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**Joint effects of rising temperature and the presence of introduced
predatory fish on montane amphibian populations**

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Running title: Warming and fish predators effects on amphibians

Abstract

Amphibian populations in montane habitats are often subjected to high thermal variability, which may exacerbate anthropogenic impacts such as the introduction of exotic species. Here we present data from two experiments exploring the joint effects of rising temperatures and the presence of waterborne cues from an exotic predatory fish on the short- and long-term antipredatory responses (i.e., activity and time to metamorphosis respectively) of *Rana iberica* and *Salamandra salamandra* larvae from two montane amphibian populations. We found some evidence of a cumulative effect of an increase in temperature and the presence of predators. Although predator recognition was not precluded at rising temperatures, we observed an increase in larval activity in warmer water, which might negatively affect survival by favoring prey detectability by predators. We also observed a strong quadratic effect of temperature and a joint effect of temperature and predators on larval development: at intermediate temperatures, larvae exposed to exotic trout cues had greatly accelerated metamorphosis. These results suggest that warmer conditions might be particularly harmful for larvae in montane wetlands enduring the presence of exotic predators.

Keywords global change; exotic fish; predator recognition; *Rana iberica*; *Salamandra salamandra*; warming

Introduction

Declines in amphibian populations are a worldwide phenomenon related to multiple anthropogenic factors such as habitat destruction, climate change, the introduction of exotic species or infectious disease (Blaustein & Kiesecker, 2002; Wake & Vredenburg, 2008; Hayes, Falso & Stice, 2010). The effects of some of these factors (e.g., habitat destruction, exotic species) are well understood by conservation biologists, in part because their impacts have occurred over a long period. In contrast, a poorer –but increasing– understanding exists on how other factors (e.g., climate change, infectious disease) affect amphibian populations (Collins & Storfer, 2003). Also, the interactions of different stressors are not well known (Boone et al., 2007), partly because of their non-additive effects, which cannot be predicted from single stressor impacts (Darling & Cote, 2008). Some studies have confirmed the existence of interactive effects: for example, activity and survival of larval *Rana cascadae* significantly diminished after being exposed to low pH, high nitrate levels and ultraviolet-B (UV-B) light acting together (Hatch & Blaustein, 2000). In other experiments, predator-induced stress intensified the toxic effects of pesticides on larvae of several anuran species (Relyea & Mills, 2001; Relyea, 2004), and Reeves et al. (2010) detected amphibian limb abnormalities caused by the joint presence of predators and contaminants.

Although an increasing number of studies have examined the simultaneous effects of multiple factors on amphibian populations, demonstrations of causal relationships between amphibian declines and the interactions of impacts due to global change and natural stressors are rare (Blaustein et al., 2011). For example, temperature fluctuation is a local stressor that can negatively affect amphibian populations (Niehaus, Wilson & Franklin, 2006; Niehaus et al., 2011, 2012; Raffel et al., 2006, 2012), and its effects might exacerbate the negative outcome of anthropogenic impacts. Montane areas may be particularly affected, given their high thermal variability and temporal constraints (Navas, 1997; Navas et al., 2013). For

instance, larval and juvenile growth periods in montane or boreal habitats are constrained by the timing of snowmelt, which determines the start of the breeding season, and by temperature drop after summer, which triggers hibernation (Corn, 2005). In addition, landscape features and connectivity of aquatic habitats may be more limiting for amphibian populations in montane areas than in the lowlands (Bosch et al., 2006). For appropriate amphibian conservation, it is thus critical to assess the effects of rising temperatures in combination with other stressors on amphibian montane populations.

We explored the joint effects of rising temperatures and the presence of an exotic predatory fish, the widely introduced rainbow trout, *Oncorhynchus mykiss*, on two montane amphibian populations. In the past century, *O. mykiss* and other salmonid species have been extensively introduced in montane areas in which they were historically absent, and are one of the major causes for the decline of local amphibian populations in these areas (Knapp & Matthews, 2000; Bosch et al., 2006). We hypothesized that the outcome of the predator-prey interactions between introduced fish and amphibian larvae would be aggravated by elevated temperatures. Specifically, we tested:

- (1) Whether larval short-term antipredatory responses (i.e., reduced activity levels) are precluded at high temperatures. In the presence of waterborne cues from predators, amphibian larvae often decrease their activity, reducing the probabilities of being visually detected (Bosch et al., 2006; Ferrari, Wisenden & Chivers, 2010; Polo-Cavia & Gomez-Mestre 2014). However, at high temperatures, larval activity increases (Brown et al., 2004), possibly weakening or precluding the antipredatory behavior. High temperatures might also preclude antipredatory responses, by affecting predator recognition or swimming speed (Wilson & Franklin, 1999; Katzenberger et al., 2014).
- (2) Whether larval long-term antipredatory responses (i.e., faster development and shorter time to metamorphosis) are affected by rising temperatures. Some amphibian larvae

show faster development and earlier metamorphosis in the presence of predators (Tejedo et al., pers. comm.). A temperature increase also increases the rate of larval development (Bachmann, 1969). However, because early metamorphosis would reduce survivorship and viability of the young (Duellman & Trueb, 1986), we did not predict a cumulative effect of predators and rising temperatures on reduction of the duration of the larval stage.

Material and methods

Study animals and predator cues

We assessed the effects of rising temperature and the presence of waterborne cues from predatory fish on larvae of two amphibian species: *Rana iberica* and *Salamandra salamandra*. *R. iberica* is an Iberian endemic listed nationally and regionally as vulnerable (Esteban & Martínez-Solano, 2002). The species is mainly threatened by habitat degradation through agriculture, deforestation, tourism development, and the introduction of non-native predators (Tejedo et al., 2009). *S. salamandra* is broadly distributed on the Iberian Peninsula, although it is listed nationally as vulnerable (Buckley & Alcobendas, 2002). It typically occupies humid and shaded riverine habitats, with low tolerance of dry conditions and open environments (Velo-Antón & Buckley, 2015). For these reasons, montane populations of these two species may be especially sensitive to climate warming.

Four egg clutches of *R. iberica* and *S. salamandra* larvae were collected by hand-netting in several sites of a fishless stream in Peñalara (Sierra de Guadarrama National Park, central Spain; 40°50'N, 3°57'W), a montane area composed of several small glacial valleys, situated at 1800-2430 m asl. Natural temperatures in ponds and streams of the study area can vary greatly, even ranking from 14 to 21 °C in summer, depending of water regime (permanent vs. non-permanent), water depth and proximity to shores. Eggs and larvae were

transported to the Captive Breeding Centre for Threatened Amphibians of Guadarrama Mountains at Rascafría (14 km distance), where experiments took place. *S. salamandra* larvae and, upon hatching, *R. iberica* larvae were raised individually in 1 L containers with a gravel bottom and dechlorinated water at 15 (stream temperature at time of collection), 20 or 25 °C, under natural photoperiod (12L:12D). We used water baths to maintain water temperature constant. Larvae were fed a combination of ground rabbit chow and tubifex *ad libitum*, twice weekly. We obtained exotic rainbow trout, *O. mykiss*, and native brown trout, *Salmo trutta*, from commercial sources, to be used as predator cue donors. In the study area, both *R. iberica* and *S. salamandra* coexist with brown trout in shallow waters and in ponds and streams with shores that provide shelter –generally trunks, in which larval amphibians find protection from this native predator. Thus, brown trout were included for comparative purposes. To prepare the predator cues, three 80 L aquaria were used to house one large native trout, one large exotic trout, and no fish respectively. All aquaria were supplied continuously with water from a fishless stream section at a constant, slow flow rate, and drained at the same rate. In this way, we aimed to continuously pervade the flowing water in the fish aquaria with chemical cues from the predators. Trout were not fed during the experiments to avoid confusing effects of diet cues. Room temperature was 15 °C and photoperiod was the same as that of amphibian larvae. We conducted two mesocosm experiments between 2011 and 2012 to test the hypotheses explained above.

Experiment I. Short-term antipredatory responses

To analyze the effect of rising temperatures on the short-term antipredatory responses of amphibian larvae to waterborne cues from native and exotic predatory fish, we compared activity levels of *R. iberica* and *S. salamandra* larvae across three predator treatments (‘native trout cues’ vs. ‘exotic trout cues’ vs. ‘no predator cues’), and across three different

temperatures including the stream temperature at the time of collection (15 °C) and two higher temperatures (20 and 25 °C). This design allowed us to compare responses of larvae in different thermal environments. The ‘no predator cues’ treatment was included to estimate basal activity of larvae in a predator-free environment. We used two sets of larvae, *R. iberica* ($n = 13$; Gosner stage 35-37; Gosner, 1960) and *S. salamandra* ($n = 15$; advanced developmental stages), which had been held at 15 °C. Each larva was exposed to the nine treatment combinations in random order, with a rest period of at least 5 h between trials. Larvae were tested individually in a U-shaped PVC gutter (10 x 60 cm), sealed at both ends with plastic caps and filled with 2.5 L of dechlorinated water. Four transverse lines were marked inside the gutter to create five sections of equal surface area. When the experiment started, we placed a single larva enclosed within a cage in one end of the gutter, and depending on the predator treatment, we added in the other end 2.5 mL aliquots of water extracted from the fish or fishless aquaria containing native trout cues, exotic trout cues or no predator cues. The larva was given 10 min within the cage to acclimate to experimental conditions, and then it was released and allowed to move freely. From this moment, we recorded every 1 min during 12 min the number of times the larva crossed from one section of the gutter to another. All behavioral observations were carried out by the same observer, who remained immobile at a hidden position to avoid disturbing the larvae during the trials. Finally, we estimated larval activity as the mean number of times the larva moved between sections per min in each trial (Rohr & Madison, 2001; Gonzalo, López & Martín, 2007; Polo-Cavia et al., 2010).

Experiment II. Long-term antipredatory responses

To analyze the effect of rising temperatures on the long-term antipredatory responses of amphibian larvae to waterborne cues from native and exotic predatory fish, we compared

larval development of *R. iberica* across three predator treatments ('native trout cues' vs. 'exotic trout cues' vs. 'no predator cues'), and across three different temperatures (15, 20 and 25 °C), as above. A new set of *R. iberica* larvae of Gosner stages 25-30 (Gosner 1960) were randomly assigned to the nine treatment combinations. Larvae ($n = 126$; mean per treatment = 14) were raised individually in the 1 L containers and kept at their allocated temperature during the whole experiment using a thermostatically controlled water bath. Depending on the predator treatment, containers were continuously drip-fed with water from the fish or the fishless aquaria, and drained at the same rate. Each trial was terminated when the larva started to metamorphose. The response variable was the number of days elapsed from the beginning of the experiment to the beginning of metamorphosis (maximum 77 days).

Data analysis

To analyze data from Experiment I, we used separate two-way repeated measures analyses of variance (ANOVAs) for *R. iberica* and *S. salamandra* on mean larval activity (\log_e -transformed to improve homoscedasticity) with temperature and predator treatment ('native trout cues' vs. 'exotic trout cues' vs. 'no predator cues') as two within-subjects factors. For Experiment II we used two-way ANOVA with temperature and predator treatment as the two between-subject factors. The number of days each tadpole took to metamorphose was the dependent variable, again \log_e -transformed. A priori contrasts were used for pairwise comparisons between predator treatments, and linear and quadratic trends were fitted across the levels of temperature. Analyses were performed using Statistica 12.0 software.

Results

Short-term antipredatory responses

Activity levels of *R. iberica* larvae were significantly affected by temperature ($F_{2,24} = 5.90$, $P = 0.01$), with an increasing linear trend ($t = 3.63$, $P = 0.003$) but no evidence of a quadratic effect ($t = 0.41$, $P = 0.69$) (Fig. 1A). The predator treatment and the interaction between temperature and predator treatment were non-significant ($F_{2,24} = 1.19$, $P = 0.32$ and $F_{4,48} = 0.77$, $P = 0.55$ respectively). Activity levels of *S. salamandra* larvae were significantly affected by temperature ($F_{2,28} = 3.95$, $P = 0.03$), with an increasing linear trend in activity with increasing temperature ($t = 2.41$, $P = 0.03$) but no evidence of a quadratic effect ($t = 0.94$, $P = 0.37$) (Fig. 1B). There was also a significant effect of predator treatment on larval activity of *S. salamandra* ($F_{2,28} = 3.83$, $P = 0.03$). Compared to their basal activity in the absence of predator cues, *S. salamandra* larvae reduced significantly their activity in the presence of both native ($t = 2.21$, $P = 0.045$) and exotic trout cues ($t = 2.29$, $P = 0.04$). There was no evidence of an interaction between temperature and predator treatment affecting larval activity of *S. salamandra* ($F_{4,56} = 0.36$, $P = 0.84$).

Long-term antipredatory responses

Time to metamorphosis of *R. iberica* larvae was significantly affected by temperatures ($F_{1,117} = 24.7$, $P < 0.0001$) and predator treatment ($F_{1,117} = 7.20$, $P = 0.001$). We observed a strong quadratic trend across temperatures ($t = 6.99$, $P < 0.0001$), with the number of days to metamorphosis least at 20°C (Fig. 2). Time to metamorphosis did not significantly differ between larvae exposed to native trout cues and larvae exposed to no predator cues ($t = 0.40$, $P = 0.69$), but time to metamorphosis of larvae exposed to exotic trout cues was reduced by 8.1 % compared with larvae exposed to no predator cues ($t = 3.24$, $P = 0.002$) (Fig. 2). The interaction between temperature and predator treatment ($F_{4,117} = 2.40$, $P = 0.06$) suggested a small effect. Across temperatures (quadratic trend), time to metamorphosis of larvae exposed to native trout cues did not significantly differ from that of larvae exposed to no predator cues

($t = 0.43$, $P = 0.67$), but, in contrast, larvae exposed to exotic trout cues showed significantly shorter time to metamorphosis than larvae exposed to no predator cues ($t = 2.53$, $P = 0.01$), with the number of days to metamorphosis least at 20°C (Fig. 2).

Discussion

As expected, larval activity of *R. iberica* and *S. salamandra* larvae increased linearly when exposed to temperatures increasing from 15 °C to 25 °C. *S. salamandra* larvae also responded to the presence of predator cues by decreasing their activity, whereas the reduction in activity rates of *R. iberica* larvae was non-significant, although this species has been shown to innately respond to the presence of predators in previous studies, including native and exotic trout (Bosch et al., 2006). The absence of significant behavioral antipredatory responses in *R. iberica* larvae might be due to the wide variability in the sample. Larval activity of *S. salamandra* was reduced in response to both native and exotic trout cues. These results suggest that *S. salamandra* larvae have the ability to innately recognize introduced predators, and contrast with most studies exploring the antipredatory responses of amphibian larvae, which often demonstrate innate recognition of native but not exotic predators (Kiesecker & Blaustein, 1997; Marquis, Saglio & Neveu, 2004; Polo-Cavia et al., 2010; Gomez-Mestre & Díaz-Paniagua, 2011; Polo-Cavia & Gómez-Mestre, 2014). The responses observed in our experiment might be due to chemical similarities between cues from native and exotic trout belonging to the same family and allowing generalization of predator recognition by salamander larvae (Ferrari, Wisenden & Chivers, 2010).

Activity of larval amphibians is known to increase in warmer waters, resulting in beneficial increased metabolic rates and growth (Duellman & Trueb, 1986; Brown et al., 2004). In contrast, amphibian larvae typically reduce activity in the presence of predators, resulting in decreased growth rates, but favoring survival (Werner, 1986; Figiel & Semlitsch,

1990; Skelly & Werner, 1990; Skelly, 1992; Stauffer & Semlitsch, 1993; Kats & Dill, 1998). Therefore, rising temperatures might preclude larval behavioral defenses by impeding recognition of predator chemical cues, or as the result of the different effects of temperature and predation on activity. While we predicted that the short-term antipredatory responses of amphibian larvae would be weakened or eliminated at higher temperatures, we found only partial support for this hypothesis. Contrary to our expectations, *S. salamandra* larvae reduced their activity in response to predators independent of temperature. Nonetheless, although our results indicate that predator recognition by *S. salamandra* larvae is not precluded at rising temperatures, the positive effect on survival of reduced activity in response to predators might be offset by increased activity in warmer conditions. More active larvae would be more easily detected and captured by predators: thus there was no significant reduction in activity of *S. salamandra* larvae in the presence of predator cues at elevated temperatures compared with their basal activity in the absence of predator cues at 15 °C (Fig. 1b). Further studies are needed to confirm whether increased water temperatures indirectly affect the survivorship of amphibian larvae against predators.

Regarding the long-term antipredatory responses, we found that *R. iberica* larvae metamorphosed earlier at 20 °C than at 15 °C or 25 °C. Performance curves of physiological rates often have an inverted ‘U’ shape, with performance typically increasing with temperature until reaching a thermal optimum, beyond which performance decreases (Izem & Kingsolver, 2005). Previous studies have shown amphibian larval development to follow this quadratic trend in response to rising temperature onto a certain limit (Moore & Townsend, 1998). Although these studies have found developmental rates to decrease from 27-30 °C, with temperatures above 30 °C having deleterious effects on performance, the increased rate of larval development in our experiment occurred at a lower temperature (Fig. 2). It is expected that lower ambient temperatures in montane areas would lead to populations having

lower thermal optima, so larval growth and development would be maximized at lower temperatures. Thus, reduced growth and developmental rates might be especially evident in montane amphibian populations in warmer waters.

We observed earlier metamorphosis in larvae exposed to exotic trout cues, but not to native trout cues. Exposure to predators is known to alter developmental rates of amphibian larvae, and reduced time to metamorphosis in response to predators is interpreted as an adaptive strategy to escape from risky aquatic environments (Sih & Moore, 1993; Warkentin, 1995; Laurila, Karttunen & Merilä, 2002; Orizaola & Braña, 2004). On the other hand, theory predicts that adaptive defenses of prey will be functional against local predators but not against introduced ones with which they lack joint evolutionary history (Schlaepfer et al., 2005). Consequently, we expected to observe faster development and shorter time to metamorphosis in larvae exposed to cues from native rather than to exotic predatory trout. However, our data suggest that *R. iberica* larvae are able to shorten the larval period and accelerate metamorphosis in response to exotic predators. Interestingly, we also found evidence for a joint effect of rising temperatures and presence of predator cues on larval development at 20 °C. Thus, higher temperatures may be particularly stressful for larvae in the presence of predators and subjected to developmental constraints or stressful heating conditions limiting an earlier metamorphosis.

In conclusion, our study was able to detect partial evidence for a cumulative effect between an increase in temperature and the presence of predators. Although rising temperatures were not observed to preclude predator recognition by amphibian larvae, our data suggest that larval behavioral responses against predators might be compromised in warmer conditions, in which increased motion and activity might favor prey detectability by predators. Rising temperatures might also have complex effects on the trade-off between growing large and metamorphosing quickly to avoid predation (Werner, 1986; Rowe &

Ludwig, 1991; Abrams et al., 1996; Rudolf & Rödel, 2007). Thus, increased foraging activity in warmer waters would be expected to cause increased growth rates, greater size at metamorphosis and higher metamorphic survival (Werner, 1986; Figiel & Semlitsch, 1990; Skelly & Werner, 1990; Skelly, 1992). However, this could lead to easier detection by predators and a higher predatory risk for larvae. These interdependent effects of rising temperature on the short- and long-term antipredatory responses of larval amphibians would be expected to reduce larval and/or metamorphic survival, with strong implications for the conservation of montane amphibian populations.

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442

443 **Figure legends**

444

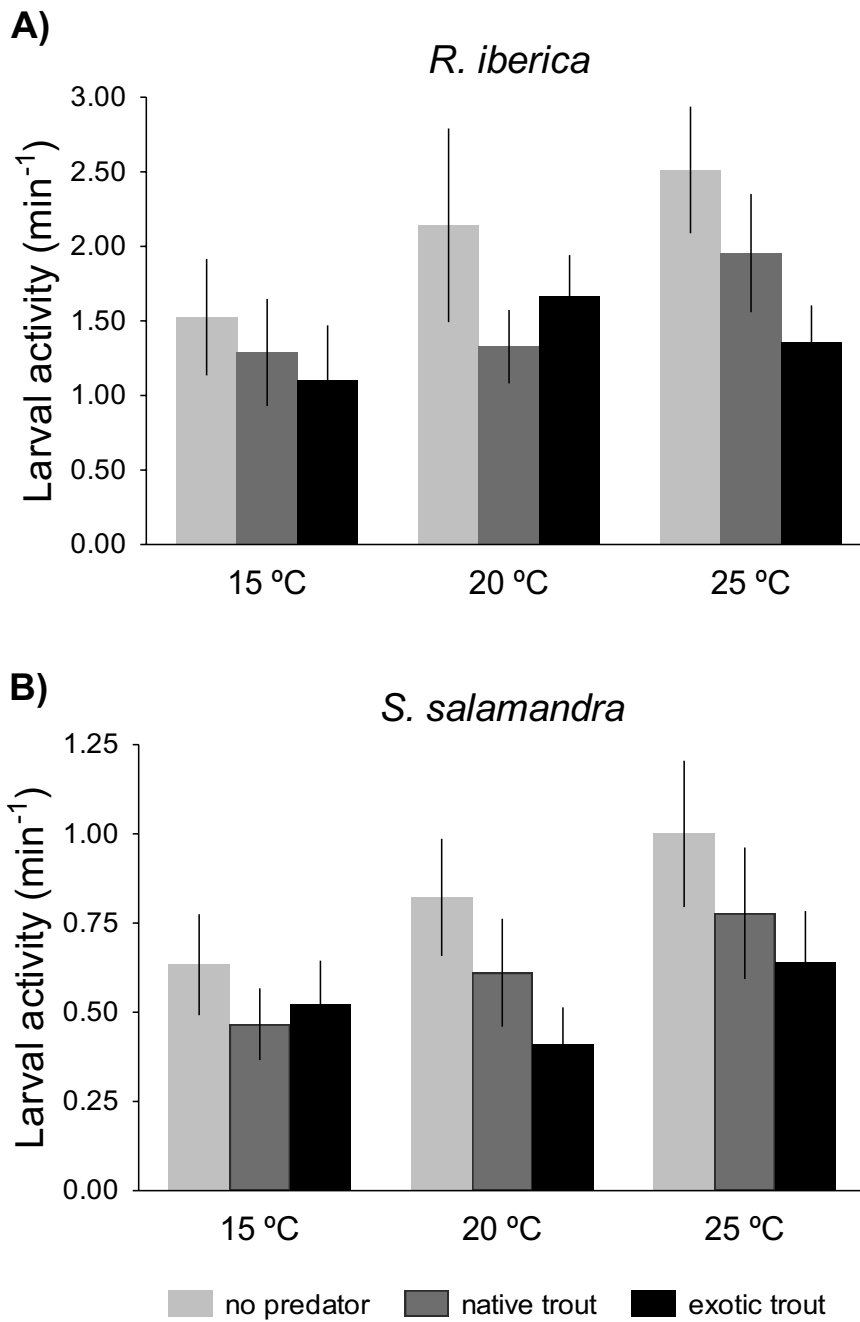
445 **Figure 1** Variation in activity levels of **A)** *Rana iberica* and **B)** *Salamandra salamandra*
446 larvae (mean \pm SE for number of times per min the larvae moved between sections of the
447 experimental container) depending on temperature and presence of waterborne cues from
448 native and exotic predatory fish (Experiment I).

449

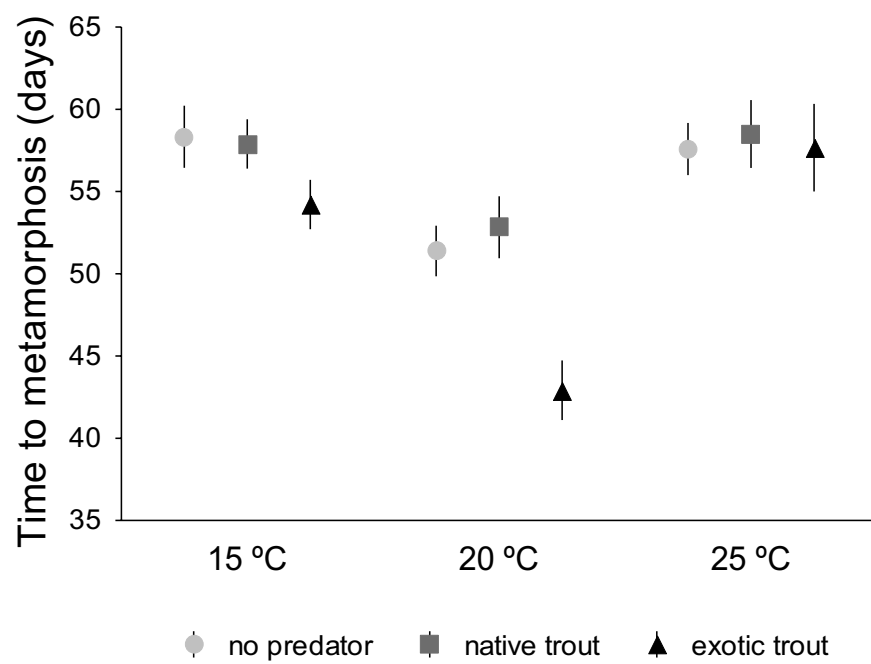
450 **Figure 2** Variation in time to metamorphosis (mean \pm SE for number of days elapsed from
451 the beginning of the experiment to metamorphosis) of *Rana iberica* larvae depending on
452 temperature and presence of waterborne cues from native and exotic predatory fish
453 (Experiment II).

454

Fig. 1



460 **Fig. 2**



461