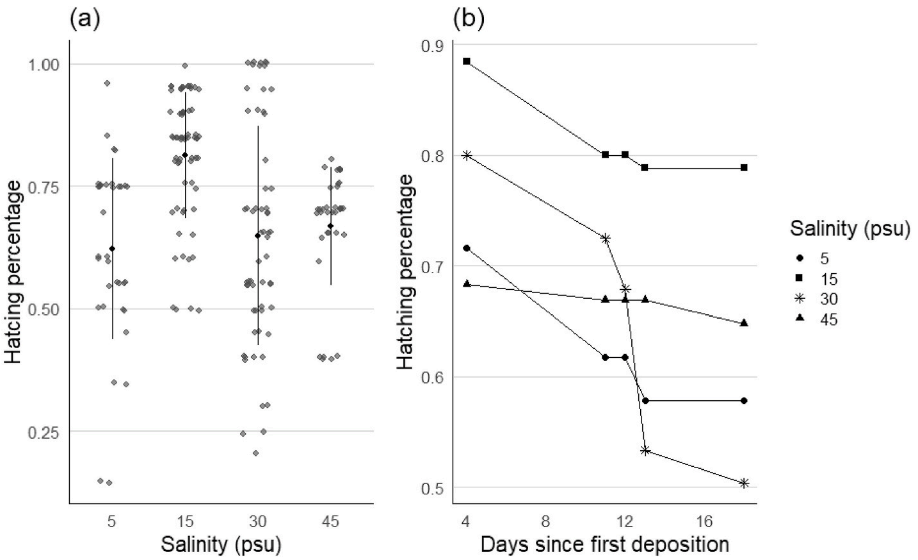

Fig. 3. a) hatching percentage, mean hatching percentage and standard deviation in the 4 analysed groups (5, 15, 30 and 45 pss). Each dot represents a single replicate (becker). Black points indicate group means, and vertical bars represent standard deviations. (b) mean hatching percentage from the spawning date (0) to 15 days after deposition at the four salinity treatments (5, 15, 30 and 45 pss).

Table 4
Results of the post hoc comparision between groups of the Linear Mixed Effects Model m2. Estimate value and P value are showed. Significant P values are highlighted in bold.

Treatments contrast (pss)	Estimate	P-value
5–15	−0.14870	**<0.01**
5–30	0.00926	>0.05
5–45	−0.04633	>0.05
15–30	0.15796	**<0.001**
15–45	0.10238	**<0.001**
30–45	−0.05559	>0.05

(Brown and Patterson, 2012; Odum, 1971, 1983; Odum and Odum, 2000). The energy systems approach allows us to visualise complex, nonlinear interactions among environmental drivers and biological responses, simulated and understood in a unified framework. Applying a similar framework to *A. fasciatus* would make it possible to simulate population responses to salinity fluctuations and test management interventions, such as habitat restoration or flow regulation. This is particularly relevant as Mediterranean coastal lagoons become increasingly vulnerable to climate-induced salinity fluctuations. Once translated into equations, such a model could be simulated to forecast future scenarios to predict the impact of prolonged hyposaline and hypersaline conditions on resident species, simulate population responses to salinity fluctuations, and test management interventions, such as habitat restoration or flow regulation.

5. Conclusion

This study highlights the role of *Aphanius fasciatus* as a valuable

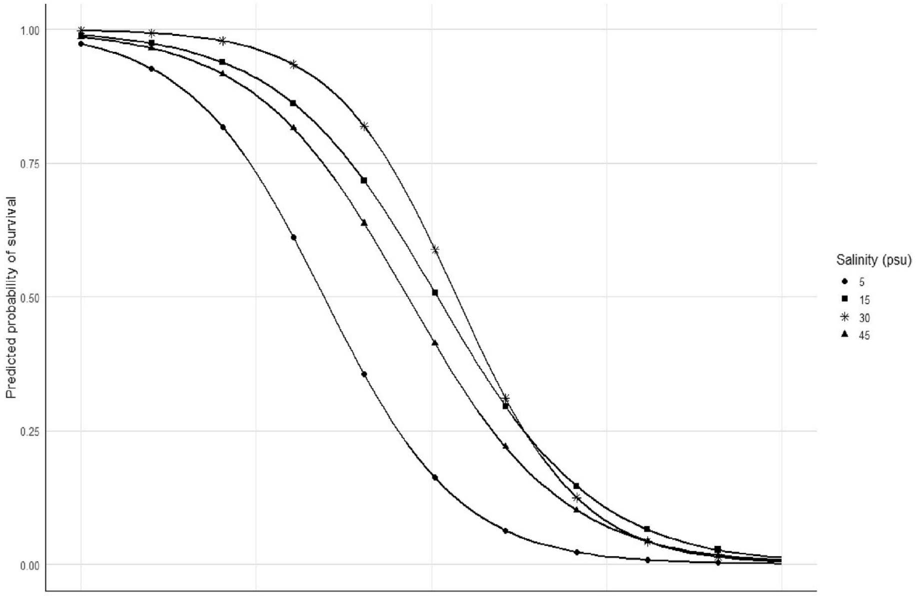

Fig. 4. Predicted probability of fry survival from 0 to 200 days after hatching at the 4 salinity treatments (5, 15, 30 and 45 pss).

model species for understanding the ecological effects of environmental dynamics in transitional waters. As a resident species, *A. fasciatus* can be a reliable indicator of habitat quality and a potential ecosystem monitoring and management tool. However, our results suggest the reconsideration of the species as a biological control agent. The poor larval survival observed in oligohaline waters indicates that *A. fasciatus* may struggle to maintain viable populations under competitive pressure in low-salinity environments, where *Gambusia* spp dominates. In light of this, habitat management strategies to preserve intermediate salinity zones become imperative to support developmental success of *A. fasciatus* and offer a buffer against the spread of invasive species.

CRediT authorship contribution statement

Luca Altavilla: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Federico Surra:** Writing – review & editing, Data curation, Conceptualization. **Chiara Facca:** Writing – review & editing, Formal analysis. **Francesco Cavraro:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Agostino Forlani:** Formal analysis. **Stefano Malavasi:** Writing – review & editing, Supervision, Methodology, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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