The role of predation risk in metamorphosis versus behavioural avoidance: a sex-specific study in a facultative paedomorphic amphibian

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Abstract

Evolutionary theory predicts the evolution of metamorphosis over paedomorphosis (the retention of larval traits at the adult stage) in response to life in unfavourable habitats and to the benefits of dispersal. Although many organisms are canalised into obligatory complex or simple life cycles, some species of newts and salamanders can express both processes (facultative paedomorphosis). Previous research highlighted the detrimental effect of fish on both metamorphic and paedomorphic phenotypes, but it remains unknown whether predation risk could induce shifts from paedomorphosis to metamorphosis, whether behavioural avoidance could be an alternative strategy to metamorphosis and whether these responses could be sex-biased. Testing these hypotheses is important because metamorphosed paedomorphs are dispersal individuals which could favour the long-term persistence of the process by breeding subsequently in more favourable waters. Therefore, we quantified the spatial behaviour and timing of the metamorphosis of facultative paedomorphic palmate newts *Lissotriton helveticus* in response to predation risk. We found that fish induced both male and female paedomorphs to hide more often, but behavioural avoidance was not predictive of metamorphosis. Paedomorphs did not metamorphose more in the presence of fish, yet there was an interaction between sex and predation risk in metamorphosis timing. These results improve our understanding of the lower prevalence of paedomorphs in fish environments and of the female-biased sex-ratios in natural populations of paedomorphic newts. Integrating sex-dependent payoffs of polyphenisms and dispersal across habitats is therefore essential to understand the evolution of these processes in response to environmental change.

Introduction

Metamorphosis and paedomorphosis are two major developmental pathways that evolved in response to trade-offs between the benefits and costs of life in larval versus adult habitats in a broad range of organisms as diverse as cnidarians, echinoderms and amphibians (Gould 1977; McKinney and McNamara 1991; McNamara 2012). Metamorphosis is a post-embryonic abrupt ontogenetic change in an individual's morphology, physiology and behaviour, which involves remodelling and adaptations typically associated with a transition of habitat. By metamorphosing, organisms exhibit a complex life cycle (Istock 1967; Wilbur 1980; Laudet 2011). In contrast, organisms with 'simple' life cycles typically use the same kind of habitat and food throughout their life without undergoing a sudden ontogenetic change (Wilbur 1980; Stocks and Cordoba-Aguilar 2012; Andrews 2017). This is the case of species that exhibit paedomorphosis, a process which corresponds to the retention of larval traits at the adult stage and, therefore promotes the use of favourable local resources, particularly in predator-free habitats (Wilbur and Collins 1973; Sprules 1974; Wilbur 1980; McKinney and McNamara 1991; Lejeune et al. 2018). Because paedomorphosis opens the possibility to breed while retaining a larval morphology much different from that one resulting from metamorphosis, this process is thought to have played a major role in macro-evolution, subsequently giving rise to several groups of organisms (Gould 1977; Laudet 2011; McNamara 2012).

In newts and salamanders, several species and even families are canalized into either metamorphosis or paedomorphosis. For instance, proteids, amphiumids and sirenids are obligatory paedomorphs, whereas some salamandrids, ambystomatids and plethodontids are obligatory metamorphs (Wells 2007; Bonett and Blair 2017). Predation and aridity are thought to have influenced the evolution of these developmental processes (Hairston 1980; Shaffer 1984; Roček 1995; Bonett et al. 2014). Facultative paedomorphic species are good models to determine the importance of such environmental variables in shaping life cycles and, to a broader extent, the fixation of these processes because both paedomorphosis and metamorphosis can be expressed in the same populations (Semlitsch 1987; Whiteman 1994; Denoël et al. 2005b; Oromi et al. 2016). Although previous research showed that individuals can go through either of the two developmental pathways depending on environmental cues, such as drying, and can move onto land to avoid inadequate aquatic conditions as a result of metamorphosis (Semlitsch 1987; Mathiron et al. 2017), predation has not yet been shown experimentally to favour the transition between paedomorphosis and metamorphosis.

The introductions of aquatic alien species, such as fish, alter all components of native communities, from consumers to producers (Sih et al. 1985; Knapp et al. 2001; Strauss et al. 2006). This has contributed to the worldwide trend of declining populations of amphibians (Knapp and Matthews 2000; Kats and Ferrer 2003; Orizaola and Braña 2006; Bucciarelli et al. 2014) but this has disproportionately affected facultative and obligatory paedomorphic species (Sprules 1974; Denoël et al. 2005a, 2019; Contreras et al. 2009; Denoël and Winandy 2015). For example, high declines were reported in facultative paedomorphic newts, even resulting in local extinctions (Denoël et al. 2005a, 2009, 2019). Similarly, a nearly obligate paedomorph, the axolotl (*Ambystoma mexicanum*), is now at the edge of extinction, partly due to fish introductions (Contreras et al. 2009). However, the causes of the highest decline of the

facultative paedomorphic phenotype in the mosaic of fish and fish-free habitats are still not well known (Denoël et al. 2005a; Denoël and Ficetola 2014). Previous experimental studies showed that metamorphs can escape fish habitats by moving onto land (Winandy et al. 2017). In contrast, because of their larval aquatic traits, paedomorphs are usually restricted to water, being unable to leave the aquatic environments without metamorphosing (Denoël 2003; Mathiron et al. 2017).

No studies have demonstrated that paedomorphs can escape predation risk through metamorphosis and, therefore, whether predation risk can favour metamorphosis over paedomorphosis. Most studies that have tested how predation risk affects metamorphosis were done on larvae and not directly on paedomorphs (Relyea 2007). They showed that larvae were either not responding (Figiel and Semlitsch 1990) or metamorphosed faster (Orizaola and Braña 2005) or slower (Van Buskirk and Schmidt 2000) in the presence of a predatory risk. In the context of facultative paedomorphosis, the only available results come from Jackson and Semlitsch (1993) who showed that larvae did not develop more into metamorphs than paedomorphs in response to predatory cues. The divergence of these results can be explained by the two alternative strategies which larvae can adopt to respond to predation: invest in growth to decrease predation risk or invest in development (i.e. metamorphosis) to escape risky habitats (Van Buskirk and Schmidt 2000; Benard 2004; Relyea 2007). Yet, in many facultative paedomorphic species, paedomorphs are larger than the minimum size at metamorphosis and, consequently, do not need to optimize their growth to be able to metamorphose (Werner 1986). An alternative anti-predator strategy exhibited by amphibians is to use aquatic shelters, thus decreasing the probability of encounters with a predator while remaining in water (Van Buskirk and Schmidt 2000; Winandy and Denoël 2013b). In such a situation, paedomorphs were found to hide more often than metamorphs (Winandy et al. 2016). Shelter use and metamorphosis could therefore be two alternative responses to avoid predation risk, but the links between these strategies have not yet been directly explored.

Through laboratory experiments, we aim to test whether (*i*) facultative paedomorphs can be induced to metamorphose by the presence of fish, (*ii*) if these paedomorphs hide more in shelters due to predation risk and (*iii*) if the probability of metamorphosis is sex-biased. We considered sex because sex has been shown to affect the probability to metamorphose (Mathiron et al. 2017) and because it is not clear how a paedomorph's sex affects the probability of metamorphosis in response to predation risk (e.g. Relyea 2007). Studying both metamorphosis and shelter use will also allow us to test the hypothesis that individuals that hide more often may avoid metamorphosis. In addition to understanding the drivers of metamorphosis, testing these hypotheses will improve our understanding of the rare coexistence of paedomorphic newts with fish and the poorly explained prevalence of paedomorphic females over paedomorphic males (i.e. female-biased sex ratios) in natural populations (see e.g. Gabrion 1976; Kalezić and Džukić 1985; Whiteman 1997). More broadly, this will help us to determine how predation and sex can affect the outcome of heterochronic processes and, therefore, complex versus simple life cycles.

Material and methods

Study organisms

Thirty-two paedomorphic palmate newts (*Lissotriton helveticus*, Amphibia, Salamandridae; 16 of each sex) were caught on 18-Mar-2015 in a fishless pond (Mas d'Azirou, Larzac, France; 43.75°N – 3.48°E, 652 m a.s.l.). Their maturity and sex were confirmed by inspection of their sexually dimorphic cloaca (Denoël 2017) (Suppl. Fig. 1). The sex-ratio in the natural population of paedomorphs was female-biased, with a ratio of 2.1 females per male (M. Denoël, pers. obs.).

This species was chosen as a model system for two main reasons: (1) it exhibits facultative paedomorphosis depending on environmental cues, such as hydroperiod and temperature, and (2) its natural populations of paedomorphs are female-biased (Mathiron et al. 2017). Previous experiments showed that the metamorphosis of this species can be completed in less than two weeks in captive conditions (Mathiron et al. 2017). Moreover, Larzac is an area in Europe where paedomorphic palmate newts are widespread (Denoël and Ficetola 2015).

In the experiment, we used goldfish (*Carassius auratus*) that came from stock bred in the laboratory. This species is a good model to investigate the effect of predation risk on paedomorphosis for four reasons: (1) goldfish are regularly introduced into the natural habitat of paedomorphic palmate newts, (2) their presence is a significant cause of paedomorphic newt decline, (3) they prey on eggs and larvae and (4) they induce behavioural anti-predatory responses, such as shelter use (Monello and Wright 2001; Denoël and Winandy 2015; Winandy et al. 2016).

Laboratory experiment

For the laboratory experiment, newts were placed in eight experimental aquariums (two males and two females per aquarium; 60 x 60 cm, 40 cm water depth). The aquariums were split into two predation risk treatments ($n = 32$ newts in total): a control treatment (i.e. without fish; $n = 16$ newts) and a fish treatment (with two fish present in each aquarium; $n = 16$ newts). Each experimental aquarium had a shelter for the palmate newts (*Lissotriton helveticus*) preventing the fish (when present) from entering (closed entrance with a grid; mesh size: 3 x 3.3 cm). All aquariums were independent of each other (i.e. no water or visual communication between them). Temperature and dissolved oxygen in the water were regulated by a room air conditioner and oxygen diffusers placed in each aquarium, respectively. Their values were maintained at a mean ± SE of 15.16 ± 0.03 °C and 9.55 ± 0.01 mg/l, respectively (HQ40D electronic multimeter: Hach, Loveland, CO, USA). There was no significant difference between the two treatments for both the temperature ($\chi^2_1 = 0.867$, $P = 0.35$) and dissolved oxygen ($\chi^2_1 = 3.003$, $P = 0.08$). The photoperiod followed the natural cycle of the population from which the newts were taken (light duration: 12:30–15:30, with changes made in regular increments). Lighting was obtained by both Lumilux de Luxe daylight tubes (OSRAM, L36W/12-950) and Sylvania Professional tubes (36W DECOR183) and, for the first and last 30 min of each day, by a daylight L36W/965 Biolux bulb (OSRAM) to simulate dawn and dusk.

The mean ± SE snout-vent length of the paedomorphic newts (i.e. from the tip of the snout to the end of the cloaca) was 3.54 ± 0.03 cm, and the standard length of the fish (i.e. from the tip of the snout to the end of the last vertebra) was 10.62 ± 0.32 cm. There was no significant

difference in the length of newts between the two treatments (males: mean ± SE = 3.45 ± 0.03 cm, range: 3.37–3.61 cm, $F_{1,14}$ = 0.075, P = 0.788; females: 3.63 ± 0.04 cm, range: 3.50–3.99 cm, $F_{1,14}$ = 0.315, P = 0.58). All paedomorphs had a larger size than the minimum size for metamorphosis, including in the studied population (M. Denoël and N. Oromi, pers. obs.). The specific fish size was chosen because it is commonly found in nature and would not hurt or have lethal effects on newts, while still being perceived as a risk (Winandy et al. 2016). Goldfish were let in direct possible contact with the newts to mimic a natural situation, specifically because previous results showed that newts can become habituated to caged fish and stop expressing anti-predator response in indirect contact (Jackson and Semlitsch 1993; Winandy and Denoël 2013a). However, newts could still avoid fish by using the shelter.

Newts were fed every day with their dietary requirements of unfrozen blood worms (50 mg of *Chironomus* per newt). Food was weighed on a Scout Pro electronic scale (OHAUS, Newark, NJ, USA) and provided to newts under their shelter to prevent foraging from fish (Suppl. Fig. 1). Fish were fed directly in open water (200 mg of *Chironomus* per individual) where they rapidly consumed the worms before the newts could access them.

Data collection and analysis

First, we quantified the shelter use of the newts during the weeks preceding the first metamorphosis event. To do so, we computed a score for the presence of each paedomorph under the shelter, based on 60 observations per individual: using a focal sampling method, we did 3 observations per day (1 min per aquarium and per observation), 5 times a week over 4 weeks, for a total of 1,920 observations. Newts were recognized within each aquarium by their external individual patterns. Therefore, they were not manipulated during the behavioural observations. We used a generalized mixed model (GLMM) with a binomial error distribution to test the effects of predation risk, sex and their interaction on the proportion of shelter use. Time (4 weeks of repeated measures) was included as a continuous variable in the model, and aquarium and individual identity were included as a random intercept (individuals nested within aquariums). We assessed the significance of factors using a likelihood ratio test (Burnham and Anderson 2002; Bolker et al. 2009). The non-significant interaction was dropped from the final model.

To assess metamorphosis, each newt was observed every 7 days over 36 weeks for signs of the completion of metamorphosis, which was determined as the closure of gill slits (see Denoël 2017). We used a GLMM with a binomial error distribution to test the effect of predation risk, sex and their interaction on metamorphosis probability (scored as 0 = still paedomorphs and 1 = metamorphosed individuals). Initial body size (i.e. snout-vent length) and body condition (i.e. initial mass/size residuals) were added as covariates and the aquarium identity was included as a random intercept. In addition, to specifically test the hypothesis of an alternative anti-predator strategy between shelter use and metamorphosis in the presence of fish, we used a GLMM (binomial error) to assess the effect of shelter use on the probability to metamorphose. The aquarium identity was included as a random intercept. We assessed the significance of factors using a likelihood ratio test. By means of a backward removal procedure,

non-significant covariates and interactions were dropped during model selection as simpler models are more adequate in such a case (see Pinheiro and Bates 2000).

Finally, we assessed if predation risk, sex and their interaction affected the timing of metamorphosis (i.e. the latency in weeks before metamorphosis) using non-parametric survival analyses for right-censored data based on the Kaplan–Meier estimation. To highlight the differences of the survival curves between the 4 groups (interactive effect between sex and predation risk), we used Wilcoxon log-rank statistics (Therneau 2017). The aquarium identity was included as a random intercept.

The significance level was 0.05. Analyses were performed in R 3.4.2 using lme4, survival, survminer and survMisc packages.

Results

Predation risk and shelter use

There was a significant effect of predation risk on shelter use (GLMM: $\chi^2_1 = 32.986$, $P < 0.001$): in the presence of fish, newts were nearly five times more often present under the shelter than in the absence of fish (Fig. 1). There were no significant effects of sex (GLMM: $\chi^2_1 = 0.311$, $P = 0.0.577$), time (GLMM: $\chi^2_1 = 2.836$, $P = 0.092$) and no significant interaction between sex and predation risk (GLMM: $\chi^2_1 < 0.001$, $P = 0.99$).
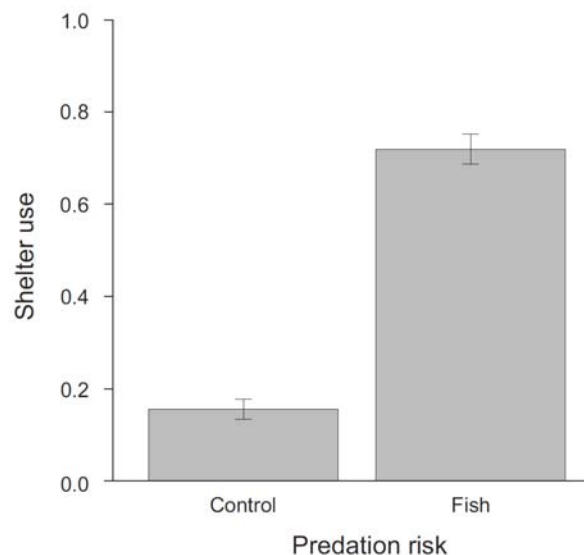

Fig. 1. Shelter use (mean ± SE) in paedomorphic palmate newts (*Lissotriton helveticus*; $n = 32$) in predation risk treatments (control versus fish).

Probability of metamorphosis

During the 36-week experiment, 62.5% of paedomorphic newts underwent metamorphosis (20 individuals on a total of 32 newts: 13 males and 7 females). There was no significant effect of predation risk (GLMM: $\chi^2_1 = 2.304$, $P = 0.129$), but a significant effect of sex (GLMM: $\chi^2_1 = 5.381$, $P = 0.02$) on the probability to metamorphose showing that males underwent metamorphosis twice as often as females (Fig. 2). The interaction between sex and predation risk on metamorphosis probability was not significant (GLMM: $\chi^2_1 = 2.541$, $P = 0.111$). The probabilities of metamorphosis in the four treatments were 0.5 ± 0.19, 0.375 ± 0.18, 1 and 0.625 ± 0.18 for females with and without fish and males with and without fish, respectively. Initial body size and body condition had also no significant effects (GLMM: $\chi^2_1 = 0.423$, $P = 0.516$; $\chi^2_1 = 0.0003$, $P = 0.986$; Suppl. Fig. 2). Finally, in the presence of fish, there was no effect of shelter use on the probability to undergo metamorphosis (GLMM: $\chi^2_1 = 0.01$, $P = 0.934$, Suppl. Fig. 3).
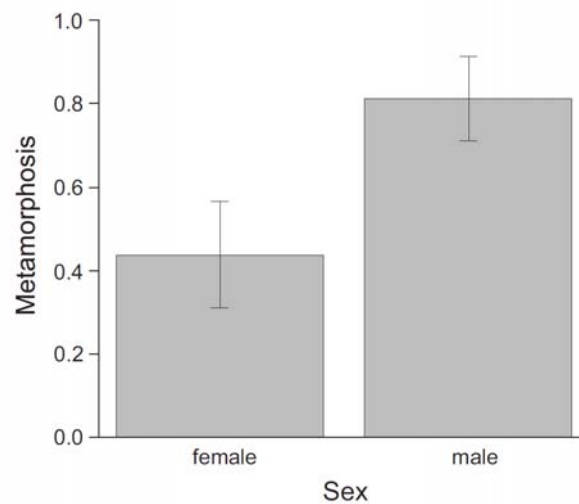

Fig. 2. Probability of metamorphosis ($\pm$ SE) of male and female palmate newts (*Lissotriton helveticus*; $n = 32$) during the 9-month experiment.

Timing of metamorphosis

The first completion of metamorphosis occurred after 4 weeks of experiment whereas late metamorphosis events were still found after 33 weeks. There was a significant effect of sex on the timing of metamorphosis, showing that males metamorphosed earlier than females (Wilcoxon test: $Z = 2.447$, $P = 0.014$). We did not detect a significant main effect of predation risk on the timing of metamorphosis (Wilcoxon test: $Z = 1.449$, $P = 0.147$). However, there was a significant interaction between predation risk and sex on the timing of metamorphosis (Wilcoxon test: $\chi^2_3 = 9.764$, $P = 0.021$; Fig. 3). Pairwise comparisons of the survival curves showed that males in the presence of fish metamorphosed earlier than females either exposed or unexposed to predation risk (Table 1).

Table 1 Pairwise comparisons of the survival curves (i.e. timing of metamorphosis of paedomorphic palmate newts; $n = 32$) between predation risk (control versus fish) and sex. Significant values are highlighted in bold (Wilcoxon test).

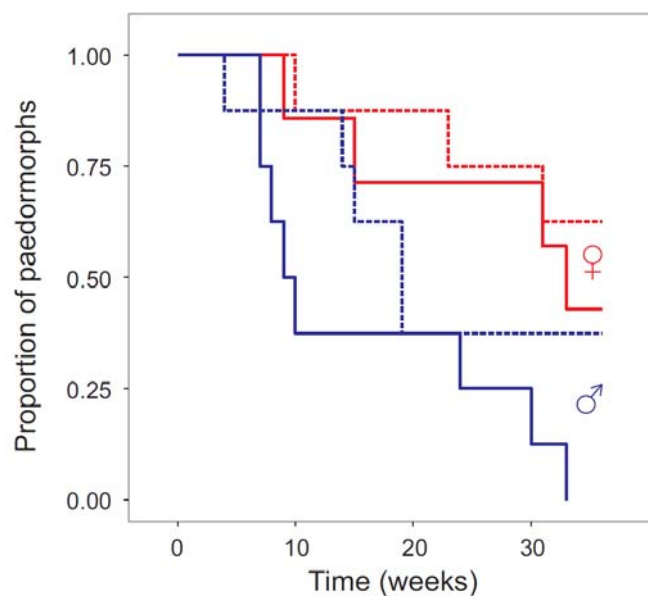| Pairwise comparisons		Z	P
♀ Control	♀ Fish	0.619	0.536
♀ Control	♂ Control	1.299	0.194
♀ Control	♂ Fish	2.683	**0.007**
♂ Control	♂ Fish	1.273	0.203
♂ Fish	♀ Fish	2.209	**0.027**

Fig. 3. Timing of metamorphosis (survival curves) for paedomorphic palmate newts (*Lissotriton helveticus*; $n = 32$) in both sexes and two predation risk treatments (fish versus control). Males: blue (lower) lines; females: red (upper) lines; fish treatment: continuous lines and control treatment: interrupted lines. See text for statistical results. This figure is available in colour in the online version of the journal.

Discussion

The present study disentangles the roles of predation risk and sex in the strategy adopted by newts, through either metamorphosis or shelter use. Whereas metamorphosis can make it possible for paedomorphs to escape waters by moving onto land (Mathiron et al. 2017), the use of shelters can decrease aquatic risk without having to leave water. Although behavioural avoidance could favour coexistence between newts and fish, the lateness or absence of metamorphosis can expose paedomorphs to a high predatory risk. Interestingly, in our study,

metamorphosis was dependent on the sex of individuals, whereas behavioural avoidance of fish before metamorphosis was similar in males and females. These results support our hypothesis of biased-sex responses in line with female-biased sex ratios in the wild, but they also showed the lack of a significant metamorphic response, particularly in females, to the presence of introduced fish.

Behavioural avoidance

Reducing activity or increasing shelter use is a typical response of larval and adult amphibians facing predation risk (Huang and Sih 1990; Van Buskirk and Schmidt 2000; Orizaola and Braña 2003; Teplitsky et al. 2003; Bucciarelli et al. 2014) and is a documented strategy for paedomorphic newts and salamanders (Jackson and Semlitsch 1993; Winandy et al. 2016). In our study, we found that both sexes of paedomorphs exhibited a similar avoidance strategy: they used shelters much more often when they were in the presence of fish (see also Winandy et al. 2016). However, contrary to our hypothesis, increasing shelter use during the paedomorphic stage did not prevent metamorphosis later during the course of the experiment. This dissociation of behavioural and metamorphic responses suggests that the mechanisms governing behavioural avoidance and metamorphosis in response to predation risk may be different. Unfortunately, the only available data in the literature on any direct link between activity and metamorphosis come from the larval stage of biphasic species for which transition onto land is usually an obligatory process (Laurila and Kujasalo 1999), whereas paedomorphs can fully bypass the transition to terrestrial life.

Sex-specific patterns of metamorphosis

Recent research showed that males can disperse more than females (Denoël et al. 2018) and that male-biased dispersal could explain phylogeographic patterns of sex-biased newt populations (Chiocchio et al. 2017). More specifically, in the context of facultative paedomorphosis, the 'male escape hypothesis' of Mathiron et al. (2017) predicts that, in newts, males are more prone to metamorphose than females. The present findings are in concordance with those of these studies and support the 'male escape hypothesis' as we found that males metamorphosed earlier and more often than females (81% versus 44%, respectively). Yet, an opposite pattern was found in an ambystomatid salamander (Whiteman 1997) but, in both newts and salamanders, the probability to metamorphose was a likely driver of the sex-ratio biases found in natural populations (Gabrion 1976; Kalezić and Džukić 1985; Denoël et al. 2007; Whiteman et al. 2012). For example, there were two times as many paedomorphic females than males in the studied population of palmate newts (M. Denoël, pers. obs.). On the other hand, a higher female dispersal after overwintering was also found in some newt populations (Grayson and Wilbur2009). Therefore, studying the links between sex-biased dispersal patterns and those of metamorphosis by integrating the environmental context and reproductive strategies could reveal interesting patterns for our understanding of the evolution of dispersal and metamorphosis.

Metamorphosis and predation risk

By coexisting with fish, newts and salamanders expose their progeny to predation risk (Monello and Wright 2001) and themselves to fish harassment (Winandy and Denoël 2015), competition or even predation (Denoël et al. 2005a; Bucciarelli et al. 2014). Here, we show that 75% of the paedomorphic newts that were in the presence of fish metamorphosed and could therefore avoid predation risk as metamorphosis provides the potential to leave water (see Mathiron et al. 2017 for the correspondence between metamorphosis events and escape of waters by metamorphosed paedomorphs). However, as this metamorphosis also occurs in fish-free habitats, it is not primarily the presence of fish that induces newts to metamorphose. Yet, in our experiment, 100% of the paedomorphic males metamorphosed in the presence of fish versus 60% who did so in their absence. In contrast, 50% of females remained in water in the presence of fish.

Metamorphosis gives the advantage to metamorphosed paedomorphs of possibly finding an alternative fish-free habitat in which to reproduce, which could favour the persistence of facultative paedomorphosis as metamorphs lay eggs which can later become paedomorphs (Denoël and Winandy 2015). However, in the absence of favourable habitats during several generations, paedomorphosis can be counter-selected, and its genetic underpinning can be altered, as shown by artificial selection experiments in ambystomatid salamanders (Semlitsch and Wilbur 1989; Voss and Shaffer 2000). A well-known example is that of the axolotl (*Ambystoma mexicanum*), a species that does not metamorphose in the wild and is now at the edge of extinction primarily because of fish introductions (Contreras et al. 2009). The spatio-temporal configuration of landscapes is therefore an essential feature for the persistence or extinction of paedomorphosis (Denoël et al. 2016). Fortunately, in the main area where facultative paedomorphosis is expressed in palmate newts (i.e. in Larzac), there are still adequate aquatic habitats that can serve as reservoirs for the persistence of polyphenism (Denoël & Ficetola 2015, Denoël & Winandy 2015). However, this is not the case in other areas where the populations are more geographically isolated (see e.g. Oromi et al. 2019).

Inducible responses: not all cues matter

The presence of predators (i.e. dragonflies) has been shown to be a less important determinant of development rate than pond drying in frog tadpoles (Laurila and Kujasalo 1999). The results of the present study, along with those of Mathiron et al. (2017) on a nearby population of paedomorphic palmate newts (3 km) suggest the same pattern. Indeed, paedomorphs in the presence of fish metamorphosed later than in a situation of pond drying (i.e. 7 weeks for the first metamorphic event in the presence of fish versus less than 2 weeks, respectively).

On the other hand, there is now evidence that native species can either succeed or fail in responding adaptively to new invaders (Strauss et al. 2006; Ficetola et al. 2011). The present results show they responded adequately by behavioural avoidance but not by metamorphosis in the presence of goldfish in our setting. Fish introductions in Larzac are of recent origin, that is in the last decades (Denoël and Winandy 2015), whereas it can be expected that the metamorphosis of paedomorphs in response to other predators may not be adaptive as they are naturally occurring in the area. For instance, newts can face native predators such as

invertebrates, during their larval stage, as well as water snakes at both their larval and adult stages (Van Buskirk and Schmidt 2000; Luiselli et al. 2005). Particularly at the adult stage, as is the case for paedomorphs, behavioural avoidance within the aquatic habitat can then be considered to be a valuable strategy in response to predation risk. In our experiment, we used a density of 2 fish per aquarium, and the fish were in direct contact with newts. This can be considered a high but realistic pressure for newts, which can therefore be harassed but not hurt while still having the possibility to hide under a shelter. Moreover, the size of the goldfish used in our study was typical of that found in the field. Higher pressures could likely come only from larger, more aggressive or carnivorous fish such as salmonids but such fish species are very rarely introduced into ponds inhabited by paedomorphic palmate newts (Denoël & Winandy, 2015).

Perspectives

On one hand, our results show the importance of sex in understanding the long-term persistence of paedomorphosis and metamorphosis in varied natural and, more particularly, altered environmental contexts (Whiteman 1997). Integrating sex into future studies on polymorphisms, such as facultative paedomorphosis, is therefore important for our understanding of the evolution of the process. More broadly, the fact that sex-specific differentiation was found only for metamorphosis and not for behavioural avoidance suggests that metamorphosis could be particularly dependent on the sex-specific payoffs of life in water versus on land (see also Whiteman 1997).

On the other hand, we found varied individual responses to the same environmental pressure. Indeed, within the same treatments, there were some paedomorphs which metamorphosed early, late or never during the 9-month experiment, and this occurred despite that they were all large enough to metamorphose. Therefore, some facultative paedomorphic individuals do not or are slow to respond to environmental factors. This is typical of amphibian metamorphosis but suggests further questions on the causes of such variation. Previous work on ambystomatids showed that different alleles can be associated with early versus late metamorphosis (Voss and Smith 2005), whereas hormonal pathways can trigger metamorphosis in response to environmental stressors (Denver et al. 2002). Predation risk was also associated with both higher glucocorticoid levels (corticosterone) and lower activity in a plethodontid paedomorphic species (Davis and Gabor 2015). Although it is known that metamorphosis can be induced by environmental drivers (Mathiron et al. 2017) and that both coexisting paedomorphs and metamorphs form a single population in palmate newts (Oromi et al. 2016), the knowledge of the genetic and physiological underpinnings of the expression of paedomorphosis versus metamorphosis in newts and salamanders remains fragmentary. It is also likely that processes differ among species (Page et al. 2013; Keinath et al. 2017).

Therefore, our results point to the need for further studies to unravel the genetic and physiological causes of metamorphosis patterns across the sexes at both the larval and adult stages. However, massive fish introductions into natural environments make this a difficult task as they have caused extinctions of paedomorphs in many populations worldwide (Whiteman and Howard 1998; Denoël et al. 2005a, 2019; Contreras et al. 2009). Restoring favourable

fishless habitats is therefore a prerequisite for both maintaining paedomorphosis and understanding the causes of its expression and evolution.

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

Ethical approval: All applicable institutional and national guidelines for the care and use of animals were followed. The capture permit was issued by DREAL Languedoc-Roussillon (decree 2013274-0001). All experiments were approved by the University of Liège's animal ethical committee (authorization 1613).

Author Contributions: MD and LW conceived and supervised the study. MD, LD and NO collected newts in the field. LD carried out behavioural observations. LD, NO, LW, and MD participated to the logistics of the experiment. LW and MD carried out the statistical analyses. MD wrote the first draft of the manuscript, and MD, LW and NO contributed to the revisions. All authors agreed on the final version of the manuscript.

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