

Facultative paedomorphosis as a mechanism promoting intraspecific niche differentiation

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Organisms with complex life cycles are characterized by a metamorphosis that allows for a major habitat shift and the exploitation of alternative resources. However, metamorphosis can be bypassed in some species through a process called paedomorphosis, resulting in the retention of larval traits at the adult stage and is considered important at both micro- and macroevolutionary scales. In facultatively paedomorphic populations of newts, some individuals retain gills and a fully aquatic life at the adult stage (paedomorphs), while others undergo complete metamorphosis (metamorphs), allowing for a terrestrial life-stage. Because facultative paedomorphosis affects trophic structures and feeding mechanism of newts, one hypothesis is that it may be maintained as a trophic polymorphism, with the advantage to lessen intraspecific competition during the shared aquatic life-stage. Here, we tested this hypothesis combining stomach content data with stable isotope techniques, using carbon and nitrogen stable isotopes, in facultatively paedomorphic alpine newts (*Ichthyosaura alpestris*). Both stomach content and stable isotope analyses showed that paedomorphs had smaller trophic niches and were more reliant on pelagic resources, while metamorphs relied more on littoral resources, corresponding to a polyphenism along the littoral-pelagic axis and the extension of the population's trophic niche to otherwise 'underused' pelagic resources by paedomorphs. Interestingly, stable isotopes revealed that the trophic polyphenism was less marked in males than in females and potentially linked to sexual activity. Although paedomorphosis and metamorphosis are primarily seen as results of trade-offs between the advantages of using aquatic versus terrestrial habitats, this study provides evidence that additional forces, such as intraspecific trophic niche differences between morphs and trophic niche expansion, may play an important role in the persistence of this dimorphism in heterogeneous environments. Moreover, the different patterns found in males and females show the importance of considering sex to understand the evolutionary ecology of trophic polymorphisms.

The existence of polymorphisms within populations has long been suggested to play a key role in facilitating sympatric speciation (Smith 1966, Sage and Selander 1975, Schluter and Rambaut 1996), as well as promoting adaptive radiation and reducing a lineage's risk of extinction (Schluter 2000, Pfennig and McGee 2010). However, there is now increasing evidence that polymorphisms can be maintained over evolutionary time in natural populations rather than being necessarily intermediate steps of speciation (Svanbäck et al. 2008, Chevin and Lande 2013, Oromi et al. 2016), with the possibility of offering better chances of adaptation in changing environments (Murren et al. 2015). Growing efforts to identify the ecological and evolutionary factors that help maintain polymorphisms in the long-term will ultimately help describe the role of plasticity in the evolution of species, life history and paradigms of adaptive diversification and extinctions (West-Eberhard 1989, Pfennig and McGee 2010, Whiteman et al. 2012).

Trophic polymorphisms, i.e. discrete morphs that differ in their feeding and associated habitat use within a population (Smith and Skúlason 1996), are of particular interest as competition for resources has always been considered a major force in evolution (Darwin 1859, Pfennig and Pfennig 2010). Both morphological phenotypic plasticity and behavioural flexibility can generate trophic polymorphisms allowing for niche expansion and eventually differentiation through 'vacant niche' occupancy (*sensu* Van Valen 1965, Rohde 1991). Heterogeneous trophic resources and the absence of competitors are, therefore, ecological factors that should promote such polymorphisms (Wimberger 1994). Examples have been provided in lacustrine fish where intraspecific diversification processes along the littoral-pelagic axis appear to be very common (Malmquist et al. 1992, Swanson et al. 2003, Woods et al. 2013). Trophic polymorphisms can also promote survival by allowing the exploitation of alternative profitable prey within shared habitats. Classic examples are found in snail eating versus detritivore phenotypes in fish (Kornfield et al. 1982) or the development of cannibal morphs in amphibian tadpoles living in temporary ponds (Pfennig 1992).

Complex life-histories, such as those found in amphibians, provide interesting templates for the development of important morphological differences without the need for genetic modification through developmental plasticity and, especially, heterochrony (McKinney and McNamara 1991). Organisms with complex life cycles are characterized by metamorphosis that allows for a major habitat shift and the exploitation of alternative resources in organisms as varied as cnidarians, insects or amphibians (Laudet 2011). However, metamorphosis can be bypassed in some species through a process called paedomorphosis, resulting in the retention of larval traits at the adult stage and considered important at both micro- and macroevolutionary scales (McKinney and McNamara 1991). Whereas some species are fixed in being able to develop only through paedomorphosis or metamorphosis, others retain the ability to express both ontogenetic processes through facultative paedomorphosis. This is the case in several families of newts and salamanders where a single population can be composed of paedomorphs that retain larval gills, allowing for a fully aquatic life, and metamorphs that undergo complete metamorphosis and are able to use terrestrial habitats (Whiteman 1994, Denoël et al. 2005). The evolution and persistence of paedomorphosis and metamorphosis have been considered to be reliant on the specific advantages of life in the aquatic versus terrestrial habitat (Wilbur and Collins 1973) or to be a result of constraints forcing some individuals to make 'the best of bad lot' in the aquatic habitat (Whiteman 1994). Both models were supported by ecological modelling and mesocosm experiments (Semlitsch 1987, Whiteman et al. 2012, Denoël and Ficetola 2014). Paedomorphosis can be advantageous in avoiding the cost of metamorphosis and life in detrimental terrestrial habitats but also in allowing an early reproduction. In contrast, metamorphosis remains adaptive in the case of deterioration of the aquatic habitat (e.g. drying that induces larvae to become metamorphs) and in terms of dispersal (Semlitsch 1987;

Whiteman 1994; Denoël et al. 2005). As it is a polyphenism, metamorphic dispersers may therefore give birth to future paedomorphs, then reconstituting facultatively paedomorphic populations (Denoël & Winandy 2015). However, morphological differences between paedomorphs and metamorphs are not strictly linked to adaptations to aquatic versus terrestrial life. Indeed, metamorphs of biphasic species still need water for breeding, and, in some populations of newts, they remain mostly, if not completely, in water during their active period. Additionally, facultative paedomorphosis affects trophic structures of newts with direct impacts on feeding mechanisms in aquatic habitats. In the aquatic habitat, metamorphs rely on a bi-directional flow to catch prey, whereas paedomorphs use a unidirectional 'suction' flow by expelling water through the preserved gills slits (Joly 1987, Lauder and Shaffer 1993, Denoël 2004). Spatial segregation patterns and stomach contents of facultatively paedomorphic populations suggest that resource partitioning among morphs may play a role in the long-term maintenance of this polyphenism in alpine lakes (Denoël and Joly 2001).

Although males and females differ in many ways, such as size but also reproductive traits, the influence of sex-dependent traits in trophic polymorphisms, and more particularly in facultative paedomorphosis, remains a neglected but important aspect to understand its persistence and evolution as polymorphisms can be maintained by sex-dependent payoffs (Whiteman 1997). Morphological differences have been more studied in fish, showing either sexual similarities (Meyer 1990) or differences responsible for a large portion of the variation observed in trophic-related traits (Proulx and Magnan 2004). Previous work, focused on feeding itself of paedomorphic and metamorphic newts from alpine lakes, showed some consistencies in both sexes (Denoël and Joly 2001) in line with their feeding efficiencies (Denoël 2004). However, sex differences within each phenotype and potential consequences of breeding strategies (see e.g. whiteman 1997) on trophic ecology in the context of facultative paedomorphosis in newts remain unknown.

Because stomach content analysis is only a snapshot that does not guarantee a consistency of behaviour over time (see e.g. Denoël and Joly 2001) or that all ingested material is assimilated, the integration of multiple tools is necessary to address the existence of long-term trophic niche differences among morphs within populations. Therefore, stable isotope analyses are particularly reliable, as they are time- and space-integrative ecological tools that have been widely used to address trophic niche differences at the community and population levels (Layman et al. 2007, Newsome et al. 2007, Jackson et al. 2011, 2012). They offer the possibility to compare isotopic niches (i.e. proxy for trophic niches) of organisms in a Bayesian framework (Jackson et al. 2011) and to trace their dependence on food sources from their environment using Bayesian mixing models in a time-integrated manner (Parnell et al. 2010). Such techniques have been successfully used to confirm long-term intraspecific trophic niche differentiation and diet specialization in fish (see e.g. Quevedo et al. 2009; Hayden et al. 2014). Stable isotopes also allowed to reveal past changes in habitats such as in the case of life-history polymorphisms in fish (e.g. Fukumori et al. 2008). However, they have never been applied to study the trophic implications of facultative paedomorphosis.

In this study, we first aimed to test the hypothesis that facultative paedomorphosis can be adaptive by promoting niche differentiation among alternative adult phenotypes over the course of the shared aquatic life-stage. This advantage would therefore account for the long-term persistence of two alternative developmental pathways in the same population. In an alpine lake that is devoid of interspecific competitors, we predicted that this polyphenism would allow for the extension of the niche of paedomorphs (gilled adults) into the pelagic zone, an otherwise potentially 'vacant niche' (*sensu* Van Valen 1965; Rohde 1991), as a way to escape resource competition with metamorphs in the littoral zone. Second, we expected that resource polymorphism is exhibited in both males and females, but with potential

differences across them due to possible sex-specific constraints during the reproductive period. To test these two hypotheses, we coupled stomach content data with stable isotope mixing models (Parnell et al. 2010) and isotopic niche approaches (Jackson et al. 2011) on both sexes and phenotypes of an amphibian model species, the alpine newt (*Ichthyosaura alpestris*, previously named '*Triturus alpestris*' in the literature).

Materials and methods

Study site and sampling

The study site is an alpine lake located in the southern French Alps (Le Lauzet-Ubaye, Alpes-de-Haute-Provence, France; 44°24' N/6°24' E) at an elevation of 1950 m a.s.l. It is home to the last remaining alpine lake population of facultatively paedomorphic newts of the nominative subspecies *Ichthyosaura a. alpestris* (Recuero et al. 2014; Denoël et al. 2001a). The lake is oligotrophic, with a rocky bottom. Its water level decreases during the summer, and that eventually leads the lake to split into two parts. At the time of this study in late July 2014, the two basins were still connected by a shallow area (0.5 m depth). Maximum water depth was 5.6 m and surface area of the lake was 5300 m². Although not assessed during this study, the population's density appeared to be low. A total of 78 adult newts (26 metamorphic and 16 paedomorphic females, and 24 metamorphic and 12 paedomorphic males) were captured by dip netting from the shore in littoral zones and from an inflatable boat or by diving in deep parts of the lake. Both paedomorphs and metamorphs are adult individuals, identified as such by the presence of a developed cloaca, which is sexually dimorphic in the studied species (Denoël 2017). In contrast to metamorphs, the paedomorphs have developed gills and present all open gill slits (Fig. 1). Differences in size between phenotypes and sexes were assessed using a two-way univariate permutational analysis of variance (PERMANOVA) that employed a similarity matrix based on Euclidean distances (Anderson 2001). There was no significant difference in snout-vent length (SVL) between paedomorphs and metamorphs (pseudo-F_{1,74} = 0.917, p = 0.334) but females were significantly larger than males in both phenotypes (54.4 ± 4.5 mm and 53.6 ± 5.3 mm for paedomorphic and metamorphic females, respectively, compared to 50.9 ± 1.7 mm and 49.9 ± 2.8 mm for paedomorphic and metamorphic males, respectively; pseudo-F_{1,74} = 13.983, p = 0.0005; Supplementary material Appendix 1 Table A1). Body condition was calculated by computing the residuals of the regression of ln-transformed body mass on ln-transformed SVL of newts (Jakob et al. 1996). Alpine newts were the top predator in the fishless lake.

Stomach contents were sampled in late July after the reproduction period of the alpine newt. Individuals were anaesthetized in a bath of phenoxyethanol (0.5 ml/l; 1-l tank) and processed within 30 min to conduct a stomach flushing according to the method described by Joly (1981) and to prevent digestion (Schabetsberger 1994). Stomach contents of each individual were stored individually in 70% ethanol. A caudal skin sample was taken from each individual and preserved at -80°C for further stable isotope analysis. Individuals were sampled only once. These techniques do not affect newts (Joly 1987, Arntzen et al. 1999). All newts were kept in tanks filled with water from the lake until they were completely awake, and then they were released into the lake.

Macroinvertebrates (adult and larval Coleoptera) and pelagic organisms (Cladocera, Copepoda, Chaoboridae pupae and fairy shrimps, *Chirocephalus* sp.) were collected by dip netting (mesh size: 250 µm) in the littoral and pelagic zones, respectively, to be used as potential food sources (n = 6 per taxa) for stable isotope analysis. Terrestrial prey drowning on the water surface were collected by dip netting (mesh size: 600 µm) from a boat.

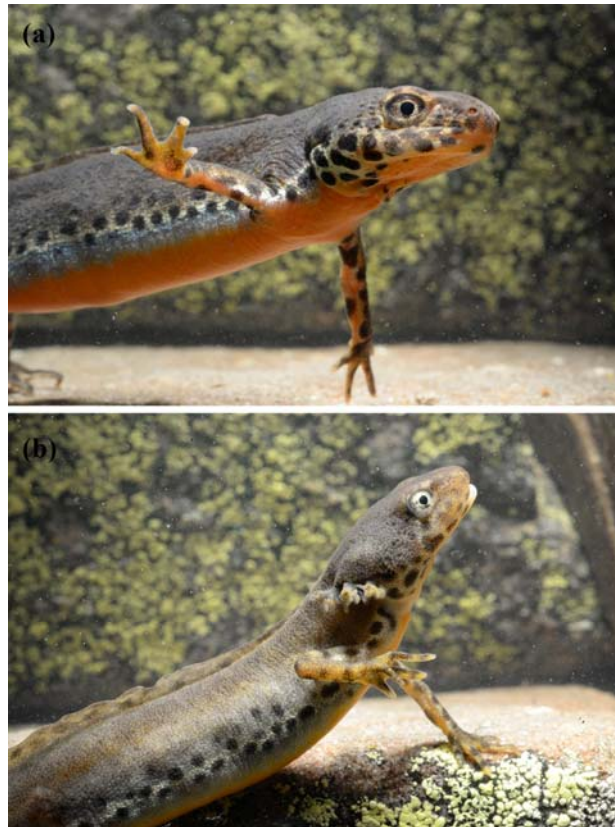


Figure 1. The two alternative phenotypes in the alpine newt coexist in water but segregate along the littoral-pelagic axis of lakes. (a) The metamorph is metamorphosed and forages predominantly in the littoral zone. (b) The paedomorph is fully aquatic thanks to its gills and can forage in the pelagic zone and deeper parts of the lake. Each picture represents a male from La Cabane Lake (France) in July 2014. Photos: Mathieu Denoël.

Stomach content analysis

Prey were identified and counted under a stereoscopic microscope (Zeiss Stemi 2000-C; Carl Zeiss, Jena, Germany), and they were then grouped into categories reflecting foraging constraints: ‘terrestrial insects’ (which had fallen on the water surface), ‘zooplankton’ (i.e. copepods and cladocerans), ‘Chaoboridae pupae’ and ‘fairy shrimps’ (pelagic) and ‘adult Coleoptera’, ‘Coleoptera larvae’ and ‘Trichoptera larvae’ (littoral). Other littoral prey, occurring in fewer than five stomachs in the population, were grouped in ‘minor benthic taxa’. Total stomach content of each individual newt was oven-dried at 60°C for 48 h and the exact mass was determined using a mass balance (Pioneer PA64C; OHAUS, Pine Brook, NJ, USA).

We calculated the trophic niche width based on prey abundance in the stomach contents for each individual using Shannon (1948) index ($H' = -\sum p_i \times \ln p_i$, where p_i is the proportion of prey i for a given individual relative to the total number of this prey in the population). Differences in niche width and prey biomass intake (total dry weight of prey in the stomach, corrected by newts’ size, i.e. snout-vent length in mm) between phenotypes and sexes were assessed using a two-way univariate PERMANOVA that employed a similarity matrix based on Euclidean distances (Anderson 2001). Because females were significantly larger than males, we included size (ln-transformed SVL) as a covariate in PERMANOVA tests in order to subtract the variance explained by this factor when comparing diet and trophic niche width across phenotypes and sex. Trophic niche overlap between pairs of newt

categories were calculated using the Schoener (1968) index on proportions of prey per stomach for each category, following the equation: $S = 1 - 0.5 \times (\sum_i |p_{xi} - p_{yi}|)$, where p_{xi} is the proportion of prey i in the diet of group x , and p_{yi} is the proportion of prey i in the diet of group y . We performed a two-way PERMANOVA based on Bray-Curtis distances calculated from square-root transformed proportions of prey per stomach to assess diet differences between phenotypes and sexes, including SVL as a covariate (Anderson 2001, Anderson and Walsh 2013). This data transformation was carried out to account for differences in stomach capacity and to balance the contribution from the rarer preys. We subsequently used a similarity percentage (SIMPER) analysis to assess the average percent contribution of each prey type to the dissimilarities between diets corresponding to significant differences according to PERMANOVA (Clarke 1993). PERMANOVA and SIMPER were performed using PRIMER ver. 6 software (Clarke and Gorley 2006) and the PERMANOVA+ add-in (Anderson et al. 2008). PERMANOVA were computed with 9999 permutations to assess significance of the results, and the homogeneity of multivariate dispersions within groups was tested prior to all analyses using permutational analysis of multivariate dispersion (PERMDISP) in PRIMER ver. 6 (Clarke and Gorley 2006).

Stable isotope analysis

Because of their small size, newts offer few tissues that can be sampled without killing the individual. To avoid having to take into account the potential terrestrial life-stage while studying newts in their aquatic environment, we selected skin samples from the dorsal tail fin because it develops during the aquatic life-stage in newts (Winandy and Denoël 2015), can fully regenerate in natural conditions over two months (B. Lejeune, *pers. obs.*) and does not affect newts (Arntzen et al. 1999).

All samples were oven-dried at 60°C for 72 h and ground into a homogeneous powder. Stable isotope ratios of carbon and nitrogen were measured using an isotope ratio mass spectrometer (Isoprime 100; Isoprime, Cheadle Hulme, UK) coupled in continuous flow to an elemental analyser (Vario MICRO cube; Elementar, Langensbold, Germany). Stable isotope ratios were conventionally expressed as δ values in ‰ (Coplen 2011). Certified reference materials from the International Atomic Energy Agency (IAEA, Vienna, Austria) used were ammonium sulphate (IAEA-N₂; $\delta^{15}\text{N} = 20.3 \pm 0.2$ ‰) and sucrose (IAEA C-6; $\delta^{13}\text{C} = -10.8 \pm 0.5$ ‰). Both these reference materials are calibrated against the international references Vienna Pee Dee Belemnite for carbon samples and atmospheric air for nitrogen. Internal standards (glycine) were inserted into all runs at regular intervals to assess potential drift over time. Repetitive measurements of glycine ($\delta^{15}\text{N} = 2.3 \pm 0.3$ ‰; $\delta^{13}\text{C} = -47.5 \pm 0.3$ ‰) were also used to calibrate isotopic data and as an elemental standard. One of the samples was randomly selected and analysed multiple times (once every 15 analyses). Analytical precision (\pm standard deviation) on replicated samples equalled 0.3 ‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Elemental data are expressed in ‰ dry mass, and C/N ratios are weight-based.

Isotopic niche parameters were computed using Stable Isotope Bayesian Ellipses in R (SIBER), SIBER package ver. 2.1.0 (Jackson et al. 2011) in R ver. 3.3.1 (R Development Core Team 2016). SIBER was used to generate bivariate standard ellipses representing core isotopic niches of the four newt categories (Jackson et al. 2011). To account for sample size differences, areas of the ellipses associated with each group (Standard Ellipse Area B; SEAB) were computed using Bayesian modelling, and direct pairwise comparisons of SEAB were performed to compare isotopic niche widths (based on 10^5 draws). Pairwise comparisons were done by deriving probabilities of differences from posterior distributions of the parameters of interest. Because probabilities derived by Bayesian inference are different from p-values in frequentist statistics in that they support the actual hypothesis being tested rather

than a null hypothesis, we expressed them as percentages. Also, we considered that an observed difference derived by Bayesian inference was robust when its posterior probability was of 95% or more. Proportions of isotopic niche overlap among the four groups were calculated using a unique standard ellipse area corrected according to sample size for each group ($SEAC$). Total hull area (TA) was also calculated according to Layman et al. (2007). The percent contribution of paedomorphosis to the core isotopic niche of the population was calculated based on $SEAC$ of paedomorphs and metamorphs (both sexes combined), following the equation: $C = (SEAC(P) - \text{Overlap}(PM)) / (SEAC(P) + SEAC(M) - \text{Overlap}(PM)) \times 100$, where C = percent contribution, P = paedomorphs and M = metamorphs. Because females were significantly larger than males, the potential effect of size in the observed pattern of niche differences was addressed using a two-way PERMANOVA based on a Euclidean similarity matrix calculated from both $\delta^{13}C$ and $\delta^{15}N$ of newts, including newts' size as a covariate. $\delta^{13}C$ and $\delta^{15}N$ were ln-transformed prior to the analysis to reduce heteroscedasticity. Subsequent post hoc permutational t-tests were used to examine the interaction term (Clarke and Gorley, 2006). Secondly, we used Stable Isotope Analysis in R (SIAR) mixing models (SIAR ver. 4.2.2; Parnell et al. 2010) to estimate the contributions of each prey item to the isotopic composition of newts (based on 10^5 draws). We selected several sources to include in the SIAR model, based on their occurrence in stomach contents. However, because some of them had very similar isotopic compositions, we ran multiple models, gradually pooling or excluding them in an ecologically relevant way to improve the discriminatory ability of SIAR (Mantel et al. 2004). All models gave similar results and different ways of pooling sources had no consequences on their interpretation. Nevertheless, we selected the most performant model, which included 4 food types: 'Zooplankton', 'Terrestrial insects', 'Pelagic invertebrates' (including Chaoboridae pupae and fairy shrimps) and 'Littoral taxa' (including aquatic adult coleoptera and larvae) (Supplementary material Appendix 1 Fig. A1; Table A2).

Trophic enrichment factors (TEFs), which predict the change in stable isotope ratios between a consumer and its prey for a given tissue, are needed to properly run stable isotope mixing models (Parnell et al. 2010), but have not been determined in any European newts. In such case, using TEFs of the same tissue from phylogenetically close or trophically similar species is advised (Newsome et al. 2007). We applied the only trophic enrichment factors from amphibian skin available: 2.3 ± 0.5 (‰) for $\delta^{15}N$ and 0.1 ± 0.4 (‰) for $\delta^{13}C$ (Cloyed et al. 2015). Although coming from an anuran species, we are confident about their suitability to alpine newts as they are almost identical to TEFs estimated in aquatic plethodontid salamanders, having a more similar ecology, but calculated on samples constituted of a mix of different tissues (Gillespie, 2013).

Solutions from SIBER analysis and the SIAR mixing model are presented using mode and credibility intervals of distributions of the probability density function.

Results

Stomach contents

There were no empty stomachs in the sampled population. Both paedomorphs and metamorphs consumed all types of aquatic prey (Table 1). Paedomorphs relied almost exclusively on zooplankton, fairy shrimps and Chaoboridae pupae (more than 99% of the cumulative contribution). Zooplankton alone constituted 85% of the females and 79% of the males' diets. Conversely, the metamorphs' diet included, as percentage of the diet, more littoral prey (around 11% and 8% for the females and males, respectively), less zooplankton (54% and 68%) and proportionally more Chaoboridae pupae (15% and 13%) and fairy shrimps (20% and 12%).

Table 1. Abundance (Mean \pm SE) and proportion of each prey type in the stomach of alpine newts. P $\text{\textcircled{f}}$ = Paedomorphic females (n = 16), P $\text{\textcircled{m}}$ = paedomorphic males (n = 12), M $\text{\textcircled{f}}$ = metamorphic females (n = 26), M $\text{\textcircled{m}}$ = metamorphic males (n = 24).

Prey taxa	P $\text{\textcircled{f}}$	P $\text{\textcircled{m}}$	M $\text{\textcircled{f}}$	M $\text{\textcircled{m}}$
Zooplankton ¹	47.1 \pm 17.5 84.8%	28.6 \pm 10.2 79.4%	11.2 \pm 4.6 54.0%	15.8 \pm 7.1 67.6%
Fairy shrimp ²	3.6 \pm 0.7 6.4%	3.0 \pm 0.9 8.3%	4.0 \pm 0.4 19.5%	2.7 \pm 0.5 11.6%
Chaoboridae pupae	4.5 \pm 1.8 8.1%	4.1 \pm 1.6 11.3%	3.1 \pm 0.8 15.0%	3.0 \pm 0.7 12.7%
Aquatic Coleoptera	0.1 \pm 0.1 0.1%	0.0 \pm 0.0 0.0%	0.7 \pm 0.3 3.5%	0.6 \pm 0.2 2.5%
Coleoptera larvae	0.1 \pm 0.1 0.2%	0.3 \pm 0.1 0.7%	1.1 \pm 0.2 5.2%	0.8 \pm 0.3 3.2%
Minor benthic taxa	0.2 \pm 0.1 0.3%	0.1 \pm 0.1 0.2%	0.4 \pm 0.3 1.9%	0.4 \pm 0.2 1.6%
Trichoptera larvae	0.0 \pm 0.0 0.0%	0.0 \pm 0.0 0.0%	0.1 \pm 0.1 0.6%	0.1 \pm 0.1 0.5%
Terrestrial insects ³	0.0 \pm 0.0 0.0%	0.0 \pm 0.0 0.0%	0.1 \pm 0.1 0.4%	0.0 \pm 0.0 0.2%

¹Almost exclusively *Daphnia* sp.; ²*Chirocephalus* sp.; ³Flying insects that had fallen on water surface. See text for results of statistical analyses.

The trophic niche of metamorphs was significantly larger ($H' = 0.89 \pm 0.07$ and 0.79 ± 0.08 for females and males, respectively) than that of paedomorphs ($H' = 0.47 \pm 0.08$ and 0.54 ± 0.11 for females and males, respectively), as shown by PERMANOVA (pseudo- $F_{1,74} = 17.753$, $p = 0.0002$), with no effect of sex (pseudo- $F_{1,74} = 0.128$, $p = 0.724$) or their interaction (pseudo- $F_{1,74} = 0.933$, $p = 0.339$), and with no effect of the covariate size (pseudo- $F_{1,74} = 2.636$, $p = 0.102$) (Supplementary material Appendix 1 Table A3). The Schoener index showed a higher proportion of trophic niche overlap between sexes (0.98) than between phenotypes (0.58) (Supplementary material Appendix 1 Table A4). Within the four categories, the highest overlap was found between paedomorphic females and males (0.94). The lowest overlap was found between metamorphic females and paedomorphic females (0.69). Accordingly, the PERMANOVA showed a significant difference between paedomorphs and metamorphs' diets (pseudo- $F_{1,74} = 5.287$, $p = 0.004$) but no effect of sex (pseudo- $F_{1,74} = 0.345$, $p = 0.772$) or interaction (pseudo- $F_{1,74} = 0.861$, $p = 0.459$), and no effect of the size covariate (Pseudo- $F_{1,74} = 2.707$, $p = 0.061$) (Supplementary material Appendix 1 Table A5). The subsequent SIMPER analysis showed that zooplankton was the main driver of the dissimilarities between paedomorphs and metamorphs' diets (29.3%), followed by fairy shrimp (22.3%), Chaoboridae pupae (21.9%), Coleoptera larvae (11.6%), adult Coleoptera (7.1%), minor benthic taxa (5%), Trichoptera (1.9%) and, finally, terrestrial insects (1%).

Biomass intakes (total dry weight of prey in the stomach) of newts corrected by individuals' size (Mean \pm SD) were: 0.22 ± 0.14 mg and 0.22 ± 0.17 mg for the paedomorphic females and males, respectively and 0.28 ± 0.19 mg and 0.33 ± 0.33 mg for the metamorphic females and males, respectively. PERMANOVA showed no significant differences between phenotypes (pseudo- $F_{1,74} = 2.092$, $p = 0.151$), sex (pseudo- $F_{1,74} = 0.157$, $p = 0.703$) or their interaction (pseudo- $F_{1,74} = 0.215$, $p = 0.666$) (Supplementary Material

Appendix 1 Table A6). The residuals of body condition were: 0.009 ± 0.144 and -0.012 ± 0.133 for the paedomorphic females and males, respectively and -0.008 ± 0.116 and -0.056 ± 0.100 for the metamorphic females and males, respectively. There were no significant effects of phenotype (pseudo- $F_{1,74} = 0.0004$, $p = 0.982$), sex (pseudo- $F_{1,74} = 3.710$; $p = 0.059$) and their interaction (pseudo- $F_{1,74} = 1.679$, $p = 0.198$) on the body condition of newts, as tested by PERMANOVA (Supplementary Material Appendix 1 Table A7).

Stable isotopes

The isotopic niche comparison among groups revealed that paedomorphs had a smaller isotopic niche than metamorphs, with paedomorphic females having the smallest and metamorphic males the largest niche in the adult population. The SEA_B modes with 95% CI, from the smallest ellipse to the largest, were as follows: paedomorphic females = 0.70 (0.40–1.16); paedomorphic males = 0.66 (0.38–1.25); metamorphic females = 1.25 (0.86–1.89); and metamorphic males = 1.50 (1.02–2.36). Results are comparable for all estimates of the isotopic niche areas (SEA , $SEAc$, SEA_B and TA : Supplementary material Appendix 1 Table A8). Pairwise comparisons of SEA_B provided robust evidence that differences in isotopic niche width exist between paedomorphs and metamorphs, but not between sexes within each of these alternative phenotypes. The isotopic niche of paedomorphic females was smaller than that of metamorphic females and males in 96% and 99% of the posterior draws, respectively, but was not smaller than the paedomorphic males' isotopic niche (49%). Similarly, the paedomorphic males' niche was smaller than that of metamorphic males and females in 98% and 95% of the cases, respectively. The metamorphic males' niche was the largest in 99%, 98% and 76% of the posterior draws when compared to paedomorphic females, paedomorphic males and metamorphic females, respectively. Patterns of isotopic niche overlap among the four groups assessed using $SEAc$ (standard ellipse area corrected for small size samples) indicated a high overlap between the two metamorphs' niches (females shared 82.8% of their niche with the males), but a marked difference between the two paedomorphs' niches (females only shared 15.2% of their niche with the males) (Fig. 2; Supplementary material Appendix 1 Table A9). In fact, paedomorphic males overlapped more with metamorphs than with paedomorphic females. They shared 74.1% and 49.7% of their niche with metamorphic females and males, respectively, while they only shared 14.9% of their niche with paedomorphic females. Hence, paedomorphic females had the most different niche within the adult population, with only 16.7% and 6.4% of overlap with metamorphic females and males, respectively, and a marked difference in position in the isotopic space. There was a global effect of size on the isotopic composition (centroid location in the bivariate space of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes) of newts, as shown by a significant effect of the covariate in the PERMANOVA test (Pseudo- $F_{1,74} = 4.194$, $p = 0.04$) (Supplementary material Appendix 1 Table A10). However, after controlling for this effect there were still significant effects of the phenotype (Pseudo- $F_{1,74} = 13.688$, $p = 0.0003$) and phenotype-sex interaction (Pseudo- $F_{1,74} = 7.133$, $p = 0.007$) on the isotopic composition of both $\delta^{13}C$ and $\delta^{15}N$ of newts, but no main effect of sex (Pseudo- $F_{1,74} = 2.217$, $p = 0.131$). Post hoc permutational t-tests indicated that paedomorphic females were significantly different from paedomorphic males ($t_{25} = 3.216$, $p = 0.003$) and metamorphic females ($t_{39} = 4.453$, $p = 0.0001$), while there were no differences between paedomorphic and metamorphic males ($t_{33} = 0.789$, $p = 0.462$) or between metamorphic females and males ($t_{47} = 0.472$, $p = 0.715$). Overall, paedomorphs contributed to the expansion of the core isotopic niche of the population by 30%, based on estimates of $SEAc$ (paedomorphs = 0.985, metamorphs = 1.525 and their overlap = 0.333).

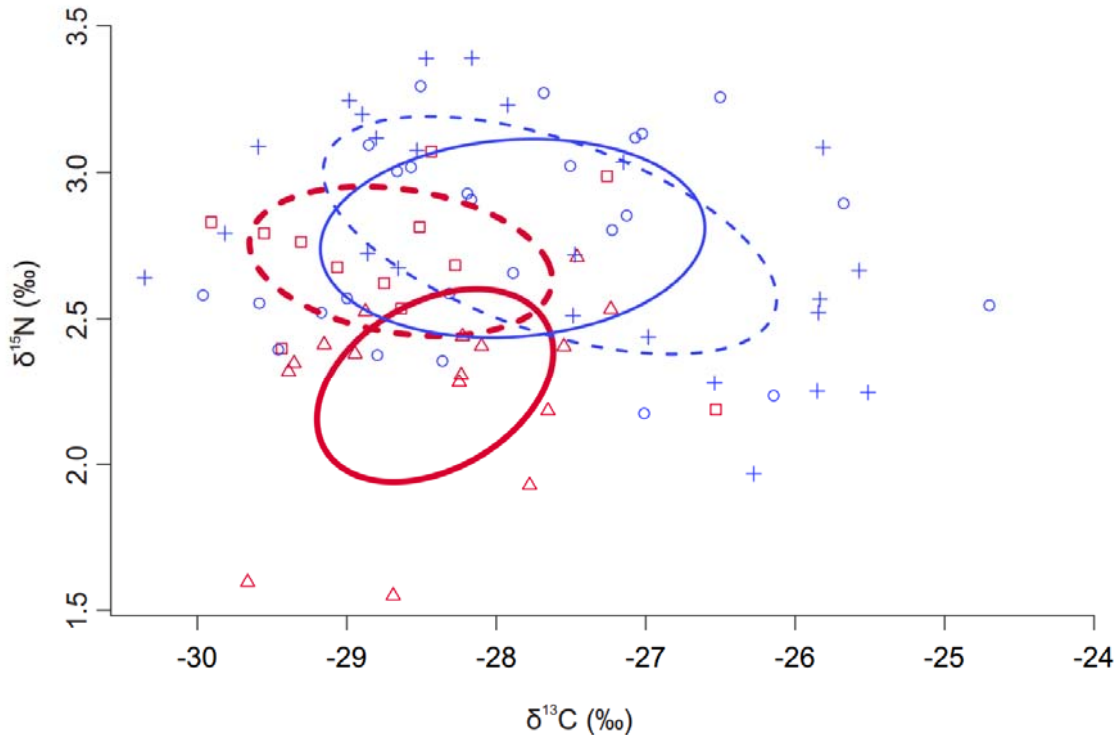


Figure 2. Stable isotope bi-plot illustrating the isotopic niche of alpine newts. The lines enclose the standard ellipse area (SEAC) of each group. Paedomorphs: thick red lines, metamorphs: thin blue lines. Females: solid lines, males: dashed lines. Symbols used for individuals of each group are: triangles for paedomorphic females, squares for paedomorphic males, circles for metamorphic females and crosses for metamorphic males.

The SIAR mixing model indicated that zooplankton marked the main difference among the four groups. It was clearly the dominant food source for paedomorphic females (mode = 62%, CI_{95} = 43–82%), but not for the males (mode = 39%, CI_{95} = 20–60%), which had values similar to that of metamorphic females (mode = 36%, CI_{95} = 21–58%) and males (mode = 34%, CI_{95} = 17–61%) (Fig. 3; Supplementary material Appendix 1 Table A11). Pairwise comparisons showed that the contribution of zooplankton to paedomorphic females was higher in 96, 95 and 94% of the posterior draws of the mixing model when compared to metamorphic females, metamorphic males and paedomorphic males, respectively (Supplementary material Appendix 1 Table A12). The contribution of littoral taxa was roughly equivalent in metamorphic females (mode = 28, CI_{95} = 11–42%) and males (mode = 33, CI_{95} = 17–61%), and higher in 88% and 92% of the posterior draws when compared to paedomorphic females. Paedomorphic males' reliance on littoral taxa was intermediate. Reliance on pelagic invertebrates was roughly equivalent for metamorphic females (mode = 28%, CI_{95} = 12–40%) and metamorphic males (mode = 26%, CI_{95} = 7–40%), but substantially higher in paedomorphic males (mode = 35%, CI_{95} = 20–50%) and the lowest in paedomorphic females (mode = 19%, CI_{95} = 5–33%). Reliance on terrestrial taxa was generally low. Overall, differences in estimated source reliance were mostly visible between paedomorphic females and the three other groups as they had the highest reliance on zooplankton and the lowest reliance on pelagic invertebrates, littoral and terrestrial taxa.

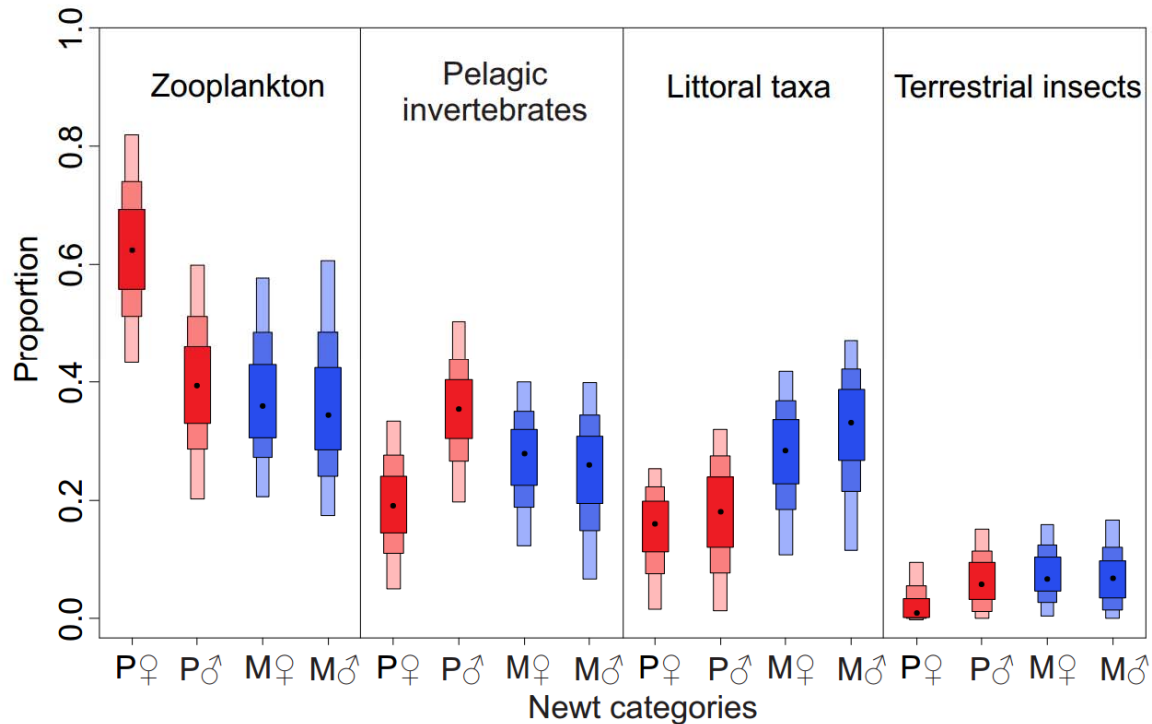


Figure 3. Relative contribution of four food sources to the four alpine newt categories. Red: pedomorphs, blue: metamorphs. P♀ = pedomorphic females, P♂ = pedomorphic males, M♀ = metamorphic females, M♂ = metamorphic males. Black dots represent the mode. Credibility intervals (CI): CI₅₀ = dark red/blue, CI₇₅ = medium red/blue, CI₉₅ = light red/blue. ‘Terrestrial insects’ are preyed upon by newts in the aquatic environment and not on land.

Discussion

Using both stomach contents and stable isotopes, along with niche comparisons and mixing models, we have found evidence that facultative pedomorphosis can promote both niche expansion and trophic differentiation throughout the aquatic life-stage of an amphibian species, the alpine newt. However, the use of stable isotopes revealed an unexpected pattern of niche overlap, bringing pedomorphic males closer to metamorphs in terms of trophic ecology and consequently showing that the resource partitioning was more pronounced in females than in males, on the long-term. This pattern may have important consequences for understanding the evolution and persistence of facultative pedomorphosis and highlights the importance of considering sex differences when studying polyphenisms.

Trophic niche differentiation through facultative pedomorphosis

Trophic niche differentiation at the intraspecific level allow risk-spreading among different feeding strategies, ultimately allowing higher reproductive success, accounting for their adaptiveness and long-term maintenance in some populations (Schmidt et al. 2000, Bolnick 2001, Denoël et al. 2004). Here, stomach content analysis revealed that, regardless of sex, pedomorphs were almost exclusively reliant on pelagic prey and mainly on zooplankton, while metamorphs had a significantly different diet, relying substantially more on littoral prey. Consequently, the latter had a larger trophic niche, as expressed by a significantly higher Shannon index. The feeding strategies found here are consistent with previous comparisons of stomach contents and differences in microhabitat use between pedomorphs

and metamorphs (Denoël and Joly 2001). Metamorphs are more constrained to the littoral zone or the water surface because they find there the prey for which they are better foragers than paedomorphs (Denoël, 2004) and likely because of their need to breathe on atmospheric air whereas paedomorphs rely more on their gills (Seliskar and Pehani 1935) and then spend more time in the pelagic zone and in the deeper parts of the lake. Despite important differences in individual biomass of zooplankton compared to larger pelagic, littoral or terrestrial prey (see e.g. Dumont et al. 1975, Benke et al. 1999), both strategies provided similar gains as the total dry mass of ingested prey, corrected by newt size, and the body condition of newts were equivalent in both phenotypes. However, body condition can change over time and therefore reflects the seasonal advantage and not the lifetime pattern (Denoël et al. 2002). Stable isotopes confirmed some of the results of the analysis of stomach contents, i.e. in showing that metamorphs had the highest reliance on littoral taxa, while paedomorphs were mainly dependent on pelagic prey. They also allowed to globally determine the niche width of the two phenotypes, with metamorphs having a larger isotopic niche than paedomorphs. Overall, they showed that paedomorphosis contributed to the expansion of the core isotopic niche of the population by 30% compared to metamorphs alone. Cloyed et al. (2015) showed that amphibian skin integrates diet information from ~2-3 months before capture, which in this case represents a large part of the aquatic active period of newts, including the reproductive period. Conversely, stomach contents are a snapshot representation of the period after the reproduction, as they were sampled in late summer when all newts were still in the water but not reproducing anymore. Stomach contents are also more sensitive to changes in local availability of resources in both time and space. For instance, during our study, we almost did not find predation on terrestrial invertebrates whereas stable isotope analyses showed their importance in the diet, particularly of metamorphs. This discrepancy is likely due to rainy conditions during our sampling as a previous study already showed that newts do not always forage on this resource, likely because of its low availability during rainy periods (Denoël and Joly 2001). It has to be noted that foraging on terrestrial invertebrates occurs in water only as both metamorphic and paedomorphic newts are aquatic in this population and were never seen foraging on land (M. Denoël & B. Lejeune, pers. obs.).

These results are also concordant with assumptions from the trophic morphology of the two phenotypes. Paedomorphs use a suction mechanism with a uni-directional flow and have gill rakers to help retain small prey while expelling water through the gills (Lauder and Reilly 1994). This morphological difference allows them to more efficiently catch small aquatic prey and zooplankton than metamorphs, as previously demonstrated experimentally by Denoël (2004). This trait is also correlated with zooplanktivory in fish, where, in polyphenic species, phenotypes adapted to the pelagic zone have longer gill rakers (Malmquist 1992). The superior efficiency of paedomorphs at catching aquatic prey, compared to metamorphs, was also demonstrated in the amphibian species *Ambystoma tigrinum*, which also displays heterochronic polyphenism, but with the difference that, in this case, paedomorphs had the complete advantage over metamorphs (Whiteman et al. 1996). In the case of alpine newts, previous field (Denoël & Joly 2001, Denoël 2004) and experimental studies (Denoël 2004) showed that metamorphs were more efficient at catching large prey and, especially, terrestrial insects floating on the water surface. In ambystomatids, the reverse was found, most likely because of the large size of ambystomatid paedomorphs which enables them to forage on a wide variety of prey (Whiteman et al. 1996).

Sex differences and maintenance of the trophic polyphenism

Interestingly, the use of stable isotopes revealed unexpected differences between paedomorphic males and females, with paedomorphic males having a trophic niche closer to metamorphs than to paedomorphic females, while there is no difference between sexes in metamorphs. Although size was shown to have a global effect on the isotopic composition of newts, the differences between phenotypes and between sexes within paedomorphs were still significant after controlling for snout-vent length of newts, suggesting that other sex-dependent traits might play a role in bringing paedomorphic males closer to metamorphs than to paedomorphic females. The mixing models suggested that paedomorphic males relied significantly less on zooplankton, but substantially more on 'pelagic invertebrates' (Chaoboridae pupae and fairy shrimps) than the paedomorphic females, and that they had a reliance on terrestrial insects (which had fallen on the water surface) and littoral prey more similar to that of metamorphs. We argue that some of the inconsistency of paedomorphic males' isotopic composition compared to their stomach contents suggests a change in their trophic habits that might be partly explained by their past reproductive activity. Indeed, during the reproductive period, males of both phenotypes exhibit a time-consuming courtship behaviour to attract the females which often happens in the littoral zone and cannot be completed in open waters because the spermatophore needs to be deposited on the substratum of the lake before its transfer to the female (Denoël 2002; M. Denoël, pers. obs.). Moreover, male newts have a continued breeding period, whereas females are often in 'time-out' (i.e., temporarily not available for new breeding) because of egg laying and likely because of foraging to restore their energy loss (Gabor & Halliday 1997). Therefore, we assume that the proximity of the paedomorphic males' isotopic niche to the metamorphs' one may be influenced by the fact that, during the reproductive period, they spend more time together in the littoral zone to exhibit courtship behaviour. Following this hypothesis, the fact that either Chaoboridae or fairy shrimps (from the mixed food source 'pelagic invertebrates') contributed substantially more to paedomorphic males' isotopic composition than any other group, might be explained by the presence of large quantities of Chaoboridae pupae close to the shoreline. Probably pushed there by the wind as they were mostly floating under the water surface (B. Lejeune, pers. obs.), they would therefore be directly available to paedomorphic males during their reproductive activity in the littoral zone. The link to fairy shrimps is less obvious as previous work showed low amount of fairy shrimps in their diet (Denoël & Joly 2001) whereas both sexes have been shown to display similar efficiency in their capture (Denoël 2004). However, because paedomorphs are less efficient at catching larger prey (Denoël, 2004), the obligate presence of paedomorphic males on the littoral zone for reproduction may constitute a sex-specific cost of paedomorphosis (Whiteman, 1997), unless they prove more plastic during this period or focus on smaller littoral or terrestrial prey than the metamorphs. A previous study also highlighted that paedomorphic males had a similar body condition to metamorphic males whereas paedomorphic females had a higher body condition than metamorphic females (Denoël et al. 2002). On the other hand, the more divergent trophic ecology of paedomorphic females is in line with their morphology, allowing optimal access to underused resources, i.e. in open waters and in the deep parts of the lake, therefore escaping intraspecific competition in the littoral zone. Moreover, because newts, and especially females, consume eggs of their own species (Miaud 1993), the extension of the paedomorphic females' niche to the pelagic zone may be adaptive not only as a trophic strategy, but as it would give them the opportunity to lay eggs in the deep and, therefore, avoid cannibalism in the more frequented littoral zone. These results show the importance of considering sexes separately to understand the evolution of trophic polymorphisms. Sex-specific pay-offs of alternative strategies favouring different phenotypes

in males and females can promote the maintenance of the dimorphism as in the population of paedomorphic and metamorphic tiger salamanders studied by Whiteman (1997). However, in our case, sex-specific differences in trophic ecology were found in only one phenotype.

Previous experimental research highlighted that paedomorphs and metamorphs are sexually compatible, including in the study species from La Cabane Lake as a result of a similar courtship behaviour (Denoël 2002) and success in sperm transfer between and within morphs (Denoël et al. 2001b). However, spatial partitioning may affect this game if each morph court in different habitats, therefore favouring assortative mating and possibly ecological speciation (Whiteman & Semlitsch 2005). Indeed, niche-specific adaptation, in the case of trophic polymorphism, allows divergence that can lead the way to sympatric speciation if it creates an ecological or behavioural barrier preventing gene flow among phenotypes within a population (Smith and Skúlason 1996, Bolnick and Fitzpatrick 2007). Yet, the foraging data obtained in our study suggests an absence of sexual isolation as paedomorphic males coexist with metamorphs in the littoral benthic habitat; an observation also found in ambystomatids in one of the studied populations in Whiteman & Semlitsch (2005). In the end, limitation of the reproductive behaviour of males to the littoral zone may be a key factor holding back assortative mating and further speciation and, therefore, possibly promoting the long-term maintenance of facultative paedomorphosis as a polyphenism in alpine lakes. However, behavioural observations of courtship in the various habitats during the breeding season as well as genetic comparisons of syntopic paedomorphs and metamorphs from the study lake are needed to possibly unravel hidden isolation patterns. Indeed, although large gene flows between morphs were shown in ponds (Oromi et al. 2016), the situation in deep lakes may be different (Denoël et al. 2016).

Evolutionary ecology of trophic polyphenisms

Trophic differences observed here correspond to an extension of the trophic niche of paedomorphs to otherwise underused resources (i.e. pelagic resources). The concept of ‘underused’ resources here, is to understand as vacant niche (*sensu* Van Valen, 1965), a condition that occurs when habitat heterogeneity and the absence of interspecific competitors combine to create a situation of environmental niche vacancy that can promote niche expansion and/or differentiation (Van Valen 1965). This has been discussed in many cases of trophic polyphenisms in fish (*e.g.* Wimberger 1994; Robinson & Parsons 2002), and is exemplified in cases of niche expansion and ontogenetic diet shifts that have been described following ecological release from interspecific competition in lacustrine fish populations (Bolnick et al. 2010, Shedd et al. 2015). This concept of environmental niche vacancy, due to a relaxation of interspecific competition allowing niche expansion, seems to particularly fit the case of alpine lakes where facultative paedomorphosis occurs in newts.

We found similarities between facultative paedomorphosis in the alpine newt and cases of trophic polymorphisms along the littoral-pelagic axis that appear to be very common in lacustrine fish (Malmquist et al. 1992, Svanbäck and Eklöv 2003, Woods et al. 2013), especially in post-glacial lakes (Robinson and Parsons 2002). The occurrence and repeated nature of phenotypic divergence along the littoral-pelagic axis in lacustrine fishes suggest that littoral and pelagic habitats are common and ecologically distinct environmental niches that apply strong divergent selection favouring different phenotypes in each environment (Smith and Skúlason 1996, Robinson and Parsons 2002). Environmental variation, whether temporal or spatial, is a key component in the evolution and maintenance of polyphenisms. In the case of trophic polyphenism, ‘environmental variation’ can be defined as spatial and/or temporal fluctuations in the availability of prey in different habitats. Trade-offs in resource use efficiency between littoral and pelagic environments, combined with intraspecific

competition in the littoral zone, may therefore apply disruptive selection, thus provoking niche differentiation as an adaptive response (Smith and Skúlason 1996, Bolnick et al. 2003, Pfennig and McGee 2010). This model fits well the case of facultative paedomorphosis in alpine newts inhabiting mountain lakes where both habitats require specific performances that are met more efficiently by one phenotype or the other and where littoral resources are scarce. Therefore, specialization to better exploit pelagic resources through facultative paedomorphosis may have been selected as a way to lessen intraspecific competition in the littoral zone. This is likely specifically due to foraging abilities rather than avoidance of aggression because alpine newts are not territorial.

Further investigations should be conducted on facultatively paedomorphic populations of newts inhabiting less heterogeneous and resource rich environments, such as ponds, using integrative tools, such as stable isotopes, to test whether niche partitioning also exists between paedomorphs and metamorphs. Documenting the contexts of such cases would help identify trophic niche differentiation as a selective advantage favouring the long-term maintenance of this dimorphism on a broader scale and across different species. Moreover, it would help clarify the roles of resource diversity, intraspecific competition and the scale of habitat heterogeneity in the trophic niche differentiation between phenotypes. Particularly, it can be hypothesised that heterogeneity provided by deep lakes provides more opportunities for alternative foraging strategies than shallow ponds.

Concluding remarks

Although paedomorphosis and metamorphosis have been primarily seen as results of trade-offs between the advantages of using aquatic versus terrestrial habitats in newts and salamanders (Wilbur and Collins 1973, Whiteman 1994), we provided evidence that additional forces, such as intraspecific trophic niche differences between phenotypes, as well as trophic niche expansion, may play an important role in the persistence of this dimorphism in heterogeneous environments that are devoid of interspecific competitors, such as alpine lakes. In this case, differences corresponded to a trophic polyphenism along the littoral-pelagic axis and illustrate the fact that, while paedomorphosis inherently limits the access to terrestrial habitat in the alpine newt, it also clearly opens new opportunities in the aquatic environment. The use of stable isotopes revealed a novel aspect of the ecology of paedomorphic males, potentially linked to their reproduction, that makes them more similar to metamorphs and may be directly involved in the prevention of spatial assortative mating and ultimately sympatric speciation between the two phenotypes.

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