

RESEARCH ARTICLE

WILEY

Pushed to the edge: Environmental factors drive ecological responses of *Aphanius fasciatus* when in sympatry with invasive *Gambusia holbrooki*

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Abstract

1. Interspecific competition may be particularly exacerbated when occurring between native and invasive alien species, with environmental conditions affecting drivers of natural selection and shaping spatio-temporal dynamics of animal communities. The effects of invaders are particularly evident in aquatic ecosystems with delicate and complex balances, such as Mediterranean coastal wetlands, characterized by unpredictable water flows and variable reserves. Understanding how native species respond to human-induced biological invasions is of paramount importance for planning management measures aiming at conservation purposes.
2. This study investigated whether the occurrence of a competing invader species resulted in changes in the ecological adaptations of a native species by comparing two coastal wetland fish species: the invasive eastern mosquitofish *Gambusia holbrooki* and the native Mediterranean killifish *Aphanius fasciatus* of conservation concern. The hypothesis was tested by sampling fishes at a coastal Ramsar site in central Italy, over 2 years.
3. Generalized linear mixed models were used to examine the occurrence of killifish in relation to water parameters and the presence of its putative competitor. The aim was to determine whether salinity, temperature, and water depth influenced the presence and abundance of killifish when in sympatry with mosquitofish.
4. Both the probability of presence and abundance of killifish increased with water salinity, but this increase was stronger when mosquitofish was present. Increasing water temperature and water depth were associated with a higher probability of presence and abundance of killifish respectively, although the effect of water depth was small. Both salinity and the presence of the invader acted synergistically in characterizing and shaping the population structure of the native species.
5. Living in the most extreme and stressed environments, the killifish is potentially at higher risk of extinction. These results highlight the importance of considering the

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interplay between multiple abiotic stressors combined with comparative data between invasive and native species as increasingly critical to aid in conservation efforts.

KEYWORDS

brackish water, conservation, eastern mosquitofish, interspecific competition, invasive alien species, Mediterranean killifish, Ramsar, wetlands

1 | INTRODUCTION

In animal populations, interspecific competition is generated when the active demand by two or more species for the same limited resource (e.g. food, nest site, and/or space) occurs at the same time (Ward, Webster & Hart, 2006; Borcharding, Heubel & Storm, 2019; for fish species). Interspecific competition may be a particularly important limiting factor itself, shaping the drivers of natural selection by influencing the behaviour of individuals, health status, breeding success or fitness, and survival (Munday, 2001; Ward, Webster & Hart, 2006). Abiotic environmental conditions can further influence the outcome of competition, favouring the dominance of one species over the other(s) or generating alternative strategies/behaviours to meet the minimum requirements for survival (in the short term) and for maintaining the fitness (in the medium to long term). In the case of species with overlapping ecological niches, the absence or occurrence of competitors may induce differences in population density, distribution, habitat use, behaviour, and sex ratio (Bohn, Amundsen & Sparrow, 2008). The effects of these processes may be particularly exacerbated when occurring between native and invasive alien species, with the former generally less adapted to cope with the modified situation (Schopt Rehage, Lopez & Sih, 2020). The factors that promote coexistence of invaders and native species are poorly understood, as well as the role of abiotic factors in driving spatio-temporal dynamics of animal communities (Ho, Bond & Thompson, 2013). Non-native species, voluntarily or accidentally introduced into regions where they are not found naturally, can succeed in establishing themselves to such an extent that they become a threat to indigenous species (Gurevitch & Padilla, 2004; Azzurro et al., 2019). The latest Global Assessment Report of the Intergovernmental Science–Policy Platform on Biodiversity and Ecosystem Services identified invasive alien species as one of the top five global culprits driving negative change in nature around the world—with numbers having risen by 70% since 1970 (Brondizio et al., 2019). There is an extensive set of studies examining cases of introduction of alien species that have led to substantial changes in species assemblages and distribution (Azzurro et al., 2019; Yarra & Magoulick, 2019), to significant loss of biodiversity (Everett, 2000), damage to economic activities, or the spread of disease (Charles & Dukes, 2008). However, the effects of invaders are particularly worrying in freshwater and brackish water environments (Dudgeon et al., 2006; Miqueleiz et al., 2020), especially in systems where water availability can be a limiting resource (Smith & Darwall, 2006).

Wetlands, such as temporary marshes, ponds, ditches, slow-running streams, and canals, represent ecosystems with delicate and complex balances, characterized by seasonal variations in water provision, resulting in often unpredictable water flows and variable reserves (Gardner et al., 2015). For example, the Living Planet Index (Pollard et al., 2010; www.livingplanetindex.org) – an indicator of the state of the world's biological diversity based on population trends and adopted by the Convention of Biological Diversity – recorded a 40% decline for fish, amphibians, reptiles, and mammals when applied to Mediterranean wetlands (MWO2, 2018). In this context, understanding how native species respond to human-induced biological invasions, both from ecological and evolutionary points of view, is of paramount importance for planning adequate corrective management measures aimed at conservation of sensitive species. Moreover, investigating interactions between invasive and native species mediated by abiotic factors could be increasingly critical to aid in conservation efforts.

This study investigated whether the occurrence of a competing invader species changes the distribution patterns of a native species by comparing two coastal wetland dwelling fish species: the invasive eastern mosquitofish *Gambusia holbrooki* (hereafter 'mosquitofish') and the native Mediterranean killifish *Aphanius fasciatus* (hereafter 'killifish'). The mosquitofish is an invasive exotic euryhaline species native to North and Central America, intentionally introduced worldwide for the control of mosquito populations, and has become one of the most successful invasive fish in the world (Lowe et al., 2000). It is known to tolerate a wide range of environmental parameters, such as temperatures between 5°C and 38°C, low oxygen levels, and a broad range of salinity concentrations from 0‰ to 41‰ (Pyke, 2005; Kottelat & Freyhof, 2007). Similarly, killifish can establish viable populations between 4°C and 40°C and from freshwater environments (Gandolfi et al., 1991; Lo Duca & Marrone, 2009) to brackish environments characterized by shallow waters and with strong seasonal temperature and salinity ranges, such as lagoons, ponds, and estuaries (Maltagliati, 2002). The species is able to survive even in oxygen-poor waters characterized by higher salinity than that of seawater (hyperhaline waters), such as coastal salt pans where salt concentration can exceed 50–60‰ (Fastelli, Marcelli & Monti, 2012; Cavarro et al., 2014). Although there have been several studies on the interaction between mosquitofish and the iberian toothcarp *Aphanius iberus* (Rincón et al., 2002), no research has evaluated the effects of interspecific competition between mosquitofish and killifish in the wild, or the adaptive responses of the native species to the combined

effects of external factors, such as salinity, temperature and water depth. Previous studies, experimentally conducted in aquaria, showed how behavioural and competitive interactions between these two species might be mediated by salinity (Alcaraz, Bisazza & García-Berthou, 2008). By setting predefined salinity concentrations at 0‰, 15‰, and 25‰ in aquaria, the mosquitofish showed a reduced aggressiveness towards the native species and a lower predation capacity at higher salinity (Alcaraz & García-Berthou, 2007; Alcaraz, Bisazza & García-Berthou, 2008). In the wild, however, the interaction of a set of external variables can contribute not only to model species presence and distribution, but also to influence population parameters, such as sex ratio and fitness. In the wild, the range of salinity encountered in temporary marshes or ponds can span over greater gradients, from oligohaline and mesohaline waters (salinity >0.5‰ and 0.5–5‰ respectively) to extremely hyperhaline waters (up to 50‰). Similarly, other environmental factors, such as water temperature, depth of the water column, and seasonal rainfall regimes can undergo considerable changes during spring and summer (a period corresponding to the breeding season). This is especially true in coastal wetlands, which experience extremes in both intra- and interannual seasonality in water availability, sometimes at the risk of desiccation.

The occurrence of killifish in relation to water quality parameters and the presence of its potential competitor was investigated. The aim was to determine whether salinity, temperature, and water depth influenced killifish presence and abundance when in sympatry with mosquitofish. More specifically, the following hypotheses were tested: (i) An increase in salinity concentrations would favour the killifish, as found with indoor experiments by Alcaraz, Bisazza & García-Berthou (2008). If so, a higher probability of killifish presence is expected at higher salinity, and even more in hyperhaline waters, with salinity mediating habitat segregation in the two species. (ii) On the basis of the assumptions already outlined, an increase in killifish abundance with increasing salinity is also predicted. Then, under the general prediction that waters characterized by shallow depths and higher temperatures are also those mostly exposed to rapid evaporation (tending to hyperhaline waters), then shallow as well as warmer waters would also be expected to favour both the presence and abundance of killifish. Furthermore, as fish species can regulate population demography and life cycles according to local environmental conditions (Alcaraz & García-Berthou, 2007), abiotic factors combined with the co-occurrence of the invasive alien species could unbalance the typical sex ratio and population fitness of killifish, in terms of the proportion of adult males/females and juveniles respectively. When the putative competitor is absent, killifish females are generally more abundant than males (Leonardos & Sinis, 1999). Males, in fact, seem to suffer higher mortality during the breeding period probably owing to their brighter colours and striking courtship behaviour (Leonardos, 2008). This leads to two further hypotheses: (i) Low to medium salinity levels and the presence of mosquitofish should further disadvantage the males of killifish, being smaller than females and suffering more from competition by mosquitofish. This would significantly reduce their number, as well as newly hatched

juveniles, that would readily suffer predation. (ii) At higher salinity, when competition with mosquitofish is less pronounced, the sex ratio of killifish would shift in favour of males and would show a greater proportion of juveniles.

2 | MATERIALS AND METHODS

2.1 | Study species

The killifish (order Cyprinodontiformes) is a small fish, endemic to the central eastern Mediterranean (Bianco, 1995; Cavraro et al., 2011). It is included in Annex II of the European Habitats Directive (Council of the European Communities, 1992) as a 'species of community interest whose conservation requires the designation of special areas of conservation', and in Annexes II and III of the Bern Convention (Council of the European Communities, 1982) as a strictly protected species. The species is distributed in the coastal habitats of the Mediterranean countries (central eastern region), with the exception of the westernmost and easternmost parts of the region, where it is replaced by *A. iberus* and *Aphanius dispar* respectively. More recently, it has also been found in inland ponds of the Sahara Desert in Algeria (Chaibi, Si Bachir & Chenchouni, 2015). In Italy, although some populations are recovering, the species has a fragmented distribution and is important for conservation owing to its documented decline in recent years (Valdesalici et al., 2015). The reasons for this decline are mainly the alterations and destruction of natural biotopes and habitat loss in coastal zones (Zerunian, 2003; Genovesi et al., 2014), as well as competition with mosquitofish. The impacts of mosquitofish on native fishes, amphibians, and invertebrates are well documented, including predation, aggressive harassment, and competition for prey and other resources (Rowe et al., 2008; Vannini et al., 2018). As a food competitor of killifish, it has caused a steep decline in killifish populations, including local extinctions (Duchi, 2006), especially in freshwater environments (Valdesalici et al., 2015).

2.2 | Study area and sampling protocol

The study area (42°39'34.95"N, 11°1'10.07"E; elevation between 0 and 3 m asl) is part of a protected area located on the Tyrrhenian coast of Tuscany (Italy), at the Ombrone River delta. The site has been declared as a Wetland of International Importance (Ramsar site no. 2284, Trappola Marshland, Ombrone river mouth), and it is completely included within the Maremma Regional Park (Figures 1 and 2a). It also includes Natura 2000 sites designated by the Italian Ministry for Environment, Land and Sea Protection in compliance with the European Habitats Directive (Council of the European Communities, 1992) and the European Birds Directive (Council of the European Communities, 2010). The area is characterized by a typical Mediterranean climate, with mild winters and hot and dry summers; rainfall is concentrated between October and December. The study site, of about 530 ha, represents one of the last remnants of a partly

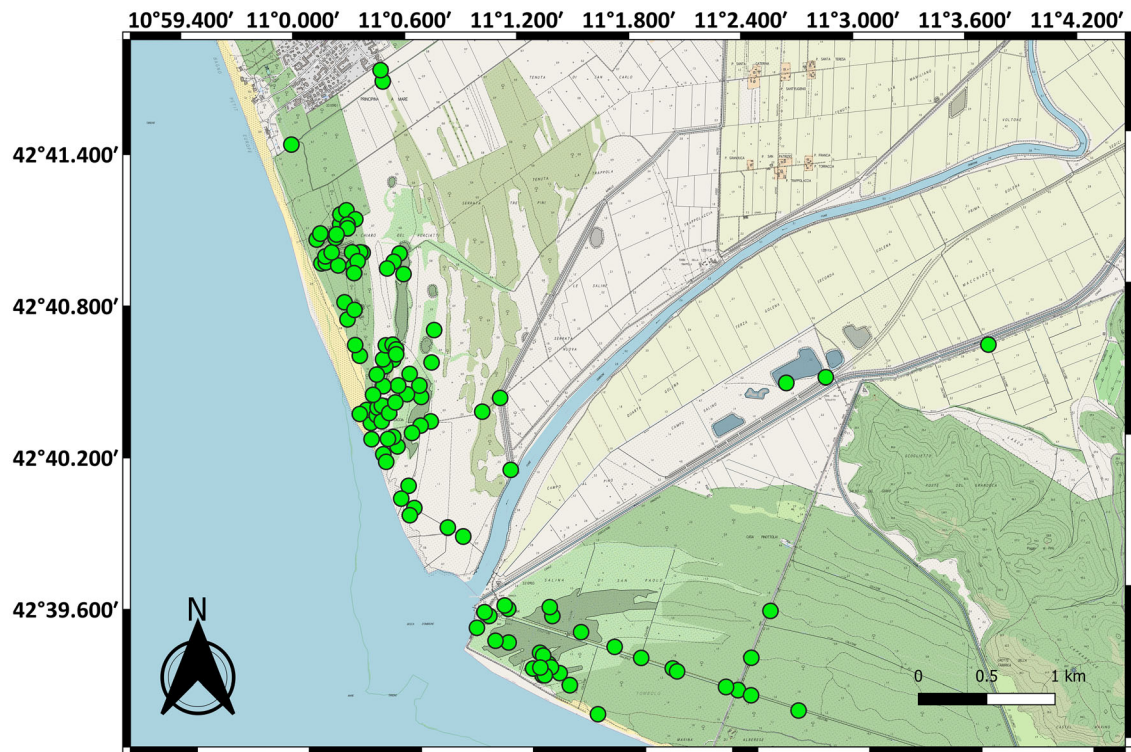


FIGURE 1 Study area located at the mouth of the Ombrone River, within the Maremma Regional Park (central Italy) and the locations of 107 sampling sites (green dots)



FIGURE 2 (a) Aerial image of the study area showing the coastal wetlands where the sampling was carried out (refer to Figure 1); (b,c) Images representing the sampling in two sampling sites: (b) a coastal pond and (c) one of the beach ponds

salty and freshwater complex of wetlands and sand dunes, with some scattered Mediterranean pinewood, pastures, and an extensive coastal wetland system located both at the north and south of the river mouth (Figures 1 and 2a). These wetlands are salty swamps characterized by a succession of emerged and depressed zones that are flooded throughout most of the year and covered mainly by halophytic vegetation. The river and larger channels built in the past to reclaim land contain fresh water throughout the year, whereas the network of secondary channels can experience desiccation during summer. Owing to a strong erosion process, the advancing sea has caused marine water intrusion into coastal groundwater and has invaded the surface of vast areas bordering the river mouth (Colombini & Chelazzi, 2010; Tarragoni et al., 2011). Thus, seawater tends to mix more or less with the fresh water in the river and channels according to the river flow rate, rain, and the conditions of the sea. For this reason, the entire area can be considered a brackish water coastal wetland, with ponds and channels of different salinity and temperature levels.

Data collection was carried out in 2011 and 2012, between late March and June, corresponding to the breeding season for both fish species (Kottelat & Freyhof, 2007) and reflecting the natural increase of water temperature during the spring–summer transition (see later). Sampling sites were randomly selected within flooded areas, which were previously identified on maps and by means of field surveys. Only locations with a maximum water depth of 150 cm were considered for sampling. The study area was sampled homogeneously, with the exception of a portion of the marsh at the south of the river mouth, excluded to limit the disturbance to a breeding pair of osprey *Pandion haliaetus* (Sforzi, Sammuri & Monti, 2019). In total, 107 locations were sampled each year. Sampling was carried out during the morning, between 9:00 a.m. and noon. Fish sampling was performed using a net with a 1.5 m extendable handle, with a mesh of 1 mm mounted on a circular frame (diameter: 45 cm). At each location, samples were taken by sweeping with the net for 5 min, covering an area of approximately 5 m² (Figure 2b,c). Each sampling event was accompanied by *in situ* measurements of water temperature, water salinity, and water depth. The water temperature was measured with an alcohol thermometer (sensitivity of 1°C). Variations in temperature levels were recorded during the study, corresponding to an increase of water temperature across Julian days, from late March to June ($r = 0.76$, $P < 0.01$). Salinity was measured using an optical refractometer (Milwaukee MR100ATC with automatic compensation of the temperature between 10°C and 30°C; resolution 1‰; accuracy ±1‰). Unlike water temperature, salinity did not markedly change seasonally ($r = 0.36$, $P < 0.01$). Water depth was measured with a 1 m bar at the point of sampling. Coordinates of each location were recorded with a global positioning system to allow repeat sampling in the same place the following year. Locations were imported into QGIS software (v. 3.6.1, Noosa) for spatial and cartographic analyses. Once captured, fishes were placed in a portable aquarium only for the time strictly necessary for counting and determining the sex and age group (i.e. less than 5 min) and then released *in situ*.

2.3 | Statistical analyses

Overall, 107 sites were sampled each year, making a total of 214 sampling events. However, in 2012, 23 sites were completely dry; thus, statistical analyses were conducted on the remaining 191 sampling events. Occurrence of killifish in relation to water parameters and presence of its potential competitor was analysed using generalized linear mixed models (GLMMs; Zuur et al., 2009). Two response variables were evaluated: probability of killifish presence was modelled with a binomial distribution (link: logit), and abundance was modelled using negative binomial errors (link: log), as customarily done when handling presence/absence and count data respectively (Zuur et al., 2009).

In both models, water salinity (continuous; mean ± SE: 28.5 ± 1.1‰), water temperature (continuous; mean ± SE: 22.5 ± 0.2°C), water depth (continuous; mean ± SE: 35.4 ± 1.5 cm) and presence of mosquitofish (categorical; reference level: absent) were included as predictors. The interactions between salinity and mosquitofish presence, and between temperature and mosquitofish presence, were also included as predictors to test whether the effect of mosquitofish on killifish was mediated by these water parameters. A preliminary data exploration suggested that only linear effects could be detected for the covariates, and no multicollinearity was found between these predictor variables ($r < |0.5|$). However, predictors were centred on their mean value to improve the interpretability of estimated coefficients, as recommended when interactions are present (Schielzeth, 2010). Years (2011; 2012) and sampling sites (107 sites) were treated as crossed random intercepts, to account for repeated sampling across sites in both years, and potential spatial correlation of the response variables across sampling stations.

For each response variable, a model selection from the full model was performed according to the information-theoretic approach (Burnham & Anderson, 2002). Each model evaluated, including a different combination of predictors, thus represented a different *a priori* hypothesis (Burnham & Anderson, 2002). The null model (i.e. that including the random intercepts only) was also evaluated to allow an assessment of model performance relative to a fixed baseline (Mac Nally et al., 2018). All the possible combinations between predictors were ranked and weighted from each global model. Following the ‘nesting rule’ (Burnham & Anderson, 2002; Richards, 2008; Richards, Whittingham & Stephens, 2011; Harrison et al., 2018), models with $\Delta\text{AICc} \geq 2$ with respect to the best model (the model with the lowest AICc value) were not selected, as well as models with an AICc value greater than that of any simpler alternative, thus achieving either a set of top-ranked models or a single best model for each response variable while accounting for nesting (Table 1). Akaike weight, standardized within the subset of selected models, and variance explained by both random and fixed effects ($R^2_{\text{GLMM}(c)}$; delta method for binomial, and lognormal method for negative binomial; Nakagawa, Johnson & Schielzeth, 2017) were also calculated for each model.

Following the approach of Richards, Whittingham & Stephens (2011), inference was based on selected models. For each

TABLE 1 Result of models selection: selected models with $\Delta\text{AICc} < 2$ while accounting for nesting

Response variable	Models retained	k	AICc	ΔAICc	$R^2_{\text{GLMM}(c)}$	Weight
(a) Probability of Mediterranean killifish presence	Water salinity \times eastern mosquitofish presence + water temperature + water depth + (year) + (site)	8	215	0	0.57	0.516
	Water salinity \times eastern mosquitofish presence + water temperature + (year) + (site)	7	216	0.13	0.55	0.484
(b) Abundance of Mediterranean killifish (no. individuals)	Water salinity \times eastern mosquitofish presence + water depth + (year) + (site)	8	-556	0	0.97	1
	[Water salinity + eastern mosquitofish presence + water depth + (year) + (site)]	7	-554	2	0.97	0]

Note: Reported values of k , AICc value, ΔAICc , $R^2_{\text{GLMM}(c)}$ and standardized weight. Random effects are shown in parentheses. For that response variable in which only a single best model was selected within $\Delta\text{AICc} < 2$, the first eliminated model and its features are also reported in square brackets.

response variable, coefficients of predictors, 95% confidence intervals and variance of random effects were estimated from the selected model. The effects of predictors were assessed by checking whether 95% confidence intervals of coefficients overlapped zero. Modelling was performed in R (R Core Team, 2013), using the packages MuMIn (for model selection; Bartoń, 2012) and lme4 (for GLMMs; Bates et al., 2015). Using the R package DHARMA (Hartig, 2020), models were validated through formal inspection of residual patterns (Supporting Information).

In the occurrence range of killifish ($n = 68$ sampling events), the proportion of juveniles was investigated and, where adults were present ($n = 57$ sampling events), the sex ratio of adult individuals (as the proportion of males) was also examined in relation to mosquitofish presence. Owing to the lower sample size of these data sets, a modelling approach that accounts for water parameters could not be used, because a sample size of at least 10 observations per independent variable would be required (Bolker et al., 2009). The effect of mosquitofish occurrence was therefore tested using a Monte Carlo permutation t test (99,999 permutations). The permutation test for equality of means uses the absolute difference in means as the test statistic, which is equivalent to using the t statistic, and only assumes equal distributions of the two samples if the null hypothesis is true. Equality of distributions was thus checked through the Kolmogorov–Smirnov test (proportion of males: $D = 0.31$, $P = 0.15$, $n = 57$; proportion of juveniles: $D = 0.28$, $P = 0.16$, $n = 68$). All tests were performed using the software PAST (Hammer, Harper & Ryan, 2001). For both species, the overall sex ratio at the study-area scale (given as males to females, M:F) was also calculated. Descriptive statistics are reported as mean \pm SD.

3 | RESULTS

In total, 438 killifish and 1,467 mosquitofish were collected. Out of the 191 sampling events, killifish and mosquitofish were found in the 38.7% and 50.2% of cases respectively. The two species were recorded together in 23.6% of cases. The killifish was found from 13‰ to 60‰ ($34.2 \pm 12.2\%$) salinity, from 10°C to 29°C

($24.06 \pm 3.8^\circ\text{C}$), and at a depth of 10–98 cm (36.5 ± 17.7 cm). Mosquitofish were recorded within a salinity range of 0‰ to 60‰ ($23.7 \pm 11.3\%$), from 15°C to 29°C ($23.4 \pm 3.2^\circ\text{C}$), and at a depth of 10–150 cm (42.0 ± 21.2 cm).

Estimates of parameters for the best models and those for the alternative selected model concerning the probability of killifish presence (yielding the same significant results as the best model) are shown in Table 2. Both the probability of presence and abundance of killifish increased with water salinity, but this increase was greater when mosquitofish were present (Table 2; Figure 3a,b). Conversely, the interactive effect of mosquitofish with temperature was not supported in either model. The probability of killifish presence increased with increasing water temperature (Table 2; Figure 4a), although the latter did not affect abundance. Water depth did not influence presence of killifish, but higher abundance occurred with increasing water depth, although its effect was small compared with other predictors (Table 2; Figure 4b).

The presence of mosquitofish affected neither the sex ratio of adult killifish (measured as the proportion of males; $t = 0.91$, $P = 0.36$, $n = 57$; Figure 5a) nor the proportion of juveniles ($t = -1.83$, $P = 0.07$, $n = 68$; Figure 5b). In the study area, the overall sex ratio of killifish was skewed in favour of males (1.9:1), whereas that of mosquitofish was skewed in favour of females (0.8:1).

4 | DISCUSSION

The results suggest that salinity is the main factor influencing the distribution and relative abundance of both the native and invasive fish species. It has been demonstrated that survival of native species under threat from invaders can be improved either by eradicating the non-native species or by maintaining resources or conditions that benefit the native species (Magoulick & Kobza, 2003; Magellan & García-Berthou, 2016). In the absence of natural shelters or resources, artificial refugia may be a viable option to promote coexistence between invasive and native species, and may serve as a viable management option, as shown for the Barrens topminnow *Fundulus julisia* in the USA (Westhoff, Watts & Mattingly, 2013) or for the

TABLE 2 Coefficients *B* and 95% confidence intervals (95% CIs) estimated by generalized linear mixed models for the best models of (a) Mediterranean killifish presence and (b) abundance, and (c) for the second best model of killifish presence

Response variable	Predictor	<i>B</i>	95% CI
(a) Probability of Mediterranean killifish presence σ^2 year <0.001 σ^2 site = 1.770	Intercept	-1.165	-1.977; -0.354*
	Water salinity	0.040	-0.001; 0.080
	Water temperature	0.158	0.014; 0.301*
	Water depth	0.019	-0.006; 0.044
	Eastern mosquitofish presence (present)	1.745	0.537; 2.952*
	Water salinity \times eastern mosquitofish presence (present)	0.159	0.046; 0.272*
(b) Abundance (no. individuals) of Mediterranean killifish σ^2 year = 0.119 σ^2 site = 4.680	Intercept	-1.460	-2.354; -0.567*
	Water salinity	0.033	0.011; 0.055*
	Water depth	0.023	0.009; 0.036*
	Eastern mosquitofish presence (present)	1.008	0.339; 1.677*
	Water salinity \times eastern mosquitofish presence (present)	0.037	0.001; 0.073*
(c) Probability of Mediterranean killifish presence (second best model) σ^2 year <0.001 σ^2 site = 1.650	Intercept	-1.248	-2.056; -0.441*
	Water salinity	0.036	-0.003; 0.075
	Water temperature	0.156	0.017; 0.295*
	Eastern mosquitofish presence (present)	1.877	0.674; 3.080*
	Water salinity \times eastern mosquitofish presence (present)	0.149	0.040; 0.258*

Note: Covariates are mean-centred. Variance of random factors (σ^2) is also shown. The reference category for eastern mosquitofish presence is 'absent'. Asterisk marks the confidence intervals that do not include zero.

Iberian toothcarp in Spain (Magellan & García-Berthou, 2016), when in sympatry with mosquitofish. In this study, sites characterized by euryhaline and extremely hyperhaline waters (up to 50‰) represented natural refugia for the killifish (but not for mosquitofish; Ruiz-Navarro, Torralva & Oliva-Paterna, 2013) that were selected in response to mosquitofish presence. In line with the expectations and previous findings from laboratory experiments (Alcaraz, Bisazza & García-Berthou, 2008), higher salinity provided a competitive refuge for the native cyprinodontiform, potentially because of competition at low to medium salinity. In addition to occurrence, killifish abundance was also higher at sites with higher salinity. In the most extreme and stressed environments, killifish is able to survive and persist, with both salinity and the presence of the invader representing two factors acting synergistically in characterizing and shaping the distribution patterns of the native species. This difference in response to salinity between the two species is consistent with their ecological characteristics and previous studies: It is known that salinity is a major limiting factor for mosquitofish (Alcaraz & García-Berthou, 2007; Ruiz-Navarro et al., 2013), whereas killifish has a much higher tolerance to hyperhaline conditions (Bianco, 1995). It has been reported that the highest salinity tolerated by mosquitofish is 58.5‰ for 30 days (Ruiz-Navarro et al., 2011), which is approximately equivalent to the highest values where the species was found in this study; however, reports of mosquitofish in hyperhaline conditions are very limited in the scientific literature (Alcaraz & García-Berthou, 2007). According to Pyke (2005), the mortality of

mosquitofish increases both with the increase in salinity and with the duration of exposure to high-salinity waters. The present study seems to confirm these reports.

In line with the second prediction, killifish presence increased with increasing water temperature; however, its abundance was not directly affected by this variable. The presence of mosquitofish interacting with temperature had no effect on either the presence or the abundance of killifish. Both species can survive over a wide temperature gradient, but are more active with increasing temperatures, as the metabolic rate of ectotherms increases with warmer waters, facilitating mobility (Leonardos, 2008; Lang, Rall & Brose, 2012). Laboratory experiments with Iberian toothcarp and mosquitofish have shown that both species exhibit increased aggression at higher temperatures, although the latter is more aggressive and has the capacity to displace the toothcarp through interference competition (Carmona-Catot, Magellan & García-Berthou, 2013). Similar temperature-dependent effects have been found between mosquitofish and Australian bass *Macquaria novemaculeata* (Lopez, Davis & Wong, 2018). These patterns, as well as the fact that the observed temperature range reflected the seasonal increase experienced during the breeding period, suggest that in warmer waters, such as in summer, the presence of mosquitofish may lead to a reduction in the numbers of the native species as well as to its displacement to non-preferred habitats characterized by non-optimal temperature. However, exclusion interference observed in laboratory studies (Caiola & de Sostoa, 2005)

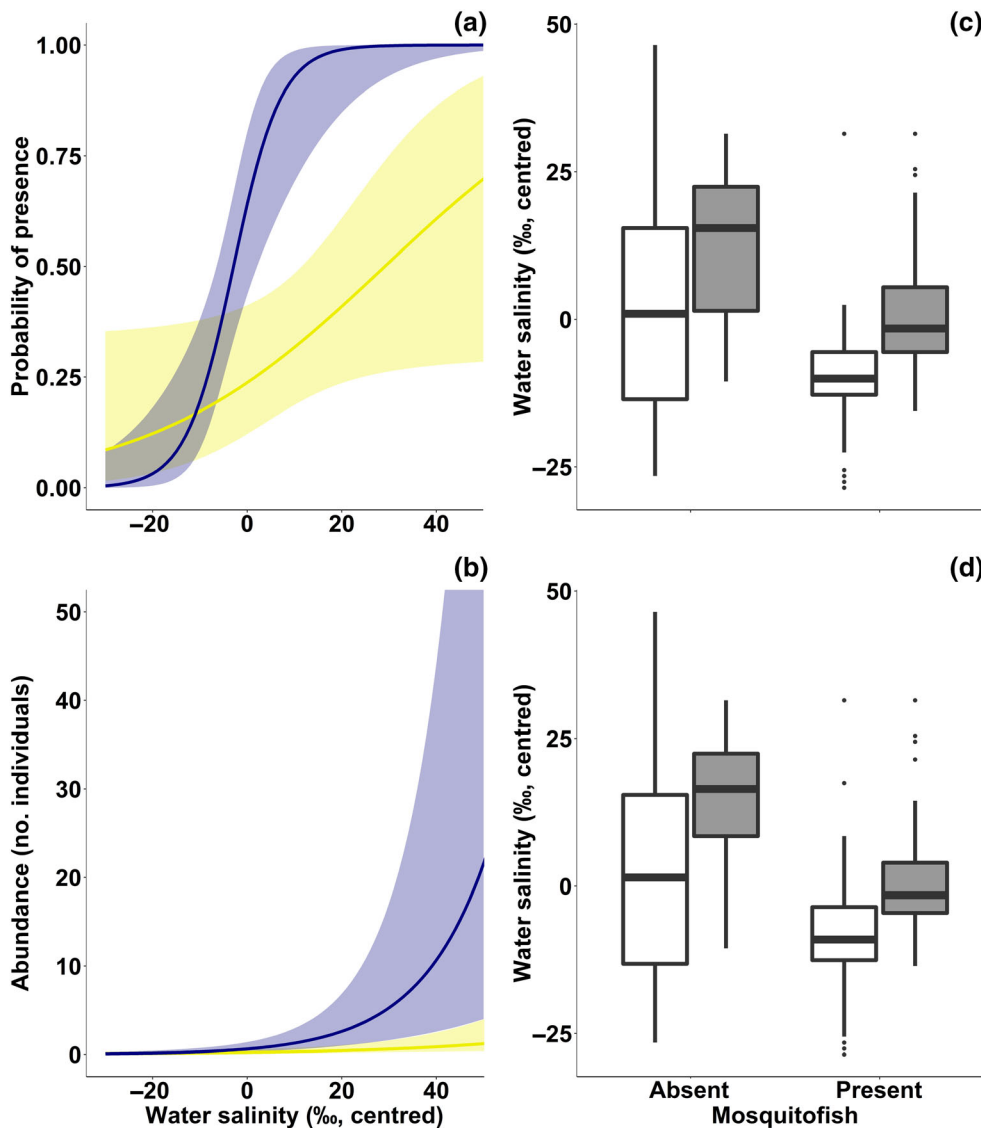


FIGURE 3 Predicted (a) probability of presence and (b) abundance of killifish in relation to water salinity and mosquitofish occurrence (yellow items: absent; blue items: present), as estimated by generalized linear mixed models. Line and bands: predicted values and 95% confidence intervals. Observed (c) occurrence (white: absent; grey: present) and (d) abundance (white: rare, 0–1; grey: abundant, >1) of killifish in relation to water salinity and mosquitofish occurrence. Boxplots depict median, quartiles, and outliers of original observations. For boxplots, note that the response variable used in the analysis is represented by the filling colour. The mean value of water salinity is reported in the text

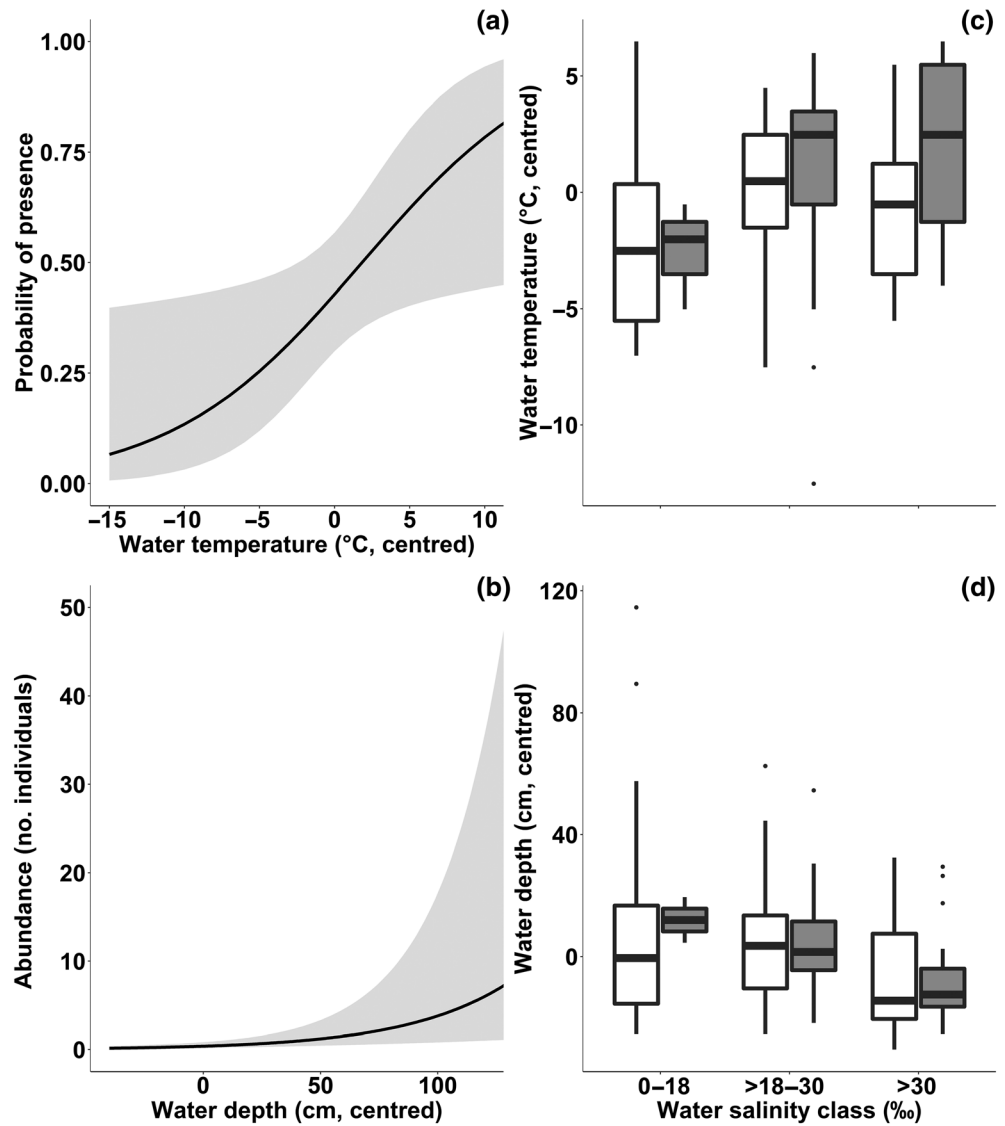
does not necessarily imply competition for the same specific factor in the wild, especially when multiple stressors interact concurrently to produce synergistic or antagonistic effects, thereby modulating invasive–native species interactions in unexpected ways (Lopez, Davis & Wong, 2018). In such cases, microhabitat segregation is more likely to occur.

In contrast to the predictions, greater water depth was associated with higher killifish abundance (but not a higher likelihood of presence), probably because deeper waters may offer more space and potential microhabitats within the water column. In the study area, greater depths were associated with an increased presence of algae and seaweeds (e.g. beaked tasselweed *Ruppia maritima*; MM, personal observation) compared with shallower sites (<10 cm deep) where vegetation was absent. Thus, deeper waters (>10 cm) potentially translate into greater possibility of finding food and shelters, explaining higher abundances compared with very shallow waters. This offers support for the hypothesis that, as water depth decreases, there is less opportunity for the species to find refugia and escape

predators. This is also in line with Zammit Mangion et al. (2011), who recorded a significant summer decrease in the number of males (probably owing to their brighter colours and striking courtship behaviour) when the habitat was subject to high temperatures and rapid evaporation. However, considering that sampling was carried out in sites with a maximum depth of 150 cm (i.e. relatively shallow waters), as well as water depth having a relatively small effect, this variable must be considered with caution and in light of other concomitant factors.

Although the present analyses were validated, providing clear evidence for the effects of salinity and mosquitofish on killifish (Figure 3c,d), it should also be noted that the net effects of water temperature in shaping killifish presence and of water depth in affecting killifish abundance were less evident when the major effect of salinity was considered. In particular, potential interactive relationships should be explored in future studies, as the effect of temperature seemed to occur mainly at high salinity levels (Figure 4c), whereas that of water depth occurred mainly at low to

FIGURE 4 Predicted (a) probability of presence and (b) abundance of killifish in relation to water temperature and water depth, as estimated by generalized linear mixed models. Line and bands: predicted values and 95% confidence intervals. Observed (c) occurrence (white: absent; grey: present) and (d) abundance (white: rare, 0–1; grey: abundant, >1) of killifish in relation to water temperature and water depth, for each salinity class arbitrarily defined following the ‘Venice system’ (Ito, 1959). Boxplots depict median, quartiles, and outliers of original observations. For boxplots, note that the response variable used in the analysis is represented by the filling colour. The mean values of water temperature and water depth are reported in the text



medium salinity levels (Figure 4d). Nevertheless, testing interactive effects can be extremely difficult in the field, requiring a large sample size and a wide range of observations for each covariate and each combination of effects (Zuur et al., 2009). Thus, laboratory experiments showing the interactions between salinity and water temperature/depth under controlled conditions could still increase the understanding of how abiotic factors affect killifish abundance and distribution.

In contrast with the last prediction, the presence of mosquitofish did not affect the sex ratio of adult killifish; however, it is important to note that the overall killifish sex ratio detected in the study area (1.9:1 males:females) seems to differ from that reported by previous studies, although a formal comparison is not possible. Generally, the strategy of this species is to invest in female individuals that usually outnumber males, especially during the reproductive period and before juvenile recruitment (Leonardos, 2008). For example, a sex ratio of 2.37 in favour of females was found in the Natural Regional Reserve of the Isonzo River mouth (Bertoli et al., 2019) and a sex ratio

of 2.44 in the Mesolongi and Etolikon lagoons (Greece; Leonardos, Sinis & Petridis, 1996), but in some cases a male-biased sex ratio has been reported (e.g. 1.5 males/females in the Venice lagoon; Cavraro, Varin & Malavasi, 2014). The female-skewed sex ratio is characteristic of the species and occurs because highly intense colouration in males during the reproductive period makes them more easily visible and consequently sensitive to predation (Gandolfi et al., 1991; Leonardos & Sinis, 1999). The sex ratio found in the present study, therefore, might suggest an effect on both the population structure and demographic parameters by non-optimal abiotic factors and by the presence of the invasive species. Mosquitofish (*Gambusia affinis* and *G. holbrooki*) is known to exhibit substantial variation in adult sex ratios across its distribution range (Fryxell et al., 2015), with female-biased populations able to cause larger changes to key community and ecosystem responses. In our study, the mosquitofish population was skewed in favour of females (0.8:1). Female mosquitofish are known to display higher feeding rates, spend relatively more time foraging, and show greater niche breadths, with a notable preference

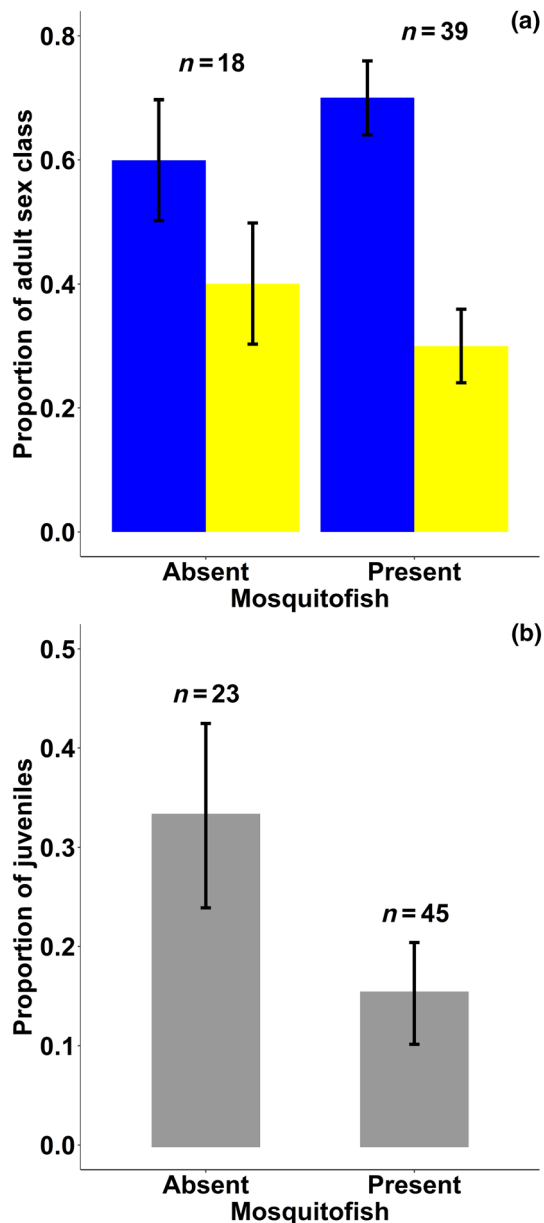


FIGURE 5 Mean differences in proportion of (a) adult sex classes (blue: males; yellow: females) and (b) juveniles of killifish in relation to mosquitofish occurrence. Error bar: standard error

for larger prey (Pilastro, Benetton & Bisazza, 2003). However, further investigations and more formal comparisons with data from other study areas are needed to clarify the drivers affecting sex ratios and changes in population structure. It might be expected that the occurrence of the invasive species might result in changes of killifish fitness through a decline in offspring production because of the displacement of native species to non-preferred habitats. Although the proportion of juvenile killifish did not change when in sympatry with mosquitofish, it was about twice lower and approached statistical significance, suggesting that further investigations are highly recommended to test this hypothesis.

In conclusion, although the two species coexist, the salinity gradient appears crucial in determining species dominance,

potentially through displacement and interactions with fish life-history traits (Alcaraz & García-Berthou, 2007; Alcaraz, Bisazza & García-Berthou, 2008).

5 | IMPLICATIONS FOR CONSERVATION

Within its distribution range, the killifish is threatened primarily by human-driven environmental changes, such as habitat alteration and water pollution (Facca et al., 2020). In particular, catchment operations, canalization, and dam construction lead to habitat fragmentation and prevent movements of fishes, thus decreasing connectivity between populations (Duchi, 2006). This process increases population isolation, with deleterious cascading effects on population genetics (Maltagliati, 2002; Angeletti et al., 2017). In addition, the invasive mosquitofish is one of the principal causes for the exclusion of killifish from the entire spectrum of salinity and temperature ranges where the species can potentially live and persist (Kottelat & Freyhof, 2007). The marked segregation of killifish to a restricted range of environmental parameters emphasizes how mosquitofish drives the ecological responses of killifish to avoid competition, resulting in a coexistence at the extreme limit of salinity ranges, leading the latter to be 'pushed to the edge'. This places the populations of killifish at increased risk of local extinction and further highlights the necessity to plan adequate conservation actions.

From a practical point of view, nature-based solutions of water management should be adopted. These should include maintaining a wide variety of microhabitats with different salinity levels, such as small tidal creeks and pools, as well as promoting connectivity within and between water bodies. Furthermore, to maintain suitable levels of salinity, reducing local freshwater inputs would allow the provision of natural refugia where the native species can avoid competition with mosquitofish. The survival and reproductive rate of killifish depend on finding sheltered areas with low hydrodynamism (Facca et al., 2020). In addition, constantly monitoring ponds and maintaining minimum water levels throughout the year could help to avoid desiccation resulting from prolonged summer droughts. These conservation measures may also be integrated with some form of biological control of mosquitofish. Although complete removal of this invader is likely to be impossible, control could be targeted towards ponds that have a limited probability of mosquitofish recolonization (Facca et al., 2020).

These practical recommendations are likely to be taken up by the responsible authorities. In the geographical area covered by this study, the Tuscany Region Administration, in compliance with European directives, promotes periodic monitoring programmes as well as site-specific management measures to be undertaken by the agencies responsible for the protected areas in its territory. Throughout the study area, the Maremma Regional Park Agency aims to maintain the complexity of natural wetland ecosystems by the management of artificial ditches and channels for the conservation of local killifish populations. These protective mechanisms could help the long-term preservation of the species and its target habitats.

ACKNOWLEDGEMENTS

We thank the Maremma Regional Park under the Tuscany Region Administration. We are also grateful to the park wardens Giuseppe Anselmi and Gianfranco Martini for initial support to fieldwork operations. Paul Stammers greatly helped us with English editing and improvement. We are also grateful to Philip J. Boon, Olaf L. F. Weyl, and another anonymous referee for their extremely useful comments on a first draft of this article. The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

FM conceived the ideas. FM, MM, and PF designed the study and carried out the fieldwork. FM led the writing of the manuscript. NF conducted statistical analyses and wrote the relevant section. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Monti F, Marcelli M, Fastelli P, Fattorini N. Pushed to the edge: Environmental factors drive ecological responses of *Aphanius fasciatus* when in sympatry with invasive *Gambusia holbrooki*. *Aquatic Conserv: Mar Freshw Ecosyst*. 2021;1–13. <https://doi.org/10.1002/aqc.3600>