



A subtle threat: behavioral and phenotypic consequences of invasive mosquitofish on a native paedomorphic newt

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Abstract Aquatic invaders often cause severe declines of native amphibian populations, either through competition, predation and/or alterations of the habitat. Such situation has raised additional concerns for the persistence of endemic species exhibiting rare and alternative phenotypes. Here, we experimentally assessed the impact of the invasive mosquitofish (*Gambusia holbrooki*) on adult newts (*Lissotriton graecus*) exhibiting paedomorphosis, the retention of larval traits such as gills, making them fully aquatic. Mosquitofish had a negative impact on paedomorphic newts by inducing both behavioral and phenotypic changes. Paedomorphic newts exhibited avoidance behavior and higher metamorphosis rates in the presence of fish. Both female and male newts

responded by decreasing mobility and foraging activity. Females stopped investing in egg-laying in presence of fish and males metamorphosed earlier than females. Hence, our results show that mosquitofish introductions, particularly in areas with populations exhibiting paedomorphosis, might have detrimental consequences on the preservation of alternative developmental pathways. Both behavioral and phenotypic effects should be assessed to understand the impacts of introduced species.

Keywords Alien species · Introduced fish · *Gambusia holbrooki* · Amphibian decline · Paedomorphosis · Anti-predator responses

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Introduction

Alien fish species constitute a serious threat to native freshwater communities, through modification of the habitat characteristics (Byers et al. 2010), exploitation of the available resources (Funk and Vitousek 2007), predation and interference with vital activities of individuals in populations (e.g. breeding, Cruz et al. 2006).

Importantly, local amphibian populations, which are already under pressure due to multiple disturbances such as climate change (Walther et al. 2002), human activities (Blaustein and Kiesecker 2002;

Beebee and Griffiths 2005; Denoël 2012) and the emergence and spread of pathogens (Garner et al. 2009; Raffel et al. 2010), are expected to encounter both the sub-lethal and lethal effects of fish invaders.

Fish invaders can prey upon larvae, eggs, and sometimes even adults of native amphibians, can compete with them for important resources such as food and shelter, and alter the chemical and structural characteristics of aquatic habitats making them hostile for many species (Orizaola and Brana 2006; Hartel et al. 2007; Joseph et al. 2011). Consequently, amphibians show avoidance of breeding habitats occupied by potential predators, which can lead to the fragmented distribution and lower population densities in amphibians (Ficetola et al. 2011; Winandy et al. 2017).

Since the early twentieth century, fish species, like guppies and mosquitofish, were widely introduced and have been established in several freshwater ecosystems worldwide, as a means of malaria control (Vidal et al. 2010; El-Sabaawi et al. 2016). The eastern mosquitofish (*Gambusia holbrooki*), part of the *Gambusia affinis* group and native to North America, utilizes a wide range of habitats and shows high ecological tolerance (Pyke 2005), making it a successful invader. The direct and indirect ecological impacts of this aggressive species have been the focus of several studies, since mosquitofish prey on different invertebrates than mosquito larvae (García-Berthou 1999; Remon et al. 2016) and reduce growth and survivorship of native fish (Mills et al. 2004). Introduced *G. holbrooki* threatens native species that lack a common evolutionary history with it (Brown et al. 2018), leading to severe declines of indigenous populations of fish and amphibians (Pyke 2008). Hence, it has pervasive impacts on native wetland communities (Shulse et al. 2013).

Many amphibians exhibit complex life cycles, utilizing both aquatic and terrestrial habitats during their life (Wilbur 1980). However, some salamander and newt species follow an alternative developmental pathway reaching adulthood without metamorphosing, thus remaining in the aquatic habitat for their entire (or most) life span; a life history strategy known as paedomorphosis (Wilbur 1980; Whiteman 1994; Denoël et al. 2005). In facultative paedomorphic species, paedomorphs retain the possibility to metamorphose, for example due to environmental drivers such as pond drying (Mathiron et al. 2017).

Paedomorphic populations have been recognized to be extremely vulnerable to fish introductions (Denoël and Winandy 2015; Denoël et al. 2019a). Fish presence reduced the size of local paedomorphic populations and caused even extinctions, such as in Montenegro where paedomorphic alpine and Greek smooth newts lost almost 99.9% of their aquatic area of occupancy, the water bodies they used to occupy before fish introductions (Denoël et al. 2019a). Although newts exhibit adaptive habitat selection and dispersal to suitable ponds (Winandy et al. 2017), populations of paedomorphs are more prone to alterations of the aquatic habitat since they spend most of their life in the water. Since mosquitofish shows high population growth rates and dispersal tendencies (Cote et al. 2010), they raise concerns for the negative impacts to the native amphibian populations, including those exhibiting paedomorphosis.

As no studies explored the behavioral and phenotypic responses of paedomorphic newts to mosquitofish, we aimed to investigate and quantify the impact of mosquitofish on newts exhibiting facultative paedomorphosis, using an experimental laboratory approach. We hypothesized that paedomorphic newts will perceive mosquitofish as a potential threat, then exhibiting anti-predatory response such as hiding in safe aquatic shelters as well as by exhibiting increased rate of metamorphosis, thus escaping the invaded aquatic habitat. Moreover, we expected that the presence of mosquitofish will reduce essential activities, such as foraging and investment on sexual reproduction.

Materials and methods

Experimental design

10 male (SVL; i.e. the length from the tip of the snout to the end of cloaca: mean SVL \pm SE: 33.6 ± 0.6 mm) and 10 female (35.6 ± 1.2 mm) paedomorphic Greek smooth newts, i.e. adult gilled individuals (*Lissotriton graecus*; previously a subspecies of *Lissotriton vulgaris* but now elevated at a specific level, Wielstra et al. 2018) were caught during the breeding season (April 2017) from an artificial fishless pond in Ioannina basin, Greece ($39^{\circ}36'$ N $20^{\circ}50'$ E, 497 m a.s.l). The sample size was selected to avoid additional pressure on native populations that

are already of high conservation concern in the area (Sotiropoulos et al. 2017). Newts' maturity was verified on the basis of a developed cloaca (Denoël 2017) and body size as well. All individuals were brought directly to the laboratory in large containers filled with pond water and were placed randomly in pairs in aquatic tanks. Additionally, 10 male (SL; from the tip of the snout to the posterior end of the last vertebra: mean SL \pm SE: 20.5 \pm 1.5 mm) and 20 female (36 \pm 1.5 mm) mosquitofish (*Gambusia holbrooki*) were collected from another natural pond in Ioannina basin (39°34' N 20°52' E, 481 m a.s.l.). Although incidents of Greek smooth newt and mosquitofish co-occurrence have been reported in the geographic area, paedomorphic individuals have not been detected to coexist with mosquitofish so far.

We separated tanks in two treatments: five tanks without fish (control) and five with fish. After 2 days habituation by newts to their new environment, we placed one male and two female mosquitofish in each tank of the respective treatment. Newts and fish were fed ad libitum with black mosquito larvae (*Culex* sp.) and bloodworms (*Chironomus* sp.) every afternoon. Aquatic tanks (38 \times 24 cm, 22 cm water depth; 20 L) were filled with dechlorinated tap water, the bottom was floored with gravel, and kept at a temperature of 16 °C and 13 h light–11 h dark photoperiod. All aquatic tanks were provided a plastic floating platform (8.5 \times 8.5 cm) and a plastic cylindrical shelter attached to the bottom of the tank (length 10 cm, diameter 4 cm). Mosquitofish prey often on the water surface and it is unlikely to compete for access in the shelter (Magellan and García-Berthou 2016). Strips of plastic were fixed on the bottom of each tank to simulate aquatic vegetation. In total, 10 tanks were distributed randomly in the lab to avoid biases, and visual contact of neighboring tanks was blocked using thick paper. The water was renewed every 5 days using dechlorinated tap water from tanks stored at the experimental temperature. The experiment lasted 4 weeks. After the end of the experiment, newts were released back to their habitat, while mosquitofish were euthanized with an overdose of MS-222 following the guidelines for handling species in scientific research according to the European Directive 2010/63/EU (Council of the European Union 2010).

Each tank was video recorded four times a day (two in the morning and two in the afternoon, with 2 h interval between them) for 10 min duration for each

video (video recorders TurboX Act-150 were set at a resolution of 1280 \times 720 pixels and 30 frames per second) over the 4-week period. Videos were analyzed using Boris software for behavioral analyses (Friard and Gamba 2016). In these videos, we scored/quantified five behavioral patterns of paedomorphic newts: moving in open areas (i.e. swimming in the water column, moving on gravel, air taking), immobility in open areas (i.e. no visible movement, motionless), hiding when newts were using the shelter, oviposition when females laid eggs, and courtship when males engaged in sexual display. In newts, including smooth newts, courtship display is very obvious as it involves varied tail movements from the male to the female (see description in Halliday 1974). In addition, we scored fish attacks to newts and noted the recipient of the attack (i.e. either the male or the female newt). For newt oviposition, scores corresponded to counts (i.e. number of eggs laid) out of each 10 min sequence.

Feeding activity was tested five times during the 4-week experiment, and involved the foraging on an earthworm (*Lumbricus terrestris*, approx. 5–6 cm long) attached to a string and immersed in each aquarium so it would be visible by all newts. We then set a video recording for 10 min in order to assess the feeding activity of newts, that is if they engaged in a feeding act (feeding vs. not feeding) and the latency by measuring the time from food delivery to the first feeding act by a newt (the minimum foraging latency).

To assess the timing of metamorphosis, we visually inspected newts daily for signs of metamorphosis (closed gill slits), which coincides with a transition to the terrestrial habitat (Mathiron et al. 2017).

Statistical analysis

We obtained measures for each behavior through the daily videos, and considered only the observations made at the paedomorphic stage (post-metamorphic data were discarded). The average \pm SE number of videos per individual was 57.8 \pm 2 (range 31–64 videos).

The data were analyzed using linear mixed models (LMM) and generalized linear mixed models (GLMM) as implemented in package “lme4” (Bates et al. 2014) in R v. 3.4.1. (R Core Team 2019). We fitted a LMM on duration for moving and immobility, using treatment (control vs. fish), sex and their interaction as fixed effects and the identity of

individuals and tanks as a random effect. A GLMM was performed on hiding data under a binomial distribution (hiding vs. not hiding), using treatment (control vs. fish), sex and their interaction as fixed effects and the identity of individuals and tanks as a random effect. Due to the rareness of events, the analysis for oviposition (number of eggs laid per female) was carried out using summarized data of the 4-week period for each tank. We fitted a GLMM for count data (Poisson distribution), using treatment as fixed factor and the identity of tank as a random effect. For courtship behavior of male newts, we did not perform any statistical analysis, since only two males in the control and only one male in the fish treatment were engaged in sexual displays, thus preventing any statistical comparisons.

To assess the feeding activity of newts, we fitted a GLMM with binomial distribution (feeding vs. not feeding) using treatment, sex and their interaction as fixed effects and individuals as a random effect. For minimum foraging latency we fitted a survival analysis for right-censored data, as implemented in the “Survival” R package (Therneau and Lumley 2015) and ordinary log rank tests were performed to test for differences between the groups.

Post hoc comparisons for significant interactions were implemented in “lsmeans” package (Lenth 2016).

In order to investigate the effect of treatment and sex on the timing of metamorphosis during the 4-week period, a survival analysis for right-censored data was performed using a non-parametric method, the Kaplan–Meier estimator, as implemented in the “Survival” R package. Ordinary log rank tests were performed to test for differences of the survival curves between the groups, using the Bonferroni adjustment to correct for multiple comparisons.

Results

A significant main effect of treatment (LMM: $F_{1,10} = 7.533$, $p = 0.02$) and sex (LMM: $F_{1,10} = 5.714$, $p = 0.037$) was found on moving. No statistically significant effect of treatment, sex and their interaction was found on immobility (Table S1). Treatment had a significant effect on hiding (GLMM: $\chi^2 = 6.634$, $p = 0.01$). In the fish treatment, both

males and females decreased moving and increased hiding (Table 1, Fig. 1).

The presence of fish had a significant impact on the oviposition behavior of female newts ($z = -3.606$, $p < 0.001$), which laid considerably fewer eggs than in the absence of fish.

Mosquitofish attacked frequently paedomorphic newts. We recorded a total of 72 attacks towards females and 40 attacks towards males over the whole observation period. A female paedomorphic newt, previously bitten by the mosquitofish, died after 3 days.

We found a significant main effect of treatment (GLMM: $\chi^2 = 7.753$, $p = 0.005$), sex (GLMM: $\chi^2 = 4.473$, $p = 0.034$) and their interaction (GLMM: $\chi^2 = 3.877$, $p = 0.048$) on the feeding activity of newts. Pairwise comparisons of the significant interaction are summarized in Table S2. Treatment and sex had a significant main effect in minimum foraging latency, $\chi^2 = 18.1$, $p < 0.001$ and $\chi^2 = 9.2$, $p = 0.002$, respectively. The interactive effect of treatment and sex was statistically significant, $\chi^2 = 50.2$, $p < 0.001$. The minimum foraging latency differed significantly between the sexes in control, and was affected by fish presence for females (Table 2). Male newts foraged less than females during the experiment (Fig. 2) and their minimum foraging latency did not differ significantly between the treatments (Table 2).

At the end of the 4-week experimental period, 75% of females and 100% of males metamorphosed in the presence of fish, whereas 0% of females and 60% of males metamorphosed in the control treatment. Sex and treatment had a significant main effect in time of metamorphosis, $\chi^2 = 5.5$, $p = 0.01$ and $\chi^2 = 6$, $p = 0.01$, respectively. The interactive effect of sex and treatment on timing of metamorphosis was statistically significant ($\chi^2 = 12.9$, $p = 0.004$, Fig. 3). The timing of metamorphosis differed significantly between the sexes in both treatments, and was affected by fish presence for females (Table 3). However, for male newts the timing of metamorphosis did not differ significantly between the treatments, and they metamorphosed earlier than female newts both in the presence and absence of fish (Table 3, Fig. 3).

Table 1 Oviposition (total number of eggs laid) and duration of each recorded behavior, as proportion of the total recorded video time (mean ± SE), during the 4-week experiment for female and male paedomorphic newts in control and mosquitofish treatment

Treatment	Sex	Oviposition	Moving	Motionless	Hiding	Courtship
Control	Female	28	0.509 ± 0.018	0.353 ± 0.018	0.044 ± 0.010	–
Fish	Female	2	0.304 ± 0.018	0.479 ± 0.021	0.129 ± 0.017	–
Control	Male	–	0.324 ± 0.017	0.532 ± 0.018	0.037 ± 0.008	0.012 ± 0.002
Fish	Male	–	0.289 ± 0.018	0.508 ± 0.025	0.108 ± 0.015	0.004 ± 0.001

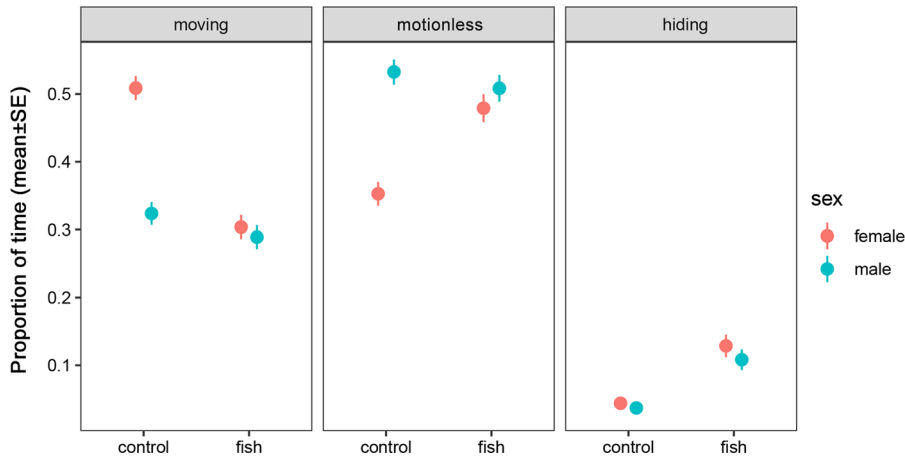


Fig. 1 Proportion of time spent moving, being motionless and hiding in the two experimental treatments (control vs. mosquitofish) for female and male paedomorphic *Lissotriton graecus*

Discussion

In this study, we assessed the detrimental effects of an invasive fish on a native amphibian species from Greece. We found that mosquitofish presence had sublethal effects on paedomorphic newts, with consequences regarding their fitness. Newts exposed to the presence of mosquitofish reduced their mobility and their foraging behavior, as well as their oviposition rates. Most notably, the presence of fish induced an increase in the frequency of metamorphosis.

Animals spend time in crucial activities for survival, exposing themselves to visibility and potential risks. Several animal taxa adopt inactive behaviors by “freezing” in order to lower the possibility of an encounter with a threat (Skelly and Werner 1990; Chelini et al. 2009; Liden et al. 2010). In our study, paedomorphic newts in the presence of fish decreased their mobility and overall activity, and considerably increased hiding, rather than adopting a freezing behavior, which might have made them less

Table 2 Pairwise comparisons of the survival curves for minimum foraging latency between sexes and treatments (control vs. mosquitofish)

Pairwise comparisons	Log rank test	
	χ^2	<i>p</i>
Female control–female treatment	23.3	< 0.001
Male control–male treatment	1.9	0.165
Female control–male control	16.4	< 0.001
Female treatment–male treatment	0.2	0.626
Female control–male treatment	31.5	< 0.01
Male control–female treatment	0.7	0.395

Significant values are indicated in bold

detectable (Stauffer and Semlitsch 1993). Given that paedomorphic newts were frequently attacked by mosquitofish in the present study, such anti-predatory response can be advantageous in decreasing the risks of predation and injury. However, this response comes

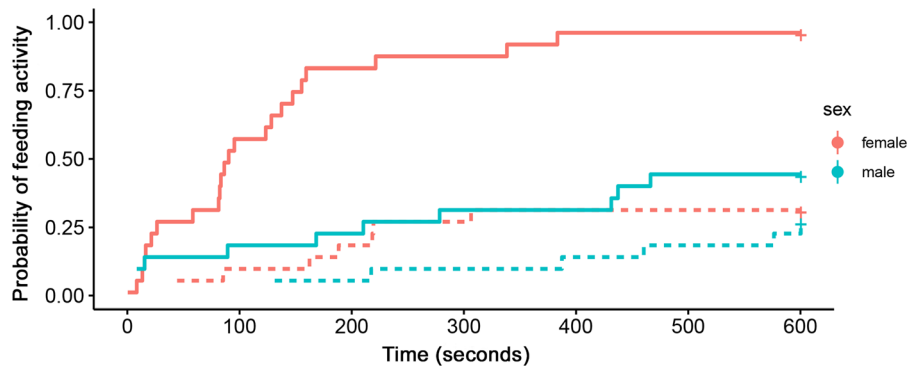


Fig. 2 Probability of feeding activity for female and male paedomorphic newts for control (solid line) and treatment with mosquitofish (dashed line) over the 10-min observation period

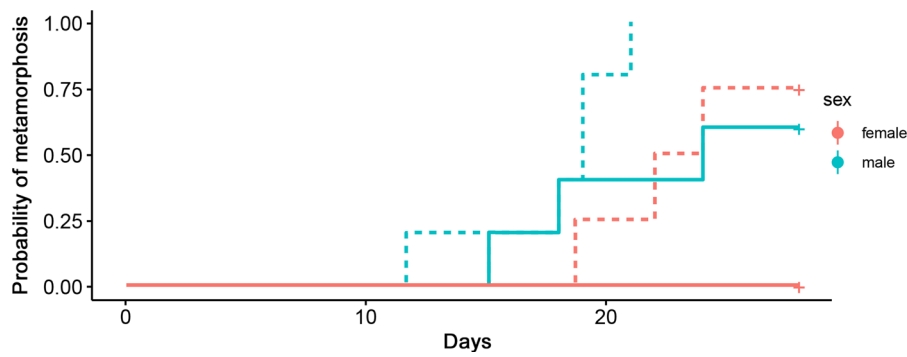


Fig. 3 Probability of metamorphosis for female and male paedomorphic newts for control (solid line) and treatment with mosquitofish (dashed line) over the 4 week experimental period

Table 3 Pairwise comparisons of the survival curves between sexes and treatments (control vs. mosquitofish) on timing of metamorphosis

Pairwise comparisons	Log rank test	
	χ^2	<i>p</i>
Sex * treatment		
Female control–female treatment	5.2	0.02
Male control–male treatment	2.2	0.14
Female control–male control	3.9	0.04
Female treatment–male treatment	4.9	0.02
Female control–male treatment	9.5	0.002
Male control–female treatment	0.0	0.8

Significant values are indicated in bold

with costs: animals will have less time for essential activities such as feeding and breeding (Bridges 2002; Stynoski and Noble 2012).

Throughout the experiment, paedomorphs reduced their foraging behavior in the presence of fish. This is in agreement with previous results where foraging activity of newts was also reduced in the presence of goldfish (Winandy and Denoël 2013a, 2015). On the long-term, reduction of the feeding activity could have harmful effects in growth and development of newts, as well as on the energy storage needed for survival and breeding, with harmful effects in fitness. For instance, Scrimgeour and Culp (1994) found that when mayflies were introduced in low food abundance environments and/or exposed to predators, they reduced their foraging behavior, resulting in the reduction of growth rates and fecundity.

During reproduction, newts secrete pheromones and exhibit elaborated courtship displays, processes that may also attract potential predators (Malacarne and Giacoma 1986; Halliday 1990). Minimizing sexual activity could imply a trade-off between predation risk and reproduction, but costing breeding

opportunities. Recent studies have showed a similar reduction of sexual activity in amphibian species in the presence of potential fish predators (Winandy and Denoël 2013b; Winandy et al. 2015; Cabrera-Guzmán et al. 2018; Denoël et al. 2019c). For example, Denoël et al. (2019c) showed that paedomorphic male palmate newts considerably reduced their courtship acts in the presence of goldfish and were more affected than females. Our study builds on these results by showing that mosquitofish affect oviposition in paedomorphic smooth newts causing a very high reduction in oviposition rates such as observed with goldfish. The question remains open regarding courtship, as male newts had a low sexual display rate both in control and fish treatment during our experiment, suggesting the need of further research in order to disentangle any effects of mosquitofish in the sexual behavior of males.

We did not test for fish consumption on eggs or aquatic larvae after hatching, which could be an additional detrimental factor. For instance, Vannini et al. (2018) showed increased mortality rates of newt larvae due to the presence of mosquitofish. Such effects could have long-term detrimental consequences on reproductive success and also contribute to the decline of local populations.

Facultative paedomorphosis is an environmentally induced polymorphism (Whiteman 1994), which gives an advantage to pond-breeding salamanders and newts to exploit different ecological niches (Lejeune et al. 2018), and in some cases, an opportunity to escape potentially hostile habitats (Semlitsch 1987; Mathiron et al. 2017). Previous work on larvae of species with complex life cycles, suggest an earlier transition to the terrestrial habitat, where more larvae metamorphose at smaller sizes to minimize the time spent in a poor aquatic habitat (Skelly and Werner 1990; Cabrera-Guzmán et al. 2017). The presence of fish in our experiment mimics a hostile environment for the paedomorphic newts, as illustrated by newts reducing their activity, food intake and egg laying (see also Winandy and Denoël 2015). Our results suggest that fish could also induce a phenotypic transition in the Greek smooth newt, and ultimately contribute to a decrease of the paedomorphs after fish introduction in the wild (Denoël et al. 2019a). Such “escape” (facilitated by the phenotypic transition) was less obvious in the case of paedomorphic palmate newts in the presence of goldfish as only sex-specific effects

were found (Denoël et al. 2019b). One explanation between these differences might be due to the nature of the attacks of mosquitofish, being possibly more detrimental than those of goldfish and triggering the metamorphosis of the paedomorphs (see also Winandy and Denoël 2015).

Our results indicate that the behavioral responses of the paedomorphic newts are partially sex-specific. In the absence of fish, females were more actively engaged in egg production as compared to the situation where fish were present. This sex-biased metamorphosis could contribute to explain the observed sex ratios in local populations of newts (Sotiropoulos et al. 2017) and strengthen the importance of sex in the preservation of alternative evolutionary trajectories (Mathiron et al. 2017; Denoël et al. 2019c).

Notably, although microcosm experiments as the present one can be a powerful tool to disentangle complex behaviors, there are some limitations due to their simplicity and the manipulation of environmental parameters (Fraser and Keddy 1997). However, several microcosm model systems have been used by ecologists in order to test ecological theories in practice and gain a deeper understanding in ecological processes (Cadotte et al. 2005). In this case, paedomorphs might show varying levels of intensity in their behavioral patterns or less prompt responses in the field, where there is a strong environmental complexity. Paedomorphic newt populations lost over 50% of their aquatic area of occupancy over a 10-year period due to fish introductions in Montenegro, which is considered a hotspot of paedomorphosis (Denoël et al. 2019a). Further field studies in natural aquatic ecosystems are necessary to investigate the extend of ecological phenomena such as predator-prey dynamics.

Despite that, our results offer some insight regarding newt conservation. Introduced alien fish induce complex disturbances to the local amphibians, promoting behavioral and phenotypic responses, which might compromise, apart the viability of local amphibian populations, also the expression and sustenance of alternative evolutionary trajectories such as facultative paedomorphosis. Future conservation strategies should focus in preserving local amphibian populations and at the same time avoid fish introductions. After all, it has been shown that amphibians are actually more capable in regulating mosquito

populations, and thus provide an effective natural agent in the control of diseases, than the introduced mosquitofish (Durant and Hopkins 2008; Bowatte et al. 2013).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experiments were approved by the Hellenic Ministry of the Environment & Energy under a special permit (code: 6ΦΠΘ4653Π8-AOT), and all used methods were carried out in accordance with approved guidelines.

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