

The ‘male escape hypothesis’: sex-biased metamorphosis in response to climatic drivers in a facultatively paedomorphic amphibian

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Paedomorphosis is a major evolutionary process that bypasses metamorphosis and allows reproduction in larvae. In newts and salamanders, it can be facultative with paedomorphs retaining gills and metamorphs dispersing. The evolution of these developmental processes is thought to have been driven by the costs and benefits of inhabiting aquatic versus terrestrial habitats. In this context, we aimed at testing the hypothesis that climatic drivers affect phenotypic transition and the difference across sexes because sex-ratio is biased in natural populations. Through a replicated laboratory experiment, we showed that paedomorphic palmate newts (*Lissotriton helveticus*) metamorphosed at a higher frequency when water availability decreased and metamorphosed earlier when temperature increased in these conditions. All responses were sex-biased, and males were more prone to change phenotype than females. Our work shows how climatic variables can affect facultative paedomorphosis and support theoretical models predicting life on land instead of in water. Moreover, because males metamorphose and leave water more often and earlier than females, these results, for the first time, give an experimental explanation for the rarity of male paedomorphosis (the ‘male escape hypothesis’) and suggest the importance of sex in the evolution of paedomorphosis versus metamorphosis.

Keywords:

climate, drying, facultative paedomorphosis, metamorphosis, newt, sex-ratio

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1. Introduction

Metamorphosis is a ubiquitous developmental process that enables habitat transition for many organisms [1]. For example, it allows larvae and juveniles to exploit different ecological niches than the adults and to escape from unfavourable environments [2, 3]. In many amphibian and insect species, the organisms cross the air-water interface at metamorphosis as the gilled aquatic larvae transform into morphs more adapted to a terrestrial lifestyle and dispersal [4, 5]. However, an alternative developmental pathway to metamorphosis is found in most zoological groups—paedomorphosis—during which larval or juvenile traits are retained in adults [6, 7]. Because of the large morphological change made possible by reproduction within a larval phenotype and the absence of habitat transition, this process is considered to have played a major role in micro- and macro-evolution [8, 9].

Paedomorphosis is particularly obvious in newts and salamanders where it corresponds to the retention of a larval traits such as gills and gill slits at the adult stage, and, therefore, to an aquatic life instead of the typical biphasic life cycle of a species going through metamorphosis [10, 11]. It is a highly complex life history trait in which exhibition in relation to metamorphosis can depend on many factors, both abiotic and biotic [12, 13]. Among them, climatic factors are thought to play a major role. Indeed, glacial and interglacial events are associated with paedomorphosis and metamorphosis, respectively [14, 15], whereas, in contemporary species, paedomorphosis is often found in permanent waters which are surrounded by arid lands [16, 17], but also in cold alpine lakes [18-20]. Recent time-calibrated phylogeny also supports the contention that paedomorphosis has evolved in relation to arid surface environments [16].

Both processes—paedomorphosis and metamorphosis—are not necessarily mutually exclusive in the same species. Some have evolved maintaining phenotypic plasticity through polyphenism (i.e. facultative paedomorphosis) where the two phenotypes coexist spatially and/or temporally [13, 21, 22]. Their expression in populations depends on the advantages of living in the aquatic and terrestrial habitats [4]. Because environmental cues play an important role in pushing amphibian larvae to metamorphose in most species [2, 23, 24], environmental variations are supposed to maintain the two phenotypes in populations because each one is adapted to specific conditions.

Facultative paedomorphosis offers the opportunity to test whether climatic factors can be the drivers of metamorphosis and the associated niche shift across sexes. This is particularly relevant to the combination of water availability and temperature because these variables have been shown to influence metamorphosis in facultative paedomorphic newts and salamanders [21, 25]. However, the combined role of the two environmental variables remains unclear. Sprules [25] found only an effect from temperature, but not an effect from water availability, whereas Semlitsch [21] showed an effect from pond drying. These studies were also conducted on larvae which may respond differently than adults because of the minimal size required in order to metamorphose [13]. For adults, only one experiment has been conducted involving complete deprivation of water, showing the possibility of metamorphosis of paedomorphic alpine newts in humid terrestrial conditions [26]. Metamorphosis would, therefore, allow survival in a total degradation of the aquatic environment, while paedomorphosis would

optimize the use of resources in the appropriate aquatic environment and quickly restructure the population size by progenesis (i.e. early maturity) [27].

In temperate climates, the environment is variable and unpredictable, and it may have a significant impact on evolutionary and ecological processes that affect and shape the genetic structure and evolution of populations [28]. European records indicate that the frequency and magnitude of heatwaves and droughts have increased over the last 45 years, especially in the southern and western regions of the continent [29]. Species such as amphibians that depend on the availability of fresh water are sensitive to fluctuations in temperature and precipitation [30]. Climate change is, thus, expected to lead to changes in population dynamics [31, 32] and community composition [33]. For example, extreme drought and/or shortened hydroperiods have been linked to the potential local elimination of paedomorphosis in salamanders [34]. Environmental pressures are also likely to decrease the proportion of paedomorphic individuals in newt populations with facultative paedomorphosis because the presence of paedomorphs is directly related to the presence of permanent water bodies [35]. Determining how both temperature and water availability affect the frequency and the timing of metamorphosis can help us to better understand how climate may influence the evolution of facultative paedomorphosis.

Various mechanistic pathways of paedomorphosis in males and females [36] and sex-specific payoffs [37] may also explain the maintenance of polymorphism which results in the sex-biased ratio of many paedomorphic populations. By studying the time interval between breeding events in tiger salamanders, Whiteman [37] showed that specific costs and benefits to each sex could explain the biased sex-ratio. However, environmental drivers have not been identified yet. Experimental determination is, therefore, needed to explain the biased sex-ratio found in many populations of paedomorphs, such as pond-breeding newts, where paedomorphic males are much rarer than females [38, 39].

Our experiment was designed to test two major hypotheses of paedomorphosis evolution. First, we expect that two environmental variables associated with climate change—high temperature and low water availability—can induce metamorphosis and major habitat change (i.e. water to land). Second, we hypothesize that such a response would be greater in males than in females. To this end, we used the palmate newt (*L. helveticus*) as the model species because of these factors: i) paedomorphosis is facultative [22], ii) paedomorphosis is frequently expressed in both permanent and temporary ponds with varied water levels [35, 40], iii) paedomorphic males are rarer than females in the field [38] and iv) summer drought has been associated with the disappearance of paedomorphs [40].

2. Material and methods

(a) Newt sampling

On 16 April 2016, 80 paedomorphs (40 individuals of each sex) of palmate newts were caught by dip-netting in a pond devoid of fish (Bergerie de Tédénat, Department of Hérault, France; 43.778086° N – 3.455234° E, 792 m elevation a.s.l.). This pond is located on the Larzac limestone plateau. Although it can hold water and newts all year round, it was also found almost dried up without newts in summer [40; M. Denoël, pers. obs.]. Capture took place at the start

of the reproductive period. At the time of collection, the sex-ratio of paedomorphs was sex-biased with 32% male ($n = 161$). Paedomorphs were distinguished from larvae by the presence of a developed cloaca; sex was determined by the shape of the cloaca [41]. Newts were transported to the laboratory in six 3 L tanks ($30 \times 20 \times 18$ cm) and placed in a refrigerated box (230 L).

(b) Laboratory maintenance

On 18 April 2016, we distributed the newts individually in 80 identical and independent tanks (30×20 cm) following four experimental treatments (10 individuals of each sex per treatment): a high water level (4 L: water depth = 10 cm; tank height = 22 cm) or a low water level (0.5 L: water level = 1.5 cm; tank height = 14 cm), with a low temperature (mean \pm SE = 16.7 ± 0.02 °C) or with a high temperature (mean \pm SE = 21.1 ± 0.03 °C). The position of the tanks on the shelves was randomized to avoid differences of temperature between the low and high water level treatments. The temperature was controlled by air conditioners regulated by a thermostat (0.1°C resolution). The experiments at the two temperatures occurred in two contiguous laboratory rooms in which nothing else differed except temperature. The terrestrial habitat of the tanks consisted of the walls of the tanks above water level and had the same dimension in all treatments. Newts are good climbers and adhere very well to the walls of the tanks (M.D., pers. obs.). The size of newts did not differ significantly across treatments (snout-vent-length, mean \pm SE = 34.6 ± 0.3 mm; ANOVA-2, all $P > 0.35$; Suppl. Table S1) and was larger than the usual size at metamorphosis in palmate newts [42].

The water was renewed every two days using water from tanks stored at the experimental temperatures. An oxygen diffuser was placed in the tanks to oxygenate water. We also established a photoperiod (Lumilux de luxe 2350-lm daylight tube, L36W/12-950, OSRAM; 156 ± 2.8 lux at the level of tanks) that reflected the natural cycle at the capture location (13 h light/11 h dark). To avoid a sharp transition between day and night, a dawn was simulated for 30 min in the morning and dusk for 30 min in the evening (Daylight L36W/965 Biolux, OSRAM). All individuals were fed ad libitum at the same time with 100 mg of thawed *Chironomus larvae* per newt. This food represents a typical newt prey, and the quantity provided corresponded to the newts' needs [43].

(c) Data collection

The experiment lasted 85 days, from 20 April to 14 July 2016. Every two days, when the water was renewed, newts were checked for signs of full metamorphosis, i.e. the closure of gill slits as this corresponds to an irreversible developmental shift [12]. In addition, their behaviour state, as indicated by their position in the tanks—i.e. aquatic vs. terrestrial—was noted daily (from 9:00 am to 11:00 am) to ensure that the timing of metamorphosis correlated to the shift of habitat preference (supplementary data S1). Both the timing of metamorphosis and the shift of the behavioural state were strongly correlated ($R^2 = 0.72$, $n = 33$; supplementary figure S1).

Therefore, we inferred habitat shift on the basis of metamorphosis as it a clear irreversible state shift.

(d) Statistical analyses

A first check of the data indicated an overriding effect of the water level on the proportion of paedomorphs that metamorphosed (see results section for details), thus preventing a survival analysis from being performed on the whole dataset. For this reason, we first investigated the effect of sex and both treatments (the water level and the temperature one) on the frequency of metamorphosis (recorded at the end of the experiment) using a generalized linear model. For this purpose, all the above main effects as well as their first-way interactive effects were introduced as explanatory terms in the model and the individual metamorphosis state was treated as the dependent binary variable using a binomial distribution for the error term. Because of the sparse data structure resulting from the overriding effect of the water level treatment, we used a Bayesian approach implemented in the GENMOD procedure (SAS 9.4, SAS Institute Inc. 2014) which is more convenient to investigate the effect of each explanatory term in such a situation. More specifically, we used the Jeffrey's distribution as a non-informative prior with a burn in period of 10000 and 100000 subsequent MCMC repetitions. The convergence of the MCMC to the posterior distribution for each explanatory term was assessed using three diagnostic tools: the parameter trace, the Geweke test and the autocorrelation among Markov chain samples. For each explanatory term, the 95% credible interval was constructed using the highest posterior density interval. Timing of metamorphosis was investigated in the low water level treatment groups using nonparametric survival analyses for right censored data based on the Kaplan-Meier estimation (Lifetest procedure, SAS 9.4)[44]. Both ordinary and weighted (i.e. Wilcoxon) log-rank statistics were used to test for the difference of the survival curve between the groups since these statistics are more powerful in detecting late and early differences in survival curves, respectively [44]. All pairwise comparisons were done using a Šidák adjustment to take into account the error risk of multiple comparisons.

3. Results

(a) Frequency of metamorphosis

Of the 40 animals in each water level, only five metamorphosed in high water whereas 33 metamorphosed in the low water treatment (see figure 1 for details). A large effect of the water level is confirmed by its 95% Bayesian credible interval (which did not include zero): the metamorphosis odds of animals experiencing a low water level was at least 2.81 times higher than the one of those experiencing the high water level (table 1). Metamorphosis also varied between sexes: the metamorphosis odds of males was at least 1.99 times higher than the one of females (table 1). However, neither the temperature treatment nor any interactive effects between treatments or sex and treatments significantly altered the frequency of metamorphosis as indicated by their respective credible interval (table 1).

Table 1. Bayesian estimates of the posterior distribution for the effect of sex, water level, temperature level as well as their first-way interaction on the metamorphosis frequency of paedomorphic palmate newts.

| Explanatory terms | Median | 95% Credible Interval | |
|------------------------|--------|-----------------------|---------------|
| Intercept | -1.314 | -2.943 | 0.118 |
| Sex (female/male) | -5.159 | -10.794 | -0.689 |
| Water (low/high) | 3.401 | 1.032 | 6.037 |
| Temperature (low/high) | 0.487 | -1.537 | 2.554 |
| Sex * Water | 3.828 | -0.341 | 9.102 |
| Sex * Temperature | 1.962 | -1.179 | 5.501 |
| Water * Temperature | -1.208 | -4.760 | 1.812 |

All estimates are indicated on the logit scale: Intercept is the Log(odds) for the male experiencing the low water level and the low temperature treatment, all other terms are log(odds ratio). For each main effect, the modalities respectively used as the numerator and the denominator in the odds ratio are indicated in the brackets. In bold: all log(odds ratio) for which the highest posterior density does not include the zero correspond to odds ratio for which odds equality (i.e. null effect) can be rejected with 95% of confidence. See the statistical section for details on the bayesian analysis performed.

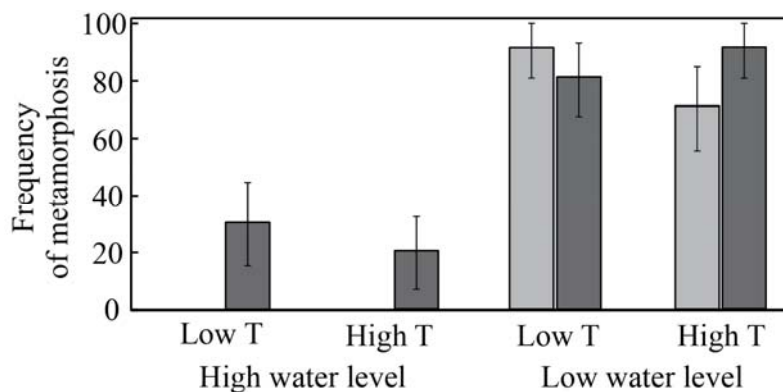


Figure 1. Proportion (\pm SE) of paedomorphic palmate newts that metamorphosed in high and low water level tanks and at high and low temperatures ($n = 20$ paedomorphs, 10 of each sex per treatment). Dark grey boxes: males; light grey boxes: females, T: water temperature. See text for statistics.

(b) Timing of metamorphosis

We detected a significant interactive effect between sex and temperature on the timing of metamorphosis at the low water level. This effect was significant using both the Wilcoxon test and the ordinary log-rank test (figure 2; $\chi^2_3 = 18.31, P < 0.001$; $\chi^2_3 = 10.43, P = 0.015$, respectively). The timing of metamorphosis did not differ between sexes at the low temperature, and it was not altered by the temperature elevation in the females (table 2, figure 2). However,

males metamorphosed earlier at the high temperature than at the low temperature; they also metamorphosed earlier than females exposed to the high temperature treatment (table 2, figure 2).

Table 2. Pairwise comparisons of the survival curves (i.e. timing of metamorphosis of paedomorphic palmate newts) between sexes and temperature treatments at the low water level treatment.

| Pairwise comparisons | | Ordinary LogRank test | | Wilcoxon test | | |
|----------------------|----------------|-----------------------|------------|---------------|------------|--------------|
| Sex (♀,♂) | T° (High, Low) | χ^2 | <i>P</i> * | χ^2 | <i>P</i> * | |
| ♀ | Low T° | ♀ High T° | 0.37 | 0.991 | 0.0 | 1 |
| ♀ | Low T° | ♂ Low T° | 0.26 | 0.996 | 0.11 | 0.999 |
| ♀ | Low T° | ♂ High T° | 2.95 | 0.416 | 8.44 | 0.022 |
| ♀ | High T° | ♂ Low T° | 0.0 | 1 | 0.10 | 0.999 |
| ♀ | High T° | ♂ High T° | 5.98 | 0.084 | 8.73 | 0.019 |
| ♂ | Low T° | ♂ High T° | 5.31 | 0.121 | 10.85 | 0.006 |

*: Šidák adjustment for multiple comparisons, T°: temperature, significant values are indicated in bold.

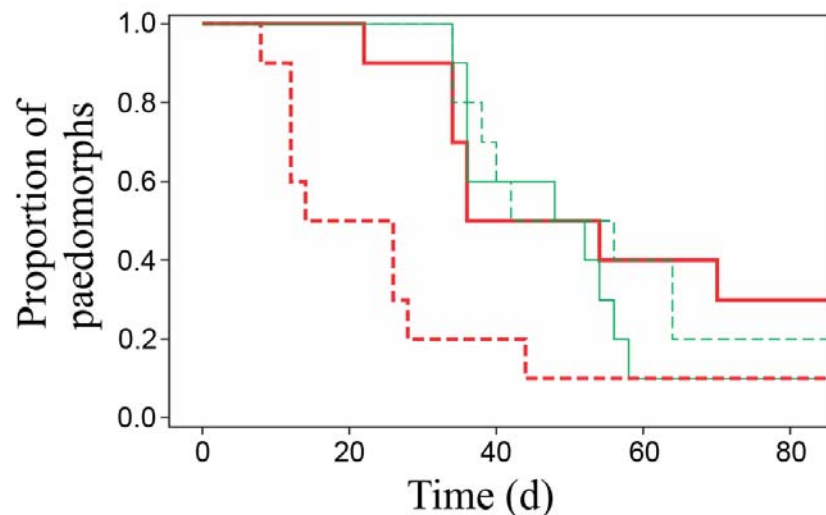


Figure 2. Timing of metamorphosis (survival curves) for paedomorphic palmate newts in both sexes and two temperature treatments (all at low water level). High temperature: thick red lines, low temperature: thin green lines, males: interrupted lines, females: continuous lines. See Table 2 for statistical pairwise comparisons.

4. Discussion

The manipulation of environmental factors in this study demonstrates that two climatic drivers—temperature and water availability—each play a significant role in metamorphosis expression and in the persistence of paedomorphosis. These results have major implications as they support the Wilbur and Collins [4] model of amphibian metamorphosis in detrimental waters and paedomorphosis in favourable aquatic habitats. More specifically, they show experimentally the importance of droughts as a primary determinant in the evolution of paedomorphosis as suggested by paleontological records or time-calibrated phylogenies [14, 16]. Moreover, these results evidenced a sex-biased effect of the metamorphic response, highlighting the role of sex in the metamorphosis of paedomorphs.

(a) Environmental drivers of paedomorphosis

Although climatic parameters are thought to act as selective agents of paedomorphosis versus metamorphosis [16], their effects are complex. Droughts can make life on land risky for amphibians because they are ectothermic organisms that cannot live in temperatures that are too cold or too hot, and they rely on a minimum level of humidity given their soft, permeable skin [45]. From this perspective, the terrestrial landscape surrounding the water habitats of paedomorphic species, such as in Texan plateaus, is often dry and hostile to metamorphs [16]. The same observations were carried out for the ancestors of pond-breeding newts, which were paedomorphic during cold events that caused low growth in water and aridity on land [14, 15]. However, metamorphs can be found on arid lands surrounding facultative and obligate paedomorphic populations in ambystomatids [17, 46], and droughts can also affect aquatic habitats by increasing their likelihood to dry, therefore, possibly favouring metamorphosis [40, 47, 48]. Some Mexican lakes and Texan rivers inhabited by paedomorphic ambystomatids and plethodontids, respectively, can retain water all year round, and are, therefore, favourable for the maintenance and fixation of paedomorphosis [17, 46, 49]. In contrast, most facultatively paedomorphic species subsist in aquatic environments, such as ponds that can be either temporary or permanent; therefore, droughts are expected to have contrasting effects depending on water availability [40, 50].

Indeed, our results on palmate newts showed that water availability induces phenotypic transition; paedomorphic individuals metamorphosed more when they were maintained at a low rather than high water level. This is the first time that a low water level has been shown to affect the metamorphosis of paedomorphic newts, as in the alpine newt, where the decreasing water level did not induce paedomorphs to metamorphose more than at a stable water level [26]. However, previous work on facultatively paedomorphic species suggested the effect of water availability, first, at the adult stage when fully deprived of water [26], and then at the larval stage when more individuals opted for paedomorphosis in permanent waters than in temporary waters [21, 50]. Our experiment mimics an extreme, but natural and frequent situation, i.e. the end of pond drying [see also 24 for a similar setting]. The fact that very few newts metamorphosed in the high water level treatment—which remains relatively low in comparison with the range of values found in the field [35]—is likely adaptive. Indeed, the water level of many ponds experience fluctuating water levels without completely drying out; rainfall can refill drying ponds, allowing the maintenance of favourable aquatic conditions.

On one hand, the low temperature of water, such as in high mountain lakes, has been seen as a constraint, preventing development and metamorphosis and, thus, favouring paedomorphosis [18, 25]. On the other hand, high temperatures that occur in ponds increased the trigger of metamorphosis [51]. However, because pond drying in natural conditions is associated with both water reduction and a temperature elevation, these two factors could mediate metamorphosis as suggested by studies manipulating drying risk in mesocosms [21]. By controlling both water availability and temperature in the present study, we separated their respective effects. The effect of temperature was significant but overridden by water availability as it affected the timing of metamorphosis only when water availability was low.

Because scenarios of climate change predict lower precipitation and higher temperatures [29], particularly in summer in Mediterranean areas where facultative paedomorphosis is expressed, it is likely that paedomorphosis would be counter-selected in ponds that are not deep enough to maintain water all year round in the dry years [34, 40]. The large gene flow between paedomorphs and metamorphs within populations [22], and the fact that metamorphs can lay eggs that hatch in larvae becoming paedomorphs [52], may still allow the persistence of the process across dry years. It is only if drought frequency increases that payoffs of metamorphosis and paedomorphosis may change as progenies of paedomorphs would not have time to reach adulthood. Indeed, previous research on paedomorphic ambystomatids suggests that fixation of alleles associated with metamorphosis timing and paedomorphosis can be rapid [53, 54]. Future studies should, therefore, examine how climatic parameters influence the long-term persistence of paedomorphosis and metamorphosis in natural populations of facultatively paedomorphic species.

(b) Sex-biased metamorphosis

An intriguing pattern in pond-breeding newts is that populations of paedomorphs are usually female-biased [38, 39]. A capture-mark-recapture study showed that paedomorphic newt males metamorphosed more than females [55], but these results lacked the experimental determination of the causative agents. Previous manipulations of temperature did not evidence the effect of sex in the timing of metamorphosis [51], whereas experimental breeding produced similar numbers of paedomorphic males and females in salamanders [56]. A particularly novel finding of this study is, therefore, to give proximate bases that contribute to explain the sex-biased sex-ratios of paedomorphs in natural populations. Both water level and temperature affected the metamorphosis of males more than females. More paedomorphic males metamorphosed than females and, in drying conditions, started metamorphosis earlier than did females, which resulted in a female biased sex-ratio of paedomorphs. This pattern reflects well the natural situation in Larzac where the full drying of ponds is not a rare phenomenon [40]. However, habitat specificities may give rise to varied patterns as some ponds remain permanent [35] whereas other factors may also be at the basis of biased sex-ratio [37]. Moreover, as females start to metamorphose later than males, they may benefit of rains refilling ponds and thus avoid metamorphosis. According to our experimental results, we hypothesize that sex-ratios should be less female-biased in the most permanent ponds inhabited by paedomorphic palmate newts. Field studies are thus awaited to test this hypothesis.

Because metamorphosed paedomorphs shift habitats, moving from water to land, metamorphosis is inherently associated with niche shift and possibly with dispersal. Because ponds with facultative paedomorphosis form clusters that can be colonized by dispersers [52, 57], dispersal is a likely mechanism for maintaining paedomorphosis in a mosaic of temporary and permanent habitats [58]. However, the reason why males metamorphose more than do females and leave water before them remains an unanswered question. Yet, we hypothesize there are at least two possible reasons for this. First, because hormones control metamorphosis [59], and because hormonal pathways differ in males and females [60], different responses to environmental disturbers may be expected between the sexes. In support of this hypothesis, Hayes et al. [61] showed that, in frog tadpoles, testosterone induced an earlier emergence of forelimbs. Second, looking more broadly into sex-biased dispersal, there are variations in the propensity of males or females to be the most dispersing or philopatric sex. For instance, in red-spotted newts, males are more resident than females in overwintering in ponds, whereas females move to land [62]. Similarly, in tiger salamanders, paedomorphic males are more philopatric than females to their ponds. The philopatry of one sex is thought to result from the asymmetry in the costs of dispersal, as well as the greater benefits of familiarity with a natal area, resulting in greater acquisition of resources [63, 64]. The acquisition of resources of individuals using temporary and permanent ponds, but also of those remaining in water, versus those moving to land, is likely to be very different; therefore, variation across studies may lie in the relative advantages of life in the aquatic and terrestrial environments. In Larzac, aquatic habitats most likely provide more resources than their terrestrial dry surroundings, and pond drying often follows the breeding period. Females may need to stay in water longer than males to fuel up their reserves after they have laid eggs during breeding [65]. In contrast to studies in permanent ponds, where it can be favourable for males to stay in water to get earlier access to females at the next breeding season [62], males living in temporary ponds do not have competitive sexual advantages to remain in water.

(c) Conclusions

Our study clearly shows how climatic drivers affect phenotypic transition in a facultatively paedomorphic amphibian and illustrates the importance of sex in the metamorphosis of paedomorphs. Our results particularly highlight that, although climate warming can favour aquatic life in permanent waters, those species evolving in shallow aquatic habitats may be affected with the dispersal, metamorphic phenotype being likely selected. The ‘male escape hypothesis’ contributes to explain the bias in the sex-ratio of the paedomorphic palmate newt, but the specific benefits for males and females for living in water versus on land remain to be explored. Our study, therefore, calls for further work to examine the underlying effects of such climatic drivers on phenotypic transition and the links between the individual physiology state and the ‘male escape hypothesis’.

Ethics. The newt capture permit was issued by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement (DREAL) de Languedoc-Roussillon, following approval by the Conseil National de la Protection de la Nature (decree 2013274-0001). All experiments

reproduced natural environmental conditions and were conducted in accordance with all relevant guidelines and regulations. They were approved by the animal ethical committee of the institution where the study was conducted, the University of Liège, Belgium (authorization 1613). After sampling, the newts were released in healthy condition in their capture location following the recommendation of the capture permit.

Data accessibility. Data are available in Dryad: <http://dx.doi.org/10.5061/dryad.60mk6>.

Authors' Contributions. M.D. designed and supervised the experiments; M.D. and S.B. collected the newts; A.M. and S.B. conducted the experiment; J-P.L. computed the statistical tests; M.D. and A.M. wrote the first draft of the paper; all authors contributed to the final version of the manuscript.

Competing Interests. We declare we have no competing interests.

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