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Population dynamics of the Pyrenean newt (Calotriton asper): demography, morphometry and population genètics

Sebastià Camarasa i Arbós

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Universitat de Lleida

TESI DOCTORAL

Population dynamics of the Pyrenean newt (*Calotriton asper*): demography, morphometry and population genetics

Sebastià Camarasa i Arbós

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Gestió Forestal i del Medi Natural

Directors
Delfí Sanuy Castells
Neus Oromí Farrús

2021

AGRAIMENTS

Molt agraït al Delfí Sanuy, per obrir-me les portes del departament, pel seu suport incondicional, assessorament i consells en cada una de les etapes d'aquesta tesi. També a la Neus Oromí i el Fèlix Amat per mostrar-me i guiar-me en fer ciència. També a tota la gent del departament de Ciència Animal de la Universitat de Lleida.

També agraïments a la Universitat de Tiaret (Algèria), i de Lviv (Ukraïna), a tota la gent que em va acollir durant els mesos d'intercanvi.

Gran agraïment pel Claude Miaud, la Claudine Montgelard i la Veronique Arnal, per tots els coneixements transmesos durant les meves estances a Montpellier, al Centre d'Ecologia Funcional i Evolutiva (CEFE) i per la paciència que han tingut.

Als diferents governs implicats pels permisos de captura científica.

Als revisors externs per la seva tasca desinteressada.

Al tribunal per la lectura i posterior avaluació.

Finalment agrair al company de despatx, el Xurde, per tots els dubtes que m'ha resolt i el suport moral.

A la bona gent. De tot cor... molt agraït, sense ells aquest treball no hauria estat possible.

DEDICACIÓ

Dedicat a la vida; pels plaers que ens regala a cada racó d'aquest món fascinant. A la meva "crush", la família i amics.

> La natura mai fa res sense motiu.
>
> (Aristòtil, Estagira 384 aC - Eubea 322 aC, Grècia)

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RELATED WORKS AND MANUSCRIPTS

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i. **Sebastià Camarasa**, Fèlix Amat, Delfí Sanuy & Neus Oromi (2018) The occurrence of facultative paedomorphosis in a lacustrine population of the Pyrenean newt (*Calotriton asper*): morphology and age structure, *Journal of Natural History*, 52:33-34, 2133-2145, DOI: 10.1080/00222933.2018.1516831

ii. **Sebastià Camarasa**, Neus Oromi, Delfí Sanuy & Fèlix Amat (2020). Demographic Traits Variation in a Pyrenean Newt (*Calotriton asper*) among Lacustrine and Stream Populations, *Diversity* 12, no. 12: 471. DOI: 10.3390/d12120471

iii. **Sebastià Camarasa**, Neus Oromi, Delfí Sanuy & Fèlix Amat (2021). Morphological variation in Pyrenean newts (*Calotriton asper*): does habitat and ontogeny determine size and body shape more than sexual dimorphism? *Accepted manuscript, under review in the journal Diversity.*

iv. **Sebastià Camarasa**, Delfí Sanuy, Véronique Arnal, Claude Miaud & Claudine Montgelard. Genetic diversity, structure and connectivity of the populations of *Calotriton asper* in the Eastern Pyrenees. *Manuscript in preparation.*

Contributions in other manuscripts related to the thesis published by the author:

i. Fèlix Amat, **Sebastià Camarasa**, Jaime Bosch i Delfí Sanuy (2018) Evidència de la davallada demogràfica en la població de tritó pirinenc (*Calotriton asper*) al llac de Perramó, Parc Natural de Posets-Maladeta (Benasc). *Butlletí de la Societat Catalana d'Herpetologia,* 26: 13-18.

ii. Emilio Valbuena-Ureña Neus Oromi, Anna Soler-Membrives, Salvador Carranza, Fèlix Amat, **Sebastià Camarasa**, Mathieu Denoël, Olivier Guillaume, Delfí Sanuy, Adeline Loyau, Dirk S. Schmeller & Sebastian Steinfartz (2018). Jailed in the mountains: Genetic diversity and structure of an endemic newt species across the Pyrenees. PLoS ONE 13(8): e0200214. https://doi.org/10.1371/journal.pone.0200214

iii. Neus Oromi, Emilio Valbuena-Ureña, Anna Soler-Membrives, Fèlix Amat, **Sebastià Camarasa**, Salvador Carranza, Delfí Sanuy & Mathieu Denoël (2018). Genetic structure of lake and stream populations in a Pyrenean amphibian (*Calotriton asper*) reveals evolutionary significant units associated with paedomorphosis. *Journal of Zoological Systematics Evolutionary Research*, 57, 418–430, DOI:10.1111/jzs.12250.

RESUM EN CATALÀ

L'objectiu d'aquest estudi és analitzar la dinàmica poblacional del tritó pirinenc (*Catotriton asper*), un endemisme àmpliament distribuït per les muntanyes pirinenques, amb la finalitat d'aportar nous coneixements útils per a la seva conservació. En primer lloc, es va avaluar el fenomen de la paedomorfosis facultativa, en una població lacustre en què es va detectar la presència d'individus pedomòrfics (Ibón de Perramó). Entre les poblacions paedomòrfiques i les metamòrfiques, es van trobar diferències en la mida corporal i en alguns caràcters del cicle vital, com la longevitat i l'edat de maduresa. Els mecanismes que poden explicar la presència de la paedomorfosis facultativa són complexos i comprenen la interacció de múltiples factors relacionats amb el cost de la reproducció i el creixement. Per avaluar la variabilitat de l'espècie, es va analitzar la variació dels caràcters demogràfics del cicle vital entre les poblacions de llac i de torrent. Els resultats obtinguts mostren una variabilitat significativa entre sexes i poblacions, en la mida corporal i l'estructura d'edat de *C. asper*, que no depenia de l'hàbitat. L'estudi de la variació morfològica d'un total de trenta-tres poblacions, mostra dimorfisme sexual en la mida de el cos en algunes poblacions, encara que l'àmplia variabilitat morfològica entre les poblacions, no va mostrar diferències quan es consideren totes les dades juntes. En general, els mascles tenen el cap més gran, les extremitats més llargues i la cua més alta, mentre que les femelles, tenen la cua més llarga i menys alta, l'abdomen més llarg i el cap i les extremitats més petites. La mida del cos mostra diferències entre el tipus d'hàbitat i el morfotip (metamorfs i paedomorfs). No obstant això, quan s'analitzen totes les variables morfomètriques, s'observa un complex patró de diferències entre sexes, hàbitats, morfotips i poblacions. Finalment, es va analitzar l'estructura genètica i la diversitat de *C. asper* en una zona perifèrica de la seva distribució, al massís del Canigó. Els resultats són concordants amb estudis anteriors, que van analitzar els patrons genètics de diversitat i estructura de les poblacions de tritó pirinenc al llarg de la seva distribució, suggerint que l'espècie està adaptada als hàbitats de muntanya, amb una major diversitat en les poblacions occidentals. Els nostres anàlisis evidencien la importància de la complexitat topogràfica en la determinació dels patrons de diferenciació genètica, indicant la dispersió i la connectivitat poblacional, marcada per la presència d'hàbitats favorables. En conclusió, *C. asper* és una espècie que presenta una alta variabilitat demogràfica, morfològica i genètica, que sembla que es deu principalment a les característiques intrínseques de l'hàbitat on es troben les poblacions, a les peculiaritats de les zones que ocupen. Per això, la conservació dels hàbitats que ocupa cada població és clau per a la supervivència de l'espècie.

RESUMEN EN CASTELLANO

El objetivo del presente estudio es analizar la dinámica poblacional del tritón pirenaico (*Catotriton asper*), un endemismo ampliamente distribuido por las montañas pirenaicas, con el fin de aportar nuevos conocimientos útiles para su conservación. En primer lugar, se evaluó el fenómeno de la paedomorfosis facultativa, en una población lacustre en la que se detectó la presencia de individuos paedomórficos (Ibón de Perramó). Entre las poblaciones paedomórficas y las metamórficas, se encontraron diferencias en el tamaño corporal y en algunos caracteres del ciclo vital, como la longevidad y la edad de madurez. Los mecanismos que pueden explicar la presencia de la paedomorfosis facultativa son complejos y abarcan la interacción de múltiples factores relacionados con el coste de la reproducción y el crecimiento. Para evaluar la variabilidad de la especie, se analizó la variación de los caracteres demográficos del ciclo vital entre poblaciones del lago y del arroyo. Los resultados obtenidos muestran una variabilidad significativa entre sexos y poblaciones, en el tamaño corporal y la estructura de edad de *C. asper,* que no dependía del hábitat. El estudio de la variación morfológica de treinta y tres poblaciones, muestra dimorfismo sexual en el tamaño del cuerpo en algunas poblaciones, aunque la amplia variabilidad morfológica entre las poblaciones, no mostró diferencias cuando se consideran todos los datos juntos. En general, los machos tienen la cabeza más grande, las extremidades más largas y la cola más alta, mientras que las hembras, tienen la cola más larga y menos alta, el abdomen más largo y la cabeza y las extremidades más pequeñas. El tamaño del cuerpo muestra diferencias entre el tipo de hábitat y el morfotipo (metamorfos y paedomorfos). Sin embargo, cuando se analizan todas las variables morfométricas, se observa un complejo patrón de diferencias entre sexos, hábitats, morfotipos y poblaciones. Por último, se analizó la estructura y la diversidad genética de *C. asper* en una zona periférica de su distribución, en el macizo del Canigó. Los resultados son concordantes con estudios anteriores, sugiriendo que la especie está adaptada a los hábitats de montaña, con una mayor diversidad en las poblaciones occidentales. Nuestros análisis evidencian la importancia de la complejidad topográfica en la determinación de los patrones de diferenciación genética, indicando la dispersión y la conectividad poblacional, marcada por la presencia de hábitats favorables. En conclusión, *C. asper* es una especie que presenta una alta variabilidad demográfica, morfológica y genética, que parece deberse principalmente a las características intrínsecas del hábitat donde se encuentran las poblaciones, a las peculiaridades de las zonas que ocupan. Por ello, la conservación de los hábitats que ocupa cada población es clave para la supervivencia de la especie.

ABSTRACT IN ENGLISH

The aim of the present study is to analyse the population dynamics of the Pyrenean newt (*Catotriton asper*), an endemic newt widely distributed across the Pyrenean mountains, in order to provide new knowledge usefully for its conservation. First, the phenomenon of facultative paedomorphosis was evaluated in a lacustrine population where presence of paedomorphic individuals was reported (Ibon de Perramó). Differences in body size and in some life history traits, such as longevity and age at maturity, were found between paedomorphic and metamorphic populations. The mechanisms that can explain the presence of facultative paedomorphosis are complex and comprise the interaction of multiple factors related to the cost of reproduction and growth. In order to evaluate the variability of the species, variation of demographic life history traits between lake and stream populations were analysed. The results obtained show a significant variability between sexes and populations, in body size and age structure of *C. asper* that did not depend on the habitat. The study of morphological variation from a total of thirty-three populations, shows sexual dimorphism on body size in some populations, although the wide morphological variability among populations did not show differences when all data are considered. In general, males have bigger heads, longer extremities and higher tails, whereas females have longer and less high tails, longer abdomens and smaller head and limbs. Body size show differences between habitat type and ontogenies (metamorphs and paedomorphs). However, when we analyse all morphometric variables, there is a complex pattern of differences between sexes, habitats, ontogenies and populations. Finally, the genetic structure and diversity of *C. asper* was analysed in a periphery area of its distribution, in the Canigou massif. The results are concordant with previous studies, that analysed the genetic patterns of diversity and structure of Pyrenean newt populations throughout their distribution, suggested that the species is adapted to mountain habitats, with a greater diversity in western populations. Our analyses evidence the importance of topographic complexity in determining patterns of genetic differentiation, indicating dispersal and population connectivity, marked by the presence of favourable habitats. In conclusion, *C. asper* is a species that presents a high demographic variability, morphology and genetics, that seems to be mainly due to the intrinsic characteristics of the habitat where the populations are located, to the peculiarities of the areas they occupy. Therefore, the conservation of the habitats occupied by each population is key for the species survival.

INTRODUCTION (INTRODUCCIÓ)

L’estudi que es presenta pretén analitzar la dinàmica de les poblacions del tritó pirinenc (*Calotriton asper*, Duguès 1852) des d’una òptica genètica i demogràfica. Aquesta espècie presenta un especial interès per a la seva conservació ja que és l’únic urodel endèmic dels Pirineus. Tot i que el coneixement de l'espècie no es escàs, manquen nombrosos estudis per tal d’entendre la distribució geogràfica i la dinàmica de les seves poblacions que permetin aportar nous coneixements essencials per la gestió i la conservació de l’espècie. A més a més, estudis previs sobre l'espècie han mostrat característiques peculiars, com per exemple l'aparició de paedomorfisme facultatiu en llacs d’altitud elevada. En aquest sentit, el coneixement dels processos adaptatius i evolutius són necessaris per tal de conservar les espècies, mitjançant la conservació de la seva diversitat fenotípica i adaptativa.

Calotriton asper (*C. asper*) és un amfibi urodel pertanyent a la família dels Salamandridae, endèmic dels Pirineus i Prepirineus i que es distribueix per Espanya, França i Andorra. Classificat com a gairebé amenaçat per la llista roja de la Unió Internacional per a la Conservació de la Natura, és una espècie de muntanya, principalment aquàtica. *C. asper*, està adaptat als cursos d’aigua de poc cabal i de fort corrent, tot i que algunes poblacions han colonitzat secundàriament llacs alpins i cursos d’aigua subterranis o coves [1,2]. Els trobem distribuïts en altituds compreses aproximadament entre 175 a 3000 m, ocupant tot el Pirineu [3], i les seves poblacions mostren diferencies en els trets del cicle vital entre poblacions i sexes [2,4]. Aquestes diferències poden ser degudes a diversos factors, com poden ser les característiques de l’hàbitat [5].

La característica principal dels torrents pirinencs és que el seu règim hidrològic ve determinat pel patró de nevades. En conseqüència, el cabal presenta una gran variabilitat estacional, amb màxims de primavera associats a la fosa de la neu i mínims a l’estiu, quan en alguns casos, els torrents poden assecar-se. L'altre tipus d'hàbitat que ocupa l’espècie són els llacs d'alta muntanya d'origen glacial [6]. La característica principal dels llacs és que pateixen una inversió d’estratificació de l’aigua sota la capa de gel a l’hivern. Les aigües més denses (a 4° C) cauen al fons i les aigües fredes queden a la superfície formant una capa de gel pot durar diversos mesos [7]. Aquests llacs també es caracteritzen per ambients aquàtics oligotròfics, baixes concentracions de sal dissolta a les seves aigües, productivitat limitada del fitoplàncton i absència de depredadors naturals [7,8]. Concretament en alguns llacs s’ha trobat la presència de paedormofirsme facultatiu en les poblacions de tritó pirinenc.

El paedomorfisme és una variació fenotípica que pot ser causada per una heterocronia, que és la variació en el temps o la taxa de desenvolupament d'un organisme [9]. En espècies amb cicles de vida complexos, com en els amfibis caudats, aquesta variació morfològica pot tenir conseqüències evolutives importants que poden donar lloc a la diferenciació de la població i l'especiació [10]. En aquest sentit, la paedomorfosi, la retenció de caràcters larvaris en animals sexualment madurs, es produeix en gairebé totes les famílies de salamandres [11]. La coexistència d’individus metamòrfics i paedomòrfics en la mateixa població s’ha reportat amb freqüència en moltes espècies de tritons [10,12,13,14,15,16]. En el cas del tritó pirinenc, s’ha reportat l’existència de dues poblacions amb paedomorfisme facultatiu: la població d’Ibón de Acherito [5], i la població extinta de l’estany de Manhera [17,18] on van observar gegantisme en les larves. Recentment, l’any 2012 s’ha trobat una altra població a l’Ibón de Perramó amb presència de paedomorfs que ha donat peu a l'estudi de la morfologia i estructura d’edat d'aquestes poblacions (Capítol 1).

La importància adaptativa de la presència d'individus paedomorfs ha estat discutida i s'han proposat diverses hipòtesis. S'ha plantejat que la paedomorfosi facultativa és un procés freqüent en hàbitats aquàtics estables, que permet un creixement corporal lent, en contrast amb els hàbitats on els nivells d'aigua varien, la pressió dels depredadors o la disponibilitat d'aliments variable amb el temps [10]. En aquest darrers casos, si aquests factors afecten negativament la supervivència individual, una metamorfosi o maduresa sexual primerenca podria ser una resposta adaptativa, així doncs, l'edat d’arribada a la maduresa sexual o la mida del cos pot afectar directament a l'ocurrència de la paedomorfosi facultativa [19]. Encara que hi ha molta informació sobre la variació del cicle de vida en els amfibis en diferents habitats, rangs altitudinals i latitudinals [20], se sap molt poc sobre la morfologia, demografia i ecologia de poblacions amb presència de paedomorfosi.

El processos com el creixement, desenvolupament i maduració sexual, constitueixen els elements del cicle de vida d'un organisme [21]. El cicle de vida d'un organisme és variable dins dels límits del genotip de l'individu, però en funció de l’entorn, de les pressions selectives degudes a l’hàbitat, poden canviar la seva expressió fenotípica, aleshores es diu que hi ha una plasticitat fenotípica [2,22]. En els amfibis, la plasticitat fenotípica està especialment estesa i promou una gran diversitat de cicles de vida. Aquesta diversitat és el resultat de la interacció entre els costos i els beneficis (*trade-off*) dels cicles de vida en els entorns aquàtics i terrestres [9]. Els trets dels cicles de vida en els amfibis estan fortament vinculats a través dels *trade-offs*, com l'edat a la maduresa sexual, la longevitat o la relació edat-mida corporal [23]. Aquestes característiques del cicle de vida poden variar a causa de molts factors, com ara les condicions climàtiques, els recursos tròfics, les interaccions de depredadors *versus* preses, o la competència interespecífica [24]. A més, diferents condicions ambientals, com els hàbitats de llacs i torrents, causen variabilitat i promouen la diversificació ecològica [25]. En el cas del *C. asper,* existeixen pocs estudis sobre la seva demografia i han estat objecte d'estudi del Capítol 2 de la present tesis.

Calotriton asper sembla presentar una gran variabilitat morfològica [26,27]. probablement deguda a una elevada plasticitat fenotípica en relació amb l'hàbitat. En concret les poblacions que es troben a elevada altitud, en ambients més freds, semblen ser més grans en comparació amb les poblacions d'altitud més baixes. Aquesta tendència podria ser el resultat de respostes evolutives als entorns, impulsades per cada adaptació local, processos de plasticitat o una combinació de tots dos [28]. La diversificació fenotípica entre poblacions sembla que es deu a canvis genètics ràpids, sota pressions de selecció local, en resposta a condicions ecològiques específiques de la població [29]. Relacionat a això, el dimorfisme sexual està determinat per l'equilibri i la interacció de múltiples forces selectives [30]. Per exemple, pot permetre la reducció de la competència intraespecífica a través d'especialitzacions ecològiques, morfològiques o de comportament entre els sexes. Per tant, les diferències en la mida del cos poden indicar una selecció sexual o pot ser, per exemple, conseqüència de la competència pels recursos alimentaris [31,32,33]. D'altra banda, les condicions ambientals locals poden influenciar les diferències en la longitud del cos [34]. En el capítol 3 de la present tesis s'han analitzat els principals factors que poden definir la variabilitat morfològica de *C. asper* i el dimorfisme sexual de l'espècie, que pot estar influenciat per factors ecològics, o per les condicions climàtiques o d'habitat de cada localitat [2,35,36,37].

L'estructura genètica d'una espècie és el resultat de la interacció de factors històrics, com les fluctuacions demogràfiques, i el flux o connectivitat gènica actual [38]. Les condicions climàtiques i els canvis geològics representen forces de selecció, en el passat i en l'actualitat, que han estat els principals motors que han configurat l'estructura genètica de les poblacions de les espècies [39]. Els períodes glacials i postglacials han obligat l'espècie a passar per diversos processos de contracció i expansió. Al final dels períodes glacials, als Pirineus la desglaciació va permetre la reaparició d'hàbitats adequats per l'espècie i es van colonitzar a partir de poques poblacions font [40]. Les variacions climàtiques poden afectar les espècies directament a través de la seva fisiologia (processos metabòlics i reproductius) i indirectament a través de l'ecosistema (preses, depredadors, cicles de vida, fenologia) [41]. Per a les espècies amb distribucions restringides, els canvis ambientals poden tenir conseqüències greus [42], i especialment per als ectoterms, a causa de la seva dependència de les temperatures externes de les quals deriven les seves energies [43]. De fet, les variacions ambientals poden modificar els nínxols ecològics de les espècies. Depenent de la seva capacitat de dispersió i adaptació, se solen fer tres prediccions per estimar el destí de les espècies: reducció, augment o canvi de distribució [41]. Els patrons genètics de diversitat i estructura de les poblacions de tritó pirinenc al llarg de la seva distribució evidencien que l'espècie està adaptada als hàbitat de muntanya i amb major diversitat en les poblacions occidentals [44]. En el capítol 4 del present treball, s'estudia les poblacions de *C. asper* en una zona perifèrica de la seva distribució, concretament en una zona de l'est dels Pirineus francesos. Gradients com l'altitud o la longitud configuren els hàbitats existents al llarg dels Pirineus [45]. Als Pirineus occidentals, la influència de l'oceà Atlàntic proporciona un clima més fresc i humit que a les zones orientals, més influïdes pel clima temperat mediterrani [46]. Aquests gradients proporcionen una varietat d'hàbitats adequats per a la colonització, i també poden tenir un impacte en l'estructura genètica de la població [47].

L'habilitat de *C. asper* per ocupar noves zones en el futur, dependrà de la seva capacitat de dispersió (actualment poc coneguda), així com de l'existència de vies de dispersió adequades [43]. La varietat d'hàbitats i l'efecte dels períodes glacials i interglacials als Pirineus el converteixen en un laboratori natural per a estudis biogeogràfics, evolutius i ecològics de la fauna de muntanya com el *C. asper* [44].

OBJECTIVES

The Pyrenean newt (*Calotriton asper*) is the only endemic urodel in the Pyrenees. Many aspects of its biology and ecology are still unknown. The main objective of this thesis is to contribute to knowledge about population dynamics, by means of demography, morphometry and genetics, in order to provide new knowledge usefully for the conservation of the species.

The specific aims of this work are:

1- To evaluate the life history traits and the occurrence of facultative paedomorphosis in a lake population of *C. asper*:

    - To compare the demographic life history traits (e.g. age at maturity, longevity and body size) between metamorphic and paedomorphic individuals.
    - To discuss the environmental factors that may determine the occurrence of facultative paedomorphism in lacustrine populations.

2- To analyse the effect of different types of habitats (lakes and streams) on the demographic of *C. asper*:

    - To describe the demographic life history traits of populations (e.g. age at maturity, longevity and body size).
    - To analyse the effects of lake and stream habitat conditions on populations.

3- To define the morphometry and sexual dimorphism pattern of the *C. asper* populations:

    - To evaluate the pattern of sexual dimorphism at the habitat level (lakes and streams), ontogenetic stages (paedomorphic and metamorphic) and between populations.
    - To determine the differences in body size and shape between habitats (lakes and streams), and ontogenetic stages (paedomorphic and metamorphic) in the different populations.

4- To characterise the genetic variability of the populations of *C. asper* in an area on the periphery of its distribution (Canigou massif):

  - To qualify the genetic diversity of populations.
  - To analyse the genetic structure of populations and gene flow between them.
  - To determine the dispersal pattern and analyse the possible barrier effect of existing mountains and rivers in the area.

THESIS STRUCTURE

Current literature on paedomorphosis of *C. asper* populations raise some questions to answer. Thus, our first objective was to describe this phenomenon in a new population, to understand the effects of the paedomorphosis in life history traits and morphometry between morphs and sexes (Chapter 1). Second, as a habitat effect is expected in *C. asper* populations, we have focused in evaluating the life history traits and body sizes effect in two habitats, lakes and streams (Chapter 2). Considering sexual dimorphism and the body shape, we also studied the effects of the habitat and ontogenic stages on *C. asper* populations (Chapter 3). Finally, we use genetic markers (Chapter 4) in order to infer the capacity to disperse (the distance), characterize the genetic diversity and analyse the genetic structure of the *C. asper* populations in the northeastern margin of the range. In this chapter we were able to assess how important the landscape is in the connectivity between populations.

i. CHAPTER 1: The occurrence of facultative paedomorphosis in a lacustrine population of the Pyrenean newt (*Calotriton asper*): morphology and age structure

ii. CHAPTER 2: Demographic Traits Variation in a Pyrenean Newt (*Calotriton asper*) among Lacustrine and Stream Populations

iii. CHAPTER 3: Morphological variation in Pyrenean newts (*Calotriton asper*): does habitat and ontogeny determine size and body shape more than sexual dimorphism?

iv. CHAPTER 4. Genetic diversity, structure and connectivity of the populations of *Calotriton asper* in the Eastern Pyrenees

METHODOLOGY (METODOLOGIA)

Zones d'estudi

L'àrea d'estudi de la present tesi va ser tot el Pirineu i serralades adjacents. Obtenint les poblacions dins del territori de França, Andorra i Espanya (Figura 1) i (Taula 1).

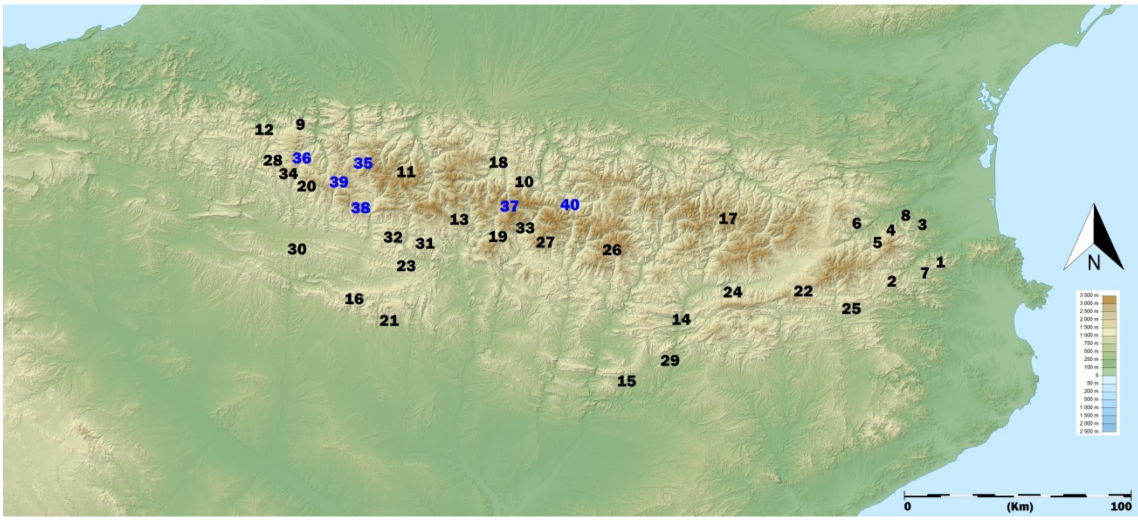

Figura 1. Localització de les poblacions mostrejades de *C. asper*. Vegeu la Taula 1 pels detalls de cada població. Els números negre són torrents i els blaus són llacs.

Taula 1. Latitud, longitud, altitud i capítol de la tesi per a cada població i hàbitat.

Nº	Population	Habitat	Latitude	Longitude	Altitude (m)	Chapter
1-	Mas Lori	Stream	42°20'48.3"N	2°31'19.0"E	805	4
2-	Prats Mollo	Stream	42°24'36.0"N	2°21'43.4"E	1605	4
3-	Valmanya	Stream	42°32'13.3"N	2°33'16.2"E	939	4
4-	Mas Malet	Stream	42°34'17.3"N	2°28'13.1"E	789	4
5-	Casteil	Stream	42°31'31.8"N	2°24'24.1"E	1139	4
6-	Serdinya	Stream	42°34'22.6"N	2°19'15.6"E	662	4
7-	Lamanera	Stream	42°21'02.4"N	2°29'31.2"E	1011	4; 3
8-	Clara	Stream	42°34'41.1"N	2°26'34.9"E	549	4; 3
9-	Ansabere	Stream	42°53'24.7"N	0°42'34.3"W	1785	3
10-	Batisielles	Stream	42°39'54.7"N	0°30'23.0"E	1815	3
11-	Bujaruelo	Stream	42°43'45.0"N	0°08'11.0"W	1687	3
12-	Contienda	Stream	42°52'34.3"N	0°49'22.4"W	1233	3
13-	Fanlo	Stream	42°36'35.1"N	0°01'07.2"E	1883	3
14-	Font Borbonera	Stream	42°12'11.2"N	1°17'59.0"E	749	3

15-	Font Figuera	Stream	42°01'16.0"N	1°01'48.3"E	803	3
16-	Monrepós	Stream	42°20'41.4"N	0°23'33.3"W	1237	3
17-	Ordino	Stream	42°37'50.4"N	1°29'56.4"E	1981	3
18-	Pino	Stream	42°40'29.6"N	0°28'20.1"E	1815	3
19-	Saboril	Stream	42°34'60.0"N	0°25'12.5"E	1683	3
20-	Selva de Oza	Stream	42°49'48.0"N	0°42'53.0"W	1181	3
21-	Sierra de Guara	Stream	42°15'35.3"N	0°14'43.1"W	935	3
22-	Torrent del Boix	Stream	42°17'25.3"N	2°00'56.9"E	1539	3
23-	Torrent de Fiscal	Stream	42°29'44.2"N	0°07'12.7"W	771	3
24-	Torrent del Pi	Stream	42°19'56.9"N	1°45'14.9"E	1231	3
25-	Vall d'en Bac	Stream	42°16'08.3"N	2°22'52.5"E	892	3
26-	Vall Fosca	Stream	42°29'29.6"N	0°59'18.7"E	1648	3
27-	Vallibierna	Stream	42°37'23.4"N	0°35'25.2"E	1854	3
28-	Zuriza	Stream	42°51'22.8"N	0°46'18.8"W	1427	3
29-	Peramola	Stream	42°04'24.7"N	1°16'20.5"E	623	3; 2
30-	San Juan de la Penya	Stream	42°30'59.0"N	0°42'02.7"W	1052	3; 2
31-	Sarvisé	Stream	42°34′51.9″N	0°4′17.70″ W	1222	3; 2
32-	Oto	Stream	42°35′47.5″N	0°7′42.56″ W	916	3; 2
33-	Benasc	Stream	42°38′2.23″N	0°34′25.21″ E	1600	2
34-	Barranc Acherito	Stream	42°52'06.1"N	0°42'38.5"W	1385	3; 2
35-	Ibón Espelunciecha	Lake	42°47'18.3"N	0°25'47.7"W	1953	3; 2
36-	Ibón Acherito	Lake	42°52'47.1"N	0°42'23.4"W	1822	3; 2
37-	Ibón Perramó	Lake	42°38'19.6"N	0°30'00.0"E	2406	3; 2; 1
38-	Ibón Bucuesa	Lake	42°42'29.6"N	0°25'52.7"W	2124	3
39-	Ibón Saman	Lake	42°44'40.7"N	0°28'44.1"W	2159	3
40-	Ibón Alba	Lake	42°39'48.3"N	0°36'20.1"E	2301	3

Treball de camp

El treball de camp per la recollida de mostres i dades es va realitzar durant el període comprés entre 2012 i el 2019. Cadascun dels mostrejos es va efectuar durant una nit i el dia següent per presa de dades per cada població. Amb els corresponents permisos, es van capturar manualment individus de l'espècie que varen ser guardats durant unes hores a l'aigua fins que van ser alliberats al indret de captura després del seu processament (Figura 2).

Figura 2. Exemplars capturats abans del seu processament.

Classificació dels individus

Els individus capturats van ser classificats en quatre grups poblacionals en base a la presencia de caràcters sexuals secundaris, concretament amb la morfologia cloacal externa que és fortament dimòrfica en aquesta espècie. Els animals que no presentaven el desenvolupament d'aquests caràcters sexuals van ser considerats com a juvenils i els que el presentaven van ser caracteritzats com a mascles o femelles en funció de la forma cloacal (Figures 3, 4 i 5). Finalment les larves tenen el cap deprimit, molt més llarg que ample, la cresta caudal gairebé es prolonga dorsalment acabant a la meitat posterior del cos i presenten tres branques branquials a cada costat, curtes i amb poques ramificacions (Figura 6). Igualment, es van examinar visualment els animals per tal de detectar la presencia de caràcters paedomòrfics: estat de desenvolupament branquial i presencia d'obertura branquial en els dos costats del cap (Figura 7).

Figura 3. Mascle de *C. asper.*

Figura 4. Femella de *C. asper.*

Figura 5. Juvenil de *C. asper.*

Figura 6. Larva de *C. asper.*

Figura 7. Adult de *C. asper* amb presencia de brànquies.

Morfometria de les poblacions

Es van prendre vuit mesures morfomètriques lineals sobre cada individu immobilitzant-lo manualment i utilitzant un calibrador digital amb un dígit de precisió. Les variables mesurades van ser les següents:

> -Longitud corporal (SVL): des de l'extrem del musell fins al marge posterior de la cloaca (mida corporal).
> -Longitud del cap: des de l'extrem del musell fins al plec gular.
> -Amplada màxima del cap.
> -Longitud de l'extremitat anterior: Des de l'extrem del dit més llarg fins al punt d'inserció.
> -Longitud de l'extremitat posteriors: Des de l'extrem del dit més llarg fins al punt d'inserció.
> -Longitud de l'abdomen: Distancia mínima entre els punts d'inserció de l'extremitat anterior i posterior d'un mateix costat.
> -Longitud de la cua: Des de la marge posterior de la cloaca fins l'extrem.
> -Alçada màxima de la cua.

Mostreig de les poblacions i demografia

Durant el mostreig es va obtenir mostres per l'obtenció de l'edat dels individus, que van consistir en extreure el dit més llarg, de l'extremitat posterior dreta. Aquest tipus d'intervenció no presenta cap perill per la vida de l'animal donat que el dit es regenera completament al cap d'unes setmanes. Aquestes mostres es van conservar individualitzades en un tub Eppendorf amb etanol al 70% i a temperatura ambient.

Desprès del treball de laboratori per l'obtenció de dades de l'anàlisi esqueletocronològic, i mitjançant les edats obtingudes en el recompte de línies de creixement es va descriure la població en funció dels caràcters o trets que configuren el cicle vital de l'espècie:

1) Distribució d'edats per sexe.
2) Edat mínima a la maduresa sexual: és el mínim nombre de línies de creixement o LAGs (lines of arrested growth) comptades en els individus reproductors.
3) Mida a la maduresa sexual: el valor mínim de la longitud corporal en els individus reproductors de mínima edat.
4) Longevitat: és el nombre màxim d' anys comptats (que s'asumeix que cada línia de creixement equival a un hivern) en individus reproductors.
5) Mitjana de vida: estimada com la mediana de la distribució d'edat de la població.
6) Relació edat-mida: mitja de longitud corporal per a cada edat

Genètica de les poblacions

Durant el mostreig es van recollir les mostres genètiques a cada individu capturat. L'ADN es va recollir de forma no invasiva, amb un bastonet d'isòtop bucal, se li posava a l'interior de la boca durant un temps de 10 segons, sense cap dany per l'animal. Les mostres de saliva es van col·locar en un tub Eppendorf de 1,5 ml. amb etanol al 70% i guardades a 4ºC fins l'extracció de l'ADN al laboratori.

CHAPTER 1

The occurrence of facultative paedomorphosis in a lacustrine population of the Pyrenean newt (*Calotriton asper*): morphology and age structure

Published in Journal of Natural History

JOURNAL OF NATURAL HISTORY
2018, VOL. 52, NOS. 33–34, 2133–2145
https://doi.org/10.1080/00222933.2018.1516831

The occurrence of facultative paedomorphosis in a lacustrine population of the Pyrenean newt (*Calotriton asper*): morphology and age structure

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ABSTRACT

Facultative paedomorphosis is a common polyphenism in newts where two alternative phenotypes can coexist: metamorphs and paedomorphs (mature with larval traits, i.e. retaining gills). This phenomenon has been reported in some lacustrine populations of the Pyrenean newt (*Calotriton asper*). The morphology and lifehistory traits were studied in a lacustrine population of this species with the occurrence of facultative paedomorphosis. The results showed that 24.3% of adults retained gills at different levels of reabsorption and had a smoother skin compared with the other populations, which were fully metamorphic. The body size and shape showed significant sexual differences, with males being larger than females. The presence of paedomorphic traits also affected the body size and shape, revealing a complex pattern of growth in which metamorphic adults were larger than paedomorphs. The age structure was different between sexes, with a median age of 10.5 and 7 years and longevity of 19 and 14 years in males and females, respectively. Considering each sex separately, the age structure was not significantly different between metamorphic and paedomorphic newts. In addition, metamorphosed and branchiate juveniles were found with a maximum age of 13 and 20 years, respectively. Remarkably, long-lived larvae with an exceptional individual of 18 years were found, which has not previously been reported in any other newt species. The mechanisms that can explain the evolution of paedomorphosis are complex and comprise the interaction of multiple factors related to the cost of reproduction and growth.

ARTICLE HISTORY
Received 16 February 2017
Accepted 8 August 2018

KEYWORDS
Calotriton asper; facultative paedomorphosis; age structure; morphology

Introduction

Phenotypic variation can be caused by a heterochrony, which is variation in the timing or the rate of development of an organism (Wilbur and Collins 1973). In species with complex life cycles such as in caudate amphibians, this morphological variation can have important evolutionary consequences that can result in population differentiation and

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speciation (Denoël et al. 2005). In this sense, paedomorphosis (i.e. the retention of larval characters in sexually mature animals) occurs in every family of salamanders (Reilly 1987). The different types of paedomorphosis can result in obligate paedomorphs (permanently aquatic larvae capable of reproduction), obligate metamorphs (either biphasic with metamorphosis or fully terrestrial, skipping the larval stage), and facultative paedomorphs with two adult phenotypes, paedomorphs and metamorphs, which can coexist in the same reproductive habitat (Whiteman 1994; Denoël et al. 2005). The coexistence of metamorphic and paedomorphic individuals in the same population has been frequently reported in many species of newts (Andreone and Dore 1991; Whiteman 1994; Denoël et al. 2005; Çiçek and Ayaz 2011; Başkale et al. 2013; Oromi et al. 2016). Several environmental factors regulate the frequency of paedomorphosis, although experimental evidence shows that the causality of this phenomenon is complex (Whiteman 1994; Denoël et al. 2005). For example, experimentally higher population densities have been related to the paedomorphosis (Harris 1987), while the manipulation of the alimentation does not show the same directional effect (Semlitsch 1987;Denoël and Poncin 2001). However, population variation in the occurrence of facultative paedomorphosis traits suggests a genetic basis (Semlitsch and Wilbur 1989).

The adaptive significance of the presence of paedomorphic individuals has been discussed and several hypotheses proposed, e.g. the paedomorphic advantage and the best of the bad lot (Whiteman 1994; Wakano and Whiteman 2008). The paedomorphic advantage hypothesis predicts that in the same population fast-growing larvae become paedomorphs by taking advantage of favourable aquatic habitats surrounded by harsh terrestrial environments (Denoël and Joly 2001). These paedomorphic individuals have higher fitness than the metamorphic individuals, which result from slowgrowing larvae (Wilbur and Collins 1973). The best of the bad lot hypothesis assumes a lower fitness of paedomorphs versus metamorphs, but the former option is the optimal for the slow-growing larvae unable to reach a minimum size required for metamorphosis (Whiteman et al. 2012). It has been hypothesised that paedomorphosis is a frequent process in stable aquatic habitats allowing a slow body growth in contrast to habitats where water levels, predator pressures, and food availability change over time (Denoël et al. 2005). In this latter case, if these factors negatively affect the individual survivorship, an early time of metamorphosis and sexual maturity may be an adaptive response. Factors such as age at maturity or body size can directly affect the occurrence of facultative paedomorphosis (e.g. Bonett et al. 2014). Although there is much information about amphibian life-history variation in different environments, altitudinal and latitudinal ranges (e.g. Sinsch 2015), little is known about the morphology, demography and ecology of populations with presence of paedomorphosis.

In this study, we evaluated the occurrence of facultative paedomorphosis in a population of the Pyrenean newt (*Calotriton asper*) living in a lake at 2270 m above sea level (asl). Lakes are a secondary habitat for the species, which is primarily adapted to live in stream waters. In this population (Ibón de Perramó), mature individuals show paedomorphic traits (i.e. presence of external gills), whereas others are typically metamorphs. Facultative paedomorphosis has previously been reported in another lacustrine population of the species (Ibón de Acherito; Oromi et al. 2014), in which some larvae are partially metamorphosed and thereby paedomorphic. Oromi et al. (2014) found variation in life-history traits between a lacustrine (with presence of paedomorphs) and a streamdwelling (without presence of paedomorphs) population of *C. asper* was considered as a consequence of the different climatic conditions. In our study, we want to examine more accurately these differences in our population with presence of facultative paedomorphosis. Therefore, the aim of this work was to evaluate the life-history traits such as body size, age structure, age at sexual maturity, longevity and the occurrence of a facultative paedomorphosis in a lacustrine population of Pyrenean newts (*C. asper*). In addition, we discuss the putative environmental factors leading this phenomenon in light of the biology of the species in Pyrenean lakes.

Material and methods

Study area and sampling

The studied population inhabits the Ibón de Perramó, a lake situated in the Central Pyrenees (42.6401° N, 00.5001° E) at 2270 m asl, with an area of 54,000 m^2 and a perimeter of 945 m (Del Castillo 2004). The lake has a glacier origin and is surrounded by alpine meadows with rocky granitic boulders and walls. Previous surveys show the location of the newts, i.e. that they are concentrated in the margin of a specific part of the lake. Fieldwork was carried out during the summer (in August) of 2012 and 2013, consisting of night surveys along the lakeshore, with two people together investing one night per year in order to capture a sample of newts. Newts were captured by hand and a hand net and kept in a mesh bag until data collection. The manipulation, data collection and samples took several hours more.

The population consisted of adults, juveniles, and larvae. The adult and juvenile newts were separated on the basis of secondary sexual characters: pointed cloaca protuberance in females and round and bulbous cloaca in males. In the adults, the paedomorphic newts were distinguished from the metamorphic ones by the presence of gill slits and external gills. The individuals without secondary sex characteristics (no external cloaca differentiation) were considered as juvenile newts. We classified as larvae those individuals with a fusiform body shape, smooth skin, and a developed dorsal and caudal fin. During 2012 sampling, a total of 336 newts (119 males, 95 females, 98 juveniles, and 24 larvae) were sampled for snout–vent length (SVL) measurement (Table 1) with a digital calliper, and we clipped one toe for skeletochronological analysis. During the 2013 sampling, a total of eight linear morphometric measurements in 144 newts (63 males, 41 females, and 40 juveniles) were obtained with a digital calliper for morphometry: 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. All the manipulations were performed in the proximities of the lake to avoid disturbance of the newts. Subsequently, all individuals were released at the site of capture.

Skeletochronological analysis

The age structure was estimated in a subsample of the population sampled during 2012 with 38 males, 34 females, 38 juveniles, and 17 larvae. Skeletochronology protocols followed the standard methods described by Miaud (1991) and modified by Amat et al. (2010). The largest toe of the left hind limb was removed, stored in 70% alcohol, and decalcified in 3% nitric acid for 45 min. Fine cross-sections (16 μm) were obtained with a freezing microtome and stained with Ehrlich's haematoxylin. The age of each individual was determined by counting lines of arrested growth (LAGs) in the diaphysis of the periosteal bone using a light microscope at 100× and 400× magnifications. LAGs counts were done by S. Camarasa and N. Oromi. Skeletochronological data were used to estimate the individual age, adult phenotype (metamorph and paedomorph), juvenile phenotype (metamorphosed and branchiate) and the following life-history traits for each group (male, female, juvenile, and larva): (1) age at maturity, as the minimum number of LAGs counted in the reproductive individuals; (2) size at maturity, as the minimum SVL of the youngest first breeders; (3) longevity, as the maximum number of LAGs counted in the reproductive individuals; and (4) potential reproductive life span (PRLS), as the difference between longevity and age at maturity. We also estimated the median age for each sex, juveniles, larvae, and phenotypes.

Statistical analysis

All variables were first tested for normality. As none of the variables conformed to normality, we used non-parametric statistics to describe distributions and applied logarithmic transformation in body size (SVL) to normalise its data distribution. The differences of frequency in metamorphic and paedomorphic newts were tested between groups using a chi-square test. The eight morphometric traits were analysed using MANOVA, testing for body shape differences between groups (females, males, and juveniles), sexes (male and females), adult phenotype (metamorphs and paedomorphs), and juvenile phenotype (metamorphosed or branchiate). Three independent tests of ANOVA were used to analyse body size (log-normalised SVL): (1) between groups (females, males, and juveniles); (2) between sexes and adult phenotypes, testing the interaction between the two factors; and (3) between metamorphosed and branchiate juveniles. Multiple comparisons for significance between phenotypes and groups were performed using the Tukey–Kramer HSD (Honestly Significant Difference) test. Moreover, a principal component analysis (PCA) was performed to explore the patterns of covariation of the morphometric variables and visualise sexual differences. Significant differences between the age structure and the median age among females, males (taking into account the effect of paedomorphosis), and juveniles and larvae were analysed using the two-sided Kolmogorov–Smirnov and Wilcoxon tests. Pearson parametric correlation between age and SVL was performed for population groups and phenotypes. All analyses were conducted using Statistica 4.5 (alpha = 0.05) (Statsoft.com 2016).

Results

Morphology

Visual examination of the newts revealed smooth skin in all individuals. The presence of gills was observed in 21.0% of the males and 28.4% of the females (24.3% of the adults) corresponding to the paedomorphic phenotype. In the juveniles, 46.9% showed gills (branchiate juveniles) at different levels of development and reabsorption. The presence of gills was significantly more frequent in juveniles than in adults ($\chi^2 = 12.963$, df = 2, $p = 0.001$), but did not differ between sexes ($\chi^2 = 0.936$, df = 1, $p = 0.333$).

The ANOVA test revealed significant differences in log-normalised SVL between groups: females, males, and juveniles ($F_{(2,309)} = 309.025$, $p < 0.001$). The post-hoc Tukey–Kramer HSD test found significant differences between the three groups, females and males versus juveniles ($p < 0.001$), and between females and males ($p < 0.036$). The ANOVA test revealed significant differences in log-normalised SVL between sexes – females and males ($F_{(3,210)} = 7.174$, $p = 0.008$) – and between phenotypes – metamorphs and paedomorphs ($F_{(3,210)} = 29.566$, $p < 0.001$) – without interaction between these two factors ($F_{(3,210)} = 0.084$, $p = 0.772$). Thus, within each group, males were larger than females, and for each sex, metamorphs were larger than paedomorphs (Figure 1, Table 3). The ANOVA test did not reveal significant differences in log-normalised SVL between metamorphosed and branchiate juveniles ($F_{(1,96)} = 2.075$, $p = 0.153$).

The MANOVA test revealed differences in body shape between females, males, and juveniles ($F_{(2,141)} = 111.041$, $p < 0.001$) and also between sexes ($F_{(1,10)} = 12.929$, $p < 0.001$) and adult phenotypes – metamorphs and paedomorphs ($F_{(1,100)} = 12.225$, $p < 0.001$) – without interaction between these two factors ($F_{(1,100)} = 0.044$, $p = 0.834$). The MANOVA test did not reveal significant differences in body shape between phenotypes (metamorphosed and branchiate) of juveniles ($F_{(1,38)} = 2.159$, $p = 0.611$). The post-hoc test

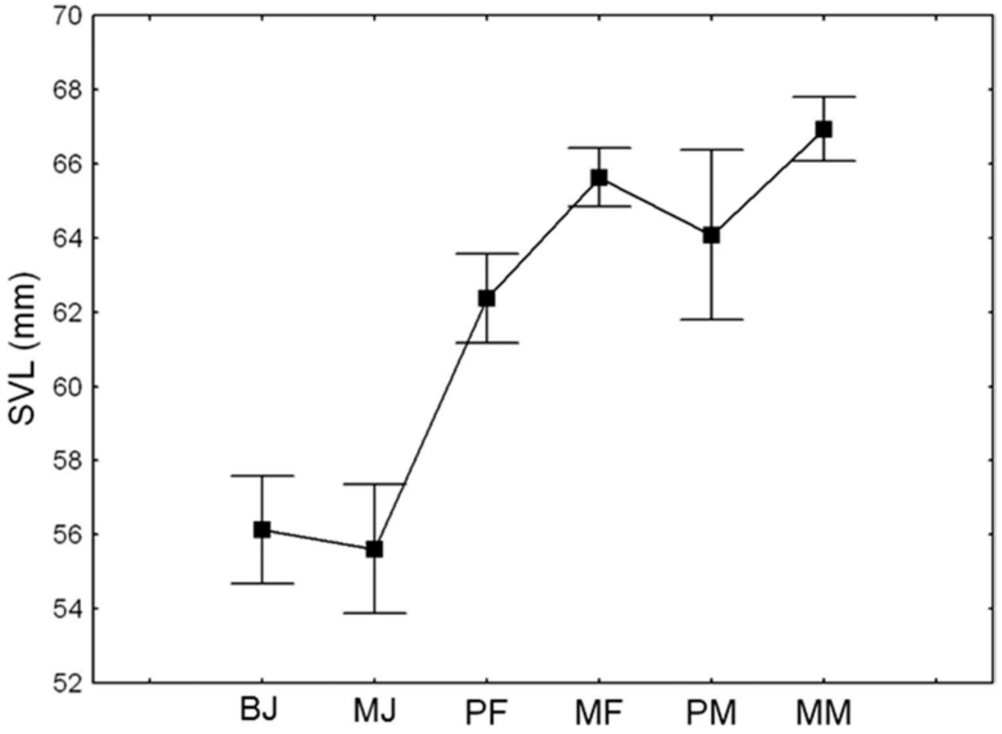


Figure 1. Snout–vent length (SVL, mean ± SE) differences between groups and phenotypes (BJ = branchiate juveniles; MJ = metamorphosed juveniles; PF = paedomorphic females; MF = metamorphic females; PM = paedomorphic males and MM = metamorphic males).

found significant differences between adults and juveniles for all the variables and significant sexual dimorphism in the width of the head, fore and hind limb, and tail and high length (Tukey HSD test, $p < 0.05$).

The first two factors of the PCA accounted for a large proportion of variance (84.9%) and showed a clear pattern of differentiation between males, females, and juveniles (Table 2). The first factor represented the variation in body size and showed two groups: adults and juveniles. Females and males were separated for the second factor, defined by the contrast between the most extremely weighted variables: tail length versus head shape and forelimb length. Thus, males were characterised by having larger heads, longer limbs, a shorter limb interval, and shorter but taller tails than females (Figure 2).

Age structure and size relationship

Annual bone marks were clearly visible in all the cross-sections. We assumed that the number of LAGs was equivalent to the number of winters experienced by each individual. Therefore, LAGs may be reliably assumed as an age measurement. The life-history traits (Table 1, Figure 3) varied depending on the adult phenotype (metamorphs and paedomorphs) and juvenile phenotype (larva and metamorphosed and branchiate juveniles). The age at sexual maturity and longevity in females and males were 4 and 6 years and 14 and 19 years, respectively. The age structure differed between sexes

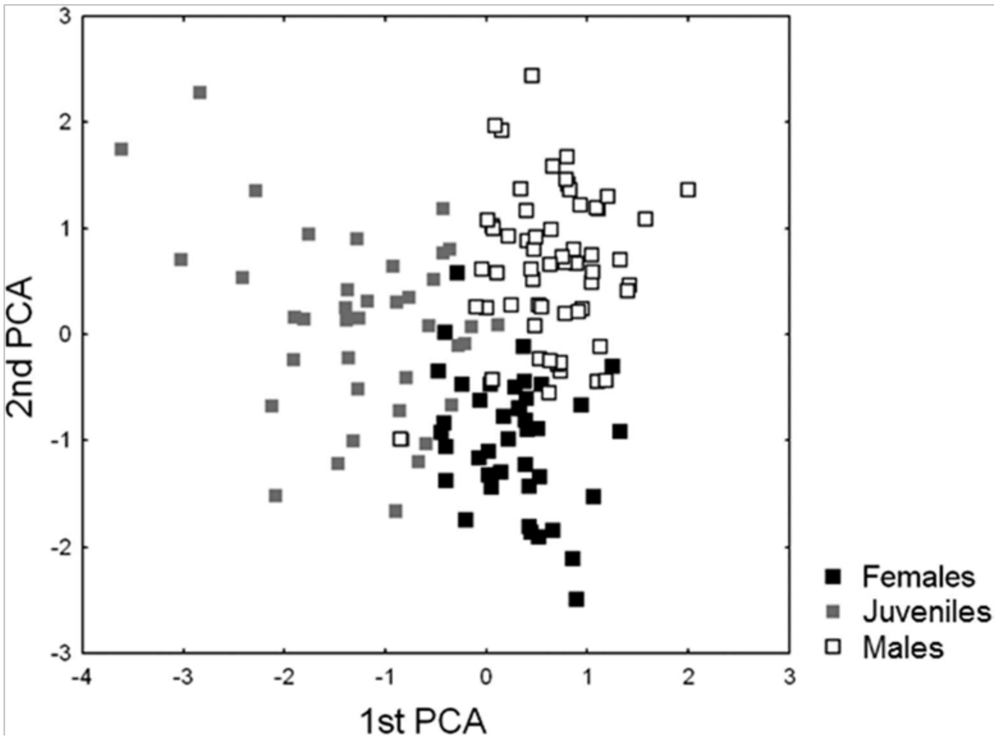


Figure 2. Plot of the first two PCs of the newts of the Ibón de Perramó population, showing differences between males, females and juveniles.

Table 1. Snout–vent length (SVL) for each group, males and females (paedomorphs and metamorphs), juveniles (branchiate and metamorphosed) and larvae of the Ibón de Perramó.

Group	Phenotype	n	Mean ± SE (mm)	Max–min (mm)
Males	Paedomorphs	25	64.8 ± 0.5	69.9–59.2
	Metamorphs	94	67.2 ± 0.3	74.0–59.2
	All	119	66.7 ± 0.3	74.0–59.2
Females	Paedomorphs	27	63.4 ± 0.5	70.7–58.0
	Metamorphs	68	66.0 ± 0.4	72.4–59.7
	All	95	65.3 ± 0.3	72.4–58.0
Juveniles	Branchiate	46	55.7 ± 0.6	64.0–47.4
	Metamorphosed	52	54.5 ± 0.7	61.6–40.1
	All	98	55.1 ± 0.5	64.0–40.1
Larvae	All	24	52.0 ± 0.9	58.5–40.1

(Kolmogorov–Smirnov test: $D = 0.485$; $p < 0.001$). The median age was also different between sexes, being 7 and 10.5 in females and males, respectively (Wilcoxon test: $Z = -4.232$; $p < 0.001$). Consequently, PRLS was also different between sexes, showing males with higher values than females (13 and 10 years, respectively).

Considering the two adult phenotypes in each sex separately, the age structure (Kolmogorov–Smirnov test: females $D = 0.260$; $p = 0.649$; males $D = 0.219$; $p = 0.970$) and the median age (Wilcoxon test: females: $Z = -0.144$; $p = 0.886$; males: $Z = 0.445$;

$p = 0.656$) were not significantly different between metamorphic and paedomorphic individuals. However, paedomorphic males mature at an older age and live shorter lives than metamorphic ones, in contrast to metamorphic and paedomorphic females (Table 3). In the females, the PRLS differed between phenotypes, being lower in metamorphic (6 years) than in paedomorphic females (10 years), in contrast to males with higher PRLS in metamorphs (13 years) than in paedomorphs (7 years). The branchiate and metamorphosed juveniles were significantly different in median age (10 and 8 years, respectively; Wilcoxon test: $Z = -2.195$; $p < 0.028$). Remarkably, the larvae age ranged between 1 and 11 years, with an exceptional individual of 18 years (Figure 4).

SVL and age were positively correlated in the total dataset ($n = 127$; $r = 0.262$; $p < 0.003$). In addition, correlation between SVL and age was significant in juveniles ($n = 38$; $r = 0.559$; $p < 0.001$) and females ($n = 34$; $r = 0.345$; $p < 0.045$), but not in males ($n = 38$; $r = 0.309$; $p < 0.059$) and larvae ($n = 17$; $r = -0.180$; $p = 0.487$). Although a correlation between these two variables was found in females and juveniles, it remained significant only in paedomorphic females ($n = 13$; $r = 0.706$; $p = 0.007$) and metamorphic juveniles ($n = 19$; $r = 0.791$; $p = 0.001$), not in metamorphic females ($n = 21$; $r = 0.182$; $p = 0.429$) and branchiate juveniles ($n = 19$; $r = 0.377$; $p = 0.111$). A significant correlation in male paedomorphs ($n = 6$; $r = 0.674$; $p = 0.142$) or metamorphs ($n = 32$; $r = 0.277$; $p = 0.124$) was not found.

Discussion

Paedomorphosis in salamanders is defined as an alternative strategy to complete or even start the process of metamorphosis that can be fixed or facultative (Whiteman 1994). Facultative paedomorphosis includes the presence of two alternative phenotypes in the same population. This phenomenon is interesting to analyse because of the evolutionary consequences of the alternative phenotype (Semlitsch et al. 1990; Oromi

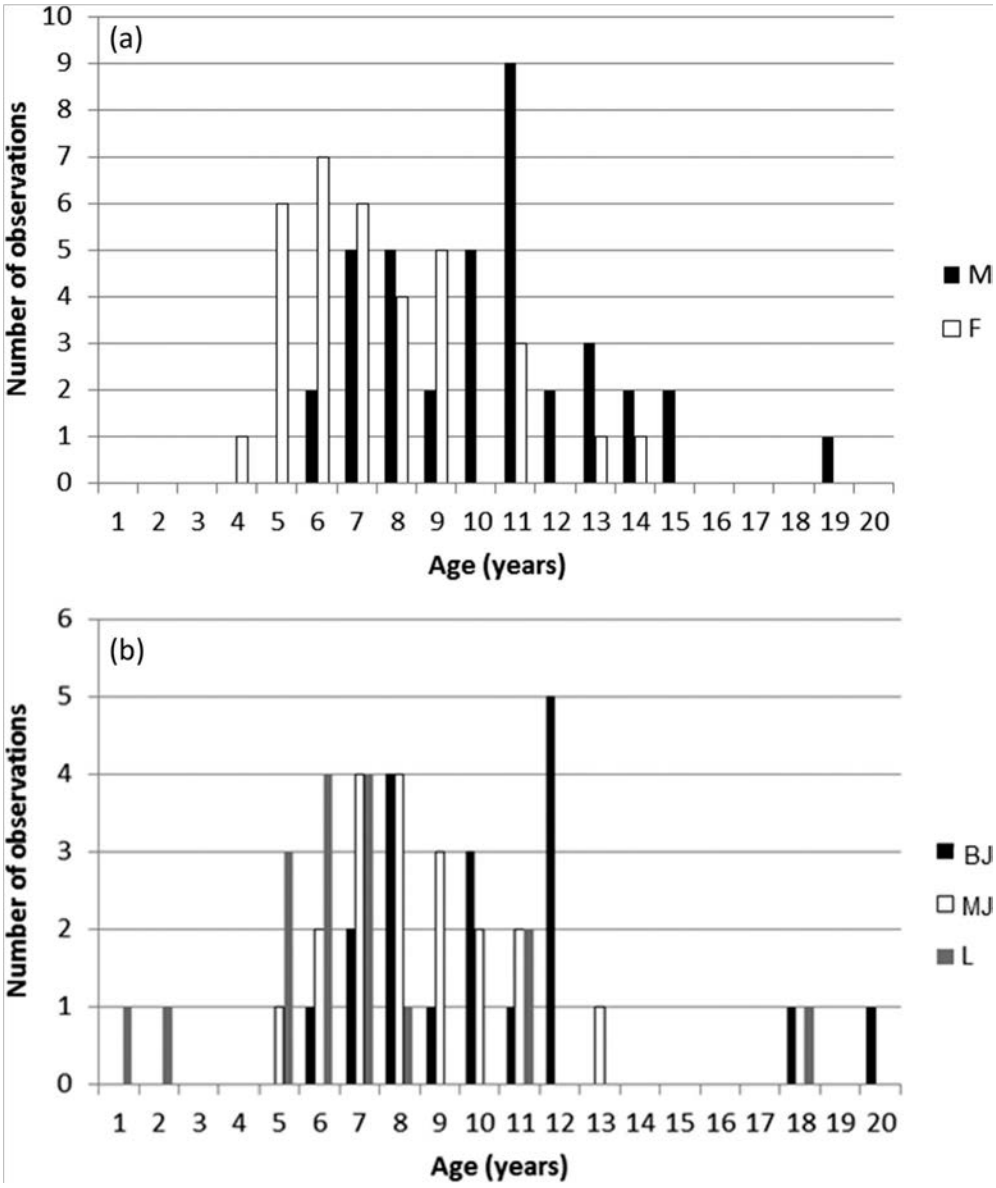


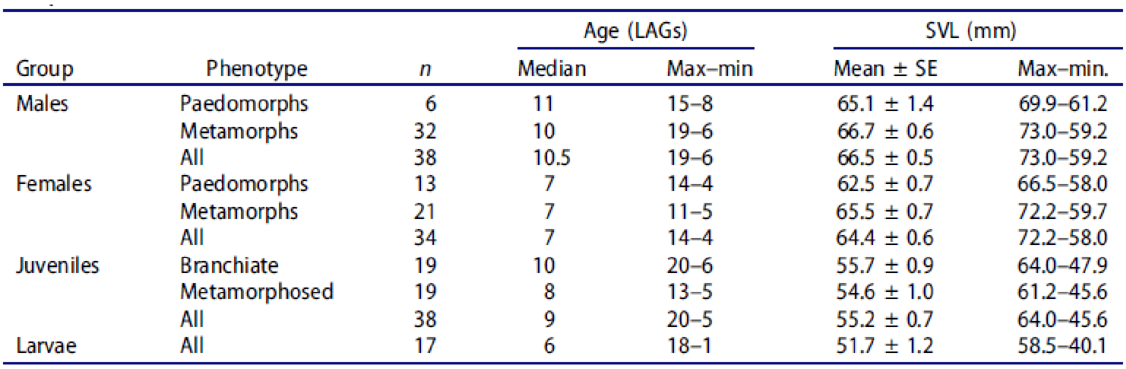
Figure 3. (a) Age structure of the newts of Ibón de Perramó population in males (M) and females (F). (b) Age structure of the newts of Ibón de Perramó population in branchiate juveniles (BJ), metamorphosed juveniles (MJ) and larvae (L).

et al. 2016). Our results indicate differences in body size and body shape and a similar age structure between adult alternative phenotypes in *C. asper*. No differences in body size and body shape were found between branchiate and metamorphosed juveniles, but there were differences in age structure. The occurrence of facultative paedomorphosis in *C. asper* has been found only in three populations in the Pyrenees (extinct population in the Valle de Arán, Campeny et al. 1986; Ibón de Acherito, Oromi et al. 2014; Ibón de

Table 2. Loadings of the morphometric variables, corresponding eigenvalues and % of cumulative variation in the first two principal components.

Variable	1st factor	2nd factor
SVL	0.960	−0.134
Head length	0.894	0.132
Head width	0.907	0.193
Limb interval	0.888	−0.117
Forelimb length	0.881	0.232
Hind limb length	0.904	0.159
Tail length	0.671	−0.719
Tail high	0.848	0.087
Eigenvalues	6.100	0.692
% Cumulative variation	76.2	84.9

Table 3. Descriptive statistics for age and snout–vent length (SVL) of the skeletochronological sample.



			Age (LAGs)		SVL (mm)	
Group	Phenotype	*n*	Median	Max–min	Mean ± SE	Max–min.
Males	Paedomorphs	6	11	15–8	65.1 ± 1.4	69.9–61.2
	Metamorphs	32	10	19–6	66.7 ± 0.6	73.0–59.2
	All	38	10.5	19–6	66.5 ± 0.5	73.0–59.2
Females	Paedomorphs	13	7	14–4	62.5 ± 0.7	66.5–58.0
	Metamorphs	21	7	11–5	65.5 ± 0.7	72.2–59.7
	All	34	7	14–4	64.4 ± 0.6	72.2–58.0
Juveniles	Branchiate	19	10	20–6	55.7 ± 0.9	64.0–47.9
	Metamorphosed	19	8	13–5	54.6 ± 1.0	61.2–45.6
	All	38	9	20–5	55.2 ± 0.7	64.0–45.6
Larvae	All	17	6	18–1	51.7 ± 1.2	58.5–40.1

Perramó, this study), and these unique populations can allow us to understand the evolution of this phenomenon in the species. These two previously investigated populations are lacustrine, and the newts have gill remnants and smooth skin, probably because the respiration of *Calotriton* newts is mostly cutaneous (García-París et al. 2004; Oromi et al. 2014). In fact, the smooth skin that characterises the paedomorphic newts is also found in other lentic populations of *C. asper* (Carranza and Amat 2005). Instead, in the stream populations of *C. asper*, individuals show extensive roughness and robust body form as an adaptation to lotic lifestyle (Oromi et al. 2014). Despite this fact, the causes that promote facultative paedomorphosis in these two lakes seems to be complex and remain unknown.

The body size differed significantly between groups (males, females, and juveniles) and between adults (metamorphs and paedomorphs), but not between juveniles (branchiate and metamorphosed) in the Perramó population. Contrarily, the other facultative paedomorphic population of *C. asper* (Ibón de Acherito, Oromi et al. 2014) did not differ significantly between sexes. The body size of the Ibón de Perramó population was influenced by sex, as found in other species such as *Ichthyosaura alpestris* and *Lissotriton vulgaris* (Denoël et al. 2009). Smaller body size has been observed in paedomorphic versus metamorphic newts in the two lacustrine populations of *C. asper* with facultative paedomorphosis (this study; Ibón de Acherito, Oromi et al. 2014). This is according to the best of the bad lot hypothesis, which assumes that small

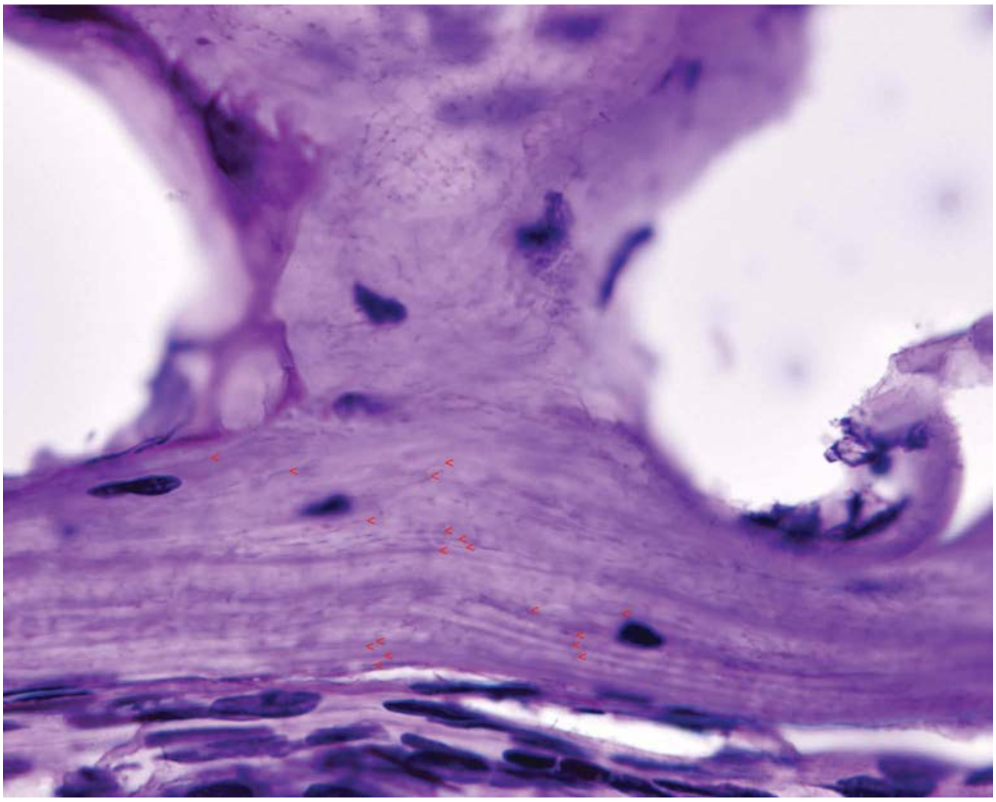


Figure 4. Stained cross-section of a phalange of an 18-year-old larva of *Calotriton asper*.

animals tend to become paedomorphs and large animals tend to metamorphose, and which occurs when the density is high (Wakano and Whiteman 2008). This is the highest population density observed and is likely produced as a direct result of selection. The role of the environment in the evolution of phenotypic variation can be adaptive, as in the best of the bad lot strategy (Whiteman 1994), inasmuch as the terrestrial phase of juveniles does not exist because the aquatic environment is better for growth than the terrestrial environment. This also occurs in other newt species such as *Lissotriton vulgaris* (Denoël et al. 2009) and *Ommatotriton ophryticus* (Başkale et al. 2013).

The selective mechanisms that can promote the evolution of paedomorphosis are complex and vary between species and populations (Reilly 1987). The existence of one or both phenotypes depends on the costs and benefits of each phenotype in its respective habitat and according to its mode of life (Whiteman 1994; Denoël et al. 2005). We suggest that only individuals with better body growth complete the metamorphosis. As a result of this trade-off, at the same age juveniles could derive energy to finish this process or attaining smaller sizes and becoming paedomorph. This supports the hypothesis of the best of the bad lot found for montane tiger salamanders as a result of cold temperatures and short growing seasons (Whiteman et al. 2012). In females, a higher cost of reproduction compared with males and strong intraspecific competition in a dense population could have produced a shorter longevity (Duellman and Trueb 1986). Early maturation of females in comparison with males can compensate for their lower longevity as a trade-off (e.g. Stearns 1992). The metamorphosis in the studied population of *C. asper* is probably gradual and, in some cases this process is not completed, i.e. in juveniles that become paedomorphic when they attain maturity.

Studies of the age structure in Pyrenean newts have shown a large variation between populations of different habitats and altitudes (Montori 1990; Miaud and Guillaume 2005; Oromi et al. 2014; this study). In general, the Ibón de Perramó population showed slightly delayed sexual maturation and higher longevity in comparison with the other lacustrine population previously studied. In Ibón de Acherito, sexual maturation took four years in both sexes (Oromi et al. 2014). The higher altitude in the Perramó lake can explain these differences, following the pattern of delayed sexual maturity and longer life in cold environments reported in other newts (Morrison and Hero 2003; Amat et al. 2010). Surprisingly, we found older larvae, one of them aged 18 years old, which, to our knowledge, is the oldest reported in a newt species. Although its sexual maturity was not checked by examining its gonads, this individual showed a typical larval morphology clearly different from juveniles and without a distinctive cloacal region or other functionally dimorphic traits related to courtship in adults (García-París et al. 2004).

Few Pyrenean lakes are inhabited by *C. asper*, and in these lakes most lacustrine populations are like stream populations, with a period where the juveniles have a terrestrial phase (observed in the lakes of Alba and Espelunciecha). The stability offered by the aquatic environment and its heterogeneous structure favours a prolonged aquatic life and, in some conditions, the occurrence of paedomorphosis (Denoël et al. 2005). Because of isolation and the harshness of some terrestrial landscapes, dispersal is also less likely than in other habitats, especially taking into account that in this species, terrestrial juveniles are in the dispersal phase (Montori 1988). However, there is no evidence of habitat differences – such as in lake altitude, size, bathymetry, or geology – between the facultative paedomorphic and the fully metamorphic populations of *C. asper* living in the lakes (Amat et al. 2010). Consequently, the combination of lentic habitat, high altitude and low predatory pressures, short activity period, and high population density generates conditions that can potentially alter development and metamorphosis (Wilbur and Collins 1973; Semlitsch and Gibbons 1985). In the lakes that follow the watercourse of the lake of Perramó there is the presence of fish and the absence of *C. asper*. Lacustrine populations represent a typical and recent habitat for the species colonised by post-glacial expansion (Valbuena-Ureña et al. 2013). This recent origin of the lake populations, a secondary habitat of *C. asper*, may have played a role in promoting facultative paedomorphosis.

Acknowledgements

This work was supported by the Biodiversity Conservation Plan of ENDESA, S.A. (ENEL Group). N. Oromi is currently a Marie Curie COFUND Postdoctoral Fellow at the University of Liege (Belgium). The research results underlying this publication have been obtained during her research stay at Lleida University. We thank the Aragón Government for providing the authorisations for scientific capture (500210/24/2014/491-500201/24/2012/12145).

Geolocation Information

Study lake, Ibón de Perramó (point): 42.6401° N, 00.5001° E

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Fundación Endesa [Biodiversity Conservation Plan of ENDESA, S.A. (EN]; Marie Curie COFUND [Postdoctoral Fellow at the University of Liege (Be].

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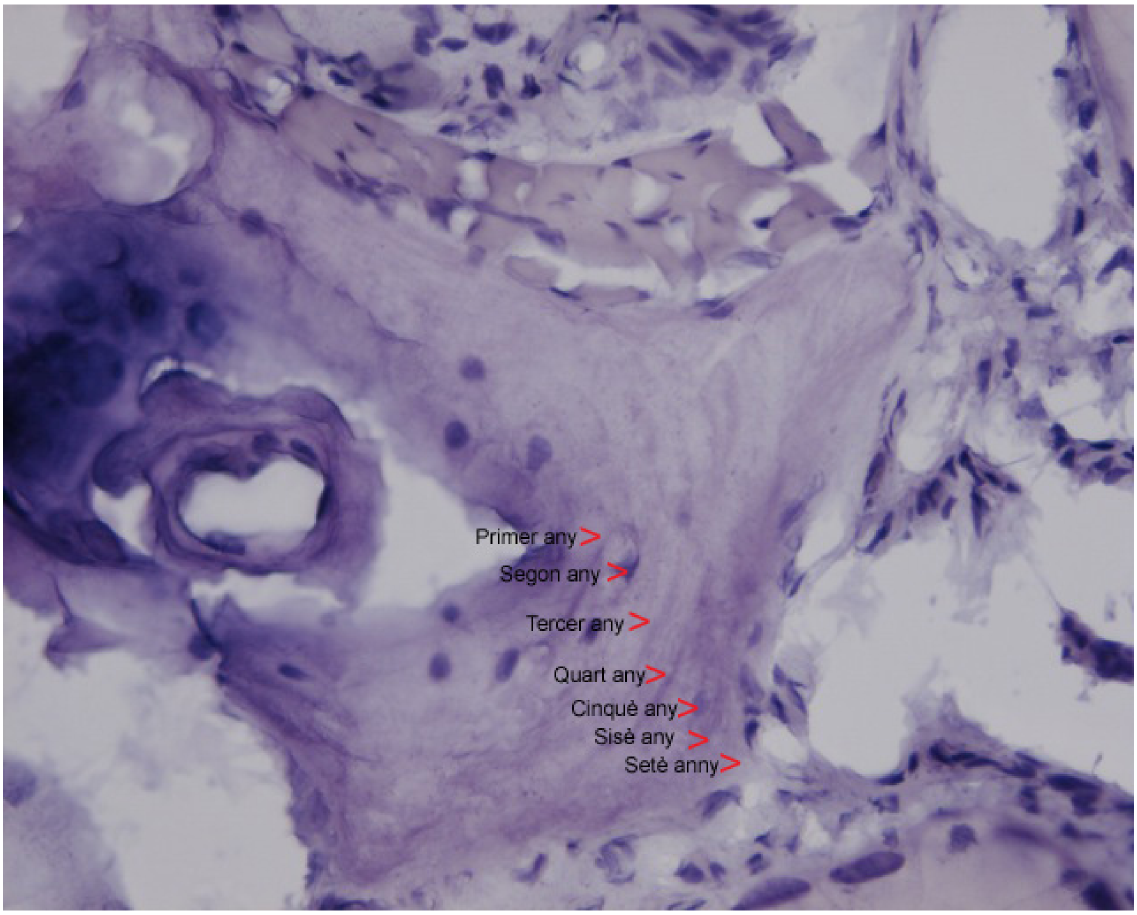

CHAPTER 2

Demographic Traits Variation in a Pyrenean Newt (*Calotriton asper*) among Lacustrine and Stream Populations

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Demographic Traits Variation in a Pyrenean Newt (*Calotriton asper*) among Lacustrine and Stream Populations

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Received: 26 October 2020; Accepted: 10 December 2020; Published: 12 December 2020

Abstract: Demographic traits were analyzed in the Pyrenean brook newt (*Calotriton asper*) to evaluate whether its variability responds to the adaptation to the different habitats. In this study, life history traits of *Calotriton asper* were studied in nine populations living in two different kinds of habitats in the Pyrenees mountains: lakes and streams. Skeletochronology was used to determine age structure and different traits such as age at maturity and longevity. Age structure was different between populations and sexes. The two lacustrine populations, with facultative pedomorphosis, attained their maturity earlier. Age at sexual maturity ranged from 4 to 9 years and in some populations was similar between sexes while in others, females matured at younger ages than males. Maximum longevity varied from 7 to 35 years among populations and was correlated with the age at sexual maturity. Body size differed among populations, was sexually dimorphic, and this disparity was not related to the kind of habitat. The maximum size was found in the lacustrine population but exhibited high variation between populations. The results obtained show a significant variability between sexes and populations, in age and body size structure of *Calotriton asper* that did not depend on the habitat.

Keywords: age structure; *Calotriton asper*; habitat type; demography; variability; evolution and body size

1. **Introduction**

Processes such as growth, development, or reproduction constitute the elements of the life history of an organism, each biological cycle being unique and vital [1]. The life history of an organism is variable within the limits of the individual's genotype and their phenotypic expression in different environments can vary, it is known as phenotypic plasticity [2]. In amphibians, phenotypic plasticity is especially widespread promoting a wide diversity of life cycles that can be observed, for example in newts and salamanders. This diversity is the result of the interaction between costs and benefits of the reproduction and development in the aquatic and terrestrial environments [3].

Life history traits in amphibians are strongly linked through trade-offs, such as age at maturity, longevity, and age–size relationship [4]. These life history traits can vary due to many factors such as climatic conditions, trophic resources, predator vs. prey interactions, or interspecific competition [5]. Besides that, these different environmental conditions, such as between lakes and streams, cause variability and promote ecological diversification [6]. The expression of a trait is determined by genetic factors, environmental influences, and interactions between genotype and environment [7]. Related to this, sexual dimorphism is determined by the balance and interaction of multiple selective forces [8]. For example, it may allow the reduction of intraspecific competition through ecological, morphological, or behavioral specializations between the sexes. Therefore, differences in body size may indicate a sexual selection or it may be the consequence of competition for food resources [9–11]. On the other hand, local environmental conditions may explain differences in body length [12]. The Pyrenean newt (*Calotriton asper*) is an endemic newt of the mountains of the Pyrenees, primarily lives in streams, although it has secondarily colonized high mountains lakes [13]. In order urodele, growth rates tend to be highest during the larval and juvenile stages [14]. In the case of the *Calotriton asper* it has been suggested that the sexes may differ in body size and other traits, influenced by ecological factors, including climatic conditions between each locality [15]. For example, *Calotriton asper* at some lacustrine localities exhibits facultative pedomorphosis [16,17]. The variation in body sizes and life history traits of the *Calotriton asper* was previously described in several populations at different habitat conditions [15,18–20]. For example, sexual dimorphism was more strongly expressed in the Central Pyrenean populations (high altitude) where females showed longer tails and smaller heads, while males had more robust tails and higher body weights compared with the Prepyrenees populations (middle altitude) [15]. Another study found that body lengths were higher for surface populations than the subterranean populations [20]. The variety of habitats and the effect of glacial and interglacial periods in the Pyrenees make it a natural laboratory for biogeographic, evolutionary, and ecological studies of mountain fauna such as *Calotriton asper* [21]. The main goal of this study is to analyze the effect of different habitat types (lacustrine vs. rheophilous) on the demographic characteristics of *Calotriton asper*. To achieve this, we evaluated life history traits such as body size, age structure, age at sexual maturity, and longevity in six torrent localities and three lacustrine localities of the species.

2. Materials and Methods

A total of 399 adult newts were sampled in nine populations (three lakes and six streams) along the southern slope of the Pyrenean mountains, from 2012 to 2015 (Figure 1, Table 1). The main characteristic of the streams is that their hydrological regime is determined by the pattern of snowfall. Consequently, the flow has an extraordinary seasonal variability, with maximums of spring associated with the melting, and the minimums in summer, when in some cases they can dry up. The other kind of habitat we analyzed was high mountain lakes of glacial origin [22]. The main characteristic of the lakes is that they suffer a stratification inversion of the water under the ice layer in winter. The densest waters (4 °C) fall to the bottom and the cold waters remain on the surface. This ice sheet can last several months [23]. Individuals were captured by hand, sexed based on the external morphology of the cloacal area, the newts were sexed based on sexual secondary characters: pointed cloacal protuberance in females and round and bulbous cloaca in males. The snout to the rear margin of the cloaca length (SVL) was measured using a digital caliper with a 0.1 mm of precision by the same person (F. Amat).

Minimum size at sexual maturity was also estimated for each sex and population, as the minimum SVL of sexually mature individuals, age at maturity, as the minimum number of lines of arrested growth LAGs counted in the reproductive individuals and longevity, as the maximum number of LAGs counted in the sexually mature individuals. The authorization numbers for scientific capture of the Government of Aragón, Spain (500201/24/2015/2747; 500210/24/2014/491; 500201/24/2012/12145). These authorizations certifying that the conditions established are approved by the Ethics Committee for this project.

Skeletochronological methods were used to estimate the individual age, and to infer age at sexual maturity and longevity for each sex and population using the protocols described [24,25]. Skeletonchronology, with precise age determinations, is widely applied to the study of sexual maturity and longevity in amphibians [24,26]. The largest toe of the left hind limb was removed and stored in 70% alcohol and the wound was disinfected. Bones were decalcified in 3% nitric acid between 35 and 75 min, cleaned by water for 1 h, and placed in phosphate-buffered saline and sucrose 30% for 48 h at 4 °C. Cross-sections (14–16 µm) were obtained with a freezing microtome and stained with Ehrlich's hematoxylin between 21 and 40 min. The age of each individual was determined by counting LAGs in the diaphysis of the periosteal bone using a light microscope at 100× and 400× magnifications. The analysis of growth marks was done by S. Camarasa and N. Oromi. Taking into account that in each individual all sections were reviewed in detail and no double growth lines were found, it was estimated that each LAG is one year.

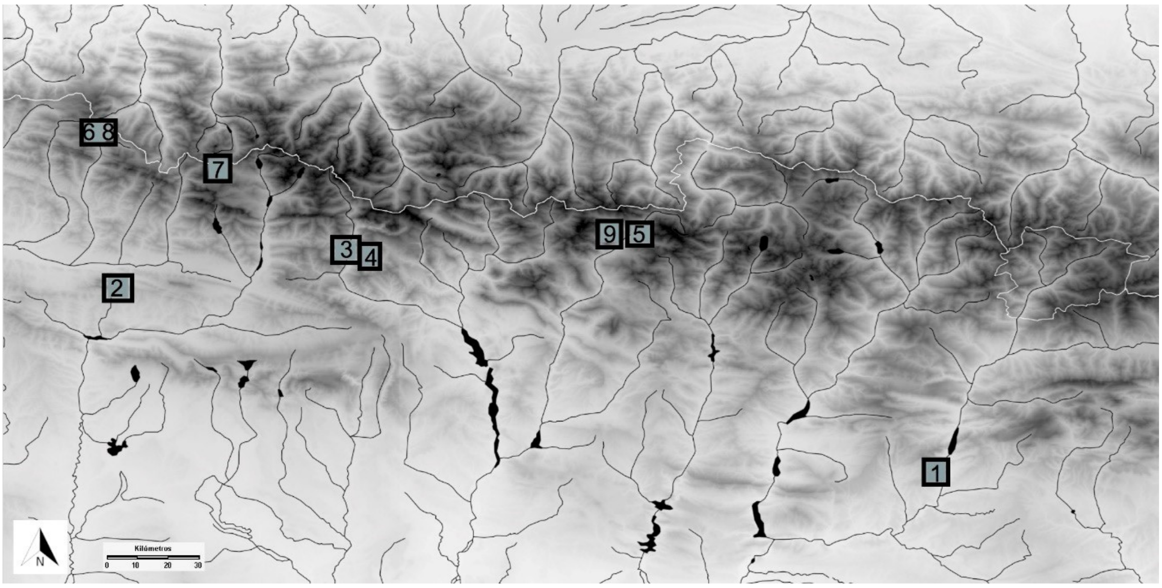

Figure 1. Localization of the sampled localities of *Calotriton asper* populations. Stream populations: 1 Peramola; 2 San Juan de la Peña; 3 Sarvisé; 4 Oto; 5 Benasc; 6 Barranc Acherito. Lacustrine populations: 7 Espeluciecha; 8 Acherito; 9 Perramó. See Table 1 for details on each locality.

Table 1. Latitude, longitude, altitude, and number of individuals (339 in total) for each locality and habitat.

Habitat	Locality	Latitude	Longitude	Altitude	*n*
Stream	Peramola	42°4′17.17″ N	1°16′30.98″ E	592	28
	San Juan de la Peña	42°30′26.94″ N	0°40′0.16″ W	1215	38
	Sarvisé	42°34′51.90″ N	0°4′17.70″ W	1222	38
	Oto	42°35′47.47″ N	0°7′42.56″ W	916	44
	Benasc	42°38′2.23″ N	0°34′25.21″ E	1600	37
	Barranc de Acherito	42°52′27.81″ N	0°43′18.64″ W	1525	47
Lake	Espeluciecha	42°47′18.06″ N	0°25′47.93″ W	1955	52
	Acherito	42°52′45.52″ N	0°42′27.48″ W	1872	44
	Perramó	42°38′19.29″ N	0°30′1.14″ E	2254	71

To test for differences in age structure among populations, we performed a two-way ANOVA on medium age using sex and populations as factors. Besides, we have a test for body size differences among populations taking into account sexual dimorphism. Thus, two-way ANOVA on mean body size was conducted using sex and populations as factors. Pearson correlation was done to test for relationships between age at sexual maturity and longevity. All analyses were done using JMP Pro 14 [27], (alpha = 0.05) on 10 log-transformed variables age and SVL.

3. Results

Populations of *Calotriton asper* exhibited significant differences between sexes, males and females, in age structure at intrapopulation ($F_{1381} = 8.667$, $p = 0.003$) and interpopulation level ($F_{8381} = 74.902$, $p < 0.001$). Three examined populations, two lacustrine (Acherito, Perramó) and one stream-dwelling (Barranc de Acherito) had a young age structure (Figure 2, Table 2). However, we found a significant interaction between these two factors (sex and locality), thus reflecting that sexual differences in age structure changed over populations ($F_{8381} = 2.470$, $p = 0.013$) (Table 3). Nevertheless, the sexual effect on demography was unrelated to the habitat (Table 2): given that the three lacustrine populations showed three different patterns, and of the six rheophilous populations, three of these showed older males than females and the other three the opposite pattern (Figure 2, Table 2).

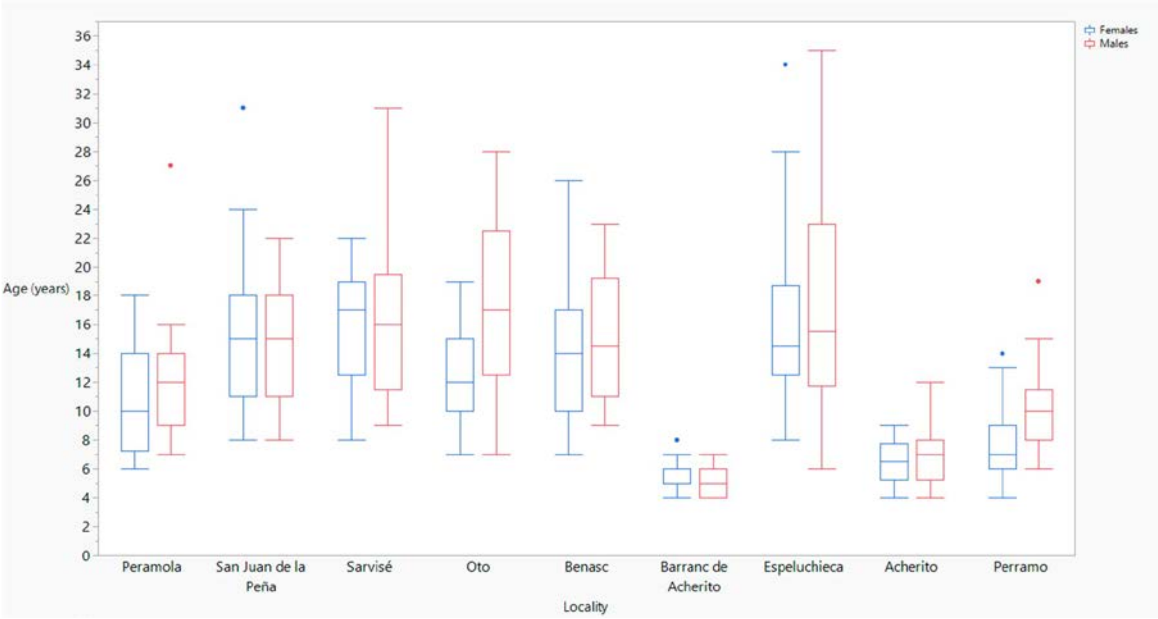

Figure 2. Box-plot of the age structure of the newts for each locality and sex. The 1st Quartile (values up to the first 25% of sample) lower box limit and the 3rd Quartile (values up to 75% of sample) upper box limit. Vertical stripes outside the box identify the maximum and minimum values. The extreme cases are represented by circles.

Table 2. Descriptive statistics for age and snout-vent length (SVL) for each sex, locality, and habitat, ES: standard error.

				Age (Years)			SVL (mm)		
Habitat	Locality	Sex	*n*	Minimum	Maximum	Median	Minimum	Maximum	Mean ± ES
Stream	Peramola	Females	16	6	18	10.0	52.3	68.8	59.0 ± 1.1
		Males	12	7	27	12.0	53.2	65.9	58.5 ± 1.3
	San Juan de la Peña	Females	19	8	31	15.0	54.8	70.3	61.4 ± 0.9
		Males	19	8	22	15.0	59.7	70.8	65.9 ± 0.6
	Sarvisé	Females	17	8	22	17.0	51.9	58.6	57.2 ± 0.6
		Males	21	9	31	16.0	57.9	67.2	62.7 ± 0.5
	Oto	Females	23	7	19	12.0	50.1	59.1	54.5 ± 0.6
		Males	21	7	28	17.0	51.1	62.6	55.3 ± 0.6
	Benasc	Females	19	7	26	14.0	56.4	71.3	63.7 ± 0.9
		Males	18	9	23	14.5	55.7	68.5	64.8 ± 0.8
	Barranc de Acherito	Females	20	4	8	5.0	63.8	77.6	73.6 ± 0.8
		Males	27	4	7	5.0	53.9	74.5	68.9 ± 0.9
Lake	Espeluchieca	Females	26	8	34	14.5	53.1	82.7	64.4 ± 1.4
		Males	26	6	35	15.5	58.7	76.2	64.7 ± 1.0
	Acherito	Females	12	4	9	6.5	56.0	68.0	61.0 ± 1.1
		Males	32	4	12	7.0	56.0	67.0	63.0 ± 0.5
	Perramó	Females	34	4	14	7.0	58.0	72.2	64.1 ± 0.6
		Males	37	6	19	10.0	59.2	73.0	66.2 ± 0.5

Table 3. ANOVA results of statistical analyses to test differences in age structure and SVL at sexual and locality level. Statistically significant values are indicate by asterisks (*).

Age Structure by Sex and Population			
Factor	**Degrees of freedom**	**F**	**Probability > F**
Sex	1	8.6671	0.0034 *
Locality	8	74.9023	<0.0001 *
Locality × sex	8	2.4706	0.0128 *
SVL by sex and population			
Factor	**Degrees of freedom**	**F**	**Probability > F**
Locality	8	62.6399	<0.0001 *
Sex	1	10.2254	0.0015 *
Locality × sex	8	6.3897	<0.0001*

Age at sexual maturity ranged from 4 to 9 years among populations. The lacustrine populations of Perramó and Acherito, and the stream-dwelling population near Acherito matured earlier than the others. In all the examined populations, age at sexual maturity was similar between sexes, or the females matured at younger age than males, the only exception was the Espeluchieca lake population (Table 2). Maximum longevity varied from 7 to 35 years among populations and correlated with the age of sexual maturity (r = 0.821, *p* = 0.010, *n* = 9). Although maximum longevities were correlated between sexes (r = 0.762, *p* = 0.016, *n* = 9), populations differed in the sexual pattern of longevity, males tending to develop high longevity. That was the case of the lacustrine populations, although it was also found in half of the rheophilous populations.

Body size significantly differed among populations (locality: $F_{8381} = 62.639$, $p < 0.001$) and was sexually dimorphic (sex: $F_{1381} = 10.225$, $p = 0.002$) (Figure 3, Table 2). Body size variation was not related to the kind of habitat of the populations (Table 2). Smallest mean body sizes for both sexes corresponded to the Oto population, and the largest to the Barranc Acherito populations. The other populations showed intermediate values.

Except in two stream localities, males show a larger mean body size than females. For example, the smallest and largest mean body sizes corresponded to stream-dwelling populations while lacustrine newts showed intermediate values. Regarding the maximum values, there was also not a clear association with habitat. Although in most populations males showed higher mean values than females, this latter sex reached higher maximum sizes in some cases. As a result, the pattern of sexual dimorphism was not the same for all the populations ($F_{8381} = 6.389$, $p < 0.001$) (Table 3) and was not related to the habitat.

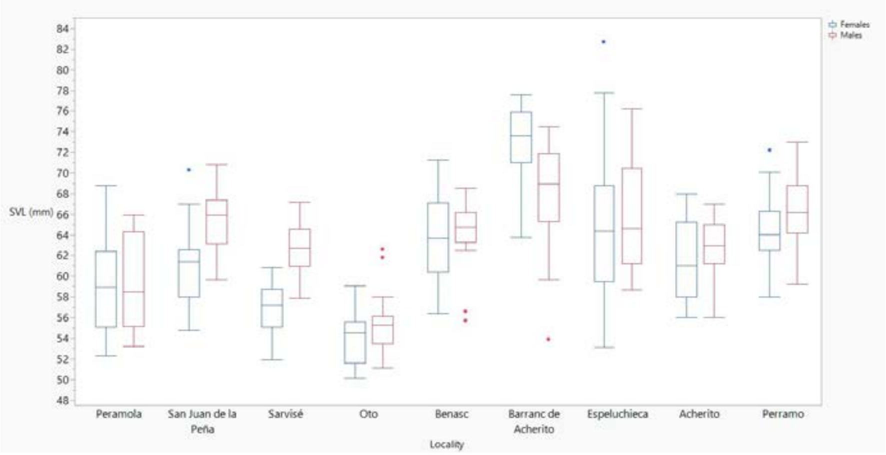

Figure 3. Box-plot of the snout-vent length of the newts for each locality and sex. The 1st Quartile (values up to the first 25% of sample) lower box limit and the 3rd Quartile (values up to 75% of sample) upper box limit. Vertical stripes outside the box identify the maximum and minimum values. The extreme cases are represented by circles.

4. Discussion

Variability in life history traits of amphibians is a result of adaptations to different environmental conditions. In our case, despite the clear differences in ecological conditions between rheophilous and lacustrine environments [28], experienced by *Calotriton asper*, our results showed a complex picture where the interactions of multiple factors, not linked with the habitat conditions, determine the demographic traits of populations. Consequently, life history traits such as age at maturity and longevity can show a variation with habitat, molded by its specificities [29]. We suggest that the environmental factors explain variation in life history traits, body size, and growth rates. This variation can be related to water temperature, annual duration of the activity period, foraging activity, and the duration of the terrestrial phase [20].

Observing the species distribution, glaciations could have limited the persistence of populations of *Calotriton asper* in lakes before the end of the last glaciation, so the origin of these would be recent colonization [30]. Quaternary glacial events, with expansion–contraction of the geographic range, have influenced and explain the current distribution of Pyrenean fauna and flora [31,32]. Although the habitats trace the great ecological lines, it is the peculiarities of each locality that define aquatic and terrestrial conditions such as the temperature, predation pressure, structure, and trophic productivity or competition with other species [3]. The different “life history traits and body size” found in the localities could be a reflex of the environmental and climatic forces that act on them. These strategies are directly influenced by the duration of the annual activity period and the duration of the terrestrial and larval phases [33]. Age or size at metamorphosis, at maturity, and longevity, result from trade-offs between advantageous conditions in environments [34]. All stream dwelling populations showed intermediate age structure among the lacustrine localities, singular conditions of each locality influences in a different way in the demography of each one of the populations. The great majority of the lakes of the Pyrenees are not populated by *Calotriton asper*, the difficult colonization of lacustrine habitats causes changes in the demography since most of them do not have the ideal biotic and abiotic conditions. The only two localities with facultative pedomorphosis, Acherito and Perramó, are characterized for not having an immature terrestrial phase, likely increasing the population density in the lake. It seems that the peculiarities in the conditions of these lakes have allowed the development of a pedomorphic phenotype [16,17,21]. In these lakes, the populations show a first sexual maturity to compensate for short longevity. The benefits of early maturation are a shorter generation time, reducing exposure to juvenile mortality [4]. In contrast, the lacustrine population of Espeluchieca delays the age of sexual maturity, reaching greater longevity and larger body size. In this population, newts live together with fishes, which share the lake but there is spatial segregation between them (personal observation F. Amat). This spatial segregation is formed on a slope of the lake, where the rocks from the landslides are submerged into the water, making a physical separation. It is known that the aquatic shelters favor coexistence between newts and fish [35]. We can also see the pressure of the fish on the newts when they live together in the stream population of Barranc Acherito, where there is short longevity and early maturity, which could be due to the presence of fish. The smaller body sizes are for the localities of Sarvisé and Oto, although they have high longevity. There is possibly a strict selection for small body individuals that can hide from floods [36], because the number and dimensions of refuges such as stones and fissures define the density and distribution of newts [37]. In *Calotriton asper* annual active life varies from 4 to 8 months or more [13], also the duration of the larval phase varies from 1 to 3 years or more [16,38]. We do not know water temperature or variations in trophic conditions for the *Calotriton asper* populations that we studied. We can, however, hypothesize that environmental conditions are the inductors of this variability, which favors its adaptation. In several urodeles, the average age, age at maturity, and body size increase as the annual activity period shortens [39]. In the duration of the terrestrial phase, there seems to be a tendency to shorten in the lacustrine habitat or does not exist in pedomorphic populations.

In summary, the results of this study show that *Calotriton asper* exhibits interpopulation variability in the demography that does not depend on the habitat. In conclusion, we reject the hypothesis that habitat (stream and lake) gives a pattern to the demography in *Calotriton asper*. The factors that shape the demographics of populations have an effect on a smaller scale, differently within each locality beyond whether the habitat is rheophilous or lacustrine.

Author Contributions: Conceptualization, S.C., N.O., D.S. and F.A.; methodology, S.C., N.O., D.S. and F.A.; software, S.C., N.O. and F.A.; validation, S.C., N.O., D.S. and F.A.; formal analysis, S.C., N.O. and F.A.; investigation, S.C., N.O., D.S. and F.A.; resources, S.C., D.S. and F.A.; data curation S.C. and F.A.; writing—original draft preparation, S.C.; writing—review and editing, S.C., N.O., D.S. and F.A.; visualization, S.C., N.O., D.S. and F.A.; supervision, S.C., N.O., D.S. and F.A.; project administration, S.C., N.O., D.S. and F.A.; funding acquisition, S.C.
and D.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Biodiversity Conservation Plan of ENDESA, S.A. (ENEL Group).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 3

Morphological variation in Pyrenean newts (*Calotriton asper*): does habitat and ontogeny determines size and body shape more than sexual dimorphism?

Under review in Diversity

Article

Morphological variation in Pyrenean newts (*Calotriton asper*): does habitat and ontogeny determines size and body shape more than sexual dimorphism?

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Abstract: The Pyrenean brook newt (*Calotriton asper)* is a primarily rheohilous endemic of Pyrenees and adjacent mountain ranges. Through its geographic range, the species experiences large phenotypic plasticity in relation to the habitat and morphology, and can even modify its biological cycle or physiology depending on the characteristics of the environment. The aim of this work has been to define the sexual dimorphism at of body shape and body size at the level of habitat (lake and stream), ontogeny (metamorphic and paedomorphic) and population. Thirty-three populations from lake and streams have been sampled with a total of 1061 individuals, of which two lake populations show the phenomenon of paedomorphosis. We found sexual dimorphism on body size in some populations, although when we tested together all the individuals and populations is not significant. However, body size show differences in habitat type and ontogeny. When we analyse the eight variables together there is a complex pattern of differences between sexes, habitat, ontogeny and populations. Although body shape did not show differences between ontogenies. The characteristics of sexual dimorphism in males are bigger heads, longer extremities and higher tails, whereas females have longer and less high tails, longer abdomens and smaller head and limbs.

Keywords: *Calotriton asper*; paedomorphosis; body size; body shape; morphology; streams; lakes; sexual dimorphism

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Diversity* **2021**, *13*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

The Pyrenean newt (*Calotriton asper*, Duguès 1852), is an amphibian urodele belonging to family of Salamandridae endemic to the Pyrenees and Prepyrenees that is distributed throughout Spain, France and Andorra.

Calotriton asper (*C. asper*), has adapted to fast-moving water environments, although some populations have secondarily colonised high mountain lakes and underground streams or caves [1,2]. Although the species occupies different environments, its characteristic habitat is mountain streams [1,3]. The main characteristic of the Pyrenean streams is that their hydrological regime is determined by the pattern of snowfall. Consequently, the flow has a hide seasonal variability, with maximums of spring associated with the melting, and minimums in summer, when in some cases they can dry up. The other kind of habitat that we analyzed was high mountain lakes of glacial origin [4]. The main characteristic of the lakes is that they suffer a stratification inversion of the water under the ice layer in winter. The densest waters (at 4 °C) fall to the bottom and the cold waters remain on the surface. This ice sheet can last several months [5]. The lakes also are characterised by oligotrophic aquatic environments, low concentrations of dissolved salt in their waters, limited phytoplankton productivity and absence of natural vertebrate predators [5, 6].

In high-altitude lakes, at the limits of the distribution of *C. asper*, there are populations in which the juveniles live a complete aquatic life [7, 8]. This is due to the fact that these are very extreme locations where the ecological conditions of the terrestrial environment are not favourable for the presence of juveniles out of the water, for instance excessively low temperatures, aridity or lack of vegetation cover. Amphibians, as ectothermic and heterothermic animals, depends on the environmental conditions for thermoregulation. Consequently, they cannot inhabit extremely cold areas and their body temperature only varies within certain ranges, outside of which they become inactive or may even die. The climatic conditions prevailing in the Pyrenees are severely limiting for the activity of reptiles and amphibians, and this becomes more important as the altitude increases. This fact severely conditions the biological cycle of the species, particularly those found above 1800-2000m [9,10].

Paedomorphism is an evolutionary phenomenon that appears in urodele (salamanders and newts) and consists in the fact that the individuals, despite being sexually adult, conserve larval characteristics. In species with complex life cycles such as in caudate amphibians, this morphological variation can have important evolutionary consequences that can result in population differentiation and speciation [11]. The coexistence of metamorphic and paedomorphic individuals in the same population has been frequently reported in many species of newts [11, 12, 13, 14, 15, 16]. Several environmental factors regulate the frequency of paedomorphosis, although experimental evidence shows that the causality of this phenomenon is complex [11, 13]. For example, experimentally higher population densities have been related to the paedomorphosis [17], while the manipulation of the alimentation does not show the same directional effect [18, 19]. It has been hypothesised that paedomorphosis is a frequent process in stable aquatic habitats allowing a slow body growth in contrast to habitats where water levels, predator pressures, and food availability change over time [11]. In this latter case, if these factors negatively affect the individual survivorship, an early time of metamorphosis and sexual maturity may be an adaptive response. Such as age at maturity or body size can directly affect in the occurrence of facultative paedomorphosis [20]. The pattern of growth, development and reproduction is fixed within limits by the genotype of the individual, but depending on the different environments that surround it can change its expression, it is said that there is a phenotypic plasticity [21]. An example of phenotypic plasticity is the great morphological variability of *C. asper* [3, 22]. This species shows great plasticity in relation to the habitat, and can even modify its biological cycle or physiology depending on the characteristics of the environment [23]. *C. asper* populations from colder climates (high-elevation) were larger compared to low-elevation populations. This trend could be the result of evolutionary responses to harsh environments, driven by each local adaptation, plasticity processes, or a combination of both [24]. Phenotypic diversification between populations seems to be due to rapid genetic changes under local selection pressures in response to population-specific ecological conditions [25].

Although there is much information about amphibian life-history variation in different environments, altitudinal and latitudinal ranges [26], little is known about the morphology, demography and ecology of populations with presence of paedomorphosis. We especially interested in two populations of Pyrerean newt in which there are individuals with paedomorphic characteristics, a very rare occurrence in this species, Ibón de Acherito [7], and Ibón de Perramó [8], also a known extinct population Lake of Manhera [27, 28].

The study of the lacustrine and lotic populations of Pyrenean newt aims to achieve the following objectives:

- Define the pattern of sexual dimorphism at the level of body shape and body size and evaluate whether this remains unchanged among populations and ontogenic stages.
- Test for differences on body size and shape at the level of habitat (lake and stream), ontogeny (metamorphic and paedomorphic) and population.

2. Materials and Methods

2.1. Study area and sampling

Fieldwork was carried out during the years from 2012 to 2019, consisting of night surveys along the lakeshore or the shore of the stream, with two people investing one night per population. Newts were captured by hand and a hand net and kept in a mesh bag until data collection. We followed a strict biosecurity protocol to protect from disease recommended by the Government of Catalonia.

Adults newts were sexed on the basis of sexual secondary characters: pointed cloaca protuberance in females and round and bulbous cloaca in males. In the populations of Ibón Acherito and Perramó, the paedomorphic newts were distinguished from the metamorphic ones by the presence of external gills at different levels of development. The individuals without secondary sex characteristics (no external cloaca differentiation) were considered as juvenile newts, and were not used in the study. During the different years a total of 1061 newts were sampled in 33 populations (6 lakes and 27 streams) (Figure 1, Table 1). A total of eight linear morphometric measurements were obtained with a digital calliper for morphometry, with a 0.1 mm of precision by the same person (F. Amat): 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. All the manipulations were performed in the proximities of the site of capture to avoid disturbance of the newts. Subsequently, all individuals were released at the site of capture. The authorization numbers for scientific capture of the Government of Aragón, Spain (500201/24/2015/2747; 500210/24/2014/491; 500201/24/2012/12145). These authorizations certifying that the conditions established are approved by the Ethics Committee for this project.

Figure 1. Localization of the sampled populations of *Calotriton asper* populations. See Table 1 for details on each population. Black numbers are streams, blue numbers are lakes and red numbers are lakes with paedomorphic individuals.

Table 1. Latitude, longitude, altitude, number of individuals (n) of females and males (1061 in total) for each population and habitat.

	Population	Habitat	Latitude	Longitude	Altitude	*n*	Females	Males
1-	Ansabere	Stream	42°53'24.7"N	0°42'34.3"W	1785	21	9	12
2-	Barranc Acherito	Stream	42°52'06.1"N	0°42'38.5"W	1385	72	47	25
3-	Batisielles	Stream	42°39'54.7"N	0°30'23.0"E	1815	6	5	1
4-	Bujaruelo	Stream	42°43'45.0"N	0°08'11.0"W	1687	24	12	12
5-	Clara	Stream	42°34'41.1"N	2°26'34.9"E	630	30	8	22
6-	Contienda	Stream	42°52'34.3"N	0°49'22.4"W	1233	52	23	29
7-	Fanlo	Stream	42°36'35.1"N	0°01'07.2"E	1883	32	11	21
8-	Font Borbonera	Stream	42°12'11.2"N	1°17'59.0"E	749	13	6	7
9-	Font Figuera	Stream	42°01'16.0"N	1°01'48.3"E	803	24	9	15
10-	Lamanera	Stream	42°20'56.5"N	2°31'17.3"E	833	25	10	15
11-	Monrepós	Stream	42°20'41.4"N	0°23'33.3"W	1237	11	3	8
12-	Ordino	Stream	42°37'50.4"N	1°29'56.4"E	1981	6	1	5
13-	Oto	Stream	42°35'47.5"N	0°7'42.56" W	916	43	23	20
14-	Peramola	Stream	42°04'24.7"N	1°16'20.5"E	623	35	20	15
15-	Pino	Stream	42°40'29.6"N	0°28'20.1"E	1815	15	9	6
16-	Saboril	Stream	42°34'60.0"N	0°25'12.5"E	1683	38	26	12
17-	San Juan de la Penya	Stream	42°30'59.0"N	0°42'02.7"W	1052	25	16	9
18-	Sarvisé	Stream	42°34'51.9"N	0°4'17.70" W	1222	22	10	12

19-	Selva de Oza	Stream	42°49'48.0"N	0°42'53.0"W	1181	15	8	7
20-	Sierra de Guara	Stream	42°15'35.3"N	0°14'43.1"W	935	26	19	7
21-	Torrent del Boix	Stream	42°17'25.3"N	2°00'56.9"E	1539	43	22	21
22-	Torrent de Fiscal	Stream	42°29'44.2"N	0°07'12.7"W	771	17	10	7
23-	Torrent del Pi	Stream	42°19'56.9"N	1°45'14.9"E	1231	39	19	20
24-	Vall d’en Bac	Stream	42°16'08.3"N	2°22'52.5"E	892	27	7	20
25-	Vall Fosca	Stream	42°29'29.6"N	0°59'18.7"E	1648	14	7	7
26-	Vallibierna	Stream	42°37'23.4"N	0°35'25.2"E	1854	43	25	18
27-	Zuriza	Stream	42°51'22.8"N	0°46'18.8"W	1427	18	8	10
28-	Ibón Acherito	Lake	42°52'47.1"N	0°42'23.4"W	1822	78	23	55
29-	Ibón Bucuesa	Lake	42°42'29.6"N	0°25'52.7"W	2124	21	12	9
30-	Ibón Espelunciecha	Lake	42°47'18.3"N	0°25'47.7"W	1953	46	21	25
31-	Ibón Perramó	Lake	42°38'19.6"N	0°30'00.0"E	2406	104	41	63
32-	Ibón Saman	Lake	42°44'40.7"N	0°28'44.1"W	2159	34	17	17
33-	Ibón Alba	Lake	42°39'48.3"N	0°36'20.1"E	2301	42	21	21

2.2. Statistical analysis

All variables were tested for normality, homogeneity of variance. As all variable distributions were significantly skewed, data were normalised by log-transformation previously to the analysis.

Differences on body size (SVL) with respect to sex (female and male) and habitat (stream and lake) were tested. Also with respect to sex (female and male) and ontogeny (metamorphs and paedomorphs) using only the lacustrine populations and with respect to sex (female and male) and population, newts were tested between groups using ANOVA test with the interaction between the two factors. Multiple comparisons for significance between factors were performed using the Tukey–Kramer HSD test. In order to asses for correlation between SVL and the other morphometric variables, Pearson parametric correlation was performed.

Therefore, the seven morphometric traits were analyzed using MANCOVA and the SVL as covariate, testing for body shape differences between sexes (male and females), ontogeny (metamorphs and paedomorphs), and habitat (stream and lake), and interaction among these factors.

Moreover, principal component analysis (PCA) was performed to explore the patterns of covariation of the morphometric variables and visualize sexual differences, also habitat and ontogeny differences. All analyses were conducted using JMP 15 Pro [29] (alpha=0.05).

3. Results

Body shape and size had an important influence in morphological differentiation. There was a large diversity of variable sizes among the populations studied. Thus, mean lengths for each variable vary between populations and between sexes within them. Descriptive analyses were performed on the whole samples and showed differences, both for males and females (Table 2) and for lakes and streams (Table 3).

Table 2. Descriptive statistics for the eight measures of morphometric variables for each sex.

Variables	**Total=1061**					
	Males=553			**Females=508**		
	Max.	**Min.**	**Mean±SD**	**Max.**	**Min.**	**Mean±SD**
SVL	78,5	51,1	64,5±5,9	84,3	48,1	64,0±7,5
Head Length	20,7	10,1	15,5±1,8	19,8	9,7	14,3±1,8
Head Width	14,8	8,2	11,5±1,2	14,1	7,5	10,4±1,2
Forelinb length	22,8	11,9	17,9±1,9	22,1	11,4	16,6±1,9
Hind Length	25,7	15,6	20,3±2,0	25,2	13,1	19,0±2,1
Limb Interval	43,4	20,8	32,3±3,7	44,6	20,5	32,1±4,7
Tail Length	60,5	32,5	47,2±4,9	76,3	32,3	52,8±7,2
Tail High	10,1	3	7,1±1,1	8,9	3,0	5,6±1,0

Table 3. Descriptive statistics for the eight measures of morphometric variables for each sex and habitat.

Variables	Stream=736						Lake=325					
	Males=363			Females=373			Males=190			Females=135		
	Max.	Min.	Mean±SD	Max.	Min.	Mean±SD	Max.	Min.	Mean±SD	Max.	Min.	Mean±SD
SVL	77,9	51,1	63,4±6,2	78,1	48,1	62,5±7,3	78,5	54	66,5±4,6	84,3	51,0	67,9±6,5
Head Length	19,4	10,1	15,0±1,9	19,8	9,7	13,9±1,7	20,7	12,1	16,3±1,3	19	11,6	15,4±1,4
Head Width	14,8	8,2	11,4±1,3	14,1	7,5	10,2±1,1	14,2	8,7	11,6±1,0	14	8,1	10,8±1,1
Forelinb length	21,8	11,9	17,3±1,8	20,9	11,4	16,0±1,8	22,8	15	19,1±1,4	22,1	14,4	18,1±1,5
Hind Length	25,5	15,6	19,7±2,0	24,1	13,1	18,4±2,0	25,7	17,8	21,4±1,6	25,2	14,8	20,4±1,7
Limb Interval	43,4	20,8	31,8±4,0	43,6	20,5	31,3±4,6	41,4	27,7	33,3±2,9	44,6	25,8	34,1±4,3
Tail Length	59,7	32,5	45,7±4,3	68,4	32,3	50,9±6,7	60,5	39,5	50,1±4,7	76,3	37,2	58,0±5,9
Tail High	10,1	4,3	7,1±1,1	8,6	3,0	5,4±1,0	9,8	3,0	7,0±1,1	8,9	3,0	6,1±1,0

With ANOVA test, we found significant differences in SVL between habitats: stream and lake ($F_{(1,1057)} = 99,3398$; $P < 0,0001$); but not between sexes: females and males ($F_{(1,1057)} = 0,0763$; $P = 0,7825$); and with interaction between these two factors ($F_{(1,1057)} = 7,1953$; $P = 0,0074$). The post-hoc Tukey–Kramer HSD test found significant differences between habitats but not among sexes inside each habitat (Figure 2), being lager lacustrine newts. In both habitats, females attained greater length than males without significant differences.

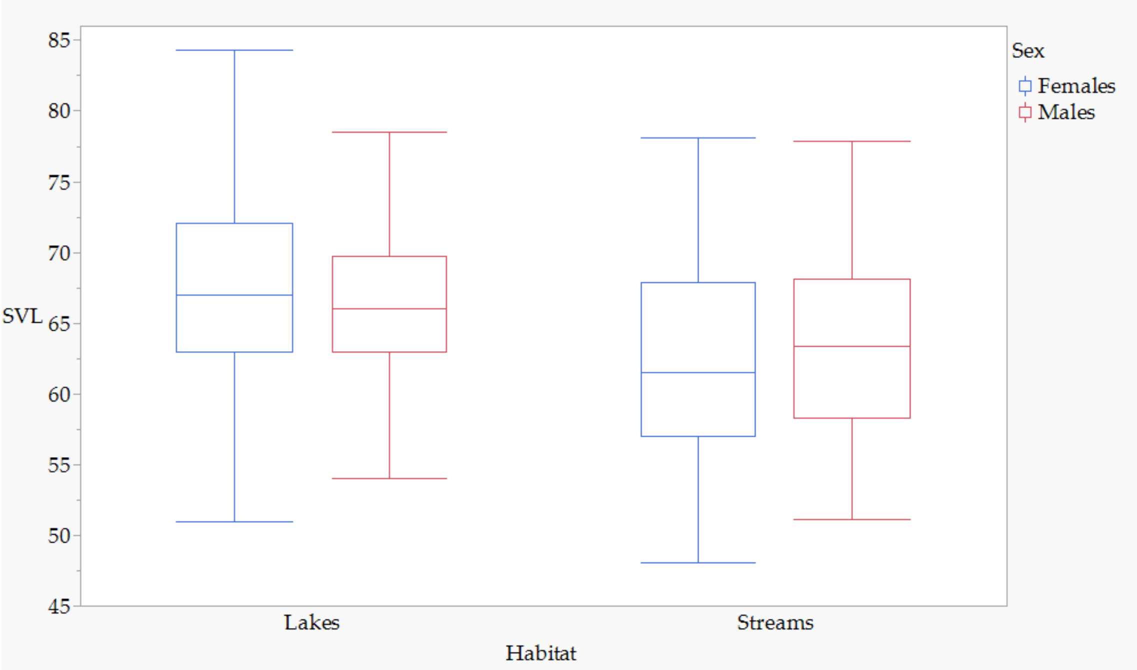

Figure 2. Box-plot of the snout-vent length (mm) of the newts for each habitat and sex. The 1st quartile (value up to the first 25% of the sample) lower box limit and the 3rd quartile (value up to 75% of the sample) upper box limit. Vertical stripes outside the box identify the maximum and minimum value.

ANOVA test revealed not significant differences in SVL, in the populations of lakes, between sexes (females and males) ($F_{(1,321)}$ = 0,3663; P = 0,5454); but significant differences between ontogeny (metamorphs and paedomorphs): ($F_{(1,321)}$ = 17,2935; P < 0.0001); without interaction between these two factors ($F_{(1,321)}$ = 2,3015, P = 0,1302). The post-hoc Tukey–Kramer HSD test found significant differences in metamorphic female individuals between female paedomorfic, males paedomorfic and males metamorphic (Figure 3). Metamorphic females are significantly larger than metamorphic males and any of the two paedomorphic sexes.

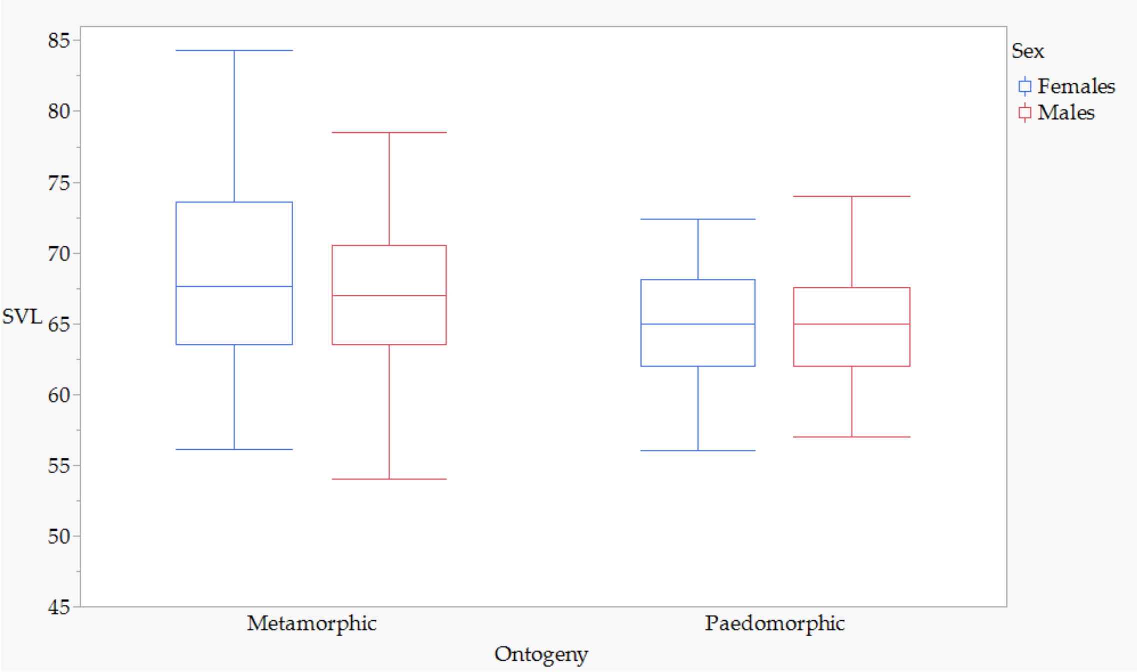

Figure 3. Box-plot of the snout-vent length (mm) of the newts for each ontogeny and sex. The 1st quartile (value up to the first 25% of the sample) lower box limit and the 3rd quartile (value up to 75% of the sample) upper box limit. Vertical stripes outside the box identify the maximum and minimum value.

We found with ANOVA test significant differences in SVL between populations: ($F_{(32,995)}$ = 53,1761; P < 0,0001); but not between sexes: females and males ($F_{(1,995)}$ = 3,4593; P = 0,0632); and with interaction between these two factors ($F_{(32,995)}$ = 1,9573; P = 0,0013) (Figure 4).

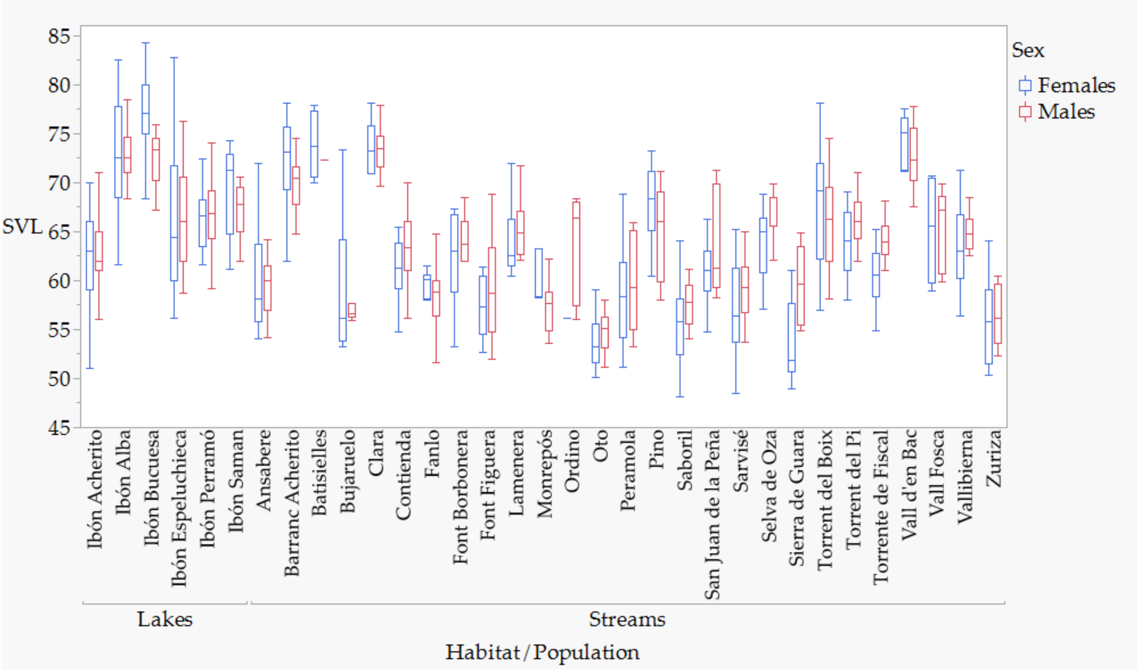

Figure 4. Box-plot of the snout-vent length (mm) of the newts for each population, habitat and sex. The 1st quartile (value up to the first 25% of the sample) lower box limit and the 3rd quartile (value up to 75% of the sample) upper box limit. Vertical stripes outside the box identify the maximum and minimum value.

The largest individuals have been found in lakes Ibon de Bucuesa, Espelunciecha and Alba. Followed by the stream populations of Batisielles, Acherito, Clara, Vall d'en Bac and torrent del Boix. The smallest individuals are from the stream populations of Saboril, Sarvisé, Sierra de Guara, Suriza and Oto.

Correlation between SVL and the variables head length, head width, hind and forelimb length, limb interval, tail length and tail high was significant.

With MANCOVA test we found significant differences in body shape between habitats (Figure 2): stream and lake ($F_{(1,1056)}$ = 67,0735; P < 0,0001); and between sexes: females and males ($F_{(1,1056)}$ = 462,4365; P < 0,0001); and with interaction between these two factors ($F_{(1,1056)}$ = 7,2570; P = 0,0072).

Ontogeny showed no significant differences with MANCOVA test, in body shape between ontogeny in the lakes populations (Figure 3): paedomorphic and metamorphic ($F_{(1,320)}$ = 1,0252, P = 0.3120); but did find significant differences between sexes: females and males ($F_{(1,320)}$ = 73,4410; P = 0,0001); without interaction between these two factors ($F_{(1,320)}$ = 1,8186, P = 0.1784).

MANCOVA test revealed significant differences in body shape between populations (Figure 4): ($F_{(32,994)}$ = 15,7769, P< 0.0001); and between sexes: females and males ($F_{(1,994)}$ = 555,3916; P< 0,0001); with interaction between these two factors ($F_{(32,994)}$ = 2,5203, P < 0,0001).

In order to visualize whether there are morphometric differences between sexes, sexual dimorphism, at a multivariate level, and also to understand the importance of each variable for each sex, a PCA has been carried out. The first two factors of the PCA accounted for a large proportion of variance 83,2% (Figure 5) and showed a clear pattern of differentiation between males and females. In the first factor all variables have positive and high coefficients, and therefore this factor represents body size. On the other hand, in the second factor there are negative coefficients for the tail high and head width, while in contrast there are positive coefficients for the tail length. This second factor can therefore be considered a body shape factor (Table S1). This factor allows us to define the characteristics of sexual dimorphism in the species.

In particular, males are characterized by having a bigger head, longer extremities and higher tails. Females, on the other hand, have longer and less high tails, longer abdomens and smaller head and limbs.

Because of clear effect of sexual dimorphism on the morphologic variation of the species and in order to detect differences between habitats (Figure 6 and Table S2) and ontogenic stages (Figure 7 and Table S3), we performed a separate PCA for each sex. Samples from lakes are mostly constituted by large newts overlapping with some, but not all the reophilous populations both in females and males. Samples from streams are characterized by having a higher tail in both sexes (Figure 6). However, no obvious differences in body shapes were detected between paedomorphics and metamorphics inspecting the PCA performed separately for each sex. PCA revealed that paedomorphic females have shorter tails, limb interval and tight heads than part of the metamorphics whereas in the males no discernible differences on body shape are found, the paedomorphic males have longer limbs than metamorphics (Figure 7).

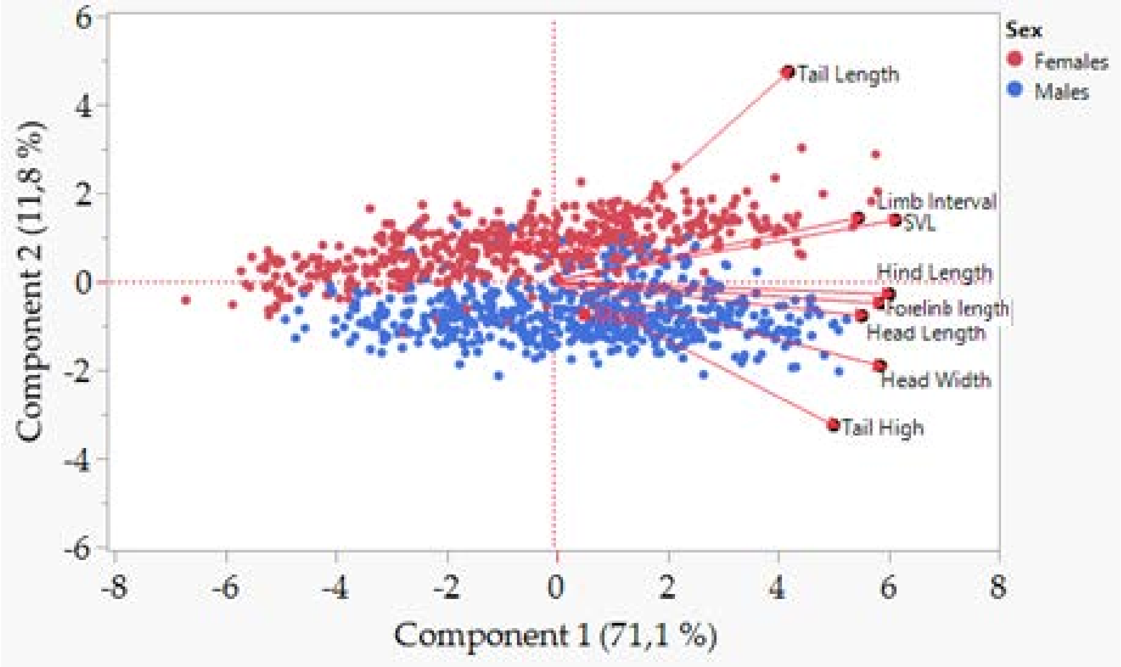

Figure 5. Graphical representation for sex of the values of the analyzed individuals and the weight coefficients of the morphometric variables in the two principal components.

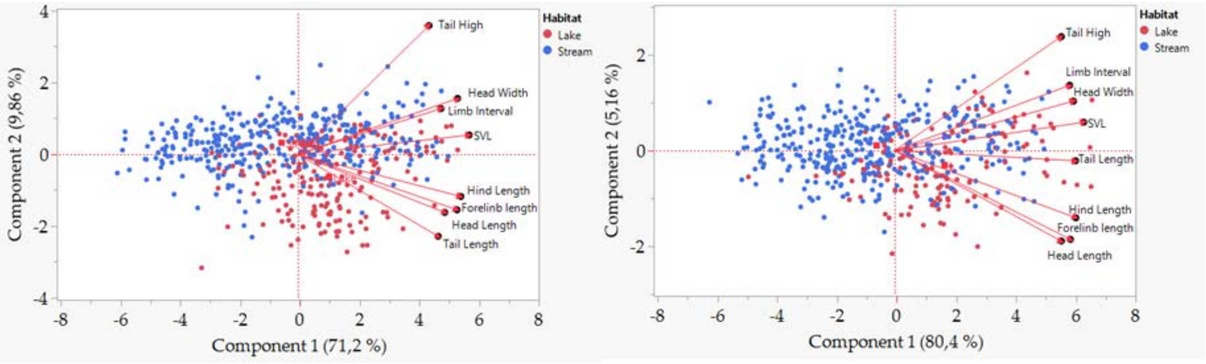

Figure 6. Graphical representation for habitat of the values of the analyzed individuals and the weight coefficients of the morphometric variables in the two principal components, males on the right and females on the left.

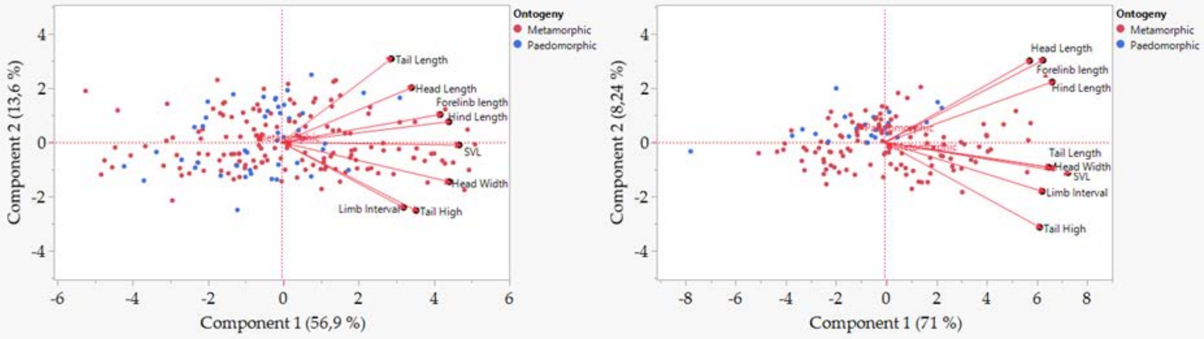

Figure 7. Graphical representation for ontogeny of the values of the analyzed individuals and the weight coefficients of the morphometric variables in the two principal components, males on the right and females on the left.

4. Discussion

Variations in ecology and body size were previously described in several populations of Pyrenean newt exposed to different environmental conditions [30]. Studies on body length in Pyrenean newts have showed a large variation between populations of different habitats and altitudes [2, 7, 8, 24, 31, 32, 33]. The interest of this work lies in investigating the morphological characteristics of the *C. asper* in lacustrine and lotic systems. In the populations of *C. asper*, in the present study, both in body size and body shape, differences are observed between habitats and ontogenies. With the whole sample together, sexual dimorphism in body size cannot be observed, due to the overlapping of measurements in the great variability that exists in each population. The results obtained show that there is a marked sexual dimorphism in body shape among and within the populations. In general, the pattern of sexual dimorphism in the populations coincides with that found in other populations [30]. Largest body size can be fixed in favour of males, feeling larger than females or vice versa, or vary between populations of the same species. In Pyrenean lakes the females are larger than the males, same pattern to *Lissotriton helveticus* in lakes of Andorra [34], except for the two populations with paedomorphosis (Ibon Acherito and Perramo). Although the opposite can be observed in most streams, we find several populations where males are larger than females. Between habitats, smaller body size has been observed in stream populations in comparison to lake populations of *C. asper*, with the higher altitude populations being larger in size [24].

The variability in body size and body shape in populations of *C. asper* can be separated into two components, the first being sexual between males and females and the second between different populations, given the environmental differences that may exist between them. There is an overlap among populations in terms of minimum body size. This is different from the range of variation in males and females, which means that for the same body size there are individuals that have already reached sexual maturity and others that have not. Therefore, the populations show a great plasticity in the SVL size. This differences in SVL depends on growth taxa, which will be sold by the success of feeding, the trophic productivity of the habitat, the density of individuals and the trophic competitiveness, the annual activity period or genetic characteristics.

The results found in the present study follow similar patterns of others studies [35], that finds no differences in SVL in relation to paedomorphism in the species *Ichthyosaura alpestris*, *Lissotriton helveticus* and *Lissotriton vulgaris*. The existence of one or both phenotypes depends on the costs and benefits gathered by each phenotype according to its mode of life [11, 13]. The smaller body size that has been observed in paedomorphic versus metamorphic newts in the lacustrine populations of *C. asper*, is according to the best of bad lot hypothesis, that assumes that small animals tend to become paedomorphs and large animals tend to metamorphose, and occurs when the density is high [36]. Ibón Perramo has the highest population density observed before the disappearance of a large part of the population [37]. The role of the environment in the evolution of phenotypic variation can be adaptive as a best of bad lot strategy [13], inasmuch as the terrestrial phase of juveniles does not exist because the aquatic environment is better for growth than the terrestrial environment, as found in other newt species such as *Lissotriton vulgaris* [35], and *Ommatotriton ophryticus* [15].

The sexual dimorphism in the species may be an adaptive response, an effect of natural selection, in comparison to other species, the general trend seems to be that males are larger than females, but there is variability between populations [30, 31]. Several hypotheses have been proposed to explain the direction of sexual dimorphism due to sexual selection in amphibians. For example, in females, the number of eggs and their size is restricted by body size [38, 39]. Therefore, females that have a larger limb interval, being larger than in the males, the selection may be acting in favour of the larger ones in order to have more offspring (selection for fecundity). In the case of the males, natural selection would be acting in favour of bigger males in order to be able to compete with other individuals of the same sex for the mating or for food between other individuals. In the males, the longer extremities are for a greater mobility to look for females, the higher and shorter tail is for the amplexus, to embrace females more efficiently. In females, the long and short tail may facilitate the mating of the males [40]. In the case of *C. asper* sexual dimorphims points to different trends of adaptation in males and females. Males have large SVL, bigger head length and width, with long legs and short tails by efficiently forage for large and evasive prey. The aggressive biting among males has been observed in the fight of *C. asper* [40]. In a comparison of the feeding behaviour of several populations of the *C. asper* [41], showed that females frequently feed on smaller-sized prey items than do males. The smaller head size may hence induce females to select smaller prey and allow a reduction in intersexual food competition.

On the other hand, males may be larger because they start from an older initial age of sexual maturity [33], so they spend more time as larvae and as juvenile but they grow more slowly than females. These animals grow throughout their lives, with two very marked growth phases: in the first phase, the growth rate is very high and grows very fast, lasting until they reach sexual maturity, and in the second phase, the growth rate is very low and grows very slowly [42]. As the cost of reproduction in females is very high, they end up dying with a smaller SVL, as the investment of energy for reproduction affects males differently than females. In principle, the assimilation of food is the same for both sexes, and also the capacity to feed is the same [41], so there is no trophic segregation between sexes. In metamorphic versus paedomorphic individuals, studies indicate habitat partitioning between morphs involving dietary differences [43].

Although sexual dimorphism in body size remains blurred, hidden in the large number of individuals and populations analysed, when we analyse the populations separately, sexual dimorphism is marked. Also for body size we can clearly observe differences in habitat type and ontogeny. If we analyse the eight variables together there is a pattern of differences between sexes, habitat, ontogeny and populations, with a very clear difference in body shape between males and females.

An effect of natural and sexual selection can be the morphological variation in *C. asper*, where habitat and ontogeny determines size and body shape. The great morphological variability of *C. asper* is the phenotypic plasticity in relation to the habitat or morph and can even modify its biological cycle or physiology depending on the characteristics of the environment.

Author Contributions: Conceptualization, S.C., N.O., D.S. and F.A.; methodology, S.C., N.O., D.S. and F.A.; software, S.C., N.O. and F.A.; validation, S.C., N.O., D.S. and F.A.; formal analysis, S.C., N.O. and F.A.; investigation, S.C., N.O., D.S. and F.A.; resources, S.C., D.S. and F.A.; data curation S.C. and F.A.; writing—original draft preparation, S.C.; writing—review and editing, S.C., N.O., D.S. and F.A.; visualization, S.C., N.O., D.S. and F.A.; supervision, S.C., N.O., D.S. and F.A.; project administration, S.C., N.O., D.S. and F.A.; funding acquisition, S.C. and D.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Biodiversity Conservation Plan of ENDESA, S.A. (ENEL Group).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 4

Genetic diversity, structure and connectivity of the populations of *Calotriton asper* in the Eastern Pyrenees

Manuscript in preparation

Genetic diversity, structure and connectivity of the populations of *Calotriton asper* in the Eastern Pyrenees

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ABSTRACT

The Pyrenees is a natural unit evolutionary and ecological for research of mountain fauna. The *Calotriton asper* is a salamandrid that mostly lives in fast running and cold mountain streams. The genetic structure of an amphibian species is strongly affected by its dispersal capacity of gene flow, that is a significant component of population structure and dynamics. Orographic features such as ridges and valleys can act as barriers or bridges to dispersal and thus give the genetic structuring. In the present work, we report in detail the genetic diversity of *C. asper* with nearby populations, the genetic structure among its populations and define whether mountains and rivers constitute a natural barrier between *C. asper* populations. With special emphasis on the degree of connectivity in small scale, using a total set of 144 individuals and 8 different localities around the mountain chain of Canigou and in its two tributary streams of “la Têt” (5 localities) and “le Tech” (3 localities) rivers, genotyped for 13 microsatellite loci.

The population genetics of *C. asper* in the Canigó massif shows a low genetic variation although with a high structure between some populations, evidencing the importance of topographic complexity in determining patterns of genetic structure. The structure of the eight populations are subdivided into two isolated populations and the other mixed populations distributed along two river basins. However, we have found connectivity between populations and a high dispersal capacity, with migrant’s individuals among populations, 6.25% of individuals. The isolation with geographical distance is not consistent with the population structure detected.

The genetic structure and diversity of *C. asper* was analysed in a periphery area of its distribution. The results are concordant with previous studies, that analysed the genetic patterns of diversity and structure of Pyrenean newt populations throughout their distribution, suggested that the species is adapted to mountain habitats, with a greater diversity in western populations. Our analyses evidence the importance of topographic complexity in determining patterns of genetic differentiation, indicating dispersal and population connectivity, marked by the presence of favourable habitats. In conclusion, *C. asper* seems to be adapted to high altitude mountain habitats, and its genetic diversity is higher in the western Pyrenees. In terms of conservation priority, we consider more

relevant the populations that represent a reservoir of genetic diversity. Therefore, the conservation of the habitats occupied by each population is key for the species survival.

1-INTRODUCTION

The genetic structure of a species is the result from the interplay of historical factors, such as demographic fluctuations, and current gene flow or connectivity (Hewitt and Butlin, 1997). For current distribution, genetic structure, and adaptations are important the phylogeographic history of species and populations (Hewitt, 2004). Climate conditions and geological changes represent selection forces, in the past and contemporary, have been the main drivers shaping the genetic population structure of species (Gutiérrez-Rodríguez, 2017), that can be used to infer its past dynamics (Hewitt *et al.*, 1997). Quaternary climatic oscillations played an important role in shaping the geographic distribution and genetic constitution of species in Europe (Hewitt, 2004). Glacial and postglacial periods have forced the species to go through several processes of contraction and expansion. Repeated contraction and expansion would accumulate genome differences and adaptations, effects of these events cause divergence and speciation to many organisms (Hewitt, 1996). These range fluctuations constitute its geographic distribution as well as its population genetic structure (Gutiérrez-Rodríguez, 2017). The Pyrenees played a major role in the dispersal routes of numerous temperate species as they were largely covered by ice during the glacial periods (González-Sampériz *et al*., 2006). The different climatic phenomena acting as a barrier or as a bridge for the migration of species. At the end of the glacial periods, Pyrenees became suitable niches and were colonized from few source populations (Hewitt, 2004). Thus represent excellent models to study the influence of climatic fluctuations on species diversification and colonisation (Schmitt, 2009). Climate change has an important impact on mountain ecosystems for various reasons, such as high-temperature variability, changes in seasonality, and variability in precipitation. This increase in the frequency of extreme weather events impacts populations (Schmeller *et al*., 2018). Climatic variations could affect species directly through their physiology (metabolic and reproductive processes) and indirectly through the ecosystem (prey, predators, life cycles, phenology) (Hughes, 2000). For species with restricted ranges, environmental changes can have serious consequences (Thomas *et al*., 2004) and especially for ectotherms, because of their dependence on the external temperatures from which they derive their energies (Araújo *et al.*, 2006). Indeed, environmental variations may modify the ecological niches of species. Depending on their dispersal and adaptive capacities, three predictions are usually made to estimate the fate of species: reduction, increase or change of distribution (Hughes, 2000).

Gradients such as altitude or longitude shape the existing suitable habitats along the mountains playing an important role in the Pyrenees (Conord *et al*., 2012). The western Pyrenees, the influence of the Atlantic Ocean provides a cooler and wetter climate than in eastern areas, which are more influenced by the Mediterranean temperate climate (Charrier *et al*., 2014). These gradients provide a variety of habitats suitable for colonization and may also have an impact on the genetic population structure by promoting genetic divergence of differentially adapted local genotypes (Savage *et al*., 2010). Dispersal of the gene flow is a significant component of population structure and dynamics (Tallmon *et al.*, 2004). The genetic structure of an amphibian species is strongly affected by its dispersal capacity (Smith *et al*., 2005). Dispersal is crucial for the survival of populations under changing conditions (Saccheri *et al*., 1998). A lack of connectivity between populations increases the risk of loss of genetic diversity (Ronce, 2007). For this reason, the genetic diversity of populations can be explained by processes such as isolation by the environment, between ecologically different habitats as a result of local adaptation, or by colonization, between all populations in the landscape caused by local genetic adaptation after colonization (Orsini *et al*., 2013). For example, bottlenecks occur in the populations, with successive range expansions from refuges when the temperature increased during interglacial periods (Valbuena-Ureña *et al*., 2013). The study of past evolutionary and phylogeographic processes and present-day gene flow and dispersal dynamics are necessary to understand the mechanisms to be the cause of contemporary genetic diversity and population structure (Epps *et al*., 2015). Orographic features such as ridges and valleys can act as barriers or bridges to dispersal and thus give the genetic structuring (Caplat *et al*., 2016). Phylopatric amphibian species with very restricted dispersal rates, such as *Calotriton arnoldi*, present highly structured and isolated populations (Valbuena-Urena *et al*., 2017). Dispersal is the unidirectional movement of an individual from a natal or breeding site to a breeding site with consequences on gene flow, with natal dispersal being the greater of the two (Clobert *et al*. 2009). It involves three successive behavioral stages: departure (emigration), movement, and installation (immigration) (Ronce, 2007; Clobert *et al*., 2009). Unlike dispersion, migration corresponds to regular movements between different places as seasonal migration, (Clobert, 2012) that is a movement of a periodic character, which implies a return to the point of departure (Cloudsley-Thompson, 1988). It should also not be confused with exploration or foraging movements (Van Dyck *et al*., 2005). Dispersal is an essential factor in maintaining species within their natural environments (Ronce, 2007), particularly in small populations where it mitigates the effect of drift. It has consequences not only on individual aptitude but also on the dynamics, population genetics, and distribution of species (Hanski, 1999).

The Pyrenean brook newt, *Calotriton asper* (Dugès, 1852)*,* is an endemic newt classified as near threatened, is a mainly aquatic montane species, inhabits different habitat types such as streams, alpine lakes, and caves at elevations ranging from 175 m asl to 3,000 m asl, occupying throughout the Pyrenees (Bosch *et al*., 2009). In this study, we use *Calotriton asper* species, to understand the role of landscape in its dispersion capacity, genetic diversity, and structure. Furthermore, *Calotriton asper* populations include hypogean (caves) and epigean (streams and lakes) habitats, exhibiting different life-history traits (Miaud *et al*., 2005; Camarasa *et al*., 2020). The ability of *Calotriton asper* to occupy new areas in the future will depend on its dispersal capacity (currently poorly known), as well as on the existence of suitable dispersal pathways (Araújo *et al*. 2006). The dispersion phase following metamorphosis occurs during the terrestrial phase, which coincides with its juvenile period, before maturity. Finally, individuals return to the aquatic phase as breeding adults. It is unclear how far individuals do disperse at this phase, and for how long they disperse. The terrestrial lands surrounding freshwater habitats can represent natural barriers to the dispersal of aquatic organisms, which can affect divergence patterns (Isselin-Nondedeu *et al*., 2017).

DePous *et al*. in 2016 have already shown using different dispersion models that future climate change would have an impact on the genetic diversity of *Calotriton asper* by reducing it as well as a loss of range. To study dispersal in *Calotriton asper*, with techniques such as Capture Marking Recapture (CMR) or radio-tracking, is complex to implement in species. Since it would have to be monitored during its juvenile stage, in the terrestrial phase, in the period between metamorphosis and sexual maturation. Genetic analysis of gene flow between populations can be an indirect way of studying it. The study of molecular markers provides data on the genetic structuring of populations to define the pattern of gene flow and dispersal of individuals (Bohonak, 1999). Microsatellite loci are still currently markers for understanding population structures (Palo *et al*., 2004). Like many amphibian species, *Calotriton asper* was thought to have low dispersal ability (Milá *et al*., 2010; Montori *et al*., 2008), although if we pay attention to this aspect, we would have to evaluate the distance and time of dispersal for the species. By contrast, other amphibians, such as *Triturus cristatus* is regarded as a species with a great capacity of dispersion (Denoël *et al*., 2018). In the case of *Calotriton asper*, following metamorphosis, a terrestrial phase of juvenile dispersal of at least 2 years is described before reaching the adult stage (Montori *et al*., 2014), but it remains unclear how far individuals can disperse. A good approximation of dispersal time is the time between metamorphosis and sexual maturity, when their terrestrial phase ends and their aquatic phase begins. In different studies (Miaud *et al*., 2005; Camarasa *et al*., 2020) using the skeletochronology technique, ages at sexual maturity have been obtained for several populations, in different habitats, obtaining values between 2 and 9 years for streams.

Currently, there are several studies published on the genetics of *Calotriton asper* that has examined its genetic population structure and phylogeography with different molecular markers: Analysis of allozymes and Cytochrome b (Montori *et al*., 2008); mitochondrial DNA and genome-wide amplified fragment length polymorphism (AFLP) (Milá *et al*., 2010); mitochondrial DNA, Cytochrome b and nuclear DNA sequence data (RAG-1) (Valbuena-Ureña *et al*., 2013); mitochondrial DNA, Cytochrome b (Oromi *et al*., 2014); and microsatellite markers (Valbuena-Ureña *et al*., 2018; Oromi *et al*., 2018; Lucati *et al*., 2020). Is expected that *Calotriton asper* exhibited relatively low levels of genetic diversity. However, high levels of genetic diversity and differentiation among localities have been found using genetic markers. However, these studies were either based on the characterization of populations in the entire range, on a large scale, and habitat types. Our study is addressed specifically on the characterization of stream populations close to each other, on a small scale of territory, in the eastern part of Pyrenees, in the Canigou massif, and its two lateral basins.

Here, we apply microsatellite loci aimed to make known the present genetic constitution of *Calotriton asper* in a small part of the periphery of its distribution range. Dispersal analyses of individuals to determine the degree of connectivity of populations. Specifically, we explore the effect of orography on the genetic diversification of populations. Furthermore, we try to understand how dispersal or the orography played a role in determining the species' current genetic structure. We want to infer the dispersal ability of the species, from the analyze the current genetic patterns of diversity and structure of the *Calotriton asper* by 8 sampling localities around the Pyrenean mountain chain of Canigou and based on 13 species-specific microsatellite loci. Our study aims to

(1) characterize the genetic diversity of each *Calotriton asper* population, (2) analyze the genetic structure among its populations, (3) to define a dispersion pattern, define whether mountains and rivers constitute a natural barrier between *Calotriton asper* populations.

2. MATERIAL AND METHODS

2.1. Sampling and DNA extraction

A total of 144 DNA samples (buccal swabs) from *Calotriton asper* were collected from 8 localities on the eastern edge of the French Pyrenees during summers of 2015 and 2016. The localities were distributed in tributary streams of “la Têt” (five localities) and “le Tech” (three localities) rivers, covering the Canigou massif (Figure 1 and Table 1). Fieldwork consisting of about 32 night surveys along the streams, investigating one night per locality in order to capture newt samples. Newts were captured by hand using a hand net and kept in a mesh bag until data collection. For each captured individual, DNA was collected in a non-invasive manner using “buccal swabs stick”, without any damage for the animal. These saliva samples were then placed in 1.5 ml Eppendorf tubes containing 70% ethanol. The samples were stored at -4°C until DNA extraction. All individuals were directly released at the place of capture a few minutes after sampling.

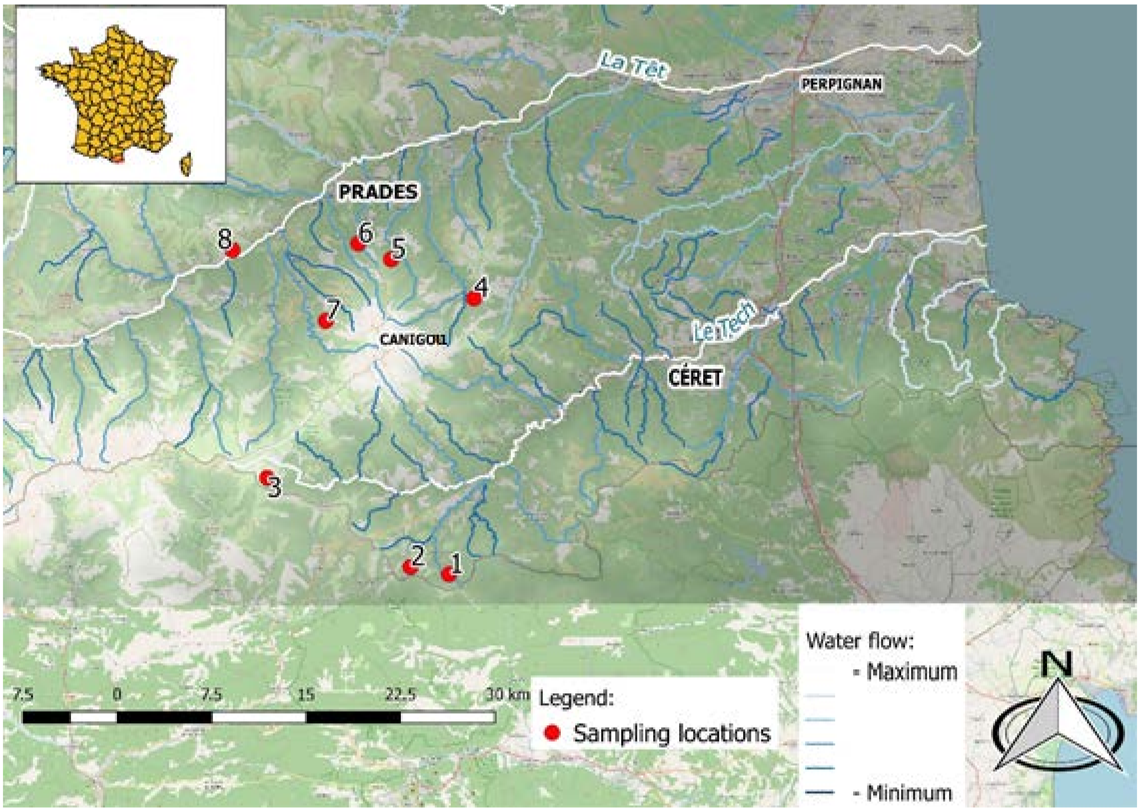


Figure 1. Geographic localization of the eight sampled localities of *Calotriton asper* (see Table 1 for details.

Locality	N	Altitude (m)	Latitude	Longitude
1- Mas Lori	20	805	42.346.743	2.521.951
2- Lamanera	18	1011	42.350.674	2.492.005
3- Prats Mollo	19	1605	42.410.007	2.362.066
4- Valmanya	21	939	42.537.021	2.554.505
5- Mas Malet	10	789	42.571.462	2.470.293
6- Clara	20	549	42.576.497	2.443.680
7- Casteil	17	1139	42.525.500	2.406.683
8- Serdinya	19	662	42.572.941	2.321.007

Table 1. Name and number of the sampled localities (see Figure 1). Number of individuals (144 in total), altitude, latitude and longitude are given for each locality.

2.2. Microsatellite analysis

DNA was extracted from buccal swabs using a QIAGEN kit, following the manufacturer's instructions at the platform GEMEX (*Génomique, Ecologie Moléculaire, Evolution eXpérimentale*) in the Center of Functional and Evolutionary Ecology (CEFE, Montpellier, France). We used 13 microsatellite loci described specifically for *Calotriton asper* (Dreschler *et al*., 2013) that were amplified by Polymerase Chain Reaction (PCR) in 3 multiplexes (see Table S1).

Locus Name	Primers	Repeat motif	Multiplex Annealing Tº	Locality with null Alleles	Number of Alleles	He	Ho	Fis
Ca1	F: TGGAACAGATGGCGTTGTAA R: TTCCTGCAACCTCCTTGTCT	(AGAT)$_{16}$	M1 60ºC		7	0.38	0.32	0.15
Ca7	F: ACCCTTACACACCCCAAACC R: GTTCCCTGCATGGCTCTAAA	(AGAT)$_{16}$	M1 60ºC	Valmanya	7	0.47	0.47	-0.00
Us7	F: CTGCACCGATTAATTGCAGA R: CTGCACCACTCGCTCCTC	(ACAT)$_{16}$	M1 60ºC		7	0.25	0.21	0.18
Ca38	F:CCTGTTAGGTGAAGGTGAGCA R: CTGGTAGCCATGCGCTTTAT	(AATG)$_{12}$	M1 60ºC		9	0.50	0.57	-0.15***
Ca21	F: AGCGTGTGCAGCAGTATCC R: GCAATGTGCCATTCATTACC	(AGAT)$_{12}$	M1 60ºC	Valmanya, Casteil, Serdinya	15	0.65	0.51	0.22***
Ca30	F: TCACACATCATGCAGCTTACC R: GACCCTCATGGGTGTGTAGC	(AATC)$_{10}$	M2 60ºC		5	0.17	0.23	-0.37**
Ca32	F: ACAGGGCAAGAGAGTCAACG R: CAGCCTATTGGCTTGTCAGC	(ACAG)$_{10}$	M2 60ºC		6	0.45	0.55	-0.22
Us2	F: TGGGCTGAAGGATTGAAAAA R: CTCAGCTGCAGTGGTGTGTT	(AGAT)$_{17}$	M2 60ºC	Mas Lori	12	0.35	0.31	0.11
Ca20	F: CAGCGGTAATACCATCAGGA R: CCACAGATCCTTCTGCAACA	(AGAT)$_{15}$	M2 60ºC	Serdinya	8	0.47	0.43	0.10*
Ca29	F:TCCATAAGCCATTATTGTGTGC R: AGTGCACTGCCTCAGCATGT	(AATC)$_{10}$	M3 64ºC		6	0.20	0.16	0.18
Ca8	F: AGAAGGGAGTCAGGCAGACA R:GGAGGATCAAATGTGTTTGGA	(AGAT)$_{13}$	M3 64ºC		10	0.46	0.46	-0.01
Us3	F:AAGTTTGTAGGTATGCATAATAGCC R: GGAAGTCCAGGCCTGTAGAC	(AGAT)$_{16}$	M3 64ºC		11	0.54	0.55	-0.01
Ca35	F: GGCGCTTTACAAGTGCTACC R: CTGCCACAAGGTAGAGGTCA	(ACTC)$_{14}$	M3 64ºC		5	0.12	0.13	-0.11

Table S1. Genetic diversity indices for each of the 13 polymorphic loci analyzed for the 8 *Calotriton asper* populations (144 individuals). Ho = observed heterozygosity; He = expected heterozygosity; Fis = inbreeding coefficient: * significant ($P < 0.05$), ** very significant ($P < 0.01$) and *** highly significant ($P < 0.001$).

Multiplex PCRs were conducted in a 10 µl reaction volume containing 2 µl of H_2O, 5 µl of the Master Mix solution from Qiagen (PCR buffer), 1 µl of each multiplexed primer at 2 µM and 1 µl of DNA. All microsatellite loci were amplified with the following cycling: a denaturation step of 20 minutes at 95 °C, followed by 35 cycles of denaturation (94°C, 30 seconds), annealing (90 seconds at 60°C for Multiplex 1 and 2, and 64°C for Multiplex 3, Table S1), elongation (1 minute at 72°C), and ending with a final elongation step of 30 minutes at 60°C. Correct DNA amplification was evaluated by agarose (2%) gel electrophoresis. PCR products were genotyped in a 16 capillary sequencer (3130 xl Genetic Analyzer, Applied Biosystems) at the “Genotyping-sequencing” platform at the LabEx CeMEB (Montpelier, France). For each sample, electropherograms were visualized and scored manually using the GeneMapper v4.5 software (Applied Biosystems). To reduce genotyping errors, two independent readings were performed by two different people (S. Camarasa and V. Arnal). As null alleles may result in an overestimation of population differentiation, all loci were checked for the presence of null alleles using the Micro-checker v.2.2.3 program (Van Oosterhout *et al*. 2004). Evidence of linkage disequilibrium (LD) was tested using Genepop v.4.2 software (Rousset, 2008) for each pair of loci in each locality.

2.3 Genetic diversity and population structure

For each sampling site (locality), the level of genetic diversity was measured with GenAlEx v 6.3 (Peakall and Smouse, 2012) through several estimators: number of alleles per locus (Na), number of private alleles (PA), the observed (Ho) and the expected heterozygosity (He; Nei, 1978) and the inbreending coefficient (F_{is}). Hardy-Weinberg Equilibrium (HWE) deviations were tested using Genepop v.4.2 software (Rousset, 2008), by estimating the differences between observed and expected heterozygosity with a Markov chain dememorization of 1000, 200 batches and 1000 iterations per batch.

The number of homogenous genetic units and population structure, was evaluated using Bayesian clustering method implemented in Structure v.2.3.4 (Pritchard *et al*., 2000). This software estimates the likelihood of a specific number of homogenous genetic clusters (K) in the data set, and the relative contribution of each individual to each cluster, without a priori information of the individual’s geographical location (no Locprior option). The data was analyzed under the admixture model with Monte Carlo Markov Chain (MCMC) run of 10^6 iterations and a burn-in period of 10^5 for K varying from 1 to 9, that is the number of sample locations plus one (8+1). Population structure was also tested for each river basin (Le Tech and La Têt) using the same procedure for K varying between 1 and 5. For each value of K, 10 runs were performed to test for the MCMC convergence with Structure. The most likely number of genetic groups (K) was inferred directly from the graph of the likelihood values as a function of K, as well as by the Evanno’s method (Evanno *et al*., 2005) based on the Delta K function calculated using Structure Harvester v0.6.8. (Earl and Von-holdt, 2012). The graphical presentation of the results was developed using the Cluster Markov Packager Across K in CLUMPAK (Kopelman *et al*. 2015).

The number of genetic cluster was also assessed by applying a Discriminant Analysis of Principals Components (DAPC) allowing to determine the most likely number of genetically close populations. The DAPC was carried out using the *adegenet 2.1.3* package with the *find.cluster* function (Jombart, 2008) on the R 3.1.1 software following the recommendations of Jombart (2015). Different clustering solutions were compared using the Bayesian Information Criterion (BIC), the optimal K corresponding to the BIC value from which the curve forms a "bend". The minimum value of BIC before the first increase or stabilization indicates the best-supported number of genetic clusters. The optimum number of clusters (K) was determined with the function *dapc* using the sampling locality as a prior (Jombart *et al*., 2010). We ran DAPC using all the available discriminant functions and calculated the assignment probability of individuals to each cluster by K-means analyses, used to infer the best-supported clustering solution.

Relationships between genetic and geographic distance matrices were calculated using Isolation by distance (IBD) was calculated and tested using the Mantel test by examining the relationship between a matrix of genetic distances (Fst) and a matrix of Euclidian geographic distances, using the function *ibd in adegenet*.

To examine the distribution of the genetic variation in the dataset, an Analysis of Molecular Variance (AMOVA) were performed using the software GenAlEx V6.5. (Peakall and Smouse, 2012). The percentage of genetic variation between populations as well as between individuals within populations was calculated, in order to quantify the fraction of total genetic variance between populations and within populations.

To analyze the differentiation between populations at allele frequencies, the level of genetic divergence between groups was estimated by calculating the pairwise F_{ST} (Wright, 1978) using FSTAT version 2.9.3.2 (Goudet, 2001).

The number of potential migrants among populations was assessed with GENECLASS2 (Piry, 2004). This program estimates the recent migration rate (first generation) using the frequency-based classification method and tests the assignment of individuals to a population other than their population of origin, based on the analysis of multilocus genotypes (Paetkau *et al*., 1995). Probability calculation was resampling Monte-Carlo using an error I (alpha) of 0.01 and 1000 simulated individuals (Paetkau *et al*., 2004).

The occurrence of genetic barriers among collection sites was evaluated using the software Barrier v.2.2 (Manni *et al*., 2004) which explore the existence and location of barriers to gene flow. The analysis used the Monmonier's maximum difference algorithm which compare geographical coordinates and genetic distances (Fst) between all populations in order to identify genetic barriers within the data set.

The effective populations size (Ne) for each population was calculated using Colony 2.0.6.4. (Jones and Wang, 2010). The method implemented used a sibship assignment taking into account possible genotyping errors and presence of null alleles. The software was run using the maximum likelihood approach for a dioceous and diploid species, with medium length runs and under the assumption of random mating, assuming polygamy for both males and females (as it is the case for most salamanders) with no sibship prior.

3- RESULTS

3.1- Genetic diversity

Regarding the microsatellite markers, all loci were polymorphic (Table S1) with a number of alleles ranging from 5 (Ca30 and Ca35) to 15 (Ca21). The presence of null alleles across all loci was detected for six loci and in four populations (Us2 in Mas Lori, Ca20 in Casteil, Ca7 and Ca21 in Valmanya, Ca20 and CA21 in Serdinya; see Table S1). A maximum of two null alleles per population was detected in two populations. We found no evidence for significant deviations from HWE or LD, does not reflect deviations from random mating or the presence of null alleles.

Overall, the level of genetic variation using several indices based on the 13 locus is presented as a summary in the Table 2. The average number of alleles is 2.82 with the lowest value (Na=2.0) was found in Mas Malet and the highest value (Na=3.92) in Mas Lori. For the allelic richness, the average was 2.30, values were found to be between 1.73 and 2.99. Values of observed (Ho) and expected (He) heterozygosity of *Calotriton asper* also differed between populations, with an average of 0.38 for both of them (Table 2). The lowest value of Ho was found for Casteil (Ho=0.24) while the highest is observed in Mas Lori (Ho=0.52). The lowest value of He was found in Prats Mollo (He=0.29) and the highest in Mas Lori (He=0.54). The average number of private alleles (PA) was 6, with the maximum number of alleles observed in the population of Serdinya (PA=13) and the minimum number in Castel (PA=1). The mean inbreeding coefficient (Fis) was estimated to be 0.01 with four populations showing significant values. Five populations presented a positive Fis values (from 0.02 to 0.19), which means a deficiency of heterozygotes whereas three populations revealed negative Fis values (from -0.01 to -0.18) indicating a heterozygotes excess. A deficit in heterozygotes with significant Fis values was noted for the populations of Mas Lori, Lamanera and Valmanya while a significant excess of heterozygote was found in Prat Mollo. The effective population sizes (Ne) estimated with Colony returned in general low values as Ne ranged between 8 (Mas Malet) and 30 (Lamanera), (Table 2).

Population	N	Na	Ar	AP	H_o	H_e	F_{is}	Ne
Mas Lori	20	3.92	2.99	6	0.52	0.54	0.04*	19
Lamanera	18	2.92	2.35	4	0.32	0.40	0.19*	30
Prats Mollo	19	2.23	2.20	6	0.32	0.29	-0.01**	29
Valmanya	21	3.15	2.39	10	0.34	0.37	0.02***	29
Mas Malet	10	2.00	1.79	1	0.38	0.32	-0.18	8
Clara	20	3.23	2.34	7	0.47	0.40	-0.16	29
Casteil	17	2.08	1.73	1	0.24	0.30	0.13	14
Serdinya	19	3.00	2.63	13	0.42	0.46	0.05	30
Total	144	2.82	2.30	6	0.38	0.38	0.01***	54

Table 2. Genetic diversity indices within each studied population of *Calotriton asper*. N= sample size; Na = number of alleles; Ar= allelic richness; AP= number of private alleles; Ho= observed heterozygosity; He= expected heterozygosity; Ne, effective population size; Fis = inbreeding coefficient: * significant ($P < 0.05$), ** very significant ($P < 0.01$) and *** highly significant ($P < 0.001$).

3.2- Genetic structure

The AMOVA analysis based on the 8 populations revealed that the genetic diversity is not equally distributed between the three groups (Figure 2). The highest percentage of diversity variation exists among individuals (48%) followed by the variance contributed within individuals (32%), while minimum diversity is observed between populations (20%).

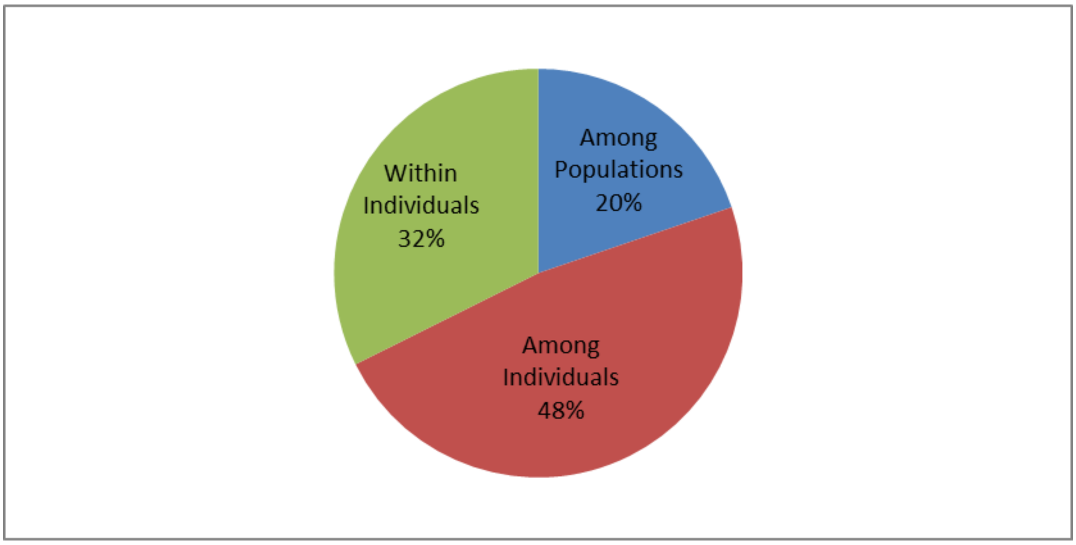
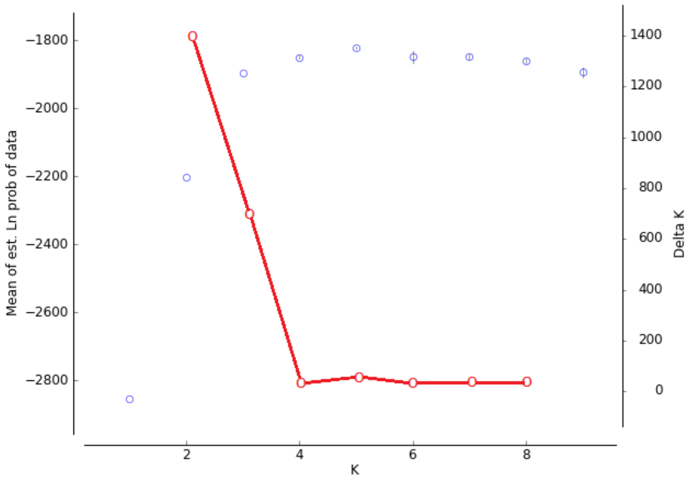


Figure 2. Percentages of Molecular Variance (AMOVA).

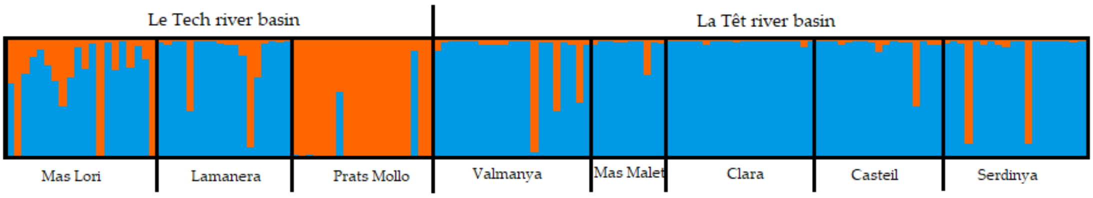
The Bayesian clustering analysis performed with Structure on the144 individuals from eight populations, indicated the highest likelihood for K=3 whereas the delta K approach grouped the samples into two genetically distinct clusters (Figure 3.a). For K=2 the analysis mostly identifed Prats Mollo as one cluster whereas the seven other populations constituted the second cluster (Figure 3.b). For K=3, (Figure 3.c) Prats Molo, (Mas Malet + Clara) and Serdinya represented separate clusters whereas other populations are more admixed. Notably, there between the two river basins as revealed by the mixted of Mas Lori, Lamanera and Valmanya populations. It can be noted that some individuals from the Serdinya cluster are found in four populations (Casteil, Valmanya, Mas Malet and Lamanera).

Refining the analysis for each river revealed a number of clusters k=2 for each basin and essentially the same results are observed. In Le Tech river basin, a strong structure is observed for Prat Mollo, and Lamanera and Mas Lori together in one cluster (Figure S1a). In La Têt river basin Serdinya is clearly differenced from the other four populations grouped in another genetic cluster (Figure S1b).

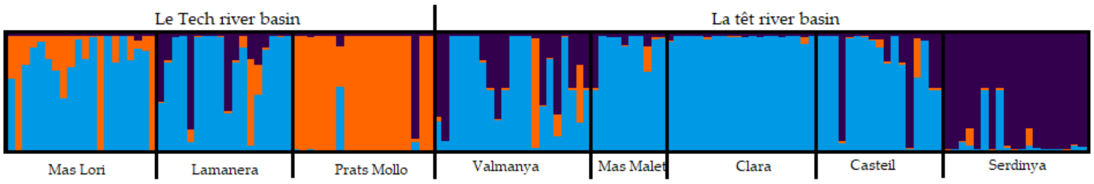
These analyses indicate that there is some admixing at the scale of each river basin, such as between Prats Mollo, Lamanera and Mas lori (Le Tech basin) or between Serdinya, Casteil and Valmanya (La Tet basin) whereas in the same basin, there is little exchange between Serdinya and Mas Malet or Clara. Finally, there is also trace of exchange between basins as suggested between Serdinya and Lamanera.



3.a.

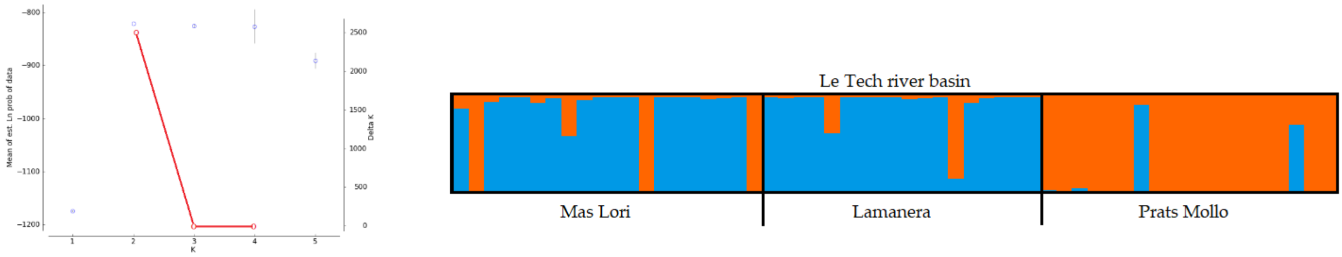


3.b.

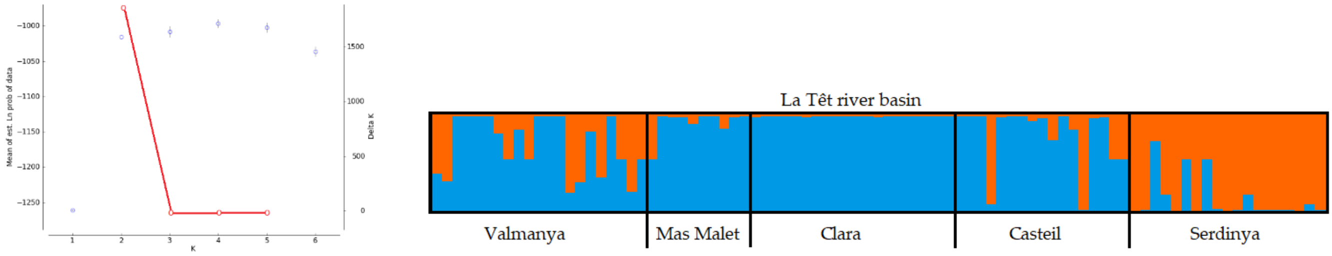


3.c.

Figure 3. Graphics representation of the estimated probability of data for each K value for global dataset of the 8 *Calotriton asper* populations. Delta K, red line and right axis. Mean log likelihood of the data (L(K)) suggesting a population structure of K, blue dots and left axis (Figure 3a). Genetic structure of the 8 populations, STRUCTURE plots. Each thick vertical bar represents an individual, and the color composition displays the probability of belonging to one of the main clusters of the Le Tech and La Têt basins with k=2 (Figure 3b), or k=3 (Figure 3c). The thin vertical black lines delineate the predefined populations in the two basins of *Calotriton asper*.



S1a.



S1b.

Figure S1. Graphics representation of the estimated probability of data for each K value for each basin. Delta K, red line and right axis. Mean log likelihood of the data (L(K)) suggesting a population structure of K, blue dots and left axis. STRUCTURE plots for each basin, each thick vertical bar represents an individual, and the color composition displays the probability of belonging to one of the main clusters. Three populations in the Le Tech basin with K=2 (Figure S1a) and, five populations in the La Têt basin with K=2 (Figure S1b). The thin vertical black lines delineate the predefined populations of *Calotriton asper*.

Population differences were also assessed using a discriminant analysis (DAPC)which shows a number of clusters of K=6 (see BIC plot) as determined with the lowest BIC (Figure S2) value obtained with the *find.clusters* function. The DAPC analysis was then carried out using six clusters (Figure 4.a). Overall, DACP was carried out by keeping 50 PCs representing more than 80% of the cumulative variance. The first component separate cluster number 6 from the other clusters, while the second component separates the remaining populations from the clusters number 2 and 6, with population number 2 being the most distant (Figure 4.a). The allocation of the individuals from the 8 populations among the 6 clusters (Figure 4.b and S3), revealed that clusters 2 and 6 mostly correspond to Serdinya and Prats Mollo, respectively. By contrast, cluster 1 is the most admixed cluster that includes individuals from all eight populations.

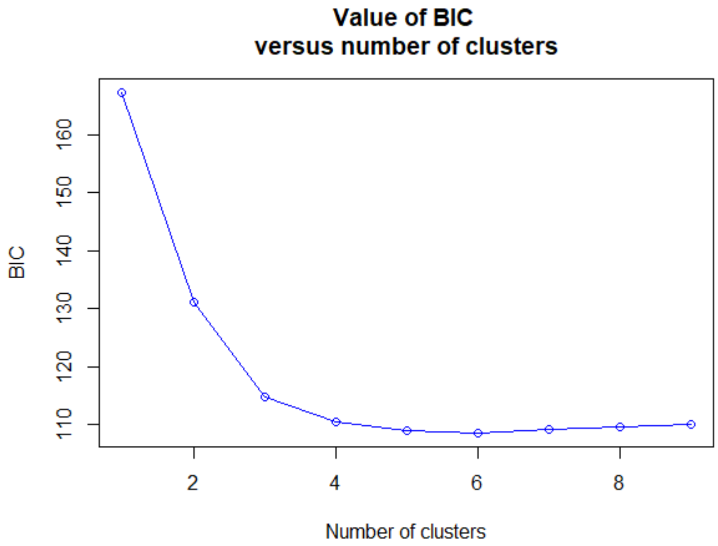


Figure S2. Bayesian Inference Criterion (BIC) values versus numbers of clusters (K), suggesting that K = 6 was the most likely number of genetically distinct clusters.

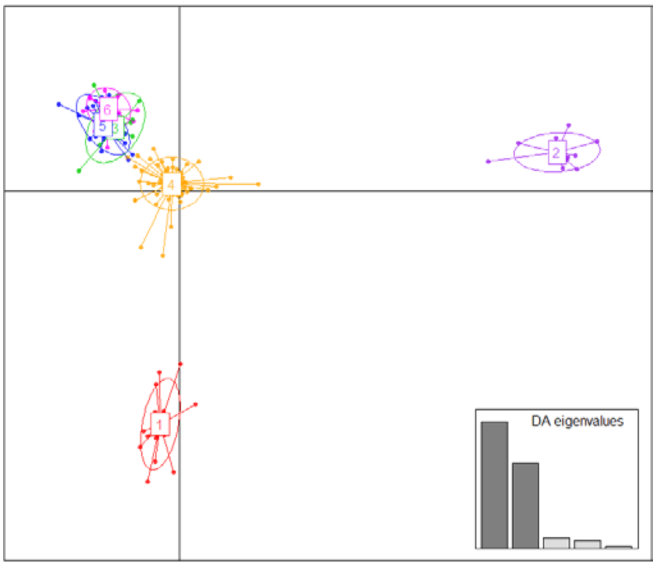


Figure 4.a. Discriminant analysis of principal components (DAPC) for 144 individuals of 8 populations. Eight PCs and six discriminant eigenvalues were retained during analyses, to describe the relationship between the clusters. The axes represent the first two Linear Discriminants (LD). Each dot represents one individual of *Calotriton asper* and colors and inertia ellipses indicate their assignment to one of the six genetic clusters inferred by DAPC. The bottom-right inset displays in relative magnitude the variance explained by the two discriminant axes plotted (in dark grey).

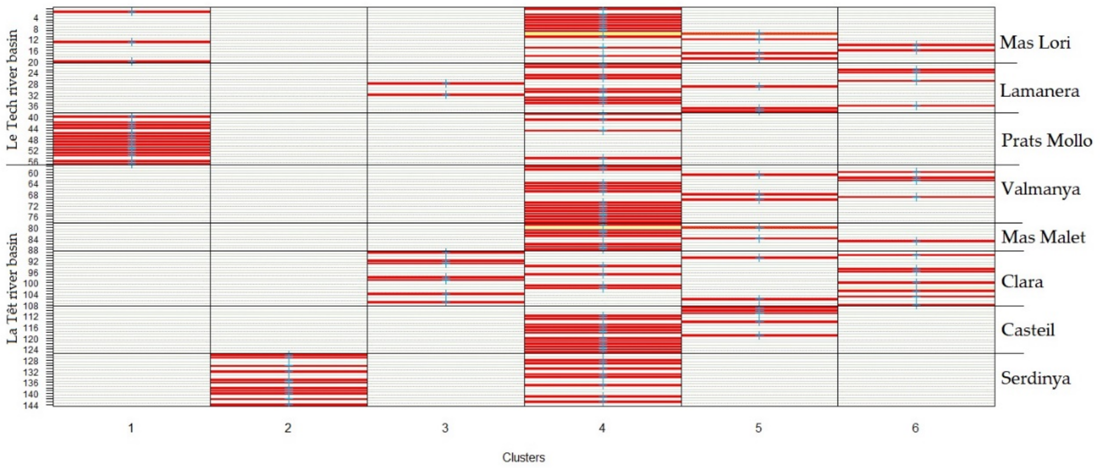


Figure S3. Summary of the results of discriminant analysis of principal components (DAPC). Panel represents the individuals (rows) were assigned to the genetic cluster where they were included (columns). Colors represent membership probabilities to each genetic cluster (red= 1, orange= 0.75, yellow= 0.25 and white= 0) and blue crosses indicate the cluster where the individuals were originally assigned by K-means analyses.

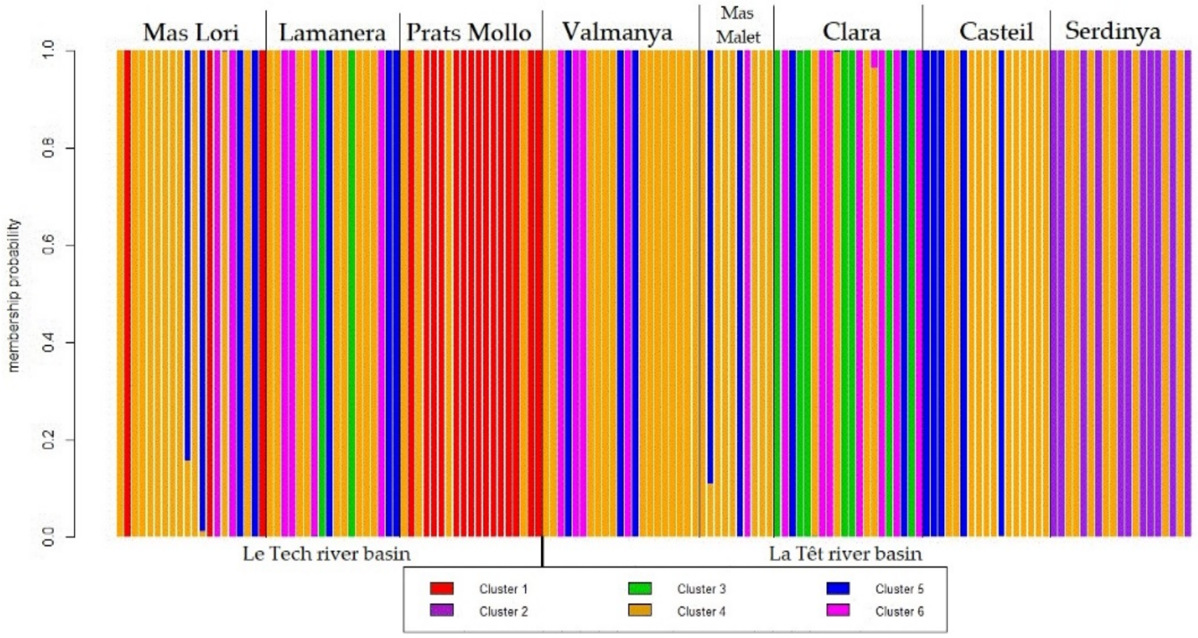


Figure 4.b. Bar plot showing for each individual: the probabilities of membership in 6 clusters determined by DAPC; the probabilities of membership in clusters. Each individual is represented as a vertical bar, with colors corresponding to membership probabilities to clusters.

The Mantel test did not show a significant correlation between genetic distance (Fst) and geographical distance between the 8 populations studied (Mantel test: 0.257, p value = 0.102), showing an unsignificant IBD (Figure 5). The scatterplot of geographic vs. genetic distance clearly shows one single consistent cloud of point, that depict the density between points. Densities between points in a scatterplot can be used to determine whether the data originated from a single genetic cline or from two or more distinct clusters.

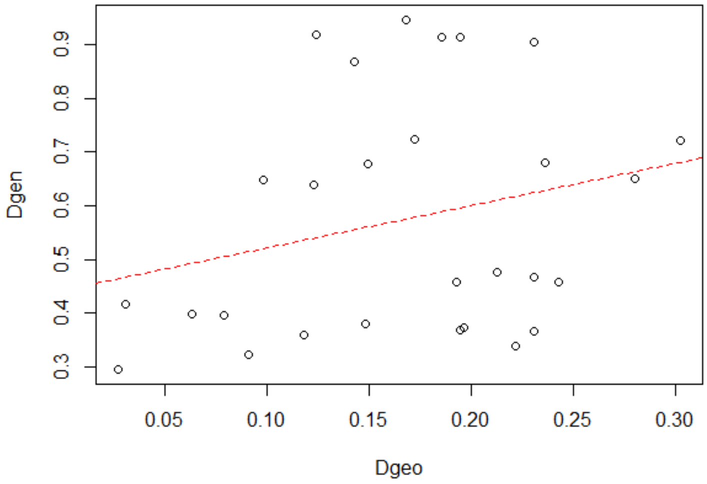


Figure 5. Graph representing the relationship between geographical distance (Dgeo) between populations and genetic differentiation (Dgen) estimated by Nei's distance. Genetic differentiation between populations (Fst) as a function of the geographical distance between these same populations in order to analyze the isolation by distance in *Calotriton asper.*

The differentiation between the 8 populations was estimated by Fst that ranged from −0.0049 (Mas Malet and Valmanya) to 0.6320 (Prats Mollo and Casteil) (Table 3). When looking at the Fst per population pair, some populations are undifferentiated with a value of Fst < 0.1 as Mas Malet with Clara, Valmanya, Lamanera, Casteil and Mas Lori. The high Fst values between populations suggest a strong structuring of the *Calotriton asper* species. There is therefore a high variance in allelic frequency between populations within their range. Moreover, Fst values greater than 0.25 are observed between very close localities as Prat Mollo with Lamanera and Mas Lori or Serdinya with all the other populations, suggesting a strong separation of populations even when geographically close, which is confirmed by the IBD. Only the Fst values for the site pairs Clara with Mas Lori, Lamanera and Valmanya, and Serdinya and Lamanera were significant genetic differentiation (Table 3).

	Mas Lori	**Lamanera**	**Prats Mollo**	**Valmanya**	**Mas Malet**	**Clara**	**Casteil**	**Serdinya**
Mas Lori	0.0000	0.1111	0.3757	0.1022	0.0910	0.1377*	0.1112	0.3337
Lamanera		0.0000	0.6114	0.0723	0.0082	0.0824*	0.0877	0.3239*
Prats Mollo			0.0000	0.6234	0.6309	0.6185	0.6320	0.5837
Valmanya				0.0000	-0.0049	0.0650*	0.0598	0.3763
Mas Malet					0.0000	0.0031	0.0895	0.3593
Clara						0.0000	0.0941	0.3434
Casteil							0.0000	0.3960
Serdinya								0.0000

Table 3. Values of the Fst fixation indexes between pairs of populations. Fst = * significant (P< 0.05).

3.3- Landscape genetics

Of the 144 individuals tested, GENECLASS2 identifies 9 potential migrants (black arrow in Figure 6) corresponding to 6.25% of the total number of individuals analysed. Most migrants come from the Mas Lori (3), and followed by Clara (2) populations. It is also remarkable that the populations of Valmanya and Casteil receive more individuals and only the population of Mas Malet does not have any kind of migrant.

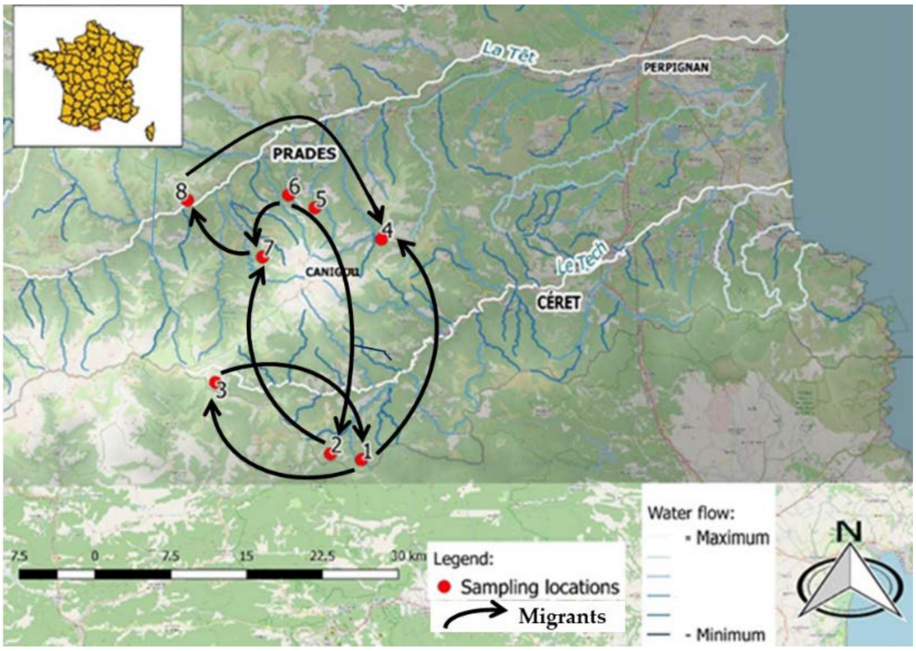


Figure 6. Map showing the different populations of *Calotriton asper* and potential migrants. The potential migrants are shown in black arrows. The numbers correspond to the populations, see table 1 for more information.

The genetic barrier prediction analysis for eight populations revealed two likely barriers against gene flow (red lines a and b in figure 7). These analyses give some clues about the possible shape of the genetic landscape. The first genetic barrier (aa, in figure 7) isolated Prats Mollo from its surrounding populations (red line). The second barrier (bb, in figure 7) separated Serdinya from neighbouring populations. The first genetic barrier does not seem to be closely related to the presence of mountains and river systems of Le Tech. On the other hand, the second genetic barrier seems to be related to the river barrier of La Têt. Barrier software revealed the occurrence of breaks in the genetic flow, analysis of all microsatellite loci simultaneously using Fst identified two possible barriers. The derived genetic barriers are closely related with the presence of mountains and river systems, corroborating the findings of structure analysis for K = 2 in each river basin.

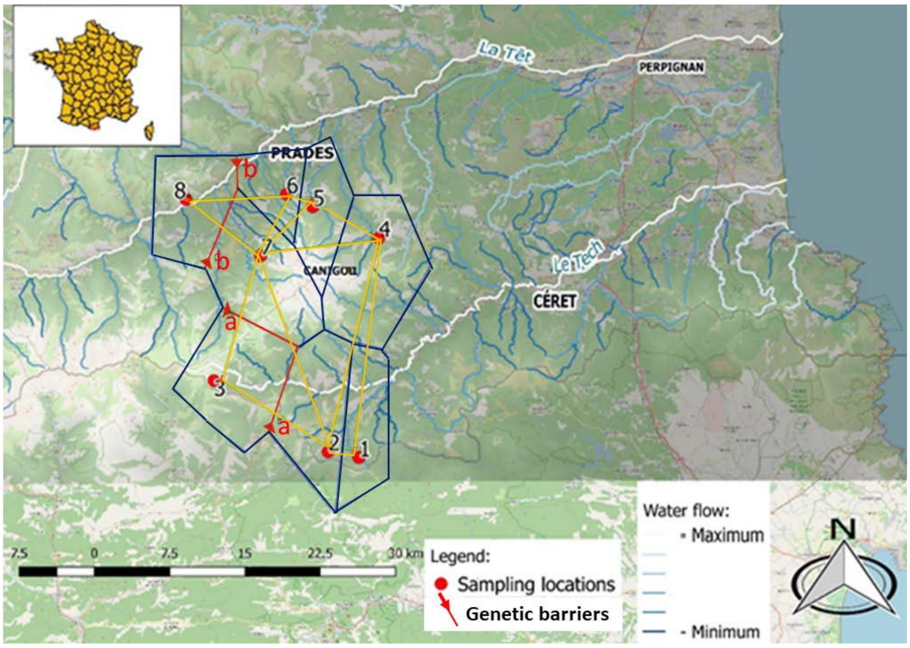


Figure 7. Map showing the different populations of *Calotriton asper* and genetic barriers identified. The genetic barriers are shown in red lines with arrows. Lines a and b indicated genetic barriers. The lines corresponding Voronoï tessellation (in dark blue) and Delaunay triangulation (in yellow). The numbers correspond to the populations, see table 1 for more information.

4-DISCUSSION

The results from population genetics allowed us to found low genetic variation although also a genetic structure. Overall, the results obtained by different approaches have provided relevant and congruent insights about the strong connectivity between populations, due to a high dispersal capacity of the species *Calotriton asper*.

In this study we assess the pattern of genetic diversity and differentiation observed between eight populations of *Calotriton asper* that we have identified on the basis of 13 polymorphic microsatellite loci. We have analysed a large genetic data set to investigate connectivity between populations and infer the dispersal capacity of the species in a mountain context.

Considering the proximity of all the populations, genetic differentiation with some populations was relatively high. Although, as the number of migrants found in this study was high, what these dispersers ensured the connectivity among populations of *Calotriton asper*.

4.1- Genetic diversity

Microsatellite loci analysis from east of the Pyrenees revealed less levels of genetic diversity in *Calotriton asper* populations (He= 0,38 and Ar= 2.30) compared to others *Calotriton asper* studies of microsatellite in the central and western part such as (Valbuena-Ureña *et al*., 2018) (He= 0,52 and Ar=2.93) and (Lucati *et al*., 2020) (He=

0,46 and Ar= 2.66). This less levels of genetic diversity is because our studied populations are at the eastern end of their distribution. This part of the Pyrenees studied, is influenced by the Mediterranean climate that provide higher temperatures and drier conditions than western oceanic influenced sectors, which provide more suitable habitats for *Calotriton asper* (Montori and Llorente, 2014). Our results are in line with that the populations at the periphery of a species distribution tend to have lower genetic diversity than the central populations, probably attributable to suboptimal habitat, greater isolation, founder effects and/or genetic bottleneck (Zhan *et al*., 2009). Some alleles are only found in certain populations (Table 2), indicating allele fixation as well as strong isolation of populations as a Serdinya with 13 private alleles, could be isolated from all other study populations by the La Têt river. Also Valmanya population has 10 private alleles. These levels of privat alleles was higth compared to populations of *Calotriton asper* study (Valbuena-Ureña *et al*., 2018). In contrast, the common alleles between the distant populations would then be remnants of ancient large population or some interconnected populations as Mas Malet and Casteil (Table 2).

Our populations show low Fis values. Indeed, He and Ho are very close, which suggests little deviation from EHW. Departure from HWE which might be due to both the systematic (selection, migration and mutation) and dispersive (genetic drift and inbreeding) forces operating in the population. Also could be attributed to the presence of unobserved null alleles (non-amplifying alleles). Low inbreeding coefficient (global Fis = 0,01) in comparison with microsatellite studies carried out for the same species (Valbuena-Ureña *et al*., 2018) (Fis = 0,36) and (Lucati *et al*., 2020) (Fis = 0,07), which suggests a very high genetic diversity within populations. A number of factors such as null alleles, nature of locus or inbeending may lead to deficiency of heterozygotes. The three populations of the Tech basin show significant values, two positive, Mas Lori and Lamanera and one negative Prats de Mollo, although close to zero, showing inbreeding and outbreeding respectively. This could be because these populations are on the periphery of the current distribution, which would favour these results. Current genetic diversity between closely populations can also be explained by their evolutionary history, like colonisations. The significance of the Fis values for Valmanya, Prats Mollo, Lamanera and Mas Lori may be explained by a possible recent founder effect, probably attributable to colonisations (Table 2). This would suggest that the river basin of Le Tech is of recent origin, supporting the idea of recent colonization, most likely by populations from the south and west.

In terms of maintaining genetic diversity, a small effective population size may not necessarily be a limiting factor, because life history strategies is another factor modulating the levels of genetic diversity of amphibians (Fouquet *et al*., 2015; Paz *et al*., 2015). Effective population sizes for the *Calotriton asper* populations were similar or higher than those of the *Calotriton arnoldi* populations or other urodele amphibians (Valbuena-ureña *et al*., 2017). Populations with a very low Ne, as Mas Malet (Ne= 8), are needed to be considered concern, because have a probability of extinction by inbreeding (Allendorf *et al*., 2007) and stochastic environmental processes.

4.2- Genetic Structure

Past evolutionary processes and present day gene flow show how shaped, the genetic structure of *Calotriton asper*. Mountain systems, like the Pyrenees, played a crucial role in determining species diversity, and the origin of intraspecific genetic structuring (Wallis *et al*., 2016). Population analyses of nuclear microsatellites, by structure software, revealed that the eight populations are subdivided into three well-supported genetic groups distributed along two river basins (K=2). The populations, Prats Mollo in the Le Tech river basin and Serdinya in the La Têt river basin are clearly differentiated. Although the other populations, very close geographically to Prats Mollo and Serdinya are clustered togheter and show wide admixture, suggests high migration rates. All these results seem to indicate a strong genetic structure for these two populations, with a strong isolation and very reduced gene flow among neighbouring populations. These assumptions are supported by the differential Fst values between populations, a higher Fst between populations shows a lower number of genetic exchanges between them (Table 3). This is likely to be explained by their evolutionary history, which may have favoured local diversification over generations and their greater genetic diversity today. If we compare our clusters found, and the anotrher clusters using microsatellite markers, for the same species, the second cluster corresponds to the population of Valmanya (Lucati *et al*., 2020), also to the cluster 2.3 corresponds the population of Valmanya (Valbuena-ureña *et al*. 2018). Then our cluster of populations mixed are likely belonging to the cluster 2 of Lucati and 2.3 of Vallbuena. Possibily, Prats Mollo and Serdinya, both populations cluster-differentiated might also be part of another new genetic system, as these populations were not sampled by these other *Calotriton asper* studies. This cluster, that grouped six populations together are not clearly structured across the Canigou massif, and the low divergence can be explained as resulting from a recent expansion of these populations from other neighbours, resulting in a founder effect.

Similar pattern we found for DAPC, with a differentiated structure for populations the Prats Mollo in the Le Tech basin and Serdinya in the La Têt basin. The grouping of the eight populations was in six clusters, and apart from these two clearly separated populations in two clusters, we can see that all populations have shared individuals, specially in cluster number 1, with their members belonging to both different river systems (figure S3). Resulting in these six populations mixed taht are only slightly different from the others.

As in the study of the same species and with the same genetic markers (Lucati *et al*., 2020), most important molecular variance was found among individuals, followed by the variance within individuals. Genetic diversity within populations is strong, and the low variability between populations suggest these strong structuring of the species *Calotrirton asper,* thus variability is within each population.

We found high values of Fst within *Calotriton asper* populations, as well as in the other studies of *Calotriton asper* (Lucati *et al.*, 2020 and Valbuena-ureña *et al*., 2018), higher that in similar temperate amphibian's species (Chan and Zamudio, 2009). But lower values than found in two cave of *Calotriton asper* localities (Valbuena-Ureña et al., 2018) with Fst = 0,771. Indeed, very high Fst values, were the genetically most distinct from the others populations in our study, and was found in Prat Mollo and Serdinya (Table 3), these populations may be less connected by suitable habitat corridors. Gene flow among localities may support the maintenance of high levels of genetic diversity (Hillman, 2014). Instead, population of Mas Lori, present lower value of population differentiation (Table 3) and is richest in terms of genetic diversity (Table 2). The high Fst values suggest a strong structuring of the population of *Calotriton asper*. Moreover, Fst values higher than 0.25 are observed in very close populations: between Prats Mollo and Lamenera or Mas Lori and between Serdinya and Casteil or Clara, suggest a long isolation of these populations. This indicates a strong separation of populations close geographically, which is confirmed by the analysis of isolation by distance. Interestingly, the high levels of nuclear genetic divergence contrast with a relatively low mitochondrial diversity of *Calotriton asper* populations (Milla *et al*., 2010), which suggests a relatively recent shared population history.

These Fst values very high observed between very close localities, suggests a strong separation, of even nearby populations geographically and on the contrary, very distant populations, even between populations in different basins, have a very low Fst, close to zero (Table 3). There is no pattern relating geographical distance to genetic distance, which is confirmed by the IBD analyse, being clearly not significant. Then, genetic differentiation between populations cannot be attributed to their spatial distance. An isolation by colonization remains a plausible explanation for the pattern of isolation that we find (Orsini *et al*., 2013). The external factors, in the case of *Calotriton asper*, can facilitates the migration of individuals by the presence of surrounding suitable corridors. Instead, seems that our populations are surrounded by less suitable habitats, and the migration might be interrupt.

4.3- Landscape genetics

We therefore assume that gene flow will most likely occur through the most suitable habitats like at higher elevations, as in the more central areas of the Pyrenees. *Calotriton asper* is a species very well adapted to high mountain environments with low temperatures, in maps of favourable habitats (de Pous *et al*., 2016). It would be interesting to see juveniles disperse, to missing the dispersal capacity of the species, essential parameter in the study of population dynamics. In our study, all populations had migrant's individuals among populations, except for the population of Mas Malet. According to Geneclass2 analyses, populations may have retained a degree of connectivity and shows 9 potential migrants, 6.25% of individuals, between populations (Figure 8), a higher percentage than found in other populations of *Calotriton asper* with 4.9% of individuals (Lucati *et al*., 2020). In contrast to these results, the data on mitochondrial markers showed very little variation genetic within different populations (Milá *et al*., 2010). Migration involved geographically close and long populations, in our case, ranged from 0 to 26 km, in Euclidean distance. Our results are in concordance with the ones found forsome individuals for the same species with a potential recent migration between localities separated by an Euclidean distance between 24 and 33 km (Lucati *et al*., 2020). This is not in agreement with Montori *et al.* (2008), which recorded short range movements in adults of *Calotriton asper* using a capture-recapture framework. Taking into account that the dispersal phase occurs in juvenile individuals, which have a terrestrial phase, in the time between completing metamorphosis and sexually maturing. This time of dispersal phase can take between one and nine years (Camarasa *et al*.,

2020) in stream populations and providing the opportunity to colonize new localities. However, although long distance dispersal of few individuals per population remains possible in amphibians (Cayuela *et al*., 2020), a possibly potential populations located in between the study sites may have been at the origin of migrants if they shared alleles with the sites of origin. Long distance dispersal, possibly some individuals in generations (Saura *et al*., 2014), is in line with our estimates of low inbreeding coefficients (Global Fis = 0.01). The dispersal distances, coupled with low small population, may explain the high levels of genetic structuring and differentiation for some *Calotriton asper* populations across the Canigou massif.

The identified two clusters using structure software, (K= 2) respond to the relation between them and the geographic landscape (Figures 3.a and 3.b). Furthermore, two putative barriers were inferred revealing that mountains and rivers played an important role in the gene flow (Figure 7). The inferred clusters in La têt river basin (K = 2) revealed a close relation with their river system, suggesting geographic isolation. And also, the inferred clusters in Le Tech river basin (K = 2) revealed a close relation with their high altitude, suggesting geographic isolation.

Mountains have strong effects on gene flow in amphibians (Sánchez-Montes *et al*., 2018). The high altitude mountains of Canigou and the big rivers as Le Tech and La Têt would be acting as a dispersal semi-permeable barrier in *Calotriton asper*. During the summer period, where the weather is adequate, passages at high altitudes can be suitable for migration. Some of the migration, however, will likely occur following the contour lines, thus, the populations of the basins of the rivers La Tech and La Têt may be genetically connected. The dispersal capability of species is also determined extrinsic factors such as landscape characteristics, climatic suitability and water quality (Hendrix, 2017). Suitable habitats can provide dispersal corridors between existing populations to promote the maintenance of gene flow between them. The mountains and rivers probably acted as a significant barrier to gene flow, limiting migration between populations in the Prats Mollo and Serdinya. BARRIER identified two strong landscape level barriers to gene flow between populations. Prats Mollo, despite the small geographic distance separating the population of Lamanera (less than 10 km), there was very high genetic distance. This may be attributable to large elevational differences between sites: nearly 600 m in elevation. The second barrier was in Serdinya, with smaller distances to the other populations, it also gives a high genetic differentiation, possibly because of the natural river barrier of La Têt. The flow of migrants might be too low to result in genetic homogenization. So emigration from this population would be in line with our hypothesis explaining the high level of genetic differentiation for these populations. Although, fine scale environmental heterogeneity and microevolutionary adaptations often allow relict populations to persist under generally harsh climatic conditions. Their location in peripheral areas of the species range, makes them the most vulnerable to climate changes or perturbations in the medium to long term.

This study emphasizes the importance of population isolation to understand their contribution to the spatial distribution of genetic variation at the eastern end of its range. Our analyses evidence the importance of topographic complexity in determining patterns of genetic differentiation, indicating that dispersal and population connectivity, is shaping the genetic structure found in our study system. Exploring the relationship between environmental features and genetic and phenotypic patterns of variation could also provide insights about the potential interplay of evolutionary and ecological processes in shaping range-wide patterns of genetic differentiation.

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GENERAL DISCUSSION (DISCUSSIÓ GENERAL)

El tritó pirinenc (*C. asper*) és un amfibi endèmic dels Pirineus, els trets biologics es troben altament influenciats per la diversitat climàtica i la disparitat d'hàbitats que ocupa. En aquest treball s'ha estudiat la dinàmica de les seves poblacions, mitjançant la demografia, morfometria i genètica poblacional, per tal d'aportar nous coneixements necessaris per a la gestió i conservació de l'espècie. Els resultats obtinguts també són extrapolables a altres espècies i permeten entendre millor el paper dels esdeveniments climàtics sobre la dinàmica de les poblacions d'amfibis de muntanya.

El *C. asper* habita principalment torrent, però el trobem també el llacs d'alta muntanya on s'ha trobat presència d'individus paedomorfs. La paedomorfosi facultativa inclou la presència de dos fenotips alternatius en la mateixa població. Aquest fenomen és interessant d'analitzar a causa de les conseqüències evolutives de l'aparició del fenotip alternatiu [16,48]. Les peculiaritats en les condicions d'aquests llacs han permès el desenvolupament d'un fenotip paedomòrfic [5,44,49] Tot i que, les causes que promouen la paedomorfosi facultativa en aquests llacs i no en altres romanen desconegudes. Els nostres resultats indiquen diferències en la mida i la forma del cos i una estructura d'edat similar entre els fenotips alternatius dels adults de *C. asper* en les poblacions amb presència de paedomorfosis facultativa.

Contràriament, a l'altra població paedomorfa facultativa de *C. asper* (Ibón de Acherito [5], la mida corporal no diferia significativament entre sexes. Aquest resultats són similars als trobats en altres estudis [50], que no troben diferències en la mida corporal en relació amb el paedomorfisme en les espècies *Ichthyosaura alpestris*, *Lissotriton helveticus* i *Lissotriton vulgaris*. S'ha observat una mida corporal més reduïda en tritons paedomòrfics vers els metamòrfics en les dues poblacions lacustres de *C. asper* amb paedomorfosi facultativa [5,49]. Aquests resultats estan d'acord amb la hipòtesi que assumeix que els animals petits tendeixen a convertir-se en paedomorfs i els grans animals tendeixen a completar la metamorfosi, i ocorre quan la densitat poblacional és alta [51].

Com a resultat d'aquest *trade-off*, a la mateixa edat, els juvenils podrien completar el procés de la metamorfosi o arribar a la maduresa sexual sense completar la metamorfosi, amb mides més petites. L'existència d'un o dels dos fenotips depèn dels costos i beneficis que reuneix cada fenotip segons el seu mode de vida [10,13]. Estudis indiquen la partició de l'hàbitat entre morfotips (metamòrfics i paedomòrfics), que impliquen diferències en l'alimentació [52]. La metamorfosi a l'Ibón de Perramó probablement és gradual i, en alguns casos, aquest procés no és completa, donat que són juvenils que esdevenen paedomorfs quan assoleixen la maduresa sexual. També vam analitzar larves i vàrem trobar-n'hi d'edat molt avançades, fins i tot una d'elles de 18 anys, que, segons sabem, és la més antiga reportada en una espècie de tritons.

Si considerem la densitat poblacional de l'Ibón de Perramó, era la més alta observada en l'espècie [53].El paper de l'entorn en l'evolució de la variació fenotípica pot ser adaptatiu com a estratègia [13], en la mesura en què la fase terrestre dels juvenils no existeix perquè l'entorn aquàtic és millor pel creixement que l'ambient terrestre, tal com es troba en altres espècies de tritons com *Lissotriton vulgaris* [50] i *Ommatotriton ophryticus* [15]. El fenomen del paedomorfisme facultatiu en *C. asper* sembla ser una estratègia on l'aparició de la forma paedomòrfica es dona en casos molt concrets probablement degut a que les condicions per a una vida terrestre són molt limitades.

Els estudis sobre l'estructura de l'edat en el tritó pirinenc han mostrat una gran variació entre poblacions de diferents hàbitats i altituds [2,4,5,36]. La població de l'Ibón de Perramó va mostrar una maduració sexual lleugerament retardada i una major longevitat en comparació amb la resta de poblacions lacustres que s'havia estudiat anteriorment, seguint el patró d'ambients freds reportats en altres tritons [54,55].

Les poblacions lacustres es consideren un hàbitat recent per a *C. asper*, que podria haver jugat un paper en promoure de la paedomorfosi facultativa. Son pocs els llacs pirinencs que estan habitats per *C. asper*, i en aquests llacs la majoria de les poblacions lacustres són com les poblacions de torrent, amb un període en què els juvenils tenen una fase terrestre (observada als llacs d'Alba i Espelunciecha). L'estabilitat que ofereix el medi aquàtic i la seva estructura heterogènia afavoreix una vida aquàtica prolongada i, en algunes condicions, l'aparició de la paedomorfosi [10]. A causa de l'aïllament i la duresa d'alguns ambients terrestres d'alta muntanya, la dispersió també és menys probable que en altres hàbitats més adients, especialment tenint en compte que en aquesta espècie, els juvenils terrestres es troben en la fase de dispersió [56]. En conseqüència, la combinació d'hàbitat de llac, gran altitud, baixa pressió depredadora, període d'activitat anual curt i alta densitat de població genera condicions que poden alterar el desenvolupament i la metamorfosi [9,57].

La variabilitat en els trets del cicle de vida dels amfibis és el resultat d'adaptacions a diferents condicions ambientals. Tot i que els hàbitats marquen les grans línies ecològiques, els nostres resultats, malgrat les clares diferències en les condicions entre ambients reòfils i lacustres [58], van mostrar una imatge complexa on les interaccions de múltiples factors, determinen els trets demogràfics de les poblacions. Les peculiaritats de cada localitat són les que defineixen les condicions aquàtiques i terrestres, com la temperatura, la pressió de depredació, competència intraespecífica i la competència amb altres espècies [9]. Per exemple, en el cas de la població lacustre d'Espelunchieca hi ha un retard en l'entrada a la maduresa sexual, que es deriva en una major longevitat i mida corporal. Aquest fet pot ser degut a la presència de peixos en el llac. Per conviure amb els peixos, els tritons es troben segregats en l'espai, és a dir, separats físicament gràcies a la presència de roques dels esllavissaments que els protegeixen dels peixos. Degut per tant, a la heterogeneïtat i característiques peculiars de cada localitat, els trets del cicle de vida com l'edat a la maduresa sexual o la longevitat poden mostrar una variació amb l'hàbitat [59]. Així doncs, els factors ambientals com la durada anual del període d'activitat o la durada de la fase terrestre [2], expliquen la variació en els trets del cicle de vida, sent un reflex de les forces ambientals i climàtiques que actuen sobre elles [13]. L'edat o la mida en la metamorfosi, la maduresa i la longevitat, és el resultat dels *trade-offs* o de les compensacions entre condicions avantatjoses en els diferents entorns [60]. Com a resultat, les condicions singulars de cada localitat influeixen d'una manera diferent en la demografia de cada una de les poblacions.

Els estudis sobre la mida corporal dels tritons pirinencs han mostrat una gran variació entre poblacions de diferents hàbitats i altituds [2,4,5,28,36,37,49]. En les poblacions de *C. asper*, tant en la mida del cos com en la forma del cos, s'observen diferències entre hàbitats i diferents fenotips (metamorfs i paedomorfs). Amb tota la mostra d'individus total, no es pot observar el dimorfisme sexual en la mida corporal, a causa de la superposició de mesures, per la gran variabilitat que hi ha a cada població. Ara bé, si analitzem separadament cada població, els resultats obtinguts mostren que hi ha un marcat dimorfisme sexual. Als llacs pirinencs, les femelles tenen major mida corporal que els mascles, el mateix patró que trobem amb *Lissotriton helveticus* als llacs d'Andorra [55], a excepció de les dues poblacions amb paedomorfosi (Ibón Acherito i Perramó). Entre els hàbitats, s'ha observat una mida corporal més petita en les poblacions de torrent en comparació amb les poblacions de llacs de *C. asper*, essent de mida corporal major en les poblacions d'altitud major [28]. La variabilitat en la mida i la forma del cos en les poblacions de *C. asper* es pot separar en dos components: el primer és sexual, es a dir, diferències entre mascles i femelles, mentre que el segon fa referència a diferències entre poblacions. Hi ha una superposició entre poblacions en quant a la mida corporal mínima, el que significa que per una mateixa mida corporal hi ha individus que ja han assolit la maduresa sexual i d'altres que no. Per tant, les poblacions mostren una gran plasticitat en la mida corporal. Aquesta diferència en la mida del cos depèn de les taxes de creixement, que vindrà donada per l'èxit de l'alimentació, la productivitat tròfica de l'hàbitat, la densitat d'individus, la competitivitat tròfica, el període d'activitat anual o les característiques genètiques.

En comparació amb altres espècies, la tendència general sembla ser que els mascles són més grans que les femelles, però hi ha variabilitat entre les poblacions [35,37]. S'han proposat diverses hipòtesis per explicar la direcció del dimorfisme sexual a causa de la selecció sexual en amfibis. Per exemple, en les femelles, el nombre d'ous i la seva mida estan restringits a l'interval entre extremitats del cos [54,61]. En el cas dels mascles, la selecció natural actuaria a favor dels de major mida corporal, per poder competir amb altres individus del mateix sexe, per aparellar-se o pel menjar. La cua més alta i més curta és per a l'amplex, per abraçar les femelles de manera més eficient. Per contra, en les femelles, la cua baixa i llarga pot facilitar l'aparellament amb els mascles [62]. Els mascles tenen una llargada i una amplada del cap majors que les femelles, que junt amb les extremitats més llargues, poden buscar preses més grans. També l'agressiva mossegada entre els mascles s'ha observat en lluites de *C. asper* [62].

Un efecte de la selecció natural i sexual pot ser la variació morfològica de *C. asper*, on l'hàbitat i l'ontogènia determinen la mida i la forma del cos. La gran variabilitat morfològica de *C. asper* és la plasticitat fenotípica, en relació amb l'hàbitat o el morfotip, i fins i tot pot modificar-ne el cicle biològic o la fisiologia en funció de les característiques del medi.

Els processos evolutius passats i el flux de gens actuals mostren com es forma l'estructura genètica de *C. asper*. Els sistemes muntanyencs, com els Pirineus, van tenir un paper crucial a l'hora de determinar la diversitat d'espècies i l'origen de l'estructuració genètica intraespecífica [63]. L'estudi de la variabilitat genètica de les poblacions de *C. asper* al llarg del seu rang de distribució i en els diferents hàbitats que ocupa mostren evidencies d'un gradient negatiu longitudinal i positiu altitudinal de la diversitat genètica [44]. *C. asper* per tant, sembla estar adaptat als hàbitat de muntanya de gran altitud amb una diversitat genètica més alta en els Pirineus Occidentals. En l'estudi de la població del Canigó, situada a la perifèria de la distribució de la població i en la zona oriental, els nivells de diversitat genètica van ser menors en comparació amb altres estudis de microsatèl·lits en *C. asper* [44,64]. Aquests nivells menors de diversitat genètica, a l'extrem oriental de la seva distribució, coincideixen amb el fet que les poblacions de la perifèria de la distribució de les espècies solen tenir una diversitat genètica menor que les poblacions centrals, probablement atribuïbles a l'hàbitat subòptim, a un major aïllament, als efectes fundadors i / o als colls d'ampolla genètics [65]. Aquesta part estudiada dels Pirineus, està influenciada pel clima mediterrani, que proporciona temperatures més altes i condicions més seques que els sectors occidentals d'influència oceànica, que proporcionen hàbitats més adequats per a *C. asper* [66].

Els resultats obtinguts en les poblacions del massís del Canigó mostren una important estructura de les poblacions, amb localitats separades genèticament tot i està geogràficament pròximes. Per tant, no podem concloure que hi hagi un efecte de de la distància geogràfica sobre la diferenciació genètica de les poblacions. Sembla doncs que l'aïllament per colonització podria ser una explicació als resultats trobats [67], en aquestes poblacions la migració es veu interrompuda per hàbitats poc adequats entre poblacions.

C. asper és una espècie molt ben adaptada a entorns d'alta muntanya amb temperatures baixes, com és mostra en els mapes d'hàbitats potencials [68]. Per tant, es suposa que el flux de gens es produiria probablement a través d'aquests hàbitats. En el nostre estudi, totes les poblacions tenien individus migrants entre les poblacions, excepte una població, Mas Malet. Les migracions inclouen migracions entre poblacions llunyanes i properes geogràficament, que oscil·laven entre els 6 i 26 km, en distància euclidiana. Aquests resultats concorden amb els que es troben per a alguns individus de la mateixa espècie, d'entre 24 i 33 km [64]. Tot i això, els resultats difereixen dels trobats en adults de *C. asper* mitjançant un model de captura-recaptura [29]. El temps transcorregut entre la finalització de la metamorfosi i la maduració sexual és la fase de dispersió, que es produeix en els individus juvenils. Aquest temps de dispersió pot durar entre un i nou anys en les poblacions de torrents [4], i proporciona l'oportunitat de colonitzar noves localitats. Encara que la dispersió a llarga distància, de pocs individus per població, és possible en els amfibis [69], també podria ser que les poblacions potencials, situades entre els llocs d'estudi, hagin estat a l'origen dels migrants, si compartien al·lels amb els llocs d'origen. Les distàncies de dispersió, junt amb un mida de població petita, poden explicar els alts nivells d'estructuració i diferenciació genètica d'algunes poblacions de *C. asper* a través del massís del Canigó.

Les muntanyes tenen forts efectes sobre el flux de gens en amfibis [70]. Les muntanyes de gran altitud del massís del Canigó i els grans rius com Le Tech i La Têt actuarien com una barrera semipermeable en la dispersió a *C. asper*. La capacitat de dispersió però també es determina per les característiques del paisatge, la idoneïtat climàtica, la disponibilitat i qualitat de l'aigua [71]. En aquest sentit, els habitats favorables, tal i com ens mostrava els anàlisis dels flux de gens, proporciones corredors i permeten la migració. Tot i que l'heterogeneïtat ambiental a petita escala i les adaptacions microevolutives sovint permeten persistir a les poblacions en condicions climàtiques generalment dures, la ubicació de poblacions en zones perifèriques de l'espai de la seva distribució fa que siguin més vulnerables a canvis o pertorbacions climàtiques a mitjà o llarg termini.

En resum el paisatge, que determina la dispersió i la connectivitat de les poblacions, està configurant l'estructura genètica i determinant els patrons de diferenciació genètica del nostre sistema d'estudi. Les nostres anàlisis evidencien la importància de la complexitat topogràfica a l'hora de determinar patrons de diferenciació genètica, cosa que indica que la dispersió i la connectivitat de la població estan configurant l'estructura genètica del nostre sistema d'estudi. L'exploració de la relació entre els trets ambientals i els patrons de variació genètics i fenotípics també podria proporcionar informació sobre la possible interacció dels processos evolutius i ecològics en la configuració de patrons de diferenciació genètica a tota la distribució.

En conclusió, el *C. asper* és una espècie que presenta una elevada variabilitat demogràfica, morfologia i genètica que sembla ser principalment deguda a les característiques intrínseques del propi hàbitat on es troben les poblacions i també de les peculiaritats de les zones que ocupen. L'aparició de pedomorfosis facultativa en algun dels llacs n'és un exemple clar. Aquesta diferenciació entre poblacions, avalada pels estudis genètics, fa que cada població hagi de ser considerada com a una unitat independent per tal de gestionar la seva conservació. És a dir, la conservació dels hàbitats que ocupa cada població és clau per la perpetuació de l'espècie, així com assegurar la presència d'habitats favorables per a la seva dispersió, i en definitiva, pel i la supervivència de l'espècie.

FINAL CONCLUSIONS

1- The ocurrence of factultative paedomorphosis in *Calotriron asper* in Ibon de Perramó seems to be a strategy where the appearance of the paedomorphic morphotype occurs in specific cases probably because the conditions for terrestrial life are very limited.

2- In the populations with facultative paedomorphosis, there are morphological and demographic differences between the two phenotypes (metamorphic and paedomorphic individuals) revealing a complex pattern of growth in which metamorphic adults were larger than paedomorphs.

3- Life history traits analysed in nine populations of *C. asper* living in lake and stream habitats show variability between sexes and populations, that did not depend on the habitat. The factors that shape the demographics of populations have an effect on a smaller scale, differently within each locality beyond whether the habitat is rheophilous or lacustrine.

4- Sexual dimorphism in *C. asper* is determined by body size and depend on habitat type (lake and stream) and ontogenetic stage (paedomorphic and metamorphic). The characteristics of sexual dimorphism are: males with bigger heads, longer extremities and higher tails, whereas females have longer and less high tails, longer abdomens and smaller head and limbs.

5- The morphological variability of *C. asper* is probably mainly due to a phenotypic plasticity in relation to the habitat conditions, that can promote the occurrence of facultative paedomorphosis when the environment conditions are limited for a terrestrial phase.

6- The study of the genetic variability of the populations of *C. asper* in an area on the periphery of its distribution (Canigou massif), shows low genetic variation with a high structure between some populations that not depend of geographical distance.

7- The landscape that determines the dispersion and connectivity of the populations, through the presence of favorable habitats, shapes the genetic structure and determines the patterns of genetic differentiation of the populations of the Canigou. The study of the relationship between environmental factors and genetic and phenotypic patterns of variation could also provide information on the possible interaction of evolutionary and ecological processes in the configuration of genetically differentiated patterns throughout distribution.

The general conclusion on this study is that *C. asper* is a species that has a high demographic variability, morphology and genetics that seems to be mainly due to the intrinsic characteristics of the habitat where the populations are located. The appearance of facultative paedomorphosis in some lakes is a clear example of this. The differentiation between populations, supported by genetic studies, means that each population must be considered as an independent unit to consider for conservation. Indeed, the conservation of the habitats occupied by each population is key to the perpetuation of the species, as well as the conservation of favourable habitats for its dispersal, and ultimately, for the species survival.

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