Life history trait differences between a lake and a stream-dwelling population of the Pyrenean brook newt (*Calotriton asper*)

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Abstract. The Pyrenean brook newt (*Calotriton asper*) is a salamandrid that mostly lives in fast running and cold mountain-streams, although some populations are also found in lakes. In the present work, we report in detail on the occurrence of facultative paedomorphosis traits in a population from a Pyrenean high altitude lake. We compare its morphology, life history traits and mitochondrial DNA variation with a nearby lotic metamorphic population. Our results indicate that the lacustrine newts are smaller and present a less developed sexual dimorphism, smooth skin, and that 53% of the adults retain gills at different degrees of development, but not gill slits. Although both populations and sexes have the same age at sexual maturity (four years), the lacustrine population presents higher longevity (12 and 9 years for males and females, respectively) than the one living in the stream (8 and 9 years). The variation on the climatic conditions at altitudinal scale is probably the main cause of the differences in life history traits found between the two populations. The food availability, which could to be limiting in the lacustrine population, is another factor that can potentially affect body size. These results are congruent with the significant mitochondrial DNA genetic isolation between populations, probably a consequence of the lack of juvenile dispersal. We found low cytochrome b variability and significant genetic structuring in the lake population that is very remarkably considering the short distance to the nearby stream population and the whole species' pattern. We suggest that a bottleneck effect and/or phenotypic plasticity may have resulted in the appearance of a paedomorphic morph in the lake.

Keywords: age structure, *Calotriton asper*, paedomorphosis, skeletochronology.

Introduction

Life history traits in amphibians, such as age at maturity, longevity and age-size relationship, are often connected by trade offs (Stearns, 2000) and can vary due to many factors, e.g. climatic conditions, food resources or interspecific competition (Adolph and Porter, 1996). Although amphibians are organisms with indeterminate growth, most of the increase in body size takes place prior to sexual maturity (Tilley, 1980; Miaud and Guyetant, 1998). Because the body size has dramatic implications for ecology and reproduction (e.g., Olalla-Tárraga and Rodríguez, 2007; Moen and Wiens, 2009), age at sexual maturity is one of the most important

traits in life histories. Skeletochronology can be effectively used for age determination in amphibians. This method is based on the number of the lines of arrested growth (LAG) – marked in any round bone – that are produced during hibernation or aestivation: e.g. a phalange obtained by toe-clipping. Skeletochronology provides an age estimation with a margin of error that mainly depends on the individual longevity with a reliability higher in short-lived amphibian species (e.g. Wagner et al., 2011). Skeletochronological assessment is widely applied to the study of sexual maturity and longevity in amphibians (e.g. Miaud, 1991; Morrison and Hero, 2003) producing accurate age estimates.

Newts and salamanders exhibit a wide diversity of life cycles, which can be understood as a result of the interplay between costs and benefits in aquatic and terrestrial environments (Wilbur and Collins, 1973; Whiteman, 1994). Although many species have a two-stage ontogenesis with a larval aquatic stage and a terrestrial post-metamorphic stage (obligate metamorphosis), others inhabit only aquatic habitats.

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A common phenomenon in newts is the facultative paedomorphosis, in which adults retain larval traits such as gills and gill slits whereas others are fully metamorphosed (Whiteman, 1994). Facultative paedomorphosis (i.e. the potential for retention of juvenile traits in mature individuals) is an adaptive strategy found in most salamander families across heterogeneous environments caused by multiple ecological factors (Whiteman, 1994; Voss, 1995; Denoël, 2003). This strategy has been reported in several European newts of the genus *Triturus* (*T. cristatus*, *T. carnifex*), *Lissotriton* (*L. italicus*, *L. helveticus* and *L. vulgaris*), *Ichthyosaura alpestris* and *Omatotriton vittatus* (Kalezic et al., 1996; Denoël and Joly, 2000; Denoël, 2003, 2007; Sindaco et al., 2006; Kaya et al., 2008; Denoël et al., 2009), demonstrating a wide occurrence among species with an apparent intraspecific geographic variation.

The Pyrenean brook newt (*Calotriton asper*) is a salamandrid adapted to a lotic life in streams and is usually metamorphic under these conditions (García-París, Montori and Herrero, 2004). However, some populations develop a lentic life because they live at high altitude lakes in Pyrenees (Clergue-Gazeau, 1965; Clergue-Gazeau and Martínez-Rica, 1978), where a paedomorphic population has been reported (Campeny, Montori and Llorente, 1986). Many aspects of the species geographic range, habitat preferences, behaviour and ecology have been studied extensively (see García-París, Montori and Herrero, 2004 for general information). For example, the demography of *C. asper* has been analysed in a variety of populations ranging from both Pyrenean slopes and comprising almost all their altitudinal range (Miaud and Guillaume, 2005). Surprisingly, there is a considerable lack of research concerning the morphology, demography and ecology of the lentic populations and more specifically about the occurrence of paedomorphosis.

In the present work we report in detail on the presence of paedomorphic traits in a population of *Calotriton asper* living in a Pyrenean high altitude lake. We compare its morphology and life history traits with a lotic metamorphic nearby population. We also analyse their genetic differentiation at the mitochondrial level taking into account the genetic variability and structure along the species' geographic range. Finally, we discuss our findings in the light of the evolutionary consequences of the colonization of such an extreme habitat by a specialized lotic high altitude salamander.

Material and methods

Study area and sampling

The lacustrine population inhabits the Ibón de Acherito, a lake in the western Pyrenees (30T 6872E 47 499N) at 1886 m a.s.l. with an area of 680 m^2, perimeter of 1150 m and a maximum depth of 20 m (del Castillo, 2004). The lake is surrounded by alpine meadows with karstic rocky boulders and walls, and has an extensive rocky and submerged platform serving as the main newt habitat. The lake supplies water to a small stream formed on the SW margin. In this stream, Pyrenean newts are found between 1350 and 1485 m a.s.l., coinciding with the altitudinal limit of the forest. Therefore, these lacustrine and stream-dwelling populations are separated by approximately 640 m of the stream section.

Fieldwork was carried out in the spring of 2010 (lacustrine population) and 2011 (stream population), and consisted of night surveys along the lake shore and the stream margins investing one night by population and, approximately three and two hours respectively. Individuals were sexed on the basis of sexual secondary characters (i.e. pointed cloaca protuberance in females and round and bulbous cloaca in males) and the presence of gills was recorded, but not for gill slits that are almost are never present. Eight linear morphometric measurements were obtained with a digital calliper: snout-vent length (SVL), head length (from the tip of the snout to the gular fold), maximum head width, hind and forelimb length (taken from the right ventral side), limb interval (minimum distance between the closest insertion points of the limbs), tail length (taken from the outer edge of the cloacal protuberance) and maximum tail high. Measurements were taken after anesthetizing the individuals with an aqueous solution of ethyl 3-aminobenzoate at 10 mg/l (MS-222, Sigma-Aldrich Co). Additional sampling included extraction of one toe for a skeletochronological and mitochondrial DNA analysis.

Skeletochronological analysis

The age structure was estimated in a subsample of the lentic population – 32 males, 12 females and 7 immatures, and the lotic population – 21 males and 20 females. Skeletochronology protocols followed the standard methods described by Miaud (1991) and modified by Amat, Oromi and

Sanuy (2010). The largest toe of the left hind limb was removed, stored in 70% alcohol and decalcified in 3% nitric acid for 5 min. Fine cross sections (12-14 μm) were obtained with a freezing microtome and stained with Ehrlich's haematoxylin. The age of each sample was determined by counting lines of arrested growth (LAGs) in the diaphysis of the periosteal bone using a light microscope at magnifications of 200× and 400×. Skeletochronological data were used to estimate age and the following life-history variables: age at maturity (the minimum number of LAGs counted in the reproductive individuals), size at maturity (the minimum snout-vent length of all first breeders with the minimum number of LAGs), longevity (the maximum number of LAGs counted in reproductive individuals), median age (median of reproductive age distribution) and potential reproductive lifespan (the difference between longevity and age at maturity).

DNA sequencing and genetic analysis

Sequences of mitochondrial cytochrome b gene of 374 base pairs were obtained from the stream-dwelling and lacustrine populations by using specific primers designed for *Calotriton asper* and visually aligning them by means of Bioedit 7.1 (Hall, 1999). These sequences are added to a *C. asper* dataset previously used in a population genetic analysis of the species (Valbuena-Ureña et al., 2013). Our final dataset includes 364 individuals from 17 populations covering the main species' geographic range.

In order to characterize genetic diversity and structure between the lacustrine and stream dwelling population in the framework of the species range, we computed the haplotype (Hd), nucleotide diversity (π) and gene flow (Fst). The population isolation was evaluated using the S_{nn} statistic (Hudson, 2000), testing its signification based on 1000 permutations (Hudson, Slatkin and Maddison, 1992). All the analyses were performed using DnaSP 3.53 (Rozas and Rozas, 1999).

Statistical analysis

We tested for body size and shape differences between sexes and populations, and the effect of paedomorphosis. An ANOVA was performed to elucidate for differences in SVL at sexual and population level, and between gilled and metamorphs in the lake population. However, in order to analyse differences on body shape at sexual and population level, we chose to remove the size effects produced by allometric growth using the following formula (Lleonart, Salat and Torres, 2000): $Y_i^* = Y_i(SVL_0/SVL_i)b$, where Y_i^* is the size-corrected proportion character of individual i; Y_i is the original value; SVL_0 is the mean SVL at which all variables are corrected; SVL_i is the body size; and b is the slope of the sex and population ordinary least-square regression. The resulting new size-corrected morphologic traits were analysed using MANOVA, testing the differences between sex and population, and among the lacustrine gilled and metamorphs. Moreover, a principal component analysis (PCA) was performed to explore the differences between sexes and populations, and paedomorphosis on patterns of covariation of size-adjusted variables.

Significant differences between the shape and median of age distributions among sexes and populations, and between the gilled and metamorphic lacustrine newts, were analysed using the two-sided Kolmogorov-Smirnov and Mann-Whitney U-tests. In addition, Spearman correlation between age and SVL was also performed. All analyses were done, using Statistica 5.4 on log-transformed variables.

Results

Morphology

The ad hoc visual examination of the lentic newts revealed the presence of gills in 53% of the adults although, much less developed than in larvae and in most cases lacking gill slits (fig. 1). Apart from the presence in these individuals of sexual secondary characters (cloacal dimorphism), they exhibit the reproductive behaviour of the species including amplexus by caudal capture of females, indicating that they are sexually active. The lacustrine population is constituted by larvae, immatures and adults, all showing a very smooth skin (fig. 1). However, in the nearby stream individuals present keratinized warts on the skin and only adults were found in the water. SVL of the newts did not differ significantly between males and females (ANOVA, $F_{1,140} = 2.569$, $P = 0.111$) showing no significant interaction with the populations (ANOVA, $F_{1,140} = 2.560$, $P = 0.114$). However, the stream newts were larger than the lake newts (ANOVA, $F_{1,140} = 173.021$, $P < 0.001$; table 1) and the average SVL of the first breeders was also larger in the lotic population (ANOVA, $F_{1,11} = 10.534$, $P = 0.007$, table 1). In the lentic newt population, SVL did not differ significantly between gilled and metamorphic adults (ANOVA, $F_{1,42} = 0.807$, $P = 0.373$).

MANOVA reveals sexual differences on the body shape in both populations ($\Lambda_{7,140} = 0.006$, $P < 0.001$) and also between them ($\Lambda_{7,140} = 0.015$, $P < 0.001$). In addition, a significant interaction between these factors indicates that the pattern of sexual dimorphism

Figure 1. Morphological differences between the lacustrine and stream-dweller newts. (A) Paedomorphic female living in the studied lake, showing the typical slender body shape and smooth skin, and with gills in some degree of reabsorption; detailed in (B). (C) Typical male from the nearby stream population showing extensive roughness and keratinized warts, and robust body form. This figure is published in colour in the online version.

Table 1. Descriptive statistics of SVL from lacustrine and stream-dwelling population of *Calotriton asper* by sex.

Population	Sex	n	Mean $\pm$ EE	Minimum-Maximum
Lake	males	55	62.8 $\pm$ 0.4	54.0-71.0
	females	23	62.9 $\pm$ 0.8	56.0-70.0
	immatures	32	48.6 $\pm$ 1.1	39.7-60.0
Stream	males	21	70.1 $\pm$ 0.5	64.8-74.5
	females	45	72.4 $\pm$ 0.5	63.8-78.1

Table 2. Factor loading of SVL-adjusted variables in the first two principal components, corresponding eigenvalues and % of cumulative variance.

Variable	Factor 1	Factor 2
Head length	−0.906	−0.185
Head width	0.267	0.665
Forelimb length	0.924	−0.329
Hindlimb length	0.959	−0.081
Limb interval	0.165	−0.834
Tail length	−0.044	0.862
Tail high	−0.362	−0.588
Eigenvalues	2.830	2.379
% Cumulative variance	40.4	74.3

differs between the lake and stream-dwelling populations ($\Lambda_{7,140} = 0.009$, $P < 0.001$). The body shape is not significantly different between gilled and completely metamorphosed individuals of the lake population ($\Lambda_{7,70} = 0.949$, $P = 0.803$). The first two factors of the PCA account for a large proportion of variance (table 2) and show a clear pattern of sexual and population differentiation. Stream-dwelling

males are characterized by proportionally larger and wider heads and higher tails, as typical in most populations of the species (fig. 2). In contrast, females from the lacustrine population have slender bodies defined by longer limbs and inter-limb distances, in relation to SVL. Stream-dwelling females have longer but narrower proportional heads and longer tails than females from the lake population. Lacustrine males show an intermediate pattern and share some similarities with stream females; showing an attenuated typical male sexual dimorphism with shorter heads and lower, but proportionally longer tails.

Skeletochronology and age-size variation

Annual bone marks were clearly visible in all cross sections, assuming that the number of LAGs was equivalent to the number of winters experienced by each individual. We did not observed the presence of reabsorption lines,
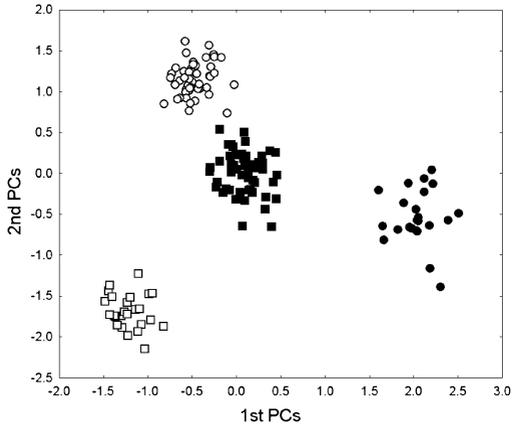
Figure 2. Plot of the first two PCs of the studied populations of *Calotriton asper*; lake: black squares (males) and black dots (females); stream: white squares (males) and white dots (females).

Figure 3. Age structure of *Calotriton asper* from the lake ($n = 51$) and the stream ($n = 41$) populations.

double lines or rapprochement of peripheral lines that could hamper the skeletochronological age determination. In both lacustrine and stream-dwelling populations the earliest age at sexual maturity was four years in both sexes. Age structure was also similar between sexes (Kolmogorov-Smirnov Test: lake population: $DN = 0.16$; $P > 0.100$; stream population: $DN = 0.05$; $P > 0.100$; fig. 3) with a total median age of 7 and 5 years in the lake and stream population, respectively (*U*-Mann Whitney Test: lake population: $Z = 0.592$; $P = 0.553$; stream population: $Z = -0.339$, $P = 0.734$). However, longevity was different between sexes and populations. In the lentic population, male longevity (12 years) exceeded that of the females (9 years), whereas in the lentic population females, longevity exceeded that of the males by one year (8 and 7 years, respectively). Consequently, the resulting potential reproductive life span (PRLS) was also different between populations and sexes, showing the males and females of lacustrine populations higher PRLS (8 and 5 years) than that of the stream population. On the other hand, gilled and metamorphic adults in the lacustrine population did not significantly differ in median age (*U*-Mann Whitney Test $Z = -1.680$; $P = 0.087$) or age structure (Kolmogorov-
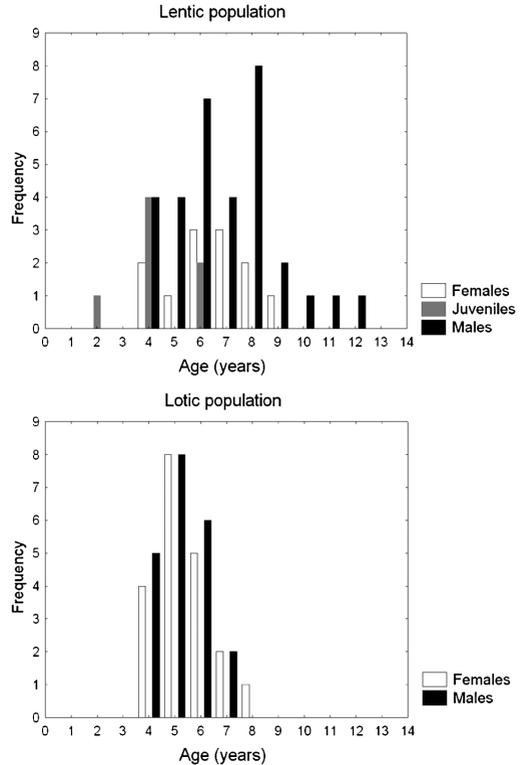
Smirnov Test: $DN = 0.043$; $P > 0.10$). Age and SVL were significant and positively correlated in the whole sample of stream ($n = 41$, $r = 0.367$, $P = 0.018$), but not in the lake ($n = 51$, $r = 0.238$, $P = 0.091$) population, and remain non significant for each sex within each population.

Mitochondrial population genetics

Genetic variability of Cyt b in the analysed populations of *Calotriton asper* is constituted by eight haplotypes. Seven populations are monomorphic and haplotypes differences are defined by changes in only one or two positions. The two mitochondrial haplotypes found in the lake and stream populations also occur in some populations of the western Pyrenees, and differ in only one nucleotidic change. One haplotype was over-represented in the lake population (96.9%) while, in the stream, this haplotype

Table 3. Estimates of genetic variability and sample sizes of the Cytochrome b in the 17 populations of *Calotriton asper* analyzed: haplotype (H_d) and nucleotide diversity (π).

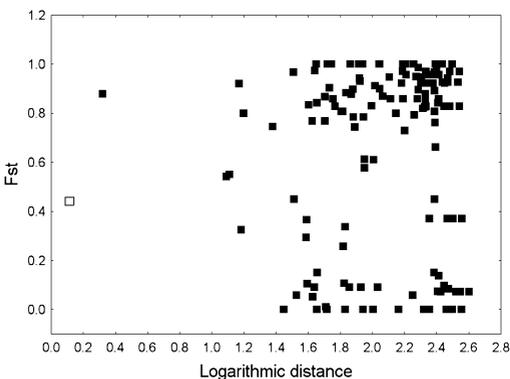| Population	n	H_d	π
Lacustrine population	32	0.063	0.00017
Stream population	21	0.524	0.00140
La Cerdanya	18	0	0
Vidrà	15	0	0
San Juan de la Peña	21	0.324	0.00087
Pto. Monrepós	24	0.083	0.00045
Bujaruelo	36	0.157	0.00084
Portalet	13	0	0
Port du Rat	12	0	0
Ordino	34	0.214	0.00114
Valle de Pineta	26	0.471	0.00126
Zuriza	20	0.505	0.00135
Isaba	18	0.111	0.00030
Vall Fosca	20	0	0
Vilanova de Meià	18	0	0
Irati	15	0.248	0.00066
Berga	21	0	0

Figure 4. Relationships between gene flow (F_{st}) and 10-logarithmic geographic distance in the 17 populations of *Calotriton asper*. Open square: lacustrine and stream-dwelling population comparison; black squares: the rest of comparisons between populations.

were roughly equal (47.6%) to the other. Haplotypic and nucleotid diversities were very variable (table 3) and lower in the lake ($\pi \pm$ SD: 0.00017 ± 0.00015; $H_d \pm$ SD: 0.063 ± 0.058) than in the stream ($\pi \pm$ SD: 0.00140 ± 0.00010; $H_d \pm$ SD: 0.524 ± 0.036). Genetic structure was detected between both populations ($F_{st} = 0.438$) and they present a significant genetic differentiation ($S_{nn} = 0.667$, $P < 0.001$). We did not found a clear pattern of covariation between geographic structure and geographic distance

(fig. 4). Remarkably, compared with other populations, our lentic and lotic populations show a strong genetic structure in relation to the close distance between them.

Discussion

The retention of gills in low extend in adults, found in newts of the lacustrine population, is the most typical and unequivocal evidence of facultative paedomorphosis. Nevertheless, our case study differs markedly of the typical found in other newts and is characterized by conserving large and functional gills in adults at the same degree of development that larvae (Healy, 1974; Kalezic et al., 1996; Denoël and Joly, 2000). Instead of this pattern, the newts simply had gills remnants as a result of the incompleteness metamorphosis, probably without any functional implication. The fact that in *Calotriton* newts respiration is mostly cutaneous, and lives in highly oxygenated waters precluding the development of large branquial apparatus (García-París et al., 2004), could have constrained the existence of this typical facultative paedomorph. Although the paedomorphic newts are characterized by their smooth skin, this trait is also present in metamorphic individuals of other lentic population and also in their sister stream-dwelling species *C. arnoldi* (Carranza and Amat, 2005). The common link between these populations is the lack of terrestrial activity in adults and even immatures. Thus, skin granulation may be an adaptation to stream water and desiccation in terrestrial habitats instead of a paedomorphic trait.

Our multivariate analysis shows that the lentic newts exhibit a divergent morphology and a less developed sexual dimorphism than the typical stream-dwelling populations of the Pyrenean newts. Such difference in body shape could be a consequence of paedomorphic processes or the development of morphology adapted to the locomotion in stagnant waters. The lotic life-style of *Calotriton asper* has promoted the evolution an amplexus male by means

of caudal capture of females by males. This remarkable behavioural specialization has enhanced a marked sexual dimorphism on the tail shape which is shorter and higher in males than in females (Serra-Cobo, Uiblein and Martinez-Rica, 2000; García-París, Montori and Herrero, 2004), as also found in this lotic population. Although *C. asper* is not considered a territorial species (Guillaume and Cavaragna, 2000), reproductive males can engage in ferocious combats if females are close by means of prolonged bites. The proportional minor head size in lacustrine newts may reduce the mandibular force and therefore the potential male aggressiveness. This could be important because the lentic population seems to be denser distributed in comparison to the lotic population.

The variation in morphology and some life history traits of the Pyrenean newt *C. asper* was previously described in several fully metamorphic populations at different habitat conditions (Montori, 1990; Guerrero-Campo, 1995; Serra-Cobo, Uiblein and Martinez-Rica, 2000; Miaud and Guillaume, 2005). The age and body size distribution of populations seem to be mainly influenced by the activity period and by the duration of the terrestrial phase before the first reproduction (Miaud and Guillaume, 2005). Life history traits such as age at maturity, longevity and body size show a co-variation with altitude and latitude in many amphibian species (reviewed by Morrison and Hero, 2003). However, multiple proximate causes such as phenotypic plasticity or genetic adaptation can promote the variation of different life history traits (Roff, 2001).

In our study, both populations mature at the same time but at a different size. However, they could differ in the time at maturity by only a few weeks or months. These differences have not been contemplated in our study because we estimated the age at maturity using skeletochronology that considers a completed year of life. The age at maturity is probably an important target of natural selection for the evolution of facultative paedomorphosis (Ryan and Semlitsch,

1998). Therefore, accurate studies of time at maturity could be interesting in order to understand the ecology of the paedomorphic traits in the *C. asper* populations.

Environmental heterogeneities and climate are potential sources of variation in traits that can increase phenotypic plasticity of life history traits among populations (Leskovar et al., 2006). In fact, there can be differences in the body size between paedomorphs and metamorphs depending on the population because environmental variables associated with the presence of paedomorphs can differ between species and populations (Denoël et al., 2009). For example, in some populations of newts (*Lissotriton vulgaris*, *L. helveticus* and *Ichthyosaura alpestris*) paedomorphic forms can be smaller than the metamorphs or vice versa, as well as approximately identical (Denoël et al., 2009). In our case, the stream population was larger than the lacustrine population, which does not show different sizes between the gilled and metamorphic adults. This fact could suggest that the different size between populations can be due to an altitudinal effect. The lacustrine population lives at higher altitude than the stream population. Therefore, the larger body sizes of the stream population may result from higher water temperatures during the longer annual aquatic phase, which allows more foraging activity and a higher growth metabolism (Miaud and Guillaume, 2006). This suggestion can also be considered to explain the difference on age structure between both populations, where the lacustrine population shows higher longevity than the stream population.

In addition, if the population size in the lacustrine population was high, food competition could also affect the rate of body growth. In contrast, metamorphosed newts in the lentic population have a terrestrial life of several years during which they could access a trophically more productive microhabitats (Whiteman, 1994; Whiteman, Wissinger and Brown, 1996; Denoël, Joly and Whiteman, 2005) because some lakes are oligotrophic (Whiteman

et al., 2012). Studies based on allozymes, mitochondrial and nuclear genes have shown a low genetic variability in *Calotriton asper* attributable to a higher genetic flow after the last glacial period (Montori et al., 2008; Valbuena-Ureña et al., 2013). Thus, much of the phenotypic variability in body size, shape and colour of the species should have been developed quickly during the last 8000 years (Carranza and Amat, 2005; Montori et al., 2008). Our genetic results show a low genetic variability in the lake newts especially in relation to the population living in the neighbouring stream. The high altitude of the lake avoided the persistence of a newt population before the end of the last glaciation, indicating that the origin of this population would be a recent colonization. Thus, we suggest that the lacustrine population may have experienced a genetic bottleneck instead of reduced genetic flow, leading to lower values of the observed mitochondrial variability.

Nevertheless, the newt populations show a contrasting pattern of nuclear and mitochondrial DNA geographic structure (Mila et al., 2010) probably derived from different rates of migration between males and females (Staub, Brown and Wake, 1995; Kutchta, Parks and Wake, 2009). Therefore, the analysis of highly polymorphic nuclear data will be valuable to quantify more accurately the genetic isolation and differentiation of the lake population of *C. asper*. A genetic bottleneck and/or phenotypic plasticity may have promoted a fast development of paedomorphosis in the lake population. Such a hypothesis deserves further research including the study of more lacustrine and paedomorphic populations. Although adaptive phenotypic plasticity can be invoked, we suggest that slow growth of larvae due to low food conditions could be the main force driving paedomorphosis in our lacustrine population. This mechanism was suggested for explaining paedomorphosis in other populations in high elevation lakes (Denöel et al., 2007; Whiteman et al., 2012). The very poor body condition observed in some individuals, the strong competition for

probably scarce food and the retention of small gills, supports this latter hypothesis, and open the door for future research.

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