Enrico Lunghi

Improving the knowledge of European cave salamanders (genus *Hydromantes*): ecological studies for conservation of a highly threatened endemic species

vom Fachbereich VI Raum- und Umweltwissenschaften der Universität Trier zur Verleihung des akademischen Grades ‚Doktor der Naturwissenschaften' (Dr. rer. nat.) genehmigte Dissertation.

Advisors and Relators:

Betreuender und 1. Berichterstattender: Prof. Dr. Michael Veith

2. Berichterstattender: Prof. Dr. G. F. Ficetola

3. Berichterstattende: Dr. Claudia Corti

23 November 2017

Trier, 2018

Table of contents

Abstract

This thesis is focused on improving the knowledge on a group of threatened species, the European cave salamanders (genus *Hydromantes*). There are three main sections gathering studies dealing with different topics: Ecology (first part), Life traits (second part) and Monitoring methodologies (third part). First part starts with the study of the response of *Hydromantes* to the variation of climatic conditions, analysing 15 different localities throughout a full year (CHAPTER I; published in PEERJ in August 2015). After that, the focus moves on identify which is the operative temperature that these salamander experience, including how their body respond to variation of environmental temperature. This study was conducted using one of the most advanced tool, an infrared thermocamera, which gave the opportunity to perform detailed observation on salamanders body (CHAPTER II; published in JOURNAL OF THERMAL BIOLOGY in June 2016). In the next chapter we use the previous results to analyse the ecological niche of all eight *Hydromantes* species. The study mostly underlines the mismatch between macro- and microscale analysis of ecological niche, showing a weak conservatism of ecological niches within the evolution of species (CHAPTER III; unpublished manuscript). We then focus only on hybrids, which occur within the natural distribution of mainland species. Here, we analyse if the ecological niche of hybrids shows divergences from those of parental species, thus evaluating the power of hybrids adaptation (CHAPTER IV; unpublished manuscript). Considering that hybrids may represent a potential threat for parental species (in terms of genetic erosion and competition), we produced the first ecological study on an allochthonous mixed population of *Hydromantes,* analysing population structure, ecological requirements and diet. The interest on this particular population mostly comes by the fact that its members are coming from all three mainland *Hydromantes* species, and thus it may represent a potential source of new hybrids (CHAPTER V; accepted in AMPHIBIA-REPTILIA in October 2017). The focus than moves on how bioclimatic parameters affect species within their distributional range. Using as model species the microendemic *H. flavus,* we analyse the relationship between environmental suitability and local abundance of the species, also focusing on all intermediate dynamics which provide useful information on spatial variation of individual fitness (CHAPTER VI; submitted to SCIENTIFIC REPORTS in November 2017). The first part ends with an analysis of the interaction between *Hydromantes* and *Batracobdella algira* leeches, the only known ectoparasite for European cave salamanders. Considering that the effect of leeches on their hosts is potentially detrimental, we investigated if these ectoparasites may represent a further threat for *Hydromantes* (CHAPTER VII; submitted to INTERNATIONAL JOURNAL FOR PARASITOLOGY: PARASITES AND WILDLIFE in November 2017). The second part is related to the reproduction of *Hydromantes*. In the first study we perform analyses on the breeding behaviour of several females belonging to a single population, identifying differences and similarities occurring in

cohorting females (CHAPTER VIII; published in NORTH-WESTERN JOURNAL OF ZOOLOGY in December 2015). In the second study we gather information from all *Hydromantes* species, analysing size and development of breeding females, and identifying a relationship between breeding time and climatic conditions (CHAPTER IX; submitted to SALAMANDRA in June 2017). In the last part of this thesis, we analyse two potential methods for monitoring *Hydromantes* populations. In the first study we evaluate the efficiency of the marking method involving Alpha tags (CHAPTER X; published in SALAMANDRA in October 2017). In the second study we focus on evaluating *N*-mixtures models as a methodology for estimating abundance in wild populations (CHAPTER XI; submitted to BIODIVERSITY & CONSERVATION in October 2017).

Own contribution to chapters:

	Design of the study	**Data collection**	**Statistical analyses**	**Manuscript writing**
Chapter 1	80%	100%	70%	60%
Chapter 2	70%	70%	-	60%
Chapter 3	20%	30%	-	10%
Chapter 4	20%	50%	-	20%
Chapter 5	50%	50%	100%	60%
Chapter 6	70%	80%	70	70%
Chapter 7	100%	80%	100%	70%
Chapter 8	50%	50%	-	60%
Chapter 9	90%	70%	90%	70%
Chapter 10	90%	80%	100%	90%
Chapter 11	40%	30%	-	10%

General summary of chapters

Background

Conservation: why it matters?

Conservation actions have as major goals the preservation of ecosystems and species biodiversity. The importance to keep "alive" all delicate natural equilibriums is generally far behind our imagination; or maybe we just do not want to see. Sure enough, with the development of a strong anthropocentric culture, we usually consider important only what is useful for us, from its medical applications to a subjective judgment of beauty. But that way of thinking is pretty far from being a valid method to protect Nature.

Nature as a whole, is a mix of multiple and complex ecosystems which, with their complementary and synergic work, sustain the life on the planet. Ecosystems provide four main essential services (Jørgensen, 2009): three of them are relating to the production, recycling and regulation of resources, while the fourth is a pleasuring cultural services mostly appreciated by humans. The importance and the value of ecosystem services is a well-known fact (Costanza et al., 1997; Balmford et al., 2002), proved also by the multiple illegal attempts to use natural resources (Kauano et al., 2017). The well operating of ecosystems is only guaranteed by the support of the local biodiversity. Indeed, biodiversity represents the most valuable good we could have (McCarthy et al., 2012). With our actions, we unbalanced the natural equilibrium involving the development of new species and extinction of some others (Barnosky et al., 2011), provoking an evident boost in extinction rates during last centuries and thus, compromising the equilibrium and the efficiency of ecosystems (Ceballos et al., 2015).

Many human actions can result in a threat for a species, from the direct kill of individuals to the destruction of its natural environment, and furthermore, from the spread of diseases due to climate change (Filippi and Luiselli, 2000; Garner et al., 2006; Ficetola and Maiorano, 2016; Akrfim et al., 2017; Borzée et al., 2017). Therefore, the main questions would be: How should we now if a species is suffering extinction threats? What is representing a threat? What can we do to limit such threat? In many cases, our lack of knowledge is the greatest barrier which prevent a complete and exhaustive species assessment (Bressi, 2004; Restrepo et al., 2017). The problem of biodiversity loss is extremely serious, so several international organisations were born with the aim of manage and gather species assessments, in order to attract public interest and provide guidelines to prevent and act against possible threats (IUCN Standards, 2016; BenDor et al., 2017). Indeed, conservationists are working on both global and small scale to improve the knowledge of their target species, aiming to discover as much as possible on their life traits, evolution and role in ecosystems (Beebee and Griffiths, 2005; Akunke Atuo et al., 2016; Brodie et al., 2016; Murray et al., 2017).

The studied species

European cave salamanders are amphibians belonging to the plethodontid family, and ascribed (with no lack of vibrant debates) to the genus *Hydromantes* (Wake, 2013). The origin of this taxon is probably attributable to the Appalachian Mountains (California), the only area in which other *Hydromantes* species are present (Wake, 1966; Lanza et al., 2006); how these species could have reached Europe is still a puzzling question, but it is likely that they moved towards West (Carranza et al., 2008). The plethodontid family is present in Europe with eight species which are endemic or sub-endemic to Italy (Sillero et al., 2014). Three species of *Hydromantes* (*H. strinatii, H. ambrosii and H. italicus*) are distributed on mainland Italy and (only *H. strinatii*) in a small part of the south-west French Provence, while the other five species (*H. flavus, H. supramontis, H. imperialis, H. sarrabusensis* and *H. genei*) are endemic to Sardinia (Lanza et al., 2006). European *Hydromantes* generally show allopatric distribution, especially in Sardinia, where each species is restricted to one single or few massifs (Chiari et al., 2012). The only known natural contact zone between species take place in the North of Tuscan Apennines, where *H. ambrosii* and *H. italicus* give birth to a viable population of hybrids (Ruggi et al., 2005).

European cave salamanders, as other plethodontid do, show peculiar features. These amphibians lack of larval stage and are totally free from aquatic environments (Lanza et al., 2006). This means that they lay eggs in terrestrial environments (Lunghi et al., 2014b) and hatchlings are just a tiny copy of adults, which usually are four to five fold bigger at their maximum development (Lanza et al., 2006). Another important feature of these salamanders is the lack of lungs. During their evolution, plethodontid salamanders switched to a total skin-mediated breathing (Spotila, 1972), and such features made them extremely sensitive to climatic conditions (Lanza et al., 2006).

Habitat

Despite their vernacular name, *Hydromantes* are epigean terrestrial salamanders which exploit different habitats, from forest floor to cracks, cervices and dry walls (Lanza et al., 2006; Manenti, 2014; Costa et al., 2016). However, due to their peculiar physiology, these salamanders are able to live only in environments which show specific characteristics (high moisture and relative cold temperature) (Lanza et al., 2006). Therefore, when external climatic conditions become unsuitable (especially during hot and dry seasons), *Hydromantes* need to find refuge in most suitable places. Indeed, these salamanders often exploit underground environments, habitats in which their physiological requirements are likely to be held (Lanza et al., 2006; Lunghi et al., 2014a). In such environments *Hydromantes* reach high density and therefore are highly detectable (Ficetola et al., 2012). Nevertheless, the high and long-lasting microclimatic suitability of underground environment offer these species the opportunity to spend there most of their lifetime, making them an essential member of underground communities (Lunghi et al., 2017).

Conservation status

The set of above mentioned features (endemism and specific ecological requirements) makes these animals very sensitive to environmental changes and thus, the International Union for Conservation of Nature (IUCN) classified them as highly susceptible to risk of extinction, and many local and international laws include them within strictly protected species. One of the main hurdles for an efficient conservation plan towards these species is the still inadequacy of information related to their ecology and life traits. Probably, the intrinsic characteristics of environments in which they are more easily detectable (caves and underground sites), do not facilitate the activities of researchers, discouraging the planning of appropriated studies. *Hydromantes* exploit two different typologies of environments, underground and superficial one, which are intimately connected by a multitude of ecological networks (Culver and Pipan, 2009). Therefore, to be effective in *Hydromantes* conservation, is crucial to have understood also the equilibriums of such environments, as they deserve conservation plans as well. This mix of missing information also hamper to develop appropriated methodologies to perform studies in the field. A multitude of different techniques are available for conservation biologists, which chose carefully the most appropriated one for their target species (Crovetto et al., 2012; Brannelly et al., 2014). Therefore, multiple studies are required to better comprehend how these species live, what they need and consequently, what we can do to prevent their disappearing.

Ecology

Phenology

Understanding species distribution and habitat use is one of the main tasks for ecologists and conservationists (Peterson et al., 2011; Bogaerts et al., 2013). Researchers aim to identify the combination of environmental features suitable for species and thus, link such information with species occurrence (Godsoe, 2010). Indeed, one of the most used approach aim to connect species presence/absence to environmental variables, in order to identify a reliable tool to predict species distribution (Guisan and Thuiller, 2005). However, a strict species-habitat association is unlikely to be held in nature (Saupe et al., 2014), and such inconstancy depend by two main non-exclusive hypothesis. On the one hand, a species may select different habitats according to their temporary requirements. Such changes are mostly determined by the activities that individuals are going to perform, or by the intrinsic differences between age classes (Seebacher and Alford, 1999; Brambilla and Saporetti, 2014; Dittmar et al., 2014). On the other hand, the constant evolution of environments may force individuals to continue "update" their preference, putting them in the position to move somewhere else, or adapt to the new local conditions (Kearney et al., 2013; Saupe et al., 2014).

In CHAPTER I we tested whether *Hydromantes* experience such changes and which may be the main causes. Such study was performed within subterranean environments, places often believed to be refuges far from any environmental fluctuation. In first part we assessed the microclimatic dynamics of underground environments, studying the main features which may influence salamanders preference: temperature, humidity and illuminance. Then, considering underground salamanders distribution throughout the year, was assessed whether individuals respond to such changes and how. *Hydromantes* showed different associations with environmental condition, and such dynamism was due to a mix of both above mentioned hypothesis. First, following different requirements, adults and juveniles showed a different pattern of habitat association, with the latter more incline to exploit sub-optimal microclimate in favour of a highest prey availability (Ficetola et al., 2013; Manenti et al., 2015). Secondly, adult salamanders follow the most suitable microclimatic conditions, which in turn shift in different part of the underground environment according to the surface local climate.

Thermal equilibrium

Within environmental features, temperature is among those which mainly affect species activities (Angilletta Jr. et al., 2002). Endothermic species evolved the ability to regulate their own body temperature through metabolism, a feature that make them able to buffer environmental thermal excursion remaining within the range of their optimal temperature (Macdonald, 2010). On the other hand, all ectotherms lack of such features and thus, they totally depend from environment to regulate their body warmth (Raske et al., 2012). Therefore, problems related to climate change are concerning a way more "cold-blooded" species, as the global rising of temperature may overcome their tolerance limits and thus, paving their way to decline (Gunderson and Leal, 2016).

In CHAPTER II we studied the thermoconformity of *Hydromantes* using one of the most advanced tools, the thermocamera, which gave us the opportunity to avoid common problematic arose using conventional methods (e.g. thermometric probes). We analyzed the relationship between air temperature and salamanders body temperature, in order to assess whether *Hydromantes* thermoconformity and how environmental changes affect their body temperature. We defined as natural study-system the underground environments, a habitat in which *Hydromantes* usually occur (Lanza et al., 2006). Considering the natural thermal gradient of such environments (see Chapter I), we performed our test during hot season, the period in which *Hydromantes* escape external unsuitable conditions (Ficetola et al., 2012; Lunghi et al., 2014a). In this scenario, underground thermal gradient shows and improvement of its suitability going from the connection with surface (low suitability) towards deepest area (high suitability). We experimentally tested and quantified the effect of thermal changes on salamanders' body temperature, estimating also the time required by individuals to reach back thermoconformity after thermal stress. *Hydromantes* showed a clear thermoconformity with surrounding environments; generally, head showed a significant slightly warmer temperature. After artificially have changed salamanders body temperature, all individuals reached back their thermoconformity in about 15 minutes; juveniles generally needed one third of that time. We identified the air temperature as a good proxy of the operative temperature for *Hydromantes*, also providing new insights on their spatial use.

Micro- vs. Macro-habitat

The idea that phylogenetically related species show similar ecological and life traits, is a common thought within ecologists (Losos, 2008). Even if phylogenetic signals are generally reliable for some features, such as morphology and life traits (Blomberg and Garland, 2002; Losos, 2008; Enriquez-Urzelai et al., 2015), was seen that relating to ecological niche, such signals are not always perfect predictors (Losos, 2008; Peterson, 2011). This mismatch is mostly related to the method used to identify species niche. The most broadly used method is the bioclimatic approach, which combine species distribution with climatic variables (Peterson, 2011). However, besides the great improvement brought by this method, it also shows some weak points. For example, data used in the bioclimatic approach usually have coarse resolution (Beck et al., 2012); therefore, an approximation of the overall abiotic features characterising the area, unlikely representing those really experienced by species, which are the result of multiple local interactions (Soberón, 2007; Kearney et al., 2014; Sunday et al., 2014). Indeed, a microhabitat approach is necessary to draw species niche and to assess the reliability of macrohabitat studies.

In CHAPTER III we analyzed the niche of the eight *Hydromantes* species using both macro- and microhabitat approach. We used caves as study system, as they have a natural microclimatic gradient which produce detailed information about *Hydromantes* requirements. The study was conducted during hot season, period in which cave microclimate shows both high and low suitability for cave salamanders, depending on cave area (see CHAPTER I). Considering that cave salamanders strongly depend by environmental features (see CHAPTER II), we collected data on occupied areas, in order to define preferred combination of microclimatic features and identify salamanders' physiological limits. We then compared microclimatic data with those obtained using bioclimatic approach. Finally, we assessed niche overlap between *Hydromantes* species, and we tested whether divergences in micro- and macrohabitat show any relationship with evolutionary or geographical distance. Within microhabitat analysis, all *Hydromantes* species showed a general similar relationship with abiotic features, preferring dark, moist and cool cave areas; however, in some cases relationships were not linear, meaning that some species have stricter tolerance limits against abiotic features. On the other hand, considering macrohabitat analysis, *Hydromantes* niche overlap was very limited, and these results were quite in contrast with those obtained considering the microhabitat. Overall, there were no evident evolutionary signals at the base of *Hydromantes* niche dissimilarities. Only for macrohabitat analysis we detected a mix of phylogenetic and geographic distance at the base of *Hydromantes* niche similarities; however, disentangle their correlation and evaluate each single effect was not possible. We provided data demonstrating a quite strong mismatch between micro- and macrohabitat analysis, underlining that phylogenetic distance is not the major cause of ecological niche dissimilarities within close related species.

Ecological adaptation of hybrids

According to its biological definition, a species represents a group of individuals which share a set of characteristic features and are able of interbreeding and producing fertile offspring, being therefore reproductively isolated from other different species (Fitzpatrick, 2004). However, in some circumstance, is it possible that individuals from close-related species are still able to interbreed, giving birth to individual characterized by a different genome: the hybrids (Tanaka, 2007; De Hert et al., 2012). Besides the copious studies performed on the hybrids genetic and on the gene flow occurring between them and parental species (Ficetola and Stöck, 2016; Grant and Grant, 2016; Bay and Ruegg, 2017), very few aimed to characterize the ecological niche of hybrids, which, together with phylogenetic data, allows to better understand evolutionary processes like adaptation and speciation (Seehausen, 2004). This information may furnish an important instrument to test whether hybrid populations have niches that are similar or intermediate to their parental species, or instead having a new different one (Ficetola & Stock, 2016). The intermediate niche theory predict that hybrids should have an intermediate niche, which represents something in between those of parental species (Moore, 1977). Conversely the transgressive niche hypothesis supposes that introgressed populations show new ecological niches that differ from those of both parental species (Ficetola & Stock, 2016).

In CHAPTER IV we studied the ecological niche of European cave salamanders' hybrids inhabiting the contact zone between *Hydromantes ambrosii* and *H. italicus*, occurring in the north of Tuscany (Italy) (Ruggi et al., 2005; Lanza et al., 2006). During five years (2012-2106) we surveyed *Hydromantes* populations inhabiting underground environments; survey were performed during hot season, as in this period cave salamanders go underground to avoid unsuitable climatic conditions (Ficetola et al., 2012; Lunghi et al., 2014a). Following the procedure described in Chapter III, we collected microclimatic data on hybrid *Hydromantes* populations. Our results demonstrated that both *H. ambrosii* and *H. italicus* introgressed populations showed a significant niche shift, with a manifest expansion toward harsher environmental conditions than those experienced by the parental populations.

Allochthonous distribution and possible source of new hybrids

Introduction of allochthonous species is always a risk for both local species and habitats, as the new species may have the ability to break ecosystems equilibriums and threat native biodiversity (Doherty et al., 2016; Gürtler et al., 2017). Such introduction may be accidental (Dyer et al., 2017), or may be made wittingly following several purposes (Kraus, 2009). Within *Hydromantes*, handful relocations occurred and always involved mainland species (*H. strinatii*, *H. ambrosii* and *H. italicus*). Relocations performed within Italian territory were mostly aimed to study syntopic dynamics (Cimmaruta et al., 1999; Forti et al., 2005; Lanza et al., 2006; Cimmaruta et al., 2013), while for other cases (not only in Italy), no source of introduction are available (Lanza et al., 2006; Lucente et al., 2016). Unfortunately, in no cases the possible impact of *Hydromantes* on native species was evaluated.

In CHAPTER V we accounted about the oldest known translocations of *Hydromantes*. Individuals from mainland Italy where brought in a subterranean laboratory in the French Pyrenees with the purpose to carry out scientific studies (Guillaume and Durand, 2003); however, after the abortion of such studies, individuals where released in an abandoned mine. We located the mine and we collected data on the exploited environment, on population structure and diet. Given that such population is viable since decades and likely composed by a mix of mainland *Hydromantes* species (Guillaume and Durand, 2003), is important to monitor both its ecological requirements and development, as the mix of *Hydromantes* species may produce potential unknown detrimental effects to both local biodiversity and parental species.

Using population dynamics to link environmental suitability and species abundance

Multiple biotic and abiotic factors play an important role in shaping species distribution. Generally, a species occurs in areas where abiotic conditions are suitable (i.e. positive intrinsic growth rate), where biotic interactions allow the persistence of viable populations (i.e. positive total growth rate), and where dispersal and colonization are possible (Soberón, 2007; Soberón and Nakamura, 2009). Within species range, the combination of both biotic and abiotic factors contribute in creating areas with different suitability and, in those with the highest suitability, it is expected that species show better performance. Therefore, exist a positive relationship between environmental suitability and species abundance (Weber et al., 2017). Such positive relationship is probably the consequence of multiple processes acting at the population level (Thuiller et al., 2014); however, until to date most of the studies just considered the most evident correlation (i.e. suitability vs. abundance), without trying to identify the population-level processes that can determine such relationship.

In CHAPTER VI we investigated the links between range-wide variation of environmental suitability and multiple population parameters (species abundance, activity pattern, feeding performance and body condition) using as model species the microendemic *Hydromantes flavus*. Specifically we predicted that: individuals will be more active in most suitable areas (The Activity Hypothesis) (Hetema et al., 2012); individuals in high suitable areas can devote more time to foraging and/or can find higher food availability (The Foraging Hypothesis); longer activity and better foraging allows improving body condition (Díaz-López et al., 2017), which is a fitness-related trait (Jakob et al., 1996) (The Body Condition Hypothesis); finally, the positive relationships predicted above will have potential cascade effects on local abundance and thus, making the connection between environmental suitability and species local abundance (Weber et al., 2017). Using Maxent we created a species distribution model (SDM) using four climatic variables knowing to affect cave salamander's distribution. Overall, we surveyed 14 caves from June 2013 to May 2017 and, to test each hypothesis, we used a different subset of collected data. The Activity Hypothesis. We performed survey during hot season, period in which salamanders move underground to escape unsuitable external climatic conditions (Lanza et al., 2006; Ficetola et al., 2012); however, also underground the activity of cave salamanders is affected by the effect of external environmental conditions (Lunghi et al., 2015). We observed that salamanders inhabiting caves located in high suitable areas were more active, occupying areas close to the cave entrance. The Foraging Hypothesis. We performed stomach flushing on salamanders from three populations, and we compared the frequency of empty stomach within such populations. Individuals from high suitable areas had less empty stomach if compared to those from less suitable areas. The Body Condition Hypothesis. Using Residual Indexes (Labocha et al., 2014), we estimated the Body Condition Index of salamanders and, as we expected, those from high suitable areas had higher Body Condition Index. Finally, we estimated salamanders' density and we found that higher densities occurred in cave located in high suitable areas.

Do ectoparasites represents a possible threat?

Hematophagous leeches are a well-known group of ectoparasites feeding on vertebrate blood (Sawyer, 1981). When are stuck to their host, leeches induce a chemical reaction aimed to attenuate the host immune response (Salzet et al., 2000; DuRant et al., 2015), promoting hosts vulnerability to further potential infections (Rigbi et al., 1987; Daszak et al., 1999). Leeches can parasitize amphibians during all their life stage, from egg to the adult form (Howard, 1978; Mock and Gill, 1984; Romano and Di Cerbo, 2007; Wells, 2007); in some this parasitism may represents an additional threat for amphibian populations (Hoffmann, 1960; Merilä and Sterner, 2002; Elliot and Dobson, 2015). The interaction between leeches and amphibians is usually studied in freshwater environments, while for mere terrestrial once, information is extremely scarce (Lanza et al., 2006; Rocha et al., 2012; Manenti et al., 2016).

In CHAPTER VII we studied the interaction between the leech *Batracobdella algira* and the Sardinian cave salamanders of the genus *Hydromantes*, also identifying for the first time ecological traits related to the presence of these leeches. From September 2015 to May 2017, we repeated survey in 26 different underground environments, monitoring at least 2 sites for each *Hydromantes* species. We took data from more than 2,600 salamanders and 130 leeches. Leeches tend to parasitize adults and they usually stuck on body parts which do not compromise salamanders movement. Parasitized salamanders showed higher Body Condition Index (BCI) than non-parasitized one; furthermore, the higher was the ectoparasite load, the higher was the salamanders BCI. Within *Hydromantes* flavus home range, *B. algira* showed a significant relationship with water hardness, making this species bounded to active karst systems. Furthermore, leeches seem to inhabit mountain areas which favor it survival and the chance to get in touch with hosts. Our study arise many questions related to the actual biodiversity of leeches parasitizing Sardinian salamanders, and about the effect they produce on the local amphibians.

Life traits

Reproduction

One of the firsts studies usually performed on species is related to their reproduction, aiming to explain mating activities, breeding behavior and potential ability to produce new generations. Such information on European *Hydromantes* are quite rare and mostly related to captive breeding (Lanza et al., 2006; Oneto et al., 2010; Oneto et al., 2014). *Hydromantes* are totally terrestrial amphibian species, not requiring the aquatic environment during any of their life stages (Lanza et al., 2006). They are oviparous species lacking of larval stage (Lanza et al., 2006); however, one species (*H. sarrabusensis*) is thought to be ovoviviparous (Lanza and Leo, 2001), but such information has not well founded base (Wake et al., 2005). Salamanders which have reached sexual maturity (around third-fourth year of life; Salvidio, 1993) give birth to a courtship in which males use their typical sexual characters (premaxillary teeth and mental gland) to arise females interest and make them willing to pick up their spermatophora (Lanza et al., 2006). Courtship has been observed throughout the year, while deposition of spemratophora only in autumn; this discrepancy produces an inevitable uncertainty about period and dynamics of courtship. Moreover, considering that in these species pachytene spermatocytes do not degenerate during the cold season (Mertens, 1923), is possible that mating may occur throughout the whole year. Gravid females look for a suitable place to lay their eggs, preferring sites far from dangers and where microclimate shows prolonged suitability (Lunghi et al., 2014b). Currently, we know about only one breeding season for *Hydromantes*, going from early spring to late summer (Stefani and Serra, 1966; Papinuto, 2005; Lunghi et al., 2014b). Mothers involved in breeding are extremely devoted to their clutch, spending several months in the care of both eggs and newborns, limiting any other activities, feeding included (Oneto et al., 2010; Oneto et al., 2014).

In CHAPTERS VIII and IX we produced new findings about breeding in natural environments of *Hydromantes*, enlightening some traits that were still unknown or thought to be possible but without any scientific proof. In CHAPTER VIII we identified a general synchrony to carry out the breeding in females from the same population. Furthermore, females also showed a common preference for laying sites, choosing those with appropriated microclimatic condition, but at the same time, not that far from feeding sites. In this section, we observed a general effect of climatic features on *Hydromantes* breeding, but such topic is well accounted in the following CHAPTER IX. In CHAPTER IX we showed how the climate influence the time in which females start their breeding (and consequently the following hatch), giving evidence of a multiple annual breeding. Nevertheless, we pointed out how breeding may represents a critical point for the conservation of *Hydromantes*, as it takes almost two years for the complete cycle and therefore, it occurs just a handful times in the life-span of these animals. Finally, oviparity in *H. sarrabusensis* was also documented.

Monitoring methodologies

Evaluating Alpha tags as individually marking method

One of the most used technique in ecological study is the Capture-Mark-Recapture, which allows researchers to collect data on single individuals and, at the same time, have an useful tool to estimate population abundance (Emata and Marte, 1992; Pierce et al., 2014). Understanding which is the most appropriated marking method represents the first crucial step, as a wrong choice may invalid the study and expose individuals to further threats (Swanson et al., 2013; Brannelly et al., 2014; Davis et al., 2014).

In CHAPTER X we evaluated if Alpha tags may represent a proper method to mark *Hydromantes* salamanders. During our study we encountered several hurdles. First, Alpha tags have standardised size, preventing to mark individuals which are not big enough. Implantation of tags was not always easy, sometimes requiring particular conditions. Retention of tags was not high and the tag wounds required long time to heal, exposing individuals to possible infections. Our results show that Alpha tags are not a valid choice to mark *Hydromantes*.

Evaluating reliability of *N*-mixture models to estimate population abundance

Estimating species abundance is a crucial task of species monitoring, as in most of cases individuals' detection is challenging. This mostly happen for several reasons, such as species elusive behaviour, cryptic habits or simply because of the limited ability of surveyors (Mazerolle et al., 2007). Therefore, the number of observed individuals rarely represents a reliable estimation of the number of individuals actually present in a given area. Several approaches have been developed to estimate the true number of present individuals, and among them, those requiring multiple capture sessions (e.g capture-mark-recaputre) have a considerable success (Chao, 2001). However, approaches requiring the capture and identification are generally labour intensive, as many individuals need to be captured and identified to obtain reliable population estimates. In the last years formal approaches have been proposed to estimate animal abundance from repeated counts at fixed sites without individual marking/identification (Royle and Nichols, 2003; Royle, 2004). The number of individuals detected in a given site is counted using standard monitoring techniques (e.g. point counts, observation in small plots, fixed area transects), and each site is generally surveyed in multiple occasions. The repeated counts in a given site are then used to jointly estimate the detectability of individuals and population size on the basis of *N*-mixture (or hierarchical) models (Royle and Nichols, 2003; Royle, 2004; Kéry et al., 2009; Dail and Madsen, 2011). As they do not require capture or manipulation of individuals, such models might allow to collect abundance information over larger areas compared to traditional approaches, can be also appropriate for protected species, and have been proposed for broad-scale assessment of populations (Kéry et al., 2009; Ficetola et al., 2017).

In Chapter XI we tested the efficiency of *N*-mixture models on the Ambrosi's cave salamander (*Hydromantes ambrosii*), comparing estimation obtained from such methodology with that obtained from removal sampling. In June 2017, we monitored ten caves of *H. ambrosii*, as during hot season underground activity of *Hydromantes* is higher (Ficetola et al., 2012). Each cave was monitored six times within a period of ten days, to ensure population closure. During first three surveys individuals were counted avoiding any type of manipulation to perform estimation using *N*-mixtures (Royle, 2004); in the following three, animals were removed to perform estimation with the removal sampling method (Chao and Chang, 1999). *N*-mixture produced an estimation of salamander ranging from 13 to 135 per site; estimation from removal sampling ranged between 13 and 244 salamanders per site. Overall, *N*-mixture models and removal provided highly correlated and consistent estimates of population densities, with overlapping confidence intervals in most of populations. The use of *N*-mixture models represents a powerful method to estimate wild population as *i*) it does not require individual manipulation, *ii*) it gives the opportunity to add covariates to the model, improving accuracy, and *iii*) costs related to this method (i.e. man/working days, instruments) are contained.

References

Akrfim, F., T. Mahmood, R. Hussafin, S. Qasfim and I.-u.-d. Zangfi (2017). "Distribution pattern, population estimation and threats to the Indian Pangolin *Manis crassicaudata* (Mammalia: Pholidota: Manidae) in and around Pir Lasura National Park, Azad Jammu & Kashmir, Pakistan." Journal of Threatened Taxa **9**(3): 9920-9927.

Akunke Atuo, F., S. T. Ivande, Z. J. Wala, S. Manu and T. J. O'Connell (2016). "Current distribution, breeding population and habitat use of the globally threatened Grey-necked *Picathartes Picathartes* oreas in south-eastern Nigeria: a call for conservation action." Ostrich **87**(2): 101-112.

Angilletta Jr., M. J., P. H. Niewiarowski and C. A. Navas (2002). " The evolution of thermal physiology in ectotherms." Journal of Thermal Biology **27**: 249-268.

Balmford, A., A. Bruner, P. Cooper, R. Costanza, S. Farber, R. E. Green, M. Jenkins, P. Jefferiss, V. Jessamy, J. Madden, K. Munro, N. Myers, S. Naeem, J. Paavola, M. Rayment, S. Rosendo, J. Roughgarden, K. Trumper and R. K. Turner (2002). "Economic reasons for conserving wild nature." Science **297**: 950-953.

Barnosky, A. D., N. Matzke, S. Tomiya, G. O. U. Wogan, B. Swartz, T. B. Quental, C. Marshall, J. L. McGuire, E. L. Lindsey, K. C. Maguire, B. Mersey and E. A. Ferrer (2011). "Has the Earth's sixth mass extinction already arrived?" Nature **471**: 51-57.

Bay, R. A. and J. K. Ruegg (2017). "Genomic islands of divergence or opportunities for introgression?" Proceedings of the Royal Society B **284**: 20162414.

Beck, J., L. Ballesteros-Mejia, C. M. Buchmann, J. Dengler, S. A. Fritz, B. Gruber, C. Hof, F. Jansen, S. Knapp, H. Kreft, A. K. Schneider, M. Winter and C. F. Dormann (2012). "What's on the horizon for macroecology?" Ecography **35**: 673-683.

Beebee, T. J. C. and R. A. Griffiths (2005). "The amphibian decline crisis: a watershed for conservation biology?" Biological Conservation **125**: 271-285.

BenDor, T. K., K. A. Vitro and J. A. Riggsbee (2017). "Pre-listing conservation of candidate species under the endangered species act: an evaluation of prevalence, accessibility, and market-based conservation efforts." Environmental Science and Policy **74**: 68-74.

Blomberg, S. P. and T. J. Garland (2002). "Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods." Journal of Evolutionary Biology **15**: 899-910.

Bogaerts, S., M. Sparreboom, F. Pasmans, A. Almasri, W. Beukema, A. Shehab and Z. S. Amr (2013). "Distribution, ecology and conservation of *Ommatotriton vittatus* and *Salamandra infraimmaculata* in Syria." Salamandra **49**(2): 87-96.

Borzée, A., K. Kim, K. Heo, P. J. Jablonski and Y. Jang (2017). "Impact of land reclamation and agricultural water regime on the distribution and conservation status of the endangered *Dryophytes suweonensis*." PeerJ **5**: e3872.

Brambilla, M. and F. Saporetti (2014). "Modelling distribution of habitats required for different uses by the same species: implications for conservation at the regional scale." Biological Conservation **174**: 39-46.

Brannelly, L. A., L. Berger and L. F. Skerratt (2014). "Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*." Herpetological Conservation and Biology **9**(2): 428-435.

Bressi, N. (2004). "Underground and unknown: updated distribution, ecological notes and conservation guidelines on the Olm *Proteus anguinus anguinus* in Italy (Amphibia, Proteidae)." Italian Journal of Zoology **71**(1): 55-59.

Brodie, G., G. M. Barker, H. Pippard, C. S. Bick and D. Ó Foighil (2016). "Disappearing jewels: an urgent need for conservation of Fiji's partulid tree snail fauna." Pacific Conservation Biology **22**: 249-261.

Carranza, S., A. Romano, E. N. Arnold and G. Sotgiu (2008). "Biogeography and evolution of European cave salamanders, *Hydromantes* (Urodela: Plethodontidae), inferred from mtDNA sequences." Journal of Biogeography **35**: 724-738.

Ceballos, G., P. R. Ehrlich, A. D. Barnosky, A. García, R. M. Pringle and T. M. Palmer (2015). "Accelerated modern human–induced species losses: Entering the sixth mass extinction." Science Advances **1**: e1400253.

Chao, A. (2001). "An overview of closed capture–recapture models." Journal of Agricultural, Biological, and Environmental Statistics **6**(2): 158-175.

Chao, A. and S.-H. Chang (1999). "An estimating function approach to the inference of catch-effort models." Environmental and Ecological Statistics **6**: 313-334.

Chiari, Y., A. van der Meijden, M. Mucedda, J. M. Lourenc, A. Hochkirch and M. Veith (2012). "Phylogeography of Sardinian Cave Salamanders (Genus *Hydromantes*) Is Mainly Determined by Geomorphology." PLoS ONE **7**(3): e32332.

Cimmaruta, R., G. Forti, D. Lucente and G. Nascetti (2013). "Thirty years of artificial syntopy between *Hydromantes italicus* and *H. ambrosii ambrosii* (Amphibia, Plethodontidae)." Amphibia-Reptilia **34**: 413-420.

Cimmaruta, R., G. Forti, G. Nascetti and L. Bullini (1999). "Spatial distribution and competition in two parapatric sibling species of European plethodontid salamanders." Ethology Ecology & Evolution **11**(4): 383-398.

Costa, A., F. Crovetto and S. Salvidio (2016). "European plethodontid salamanders on the forest floor: local abundance is related to fine-scale environmental factors." Herpetological Conservation and Biology **11**(2): 344-349.

Costanza, R., R. d'Arge, R. de Groot, S. Farber, M. Grasso, B. Hannon, K. Limburg, S. Naeem, R. V. O'Neill, J. Paruelo, R. G. Raskin, P. Sutton and M. van den Belt (1997). "The value of the world's ecosystem services and natural capital." Nature **387**: 253-260.

Crovetto, F., A. Romano and S. Salvidio (2012). "Comparison of two non-lethal methods for dietary studies in terrestrial salamanders." Wildlife Research **39**: 266-270.

Culver, D. C. and T. Pipan (2009). The biology of caves and other subterranean habitats. New York, Oxford Unuiversity Press.

Dail, D. and L. Madsen (2011). "Models for estimating abundance from repeated counts of an open metapopulation." Biometrics **67**: 577-587.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green and R. Speare (1999). "Emerging infectious diseases and amphibian population declines." Emerging Infectious Diseases **5**(6): 735-748.

Davis, J. L., M. E. Barnes, J. L. Kientz and A. G. Galinat (2014). "Effects of fish length and anatomical placement on retention of Visible Implant Alpha tags in hatchery-reared rainbow trout." North American Journal of Fisheries Management **34**: 932-937.

De Hert, K., H. Jacquemyn, S. Van Glabeke, I. Roldán-Ruiz, K. Vandepitte, L. Leus and O. Honnay (2012). "Reproductive isolation and hybridization in sympatric populations of three *Dactylorhiza* species (Orchidaceae) with different ploidy levels." Annals of Botany **109**: 709-720.

Díaz-López, G., R. Salazar-Cuytun, R. García Herrera, A. Piñeiro-Vázquez, F. Casanova-Lugo and A. J. Chay-Canul (2017). "Relationship between body weight and body condition score with energy content in the carcass of Pelibuey ewes." Austral Journal of Veterinary Sciences **49**: 77-81.

Dittmar, E. M., D. A. Cimprich, J. H. Sperry and P. J. Weatherhead (2014). "Habitat selection by juvenile Black-capped vireos following Independence from parental care." The Journal of Wildlife Management **78**(6): 1005-1011.

Doherty, T. S., A. S. Glen, D. G. Nimmo, E. G. Ritchie and C. R. Dickman (2016). "Invasive predators and global biodiversity loss." Proceedings of the National Academy of Sciences **113**(40): 1-5.

DuRant, S. E., W. A. Hopkins, A. K. Davis and L. M. Romero (2015). "Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*)." The Journal of Experimental Biology **218**: 2297-2304.

Dyer, E. E., P. Cassey, D. W. Redding, B. Collen, V. Franks, K. J. Gaston, K. E. Jones, S. Kark, C. D. L. Orme and T. M. Blackburn (2017). "The global distribution and drivers of alien bird species richness." PLos Biology **15**(1): e2000942.

Elliot, J. M. and M. Dobson, Eds. (2015). Freshwater Leeches of Britain and Ireland: Keys to the Hirudinea and a Review of their Ecology. Ambleside, The Freshwater Biological Association.

Emata, A. C. and C. L. Marte (1992). "The use of a Visual Implant tag to monitor the reproductive performance of individual milkfish *Chanos chanos* Forsskal." Journal of Applied Ichthyology **8**: 314-317.

Enriquez-Urzelai, U., A. Montori, G. A. Llorente and A. Kaliontzopoulou (2015). "Locomotor mode and the evolution of the hindlimb in Western Mediterranean Anurans." Evolutionary Biology **42**: 199-209.

Ficetola, G. F. and L. Maiorano (2016). "Contrasting effects of temperature and precipitation change on amphibian phenology, abundance and performance." Oecologia **181**: 683-693.

Ficetola, G. F., R. Pennati and R. Manenti (2012). "Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*." Amphibia-Reptilia **33**: 251-259.

Ficetola, G. F., R. Pennati and R. Manenti (2013). "Spatial segregation among age classes in cave salamanders: habitat selection or social interactions?" Population Ecology **55**: 217-226.

Ficetola, G. F., A. Romano, S. Salvidio and R. Sindaco (2017). "Optimizing monitoring schemes to detect trends in abundance over broad scales." Animal Conservation.

Ficetola, G. F. and M. Stöck (2016). "Do hybrid-origin polyploid amphibians occupy transgressive or intermediate ecological niches compared to their diploid ancestors?" Journal of Biogeography **43**(4): 703-715.

Filippi, E. and L. Luiselli (2000). "Status of the Italian snake fauna and assessment of conservation threats." Biological Conservation **93**: 219-225.

Fitzpatrick, B. M. (2004). "Rates of evolution of hybrid inviability in birds and mammals." Evolution **58**(8): 1865-1870.

Forti, G., B. Lanza, R. Cimmaruta and G. Nascetti (2005). "An experiment of artificial syntopy *ex situ* between *Speleomantes italicus* (Dunn, 1923) and *S. ambrosii ambrosii* (Lanza, 1955) (Amphibia, Plethodontidae)." Annali del Museo Civico di Storia Natualre Giacomo Doria Genova **97**: 123-133.

Garner, T. W. J., M. W. Perkins, P. Govindarajulu, D. Seglie, S. Walker, A. A. Cunningham and M. C. Fisher (2006). "The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*." Biology Letters **2**: 455-459.

Godsoe, W. (2010). "I can’t define the niche but I know it when I see it: a formal link between statistical theory and the ecological niche." Oikos **119**: 53-60.

Grant, P. R. and B. R. Grant (2016). "Introgressive hybridization and natural selection in Darwin’s finches." Biological Journal of the Linnean Society **117**: 812-822.

Guillaume, O. and J. P. Durand (2003). Les Spélerpès : genre *Speleomantes* Dubois, 1984. Évolution holocène de la faune de Vertébrés de France: invasions et disparitions. Rapport au Ministère de l'Écologie et du Développement Durable (Direction de la Nature et des Paysages). M. Pascal, O. Lorvelec, J.-D. Vigne, P. Keith and P. Clergeau.

Paris, France, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, Muséum National d'Histoire Naturelle**:** 143-144.

Guisan, A. and W. Thuiller (2005). "Predicting species distribution: offering more than simple habitat models." Ecology Letters **8**: 993-1009.

Gunderson, A. R. and M. Leal (2016). "A conceptual framework for understanding thermal constraints on ectotherm activity with implications for predicting responses to global change." Ecology Letters **19**: 111-120.

Gürtler, R. E., V. M. Izquierdo, G. Gil, M. Cavicchia and A. Maranta (2017). "Coping with wild boar in a conservation area: impacts of a 10-year management control program in north-eastern Argentina." Biological Invasions **19**(1): 11-24.

Hetema, R. S., W. M. Strauss, L. G. Fick, S. K. Maloney, L. C. R. Meyer, M. Shobrak, A. Fuller and D. Mitchell (2012). "Activity re-assignment and microclimate selection of free-living Arabian oryx: responses that could minimise the effects of climate change on homeostasis?" Zoology **115**: 411-416.

Hoffmann, J. (1960). "Notules Hirudinologiques." Archives de la section de Sciences Naturelles, Physiques et Mathematiques de l'Institut Grand-ducal de Luxembourg **27**: 285-291.

Howard, R. D. (1978). "The influence of male-defended oviposition sites on early embryo mortality in Bullfrogs." Ecology **59**(4): 789-798.

IUCN Standards, P. S. (2016) Guidelines for using the IUCN Red List categories and criteria. Version 12. DOI: Downloadable from. http://www.iucnredlist.org/documents/RedListGuidelines.pdf

Jakob, E. M., S. D. Marshall and G. W. Uetz (1996). "Estimating fitness: a comparison of body condition indices." Oikos **77**: 61-77.

Jørgensen, S. E., Ed. (2009). Ecosystem Ecology. Amsterdam, Elsevier.

Kauano, É. E., J. M. C. Silva and F. Michalski (2017). "Illegal use of natural resources in federal protected areas of the Brazilian Amazon." PeerJ **5**: e3902.

Kearney, M. R., A. Shamakhy, R. Tingley, D. J. Karoly, A. A. Hoffmann, P. R. Briggs and W. P. Porter (2014). "Microclimate modelling at macro scales: a test of a general microclimate model integrated with gridded continental-scale soil and weather data." Methods in Ecology and Evolution **5**: 27-.286.

Kearney, M. R., S. J. Simpson, D. Raubenheimer and S. A. L. M. Kooijman (2013). "Balancing heat, water and nutrients under environmental change: a thermodynamic niche framework." Functional Ecology **27**: 950-965.

Kéry, M., R. M. Dorazio, L. Soldaat, A. van Strien, A. Zuiderwijk and J. A. Royle (2009). "Trend estimation in populations with imperfect detection." Journal of Applied Ecology **46**: 1163-1172.

Kraus, F. (2009). Alien Reptiles and Amphibians a scientific compendium and analysis. Dordrecht, Springer.

Labocha, M. K., H. Schutz and J. P. Hayes (2014). "Which body condition index is best?" Oikos **123**: 111-119.

Lanza, B. and P. Leo (2001). Prima osservazione sicura di riproduzione vivipara nel genere *Speleomantes* (Amphibia: Caudata: Plethodontidae). Atti 3° Congresso Nazionale Societas Herpetologica Italica (Pavia, 14-16 settembre 2000). F. Barbieri, F. Bernini and M. Fasola. Pianura, Cremona**:** 317-319.

Lanza, B., C. Pastorelli, P. Laghi and R. Cimmaruta (2006). "A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae)." Atti del Museo Civico di Storia Naturale di Trieste **52**: 5-135.

Losos, J. B. (2008). "Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species." Ecology Letters **11**: 995-1007.

Lucente, D., J. Renet, M. Gailledrat, J. Tillet, G. Nascetti and R. Cimmaruta (2016). "A new population of European cave salamanders (genus *Hydromantes*) from west-central France: relict or introduction?" Herpetological Bulletin **138**: 21-23.

Lunghi, E., R. Manenti and G. F. Ficetola (2014a). "Do cave features affect underground habitat exploitation by non-troglobite species?" Acta Oecologica **55**: 29-35.

Lunghi, E., R. Manenti and G. F. Ficetola (2015). "Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection?" PeerJ **3**: e1122.

Lunghi, E., R. Manenti and G. F. Ficetola (2017). "Cave features, seasonality and subterranean distribution of non-obligate cave dwellers " PeerJ **5**: e3169.

Lunghi, E., R. Manenti, S. Manca, M. Mulargia, R. Pennati and G. F. Ficetola (2014b). "Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments." Salamandra **50**(2): 105-109.

Macdonald, D. W. (2010). The encyclopedia of Mammals. Oxford, Oxford University Press.

Manenti, R. (2014). "Dry stone walls favour biodiversity: a case-study from the Appennines." Biodiversity and Conservation **23**: 1879-1893.

Manenti, R., E. Lunghi, C. Canedoli, M. Bonaccorsi and G. F. Ficetola (2016). "Parasitism of the leech, *Batracobdella algira* (Moquin-Tandon, 1846), on Sardinian cave salamanders (Genus *Hydromantes*) (Caudata: plethodontidae)." Herpetozoa **29**(1/2): 27-35.

Manenti, R., E. Lunghi and G. F. Ficetola (2015). "Distribution of spiders in cave twilight zone depends on microclimatic features and trophic supply." Invertebrate Biology **134**: 242-251.

Mazerolle, M. J., L. L. Bailey, W. L. Kendall, J. A. Royle, S. J. Converse and J. D. Nichols (2007). "Making great leaps forward: accounting for detectability in herpetological field studies." Journal of Herpetology **41**(4): 672-689.

McCarthy, D. P., P. F. Donald, J. P. W. Scharlemann, G. M. Buchanan, A. Balmford, J. M. H. Green, L. A. Bennun, N. D. Burgess, L. D. C. Fishpool, S. T. Garnett, D. L. Leonard, R. F. Maloney, P. Morling, H. M. Schaefer, A. Symes, D. A. Wiedenfeld and S. H. M. Butchart (2012). "Financial costs of meeting global biodiversity conservation targets: current spending and unmet needs." Science **338**: 946-949.

Merilä, J. and M. Sterner (2002). "Medicinal leeches (*Hirudo medicinalis*) attacking and killing adult amphibians." Annales Zoologici Fennici **39**: 343-346.

Mertens, R. (1923). "Zur Biologie des Höhlenmolches, *Spelerpes fuscus* Bonaparte." Blätter für Aquarien- und Terrarien-Kunde **34**: 171-174.

Mock, B. A. and D. E. Gill (1984). "The infrapopulation dynamics of trypanosomes in red-spotted newts." Parasitology **88**: 267-282.

Murray, N. J., D. A. Keith, L. M. Bland, E. Nicholson, T. J. Regan, J. P. Rodríguez and M. Bedward (2017). "The use of range size to assess risks to biodiversity from stochastic threats." Diversity and Distributions **23**(5): 474-483.

Oneto, F., D. Ottonello, M. V. Pastorino and S. Salvidio (2010). "Posthatching parental care in salamanders revealed by infrared video surveillance." Journal of Herpetology **44**(4): 649-653.

Oneto, F., D. Ottonello, M. V. Pastorino and S. Salvidio (2014). "Maternal care and defence of young by the plethodontid salamander *Speleomantes strinatii* (Aellen, 1951)." Scripta Herpetologica. Studies on Amphibians and Reptiles in honour of Benedetto Lanza: 129-138.

Papinuto, S. (2005). "Sul ritrovamento ed il monitoraggio di una nidiata di *Speleomantes genei* (Temminck & Schlegel, 1838) (Amphibia Urodela Plethodontidae) in una galleria mineraria dell'Inglesiente (Sardegna sud-occidentale)." Sardegna Speleologica **22**: 3-6.

Peterson, A. T. (2011). "Ecological niche conservatism: a time-structured review of evidence." Journal of Biogeography **38**: 817-827.

Peterson, A. T., J. Soberón, R. G. Pearson, R. P. Anderson, E. Martínez-Meyer, M. Nakamura and M. B. Araújo (2011). Ecological niches and geographic distributions. Princeton, Princeton University Press.

Pierce, B. A., K. D. Mcentire and A. A. E. Wall (2014). "Population size, movement, and reproduction of the Georgetown salamander, *Eurycea naufragia*." Herpetological Conservation and Biology **9**(1): 137-145.

Raske, M., G. A. Lewbart, D. S. Dombrowski, P. Hale, M. Correa and L. S. Christian (2012). "Body temperatures of selected amphibian and reptile species." Journal of Zoo and Wildlife Medicine **43**: 517-521.

Restrepo, A., J. A. Velasco and J. M. Daza (2017). "Extinction risk or lack of sampling in a threatened species: genetic structure and environmental suitability of the neotropical frog *Pristimantis penelopus* (Anura: Craugastoridae)." Papéis Avulsos de Zoologia **57**(1): 1-15.

Rigbi, M., H. Levy, A. Eldor, F. Iraqi, M. Teitelbaum, M. Orevi, A. Horovitz and R. Galun (1987). "The saliva of the medicinal leech *Hirudo medicinalis*-II. Inhibition of platelet aggregation and of leukocyte activity and examination of reputed anaesthetic effects." Comparative Biochemistry and Physiology **88C**(1): 95-98.

Rocha, R., E. Borda, F. Andreone and G. M. Rosa (2012). "First reports of leech parasitism in Malagasy anurans." Comparative Parasitology **79**(2): 352-356.

Romano, A. and A. R. Di Cerbo (2007). "Leech predation on amphibian eggs." Acta Zoologica Sinica **53**(4): 750-754.

Royle, J. A. (2004). "*N*-mixture models for estimating population size from spatially replicated counts." Biometrics **60**: 108-115.

Royle, J. A. and J. D. Nichols (2003). "Estimating abundance from repeated presence–absence data or point counts." Ecology **84**(3): 777-790.

Ruggi, A., R. Cimmaruta, G. Forti and G. Nascetti (2005). "Preliminary study of a hybrid zone between *Speleomantes italicus* Dunn 1923 and *S. ambrosii* Lanza 1955 on the Apuan Alps, using RLFP analysis." Annali del Museo Civico di Storia Naturale Giacomo Doria Genova **97**: 135-144.

Salvidio, S. (1993). "Life history of the European plethodontid salamander *Speleomantes ambrosii* (Amphibia, Caudata)." Herpetological Journal **3**: 55-59.

Salzet, M., A. Capron and G. B. Stefano (2000). "Molecular crosstalk in host–parasite relationships: schistosome– and leech–host interactions." Parasitology Today **16**(12): 536-540.

Saupe, E. E., J. R. Hendricks, R. W. Portell, H. J. Dowsett, A. Haywood, S. J. Hunter and B. S. Lieberman (2014). "Macroevolutionary consequences of profound climate change on niche evolution in marine molluscs over the past three million years." Proceedings of the Royal Society B **281**: 20141995.

Sawyer, R. T. (1981). Leech biology and behavior. Neurobiology of the Leech. K. J. Muller, J. G. Nichols and G. S. Stent. New York, Cold Spring Harbor Laboratory Publications**:** 7-26.

Seebacher, F. and R. A. Alford (1999). "Movement and microhabitat use of a terrestrial amphibian (*Bufo marinus*) on a tropical island: seasonal variation and environmental correlates." Journal of Herpetology **33**(2): 208-214.

Seehausen, O. (2004). "Hybridization and adaptive radiation." Trends in Ecology and Evolution **19**: 198-207.

Sillero, N., J. Campos, A. Bonardi, C. Corti, R. Creemers, P.-A. Crochet, J. C. Isailović, M. Denoël, G. F. Ficetola, J. Gonçalves, S. Kuzmin, P. Lymberakis, P. de Pous, A. Rodríguez, R. Sindaco, J. Speybroeck, B. Toxopeus, D. R. Vieites and M. Vences (2014). "Updated distribution and biogeography of amphibians and reptiles of Europe." Amphibia-Reptilia **35**: 1-31.

Soberón, J. (2007). "Grinnellian and Eltonian niches and geographic distributions of species." Ecology Letters **10**: 1115-1123.

Soberón, J. and M. Nakamura (2009). "Niches and distributional areas: concepts, methods, and assumptions." Proceedings of the National Academy of Sciences **106**: 19644-19650.

Spotila, J. R. (1972). "Role of temperature and water in the ecology of lungless salamanders." Ecological Monographs **42**: 95-125.

Stefani, R. and G. Serra (1966). "L'oviparità in *Hydromantes genei* (Temm. et Schl.)." Bollettino di Zoologia **33**: 283-291.

Sunday, J. M., A. E. Bates, M. R. Kearney, R. K. Colwell, N. K. Dulvy, J. T. Longino and R. B. Huey (2014). "Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation." PNAS **111**: 5610-5615.

Swanson, J. E., L. L. Bailey, E. Muths and W. C. Funk (2013). "Factors influencing survival and mark retention in postmetamorphic Boreal chorus frogs." Copeia **2013**(4): 670-675.

Tanaka, Y. (2007). "Introgressive hybridization as the breakdown of postzygotic isolation: a theoretical perspective." Ecological Research **22**: 929-939.

Thuiller, W., T. Münkemüller, K. H. Schiffers, D. Georges, S. Dullinger, V. M. Eckhart, T. C. J. Edwards, D. Gravel, G. Kunstler, C. Merow, K. Moore, C. Piedallu, S. Vissault, N. E. Zimmermann, D. Zurell and F. M. Schurr (2014). "Does probability of occurrence relate to population dynamics?" Ecography **37**(12): 1155-1166.

Wake, D. B. (1966). "Comparative osteology and evolution of the lungless salamanders, family Plethodontidae." Memoir of the Southern California Academy of Sciences **4**: 1-111.

Wake, D. B. (2013). "The enigmatic history of the European, Asian and American plethodontid salamanders." Amphibia-Reptilia **34**: 323-336.

Wake, D. B., A. Salvador and M. A. Alonso-Zarazaga (2005). "Taxonomy of the plethodontid salamander genus *Hydromantes* (Caudata: Plethodontidae)." Amphibia-Reptilia **26**: 543-548.

Weber, M. M., R. D. Stevens, J. A. F. Diniz-Filho and C. E. V. Grelle (2017). "Is there a correlation between abundance and environmental suitability derived from ecological niche modelling? A meta-analysis." Ecography **40**: 817-828.

Wells, K. D. (2007). The ecology and behavior of amphibians. Chicago and London, The University of Chicago Press.

Chapter I

Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection?

Enrico Lunghi[1,2,3], Raoul Manenti[4] and Gentile Francesco Ficetola[5,6,7]

[1] Universitat¨ Trier Fachbereich VI Raum- und Umweltwissenschaften Biogeographie, Campus I, Gebaude¨ N Universitatsring,¨ Trier, Germany
[2] Natural Oasis, Prato, Italy
[3] Museo di Storia Naturale dell'Universita` di Firenze, Sezione di Zoologia "La Specola," Firenze, Italy
[4] Dipartimento di Bioscienze, Universita` degli Studi di Milano, Milano, Italy
[5] Dipartimento di Scienze dell'Ambiente e del Territorio e di Scienze della Terra, Universita` degli Studi di Milano-Bicocca, Piazza della Scienza, Milano, Italy
[6] Laboratoire d'Ecologie Alpine (LECA), Universite´ Grenoble-Alpes, Grenoble, France
[7] CNRS, LECA, Grenoble, France

ABSTRACT

Relationships between species and their habitats are not always constant. Different processes may determine changes in species-habitat association: individuals may prefer different habitat typologies in different periods, or they may be forced to occupy a different habitat in order to follow the changing environment. The aim of our study was to assess whether cave salamanders change their habitat association pattern through the year, and to test whether such changes are determined by environmental changes or by changes in preferences. We monitored multiple caves in Central Italy through one year, and monthly measured biotic and abiotic features of microhabitat and recorded Italian cave salamanders distribution. We used mixed models and niche similarity tests to assess whether species-habitat relationships remain constant through the year. Microhabitat showed strong seasonal variation, with the highest variability in the superficial sectors. Salamanders were associated to relatively cold and humid sectors in summer, but not during winter. Such apparent shift in habitat preferences mostly occurred because the environmental gradient changed through the year, while individuals generally selected similar conditions. Nevertheless, juveniles were more tolerant to dry sectors during late winter, when food demand was highest. This suggests that tolerance for suboptimal abiotic conditions may change through time, depending on the required resources. Differences in habitat use are jointly determined by environmental variation through time, and by changes in the preferred habitat. The trade-offs between tolerance and resources requirement are major determinant of such variation.

Subjects Animal Behavior, Ecology, Zoology
Keywords Tolerance, *Hydromantes italicus*, Cave, Distribution, Microclimate, Physiological niche, Spatial segregation, Biospeleology, Plethodontid

Submitted 15 April 2015
Accepted 3 July 2015
Published 13 August 2015

Corresponding author
Enrico Lunghi,
enrico.arti@gmail.com

Academic editor
Ann Hedrick

Additional Information and Declarations can be found on page 16

DOI **10.7717/peerj.1122**

How to cite this article Lunghi et al. (2015), Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection?. **PeerJ 3:e1122**; **DOI 10.7717/peerj.1122**

INTRODUCTION

The use of habitat models to evaluate factors determining species distributions is becoming increasingly prevalent in ecological research (*Peterson et al., 2011*; *Warren, 2012*; *Stein, Gerstner & Kreft, 2014*). Such models help understanding the factors determining species occurrence, and may allow predicting potential areas of occupancy, with important consequences for planning adequate conservation actions (*Dom´ıguez-Vega et al., 2012*; *Bogaerts et al., 2013*). Despite repeated calls for mechanistic modelling (*Kearney & Porter, 2009*), correlative habitat models remain the most frequently used approach. Correlative models combine data on species occurrence (e.g., presence/absence, presence-only, abundance) with information on environmental features, identifying statistical relationships which represent the basis for model predictions (*Guisan & Thuiller, 2005*). Such models are based on the assumptions that species presence is associated with favorable environmental features (species-habitat association) (*Godsoe, 2010*).

However, patterns of species-habitat association may be not consistent during time. Analyses of habitat associations generally assume that species are at quasi-equilibrium with the environment, but this assumption may not always hold (e.g., during dispersion or contraction phases) (*Saupe et al., 2014*). Furthermore, differences in habitat association patterns may occur through two distinct, non-exclusive processes: the species may select different habitats across their life-time (selection change hypothesis), and environmental features may change through time (environmental change hypothesis). According to the selection change hypothesis, a given species may be associated with different environmental features in different time periods and/or life stages. For instance, many species show seasonal activities, and select different environments depending on the activities performed (e.g., nesting, foraging, wintering) (*Seebacher & Alford, 1999*; *Brambilla & Saporetti, 2014*). In the long term, temporal variation for habitat association in a given species may also occur due to evolution of novel adaptations (*Nogu´es-Bravo, 2009*; *Stigall, 2012*). According to the environmental change hypothesis, temporal variation that exists for the many biotic and abiotic features can affect species distribution (*Kearney et al., 2013*). Such variation may occur over both short (e.g., variation of vegetation cover or temperature among the seasons) and longer timescales (e.g., climate change, habitat degradation) (*Saupe et al., 2014*). Both selection changes and environmental changes may influence the possibility of predicting species distribution in different time periods. Evaluating whether habitat association pattern changes through time, and the factors determining such variation, is extremely important to assess the transferability and generality of conclusions drawn from habitat modeling.

Among amphibians, plethodontid salamanders represent a very interesting study case. Due to their particular physiology, they need a narrow combination of environmental characteristics, and actively search places with suitable microclimatic conditions (cold temperature and high moisture; *Spotila, 1972*; *Camp & Jensen, 2007*. Cave salamanders (genus *Hydromantes*) may live both in surface and subterranean environments, but must move underground during the arid and hot Mediterranean summer, when the surface conditions become hot and dry (*Lanza et al., 2006*; *Ficetola, Pennati & Manenti, 2012*).

In subterranean environments microclimatic features are often considered to remain approximately stable, giving organisms the opportunity to inhabit caves constantly. Some studies have shown that cave salamanders are associated with caves having specific environmental features, such as low temperature, high humidity and presence of prey (*Ficetola, Pennati & Manenti, 2012*; *Lunghi, Manenti & Ficetola, 2014*), but these studies have been often performed during summer, when outdoor conditions are particularly unsuitable for salamanders, and abundance in cave is highest. However, caves are not closed systems, and environmental characteristics within caves can change over time due to external influences (*Romero, 2009*). Such fluctuations mostly affect areas near the entrance of caves (twilight zone) and can strongly influence cave communities (*Ficetola, Pennati & Manenti, 2013*; *Camp et al., 2014*; *Lunghi, Manenti & Ficetola, 2014*). Nevertheless, the few studies analyzing the seasonal variation in the distribution of European cave salamanders (*Salvidio et al., 1994*; *Vignoli, Caldera & Bologna, 2008*) did not test whether habitat selection changes through time.

The peculiar features of both caves and plethodontid salamanders make them an excellent system for species-habitat association studies. Cave environments are dominated by few, simple environmental gradients, such as light, depth, temperature, humidity and food availability (*Romero, 2009*), affording simplistic habitat characterization. Furthermore, species are easily detectable inside the delimited cave environments (*Ficetola, Pennati & Manenti, 2012*), allowing a reliable identification of occupied and unoccupied sectors.

The aim of this study was analyzing the variation through time of species-habitat association in the Italian cave salamander (*Hydromantes italicus*). First, we used habitat models to identify the relationships between the distribution of salamanders and microhabitat features, evaluating if the pattern of microhabitat association is constant through time. Second, we assessed whether the temporal variation in microhabitat occurs because the species selects different environmental features through the year, or because habitat features are affected by seasonal variation (i.e., we evaluated the support of the environmental changes vs. selection change hypotheses).

MATERIAL AND METHODS

Authorizations

All applicable institutional and/or national guidelines for the care and use of animals were followed. The study was conducted under authorization of Apuan Alps Regional Park (no 5, 4/04/2013), District of Prato (no 448, 2013), District of Pistoia (no 0022597/2013/P) and District of Lucca (no 731, 21/02/2013).

Surveys

For 12 months (from January 2013 to December 2013) we monitored 15 caves occupied by *Hydromantes italicus* in the North of Tuscan Apennines (Central Italy, between 43°52′42″N, 11°07′18″E and 43°59′51″N, 10°13′48″E). Preliminary surveys performed in 2012 indicated the presence of *H. italicus* at all sites. Surveys were conducted during day-time. The order of cave survey was chosen randomly, and the time interval between successive visits was 9–45 days. During surveys, for each cave we recorded monthly environmental data both inside and outside caves. Outside caves, we registered air temperature (accuracy: 0.1 °C) and humidity (accuracy: 0.1%) using a thermo-hygrometer Lafayette TDP92, in a shaded area 5–10 m from the entrance. The interior of each cave was divided into sectors of 3-m length, starting from the entrance and extending to the deepest explored area: our exploration was conducted until the end of the caves, or until the deepest sector reachable without speleological equipment. Three-m sectors approximately correspond to the home range of *Hydromantes* during their hypogean activity (*Salvidio et al., 1994*). Overall, we recorded data from 121 cave sectors [average development explored per cave: 24.2 m (range 6–60), corresponding to 2–20 sectors per cave]. At the time of surveys, in each sector we recorded four parameters known to influence cave salamanders. Air temperature, humidity and incident light (illuminance, measured using a Velleman DVM1300 light meter, minimum recordable light: 0.1 lux) represented the abiotic conditions of caves, which influence metabolism, water balance and activity (*Kearney et al., 2013*). The abundance of *Meta menardi* spiders was considered as a biotic variable. On the one hand, *Meta* spiders are major predators of juvenile salamanders (*Lanza et al., 2006*). Furthermore, *Meta* spiders are associated with areas showing high invertebrate abundance, and have been proposed as an indicator of prey abundance in cave environments (*Manenti, Lunghi & Ficetola, in press*). See *Ficetola, Pennati & Manenti (2013)* for additional details on the recording of cave features.

We used visual encounter surveys to assess the presence/absence of *H. italicus* and *M. menardi* spiders in each sector. This standardized technique allows to verify the presence of species in an area during a defined time (*Crump & Scott Jr, 1994*; *Jung et al., 2000*). If possible, salamanders were measured. Salamanders showing total length >6.5cm or with male secondary characters were considered adults (*Lanza et al., 2006*), the remaining salamanders were considered juveniles. All individuals were immediately released at the collection point.

Statistical analyses

Variation of environmental features of caves

We used linear mixed models (LMM) to analyze the temporal variation of cave microhabitat. We used the Akaike's Information Criterion corrected for small sample size (AICc) to identify the combination of parameters that better explain the variation of microclimatic features inside caves (*Stephens et al., 2007*). In LMM, we considered cave features (temperature, humidity, illuminance) as dependent factors, while outdoor features (temperature and humidity), linear distance from the cave entrance (hereafter, depth) and month of survey were considered as independent factors. We also considered the interaction between depth and month of survey. We also included the time of survey (hour and minute in which we began the survey) as an additional independent variable. Cave and sector identity were considered as random categorical variables, as they shows a typical combination of variables (both biotic and abiotic) independently from their position and location. For all models, Variance Inflation Factor was <5, confirming lack of collinearity issues (*Fox, 2002*). Seasonal variation also occurs for the distribution of cave spiders but was not analyzed here as it will be the focus of a separate study.

Relationships between species and environmental features

Not detecting a species during a survey does not necessarily mean that species is absent, as most species have detection probability <1 (*MacKenzie et al., 2006*). Standard approaches to the analysis of detection probability assume that sites are closed to changes in the state of occupancy for the duration of sampling (*MacKenzie et al., 2006*). However, cave salamanders quickly modify their occupancy patterns throughout the year in response to environmental variation (*Briggler & Prather, 2006*; *Camp & Jensen, 2007*; *Vignoli, Caldera & Bologna, 2008*), and therefore violate the closed population assumption. Approaches assuming open populations also exist but, in this study case, their implementation would require assumptions on population dynamics for which no data were available (*Dail & Madsen, 2011*). Sampling effort was standardized across sectors. Therefore, following recommendations by *Banks-Leite et al. (2014)*, we preferred performing analyses using standard mixed models, while verifying that low detection probability did not bias our results. First, we used generalized linear mixed models (GLMM) assuming binomial error to identify the relationships between the presence of salamanders and environmental features (air temperature, humidity, illuminance and spider abundance) of each sector, throughout the 12 months of sampling. To assess whether the habitat selection pattern is constant through time, we included the interactions between sampling month and environmental features. Sector and cave identity were included as random categorical factors. We built all possible model combinations, and ranked them using AICc. Complex models with AICc values higher than the simpler, nested models were not considered as candidate models (*Richards, Whittingham & Stephens, 2011*). We used a likelihood ratio test to assess the significance of terms in the best-AICc model. As microhabitat selection may be different among age classes (*Ficetola, Pennati & Manenti, 2013*), this analysis was repeated three times: first, considering all individuals, then considering adults and juveniles separately.

The results of the previous models may be affected by imperfect detection. We used the *MacKenzie & Kendall (2002)* approach to test detection probability of cave salamanders, on the basis of data collected in 22 sectors from three different caves. These caves were surveyed in late June-early July: during this interval, *Hydromantes* movements among sectors are expected to be limited (*Lanza et al., 2006*). For these sectors, two surveys were performed 9–14 days apart, therefore we assumed constant occupancy in this interval and estimated detection probability using single-season closed population occupancy models with the unmarked package in R (*Fiske & Chandler, 2011*). The analysis of detection probability was repeated twice: assuming constant detection across sectors, and assuming that detection probability is related to distance from cave entrance. We then used AIC to identify the best detection probability model.

Analyses (see results) showed a per-visit detection probability of 0.75, i.e., two surveys allow to ascertain presence/absences with 94% confidence (*Sewell, Beebee & Griffiths, 2010*). To assess the robustness of habitat models to imperfect detection, we also repeated the GLMM analysis by comparing two contrasting periods seasons: January–February and June–July. Movements between superficial and deep sectors are more frequent during spring and autumn (*Lanza et al., 2006*), thus we assumed that occupancy was relatively stable within these periods. We merged data from two-months periods respectively into winter (January–February) and summer (June–July), and repeated the analyses using the same variables of the best-AICc models obtained from the analyses of full dataset.

Testing the stability of habitat selection pattern

We used niche equivalency tests to assess whether salamanders select sectors with similar environmental features in different months, after taking into account differences for the availability of microhabitat conditions (*Broennimann et al., 2012*). The similarity of the habitat selection pattern in two distinct seasons was assessed using Schoener's *D*, a metric of niche similarity (*Warren, Glor & Turelli, 2008*; *Saupe et al., 2014*). For equivalency tests, salamander occurrences from different months were pooled and then randomly split in two datasets, maintaining the same number of occurrences of the original datasets; Schoener's *D* was then calculated. This procedure was repeated 300 times to assess whether niche similarity was significantly lower than expected by chance. The equivalency test was repeated for the two environmental variables (temperature and humidity) for which habitat models suggested differences among months. We focused on univariate rather than multivariate tests because we were interested on variation of habitat selection due to change of specific variables (*Saupe et al., 2014*). This analysis was performed on four months (January, February, June and July) showing contrasting patterns of habitat association (see results), and during which we do not expect major movements among cave sectors (i.e., within these intervals the quasi-equilibrium assumption is more likely to be hold than when seasonal migrations occur). The analysis was performed on all individuals together and for each age category (juveniles only and adults only). Since six pairwise tests were performed for each group and for each variable, significance values were corrected using sequential Bonferroni's correction (*Rice, 1989*).

RESULTS

Variation of environmental features inside caves

Internal temperature was strongly related to external temperature and humidity, month, depth and interaction between month and depth: all variables except depth were significant (Tables 1A and 2A). Seasonal change led to thermal inversion inside caves: from late autumn to early spring temperature increased with depth, while from late spring to early autumn temperature decreased in the deep sectors (Fig. 1A). Humidity inside caves was strongly related to external humidity, month, depth and to the time of survey. Furthermore, the significant interaction between month and depth indicated that the humidity gradient was not constant through the year (Tables 1B and 2B). The deepest sectors showed high stability of humidity through time, while fluctuations due to external variation were evident in sectors nearby the cave entrance. External humidity was particularly high in autumn and spring, determining an increase of humidity in the first sector of caves (Fig. 1B). Internal light incidence was related to depth and external humidity (Tables 1C and 2C). The deepest sectors always showed lower light

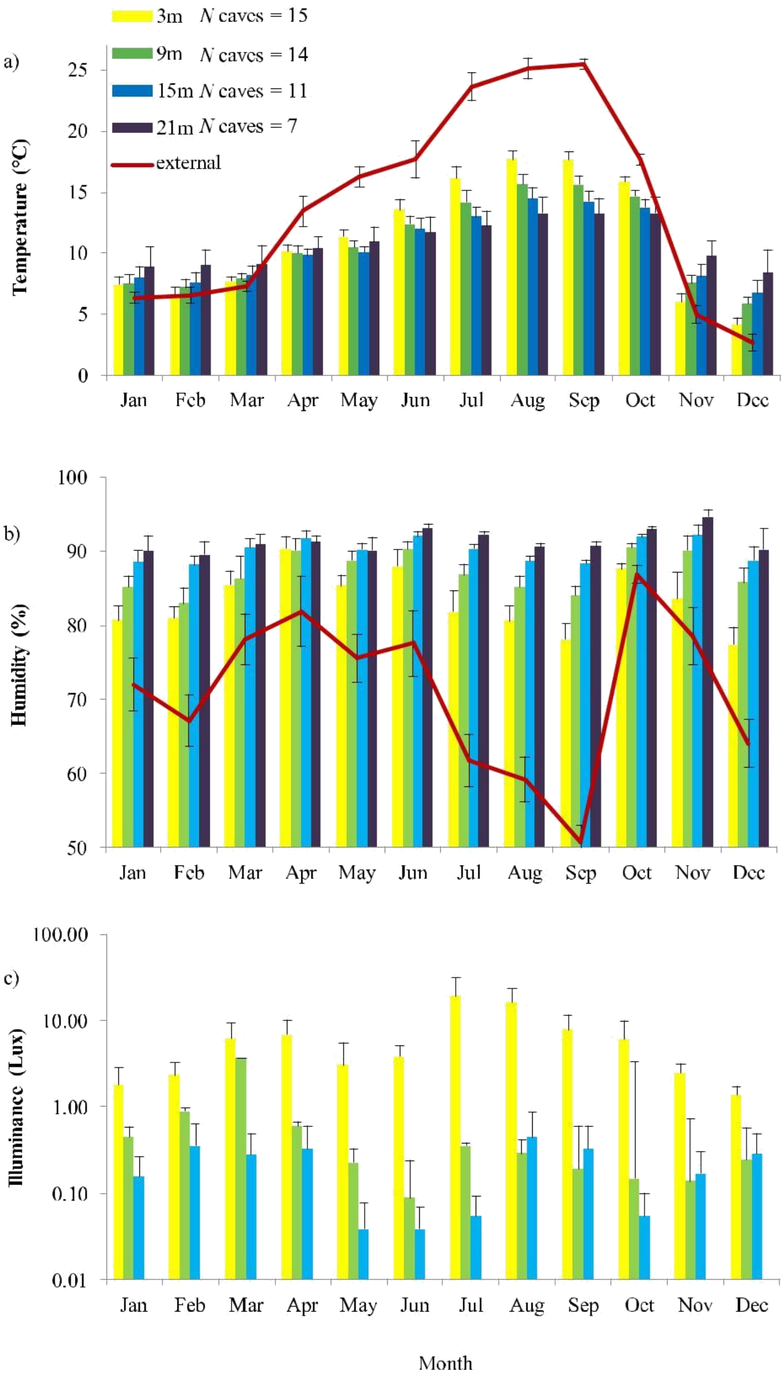


Figure 1 **Annual variation of external environment and cave microhabitat.** Internal variables are (A) temperature, (B) humidity and (C) illuminance (lux). In each graph, colored plots represent sectors located at different distance from the entrance (from 3 to 21 m). These sectors represent the area in which microclimate variability is higher; at 21 m illuminance was constantly 0 lux. Error bars are standard errors. For temperature and humidity, the trend of the respective external feature is also shown, represented by a continuous red line.

Table 1 Best AIC models explaining the variation in microhabitat features of caves. We considered as dependent variables inner abiotic features of caves: (A) Temperature, (B) Humidity and (C) Illuminance. We used as independent variables: Month of survey, Time in which the survey began, Depth of sector, External Temperature, External Humidity and interaction between Month and Depth (Prof : M). For each continuous variable, the regression coefficient is reported if the variable is included into a given model. For both categorical variables and interactions, + indicates their presence into the model. For each independent variable, we report the first five best models.

Independent variables included into the model						df	AICc	− AICc	Weight
Month	**Time of survey**	**Depth**	**External temperature**	**External humidity**	**Prof : M**				
(A) Temperature									
+		0.07	0.22	0.02	+	29	5,703	0	0.825
+	0.05	0.07	0.21	0.02	+	30	5,706.3	3.34	0.155
+		0.07	0.18		+	28	5,710.5	7.50	0.019
+	0.01	0.07	0.18		+	29	5,717.6	14.67	0.001
+	0.12	0.07			+	28	5,811.7	108.78	0
(B) Humidity									
+	0.34	0.27		0.15	+	29	8,314.1	0	0.517
+	0.31	0.27	0.11	0.16	+	30	8,314.2	0.15	0.480
+	0.26	0.24	0.15	0.17		19	8,325.2	11.09	0.002
+		0.27	0.16	0.15	+	29	8,329.8	15.75	0
+	0.31	0.24		0.16		18	8,329.8	15.75	0
(C) Illuminance									
		−0.04		−0.01		6	3,232.3	0	0.967
		−0.04	0.01	−0.01		7	3,239.7	7.41	0.024
	−0.01	−0.04		−0.01			3,241.7	9.40	0.009
	−0.01	−0.04	0.01	−0.01			3,248.7	16.39	0
		−0.04	0.01				3,256	23.72	0

than the superficial ones. However, incident light increased in summer and during periods characterized by low humidity (Fig. 1C).

Detection of cave salamanders

Through the 180 cave surveys, we obtained 1,087 detections of cave salamanders (289 adult males, 393 adult females, 49 not sexed adults and 356 juveniles). The average sampling effort was of 7.5 min/sector. Salamanders were detected throughout the year with 13% of detections in winter, 39% in spring, 30% in summer and 18% in autumn months. The model assuming constant detection probability across sectors showed a lower AIC value (AIC: 53.3) than the model assuming that detection probability is related to distance from the entrance (AIC: 53.9). Detection probability of salamanders within sectors was high (detection probability ± SE: 0.75 ± 0.12).

Analysis of occurrence of H. italicus through the year

Presence of *H. italicus* was strongly related to month, and was generally associated with sectors characterized by high humidity, low light and abundant *M. menardi* spiders

Table 2 **Parameters related to microclimatic change of caves through the year: best-AICc models.** The dependent variables were three major features of cave microclimate: (A) internal temperature, (B) internal humidity and (C) illuminance. Independent variables were: Month of survey, Depth of sector, Temp. ext (external temperature), Hum. ext (external humidity), Time (hour of survey).

Factor	***B***	χ^2	***P***
(A) Temperature (internal)			
Month		151	<**0.001**
Depth	0.07	0.81	0.368
Temp. ext	0.21	144.2	<**0.001**
Hum. ext	0.02	18.96	<**0.001**
Month × depth		680.71	<**0.001**
(B) Humidity (internal)			
Month		117.03	<**0.001**
Depth	0.27	105.91	<**0.001**
Hum. ext	0.16	205.3	<**0.001**
Time		27.95	<**0.001**
Month × depth		94.92	<**0.001**
(C) Illuminance			
Depth	−0.03	34.60	<**0.001**
Hum. ext	−0.01	49.66	<**0.001**

(Tables 3A and 4A). Furthermore, significant interactions between month and temper-ature and between month and humidity indicated different microhabitat selection patterns among months (Table 4A). Specifically, in winter periods salamanders were associated with warmest sectors, while in summer periods they were associated with coldest and most humid sectors (Figs. 2A and 2B).

The microhabitat selection pattern was similar if adults only were considered. Adults were more abundant in sectors with low light and abundant *M. menardi* (Tables 3B and 4B). Furthermore, differences among months were strong, and the interactions between month and both humidity and temperature were significant. Adults were associated with relatively cold sectors during summer, while in winter they were associated with warmer sectors (Fig. 2C). In summer, adults were associated with the most humid sectors; however, they showed a clear preference for the most humid sectors also in February (Fig. 2D).

Juveniles were more frequent in sectors with high humidity and abundant *M. menardi* spiders; furthermore the effect of month, and the interactions humidity-month and temperature-month were significant (Tables 3C and 4C). Juveniles were associated with the coldest sectors during winter and with warmer sectors during spring (Fig. 2E). From late winter until spring, juveniles were associated with sectors characterized by lower humidity, while during summer this apparent preference shifted in favor of most humid sectors (Fig. 2F).

Table 3 **Five best AIC models relating salamander distribution to environmental features.** We considered as dependent variable the presence of (A) the species, (B) presence of Adults and (C) presence of Juveniles. We used as independent variables: internal humidity (Humid), Month of survey, illuminance (Lux), *Meta* spiders abundance and internal temperature (Temp). Furthermore, we also used as independent variables interaction between month and internal humidity (Hum : M), month and illuminance (Lux : M), month and *Meta* spiders (*Meta* : M) and month and internal temperature (Temp : M). For each continuous variable, the regression coefficient is reported if the variable is included into a given model. For categorical variables and interactions, + indicates that the variable or the interaction is included into the model.

Independent variables included into the model									df	AICc	− AICc	Weight
Humid	**Month**	**Lux**	***Meta***	**Temp**	**Hum : M**	**Lux : M**	***Meta* : M**	**Temp : M**				
(A) Presence of the species												
1.12	+	**−0.34**	**0.44**	**0.27**	+			+	**40**	**1,384.8**	**0**	**0.709**
1.64	+	−0.36		0.26	+	+		+	39	1,388.9	4.15	0.089
−2.47	+	−20.74	0.45	0.25	+			+	51	1,389.3	4.5	0.075
1.41	+		0.45	0.27	+			+	39	1,390.2	5.45	0.046
7.79	+	−0.35	0.43		+				28	1,392.1	7.35	0.018
(B) Presence of adults												
1.27	+	**−0.43**	**0.39**	**0.16**	+			+	**40**	**1,253.8**	**0**	**0.721**
1.7	+	−0.44		0.16	+			+	39	1,256.2	2.44	0.213
1.67	+		0.42	0.16	+			+	39	1,261	7.25	0.019
6.83	+	−0.42	0.4		+				28	1,261.5	7.78	0.015
1.46	+	−0.44	−0.15	0.18	+		+	+	51	1,262.7	8.92	0.008
(C) Presence of juveniles												
1.46	+		**0.61**	**0.41**	+			+	**39**	**807.2**	**0**	**0.428**
1.23	+	−0.26	0.58	0.4	+			+	40	807.3	0.1	0.407
2.14	+	−0.3		0.39	+			+	39	810.5	3.22	0.085
2.57	+			0.39	+			+	38	810.8	3.57	0.072
−2.85	+	−20.8	0.59	0.35	+	+		+	51	816	8.72	0.005

Analysis between contrasting seasons

As detection probability was imperfect, we repeated the analysis by focusing on the comparison between two contrasting seasons (winter/summer), in which migration of salamanders is probably limited. During these two intervals we observed 112 salamanders in winter and 257 salamanders in summer. The results of this analysis were generally consistent with the analysis of the full dataset. If all individuals were pooled, salamanders were associated with the darkest sectors. Season strongly affected the presence of salamanders; furthermore, we detected a significant interaction between temperature and season; the interaction between humidity and season was marginally not significant (Table S1A). During these two seasons, salamanders were generally associated with sectors in which microclimate was different from outdoor climate conditions: in fact, they were associated with the most humid and cold sectors during summer, while in winter they preferred relatively warm sectors (Figs. S1A and S1B). Results were nearly identical in the analysis of adults-only (Table S1B; Figs. S1C and S1D). In the analysis of juveniles, only the interaction between season and temperature remained significant (Table S1C, Figs. S1E and S1F). However, it should be remarked that sample size was relatively small in this latter analysis (112 juveniles observed), and this may have limited statistical power, compared to the previous analyses.

Table 4 **Parameters related to presence/absence of salamanders.** The dependent variables were the presence of (A) Species, (B) Adults only and (C) Juveniles only. See Table 1 for explanation of variable names. Only the best-AICc models are shown.

Factor	***B***	χ^2	***P***
(A) Species			
Month		140.2	<**0.001**
Humidity	−2.65	4.3	**0.039**
Lux	−20.79	7.6	**0.006**
Meta abund.	0.36	6.3	**0.012**
Temperature	0.25	1.4	0.238
Hum × month		30.6	**0.001**
Temp × month		31.2	**0.001**
(B) Adults			
Month		128.7	<**0.001**
Humidity	−1.57	1.4	0.233
Lux	−1.95	9.4	**0.002**
Meta abund.	−2.31	4.6	**0.033**
Temp	1.74	0.3	0.567
Hum × month		37.3	<**0.001**
Temp × month		32.7	<**0.001**
(C) Juveniles			
Month		37.8	<**0.001**
Humidity	−3.60	5.4	**0.02**
Meta abund.	0.75	5.7	**0.017**
Temp	0.35	3.6	0.059
Hum × month		37	<**0.001**
Temp × month		39	<**0.001**

Stability of habitat selection pattern

Most of equivalency tests were not significant, suggesting that habitat selection pattern was consistent through months (Table 5). However, in the analyses of humidity considering all individuals and juveniles only, niche equivalency was significantly lower than expected by chance between February and June, and between February and July. Salamanders were more tolerant for low-humidity habitats than during winter (Fig. 3). Conversely, if adults only were analyzed, none of similarity tests were rejected (Table 5).

DISCUSSION

Caves are often described as stable environments (*Romero, 2012*), but their features and the distribution of their inhabitants shows strong fluctuations through the year, particularly in the superficial sectors. No doubt, the strong seasonal variation of salamander distribution was mostly dictated by the fluctuations of microhabitats. Nevertheless, habitat preferences and requirements may change across seasons, as in the case of juveniles that select microhabitats with slightly different conditions in different times (Figs. 2E and 2F).

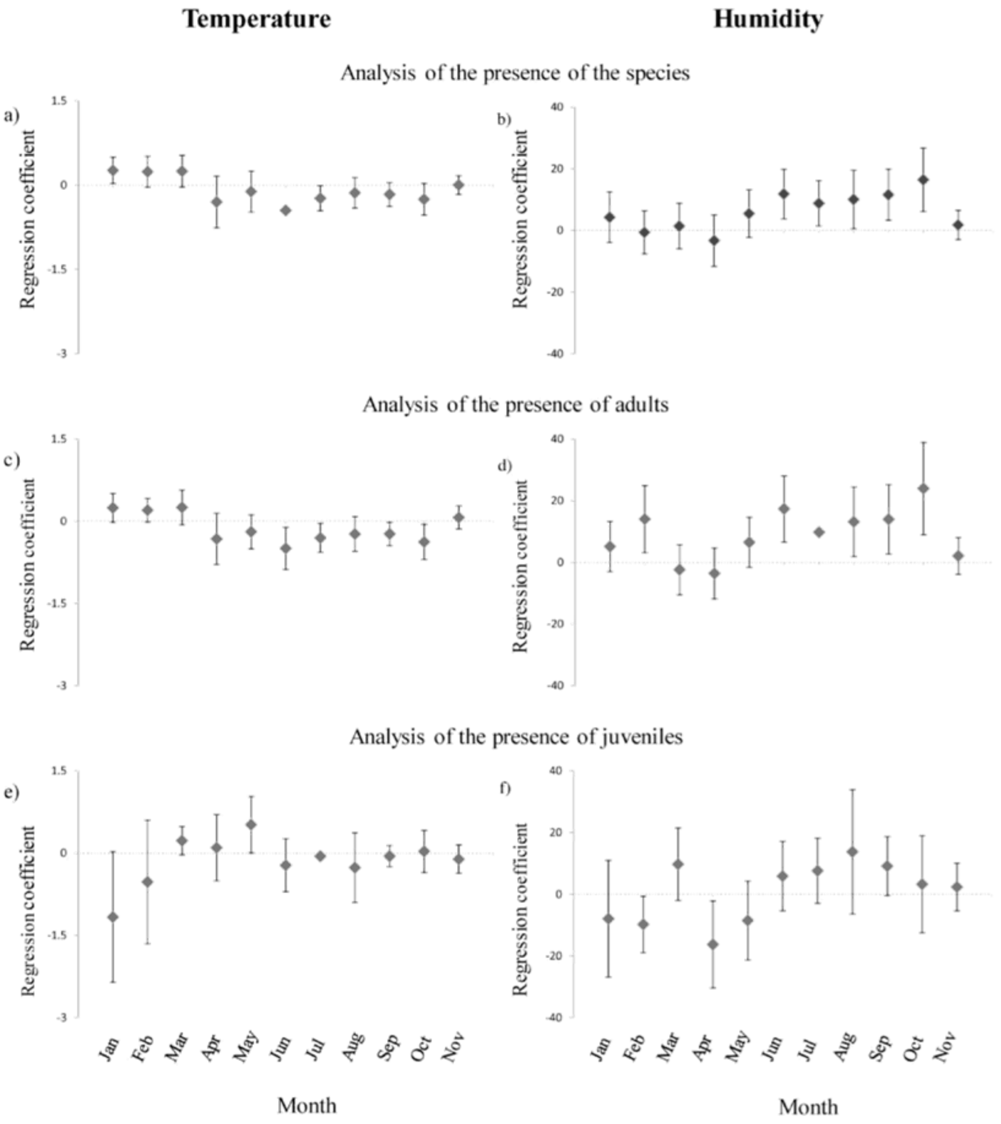


Figure 2 **Annual variation of the coefficients of regressions between presence/absence of cave sala-manders, temperature and humidity.** (A)–(B): results of regression models analyzing all individuals en-countered; (C)–(D) results of models analyzing adults only (E)–(F) results of models analyzing juveniles only. Results for December were not reported due to small sample size.

Cave depth represented the major gradient along with microhabitat features varied: as expected, humidity always increased and light decreased in the deepest sectors. The relationship between temperature and depth was more complex. During winter a positive relationship between temperature and depth was observed, while the relationship became negative during the warm months (Fig. 1A). Furthermore, all cave abiotic features (temperature, humidity and light) followed the variation of external conditions, which indeed were the major cause of fluctuations of internal microhabitats. While this influence

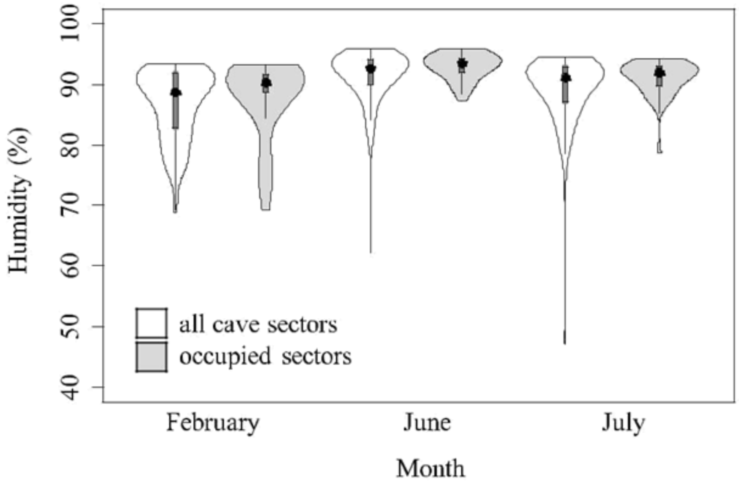


Figure 3 **Violin plots representing humidity in cave sectors available (white) and occupied by cave salamanders (grey), during three months.** The area of violin plots represents the distribution of cave sectors according to microclimate feature. Width of plots is proportional to the number of sectors showing such microclimate condition. The black points represent the medians, the grey boxes represent the second and third quartiles. The violin plots for temperature are available in Fig. S2.

Table 5 **Equivalency of species-habitat relationships (measured as Shoener's D) observed in different months.** Pairs of months for which the species-habitat relationships were not equivalent (after Bonfer-roni's correction: $\alpha' = 0.0083$) are in bold.

	Temperature			**Humidity**		
(A) All individuals						
	Feb	Jun	Jul	Feb	Jun	Jul
Jan	0.917	0.684	0.771	0.520	0.695	0.762
Feb		0.616	0.639		**0.336**	**0.375**
Jun			0.832			0.770
(B) Adults only						
	Feb	Jun	Jul	Feb	Jun	Jul
Jan	0.844	0.644	0.703	0.650	0.595	0.612
Feb		0.704	0.795		0.650	0.601
Jun			0.790			0.650
(C) Juveniles only						
	Feb	Jun	Jul	Feb	Jun	Jul
Jan	0.807	0.688	0.601	0.234	0.706	0.693
Feb		0.528	0.428		**0.077**	**0.069**
Jun			0.700			0.950

was strongest in the first meters of the caves, it remained clearly detectable at depths >20 m (Fig. 1), and therefore influenced the conditions experienced by salamanders.

During our surveys, detection probability of *Hydromantes italicus* was high, allowing us to obtain many observations, which are a necessary prerequisite for any habitat association study. The observation of *H. italicus* was strongly related to time of survey. As observed in other studies (*Ficetola, Pennati & Manenti, 2012*; *Lunghi, Manenti & Ficetola, 2014*), salamanders were strongly associated with sectors characterized by specific microhabitat features, such as high humidity, low light and abundant spiders. Individuals showed differences in their response to abiotic features, which resulted in a different distribution of salamanders inside caves (*Ficetola, Pennati & Manenti, 2013*). Adults were associated with the wettest microclimates, while juveniles were present in apparently more stressful sectors as they were also present in sectors with lower humidity and less suitable temperatures. Such presence of juveniles also in suboptimal microhabitats has been observed also in other species of cave salamanders (*Ficetola, Pennati & Manenti, 2013*), and may allow juveniles to exploit more superficial environments, where they can find more food.

Beside some differences in habitat selection between adults and juveniles, a strong interaction between temperature, humidity and time of survey was consistently observed in most analyses (Tables 3, 4 and Fig. 2). For instance, salamanders tended to be associated to the coldest and wettest sectors of caves, but this pattern was not evident during late winter/spring (Figs. 2A and 2B). Such heterogeneity in habitat selection may occur both because individuals select different conditions during different times or life stage (selection change hypothesis) or because of the strong variability of available microhabitat conditions (environmental change hypothesis). In principle, it might be also possible that in certain periods juveniles are forced to move toward suboptimal areas because of competition with adults. However, this explanation is unlikely: previous studies explicitly testing this hypotheses have found evidence that juveniles are not displaced by adults (*Ficetola, Pennati & Manenti, 2013*), while behavioral analyses suggested lack of competition for territories (*Berti & Corti, 2010*).

Our data mostly support the environmental change hypothesis. First, the temperature gradient showed a clear inversion through the seasons (Fig. 1A). If salamanders always select the same optimal temperature (about 10–15 °C; Fig. S2), they can only find such conditions in the deepest sectors of caves, in which temperature is relatively warm during winter, and coolest during summer. Actually, most of equivalency tests were not significantly different from random expectations, indicating that the species consistently selected the same microhabitat. In other words, apparent changes in species-habitat relationships (e.g., positive relationship with temperature in winter and negative relationship in summer) occurred because the habitat occupied by salamander remained the same, but environmental gradients changed through the time. As a consequence, the relationships between microclimatic conditions and salamanders were not constant with time: in summer individuals tended to select the coldest, most humid sectors of caves, while the relationship was different during winter months (Fig. 2 and Table 5). In practice, selection of the same habitat resulted in regression coefficients that were remarkably different among seasons (Fig. 2). The difficulty of extrapolating regression results and linear relationships beyond the limits of environmental gradients tested is a major issue in ecological modelling (*Randin et al., 2006*; *Zurell, Elith & Schroder, 2012*). In principle, only sampling the whole spectrum of potential habitat conditions may allow a full reconstruction of habitat preferences, but this is not feasible in the real world, because the available environmental gradients generally cover a limited range of conditions (*Soberon & Nakamura, 2009*; *Elith, Kearney & Phillips, 2010*).

Most of variation in species-habitat relationships was likely caused by the seasonal variation of temperature and humidity. Nevertheless, particularly in the analysis of humidity with juveniles, tests of niche equivalency between late winter and summer months were consistently rejected (Table 5). Cave salamanders are able to exploit the whole cave; therefore, if salamanders just require optimal abiotic conditions they can remain in farthest sectors where suitable microclimate is more stable. Conversely, in this study, salamanders during summer were associated to more humid sectors than in winter. This suggests a higher tolerance for dry sectors during winter, and supports the selection change hypothesis. Multiple, non-exclusive explanations are possible for such selection change. First, newborns *Hydromantes* normally hatch at the end of summer (*Lunghi et al., 2014*). Therefore, in the following winter, acquiring energy is a major priority for juveniles. The most superficial cave sectors are the ones with driest microclimate (Table 2), but show the highest abundance of prey. Actually, in our study caves, the potential prey richness (calculated as the summed N of species of Araneae (excluding *M. menardi*) and Diptera, as these taxa are the major food items for cave salamanders (*Vignoli, Caldera & Bologna, 2006*; *Crovetto, Romano & Salvidio, 2012*)) quickly decreases with depth (generalized linear model with Poisson error, taking into account month of survey: $B \pm SE = -0.024 \pm 0.006$, $\chi_1^2 = 201.3$, $P < 0.0001$). This indicates that juveniles may trade-off microclimatic optima for food availability (*Vlachos et al., 2014*). Actually, the end of winter may be a particularly important period, as in this period many invertebrates end their winter latency (*Bale & Hayward, 2010*). Efficient exploitation of seasonal peaks of food resources may be a key of fast development during the first years. Furthermore, the negative consequences of low humidity may be stronger in summer. Low environmental temperature reduces metabolism in ectotherms, which limits oxygen needs. As lungless salamanders exchange gasses mainly through their skin, and the efficiency of this skin function increases with high level of moisture (*Spotila, 1972*), during the cold season the individuals could be more tolerant to low humidity because of their lower respiration needs.

The peculiar physiology of plethodontids forces these salamanders to live within very narrow typologies of habitat. However, under certain circumstances, individuals may select conditions that are closer to their physiological limits (*Kearney et al., 2013*). This is likely the case for juveniles. Underground environments suffer constant food scarcity (*Romero, 2009*), but juveniles require consistent food supply in order to grow and reach maturity. Scarce access to food resources during juvenile stages poses major constraints on development, and may have prolonged consequences and even impact lifetime fitness (*Wong & Kolliker,¨ 2014*). Therefore, in certain months, young salamanders exploit superficial sectors with more stressful abiotic conditions, but they receive enough food input from the outdoor environment to offset the risk.

In principle, the "optimal" habitat of a species should match species requirements for multiple parameters, ranging from metabolism to water balance and food availability.

However, such "ideal" conditions are rarely available in the real world, and species have to deal with environmental variability, which causes frequent changes of habitat conditions and resources availability (*Seebacher & Alford, 1999*; *Araujo´ et al., 2010*; *Fredericksen, 2014*). Our study explores the complexity of habitat use patterns under variable conditions, and highlights difficulties in determining habitat selection processes. When necessary resources are inversely correlated along environmental gradients, habitat choice will be the results of a trade-off between the multiple requirements of a species. We showed that such trade-off may be not constant with time or life stage, as both species priorities and habitat features may change across time. Individuals often require different resources depending on their life stage, and thus must shift their habitat selection to exploit different environments to satisfy their needs (*Cox & Cresswell, 2014*; *Dittmar et al., 2014*; *Webb et al., 2014*). Habitat selection studies are often based on data collected over temporal snapshots. However, seasonality is a pervasive feature of natural environments, highlighting the importance to always take into account the potential seasonal variation and considering the interactions between the requirement of individuals and the variability of habitats.

ACKNOWLEDGEMENTS

We thank two reviewers for constructive comments on a previous version of this manuscript. GFF belongs to the Laboratoire d'Ecologie Alpine, which is part of Labex OSUG@2020.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

GFF was funded by Labex OSUG@2020 (Investissements d'avenir—ANR10 LABX56). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Labex OSUG@2020 (Investissements d'avenir—ANR10 LABX56).

Competing Interests

The authors declare there are no competing interests. Enrico Lunghi is representing the Association Natural Oasis as President.

Author Contributions

- Enrico Lunghi conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Raoul Manenti conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

- Gentile Francesco Ficetola conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

University of Florence approved the project by regular department's application and we followed our institutional guidelines.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Apuan Alps Regional Park (no 5, 4/04/2013),
District of Prato (no 448, 2013),
District of Pistoia (no 0022597/2013/P),
District of Lucca (no 731, 21/02/2013).

Data Availability

The following information was supplied regarding the deposition of related data:

Raw data can be found in the Supplemental Information.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/ 10.7717/peerj.1122#supplemental-information.

REFERENCES

Araujo´ CS, Candido DM, Araujo´ HFP, Dias SC, Vasconcellos A. 2010. Seasonal variations in scorpion activities (Arachnida: Scorpiones) in an area of Caatinga vegetation in northeastern Brazil. *Zoologia* **27**:372–376 DOI 10.1590/S1984-46702010000300008.

Bale JS, Hayward SAL. 2010. Insect overwintering in a changing climate. *The Journal of Experimental Biology* **213**:980–994 DOI 10.1242/jeb.037911.

Banks-Leite C, Pardini R, Boscolo D, Righetto Cassano C, Puttker¨ T, Santos Barros C, Barlow J. 2014. Assessing the utility of statistical adjustments for imperfect detection in tropical conservation science. *Journal of Applied Ecology* **51**:849–859 DOI 10.1111/1365-2664.12272.

Berti R, Corti C. 2010. Relazione del progetto: difesa del territorio in *Speleomantes italicus*. *TALP Rivista della FST* **40**:44–53.

Bogaerts S, Sparreboom M, Pasmans F, Almasri A, Beukema W, Shehab A, Amr ZS. 2013. Distribution, ecology and conservation of *Ommatotriton vittatus* and *Salamandra infraimmaculata* in Syria. *Salamandra* **49**:87–96.

Brambilla M, Saporetti F. 2014. Modelling distribution of habitats required for different uses by the same species: implications for conservation at the regional scale. *Biological Conservation* **174**:39–46 DOI 10.1016/j.biocon.2014.03.018.

Briggler JT, Prather JW. 2006. Seasonal use and selection of caves by plethodontid salamanders in a Karst area of Arkansas. *American Midland Naturalist* **155**:136–148 DOI 10.1674/0003-0031(2006)155[0136:SUASOC]2.0.CO;2.

Broennimann O, Fitzpatrick MC, Pearman PB, Petitpierre B, Pellissier L, Yoccoz NG, Thuiller W, Fortin M-J, Randin C, Zimmermann NE, Graham CH, Guisan A. 2012. Measuring ecological niche overlap from occurrence and spatial environmental data. *Global Ecology and Biogeography* **21**:481–497 DOI 10.1111/j.1466-8238.2011.00698.x.

Camp CD, Jensen JB. 2007. Use of twilight zones of caves by plethodontid salamanders. *Copeia* **2007**:594–604 DOI 10.1643/0045-8511(2007)2007[594:UOTZOC]2.0.CO;2.

Camp CD, Wooten JA, Jensen JB, Bartek DF. 2014. Role of temperature in determining relative abundance in cave twilight zones by two species of lungless salamander (family Plethodontidae). *Canadian Journal of Zoology* **92**:119–127 DOI 10.1139/cjz-2013-0178.

Cox DTC, Cresswell W. 2014. Mass gained during breeding positively correlates with adult survival because both reflect life history adaptation to seasonal food availability. *Oecologia* **174**:1197–1204 DOI 10.1007/s00442-013-2859-5.

Crovetto F, Romano A, Salvidio S. 2012. Comparison of two non-lethal methods for dietary studies in terrestrial salamanders. *Wildlife Research* **39**:266–270 DOI 10.1071/WR11103.

Crump ML, Scott Jr NJ. 1994. Visual Encounter Surveys. In: Heyer WR, Donnelly MA, McDiarmid RW, Hayek LC, Foster MS, eds. *Measuring and monitoring biological diversity: standard methods for Amphibians*. Washington, D.C.: Smithsonian Institution Press, 84–92.

Dail D, Madsen L. 2011. Models for estimating abundance from repeated counts of an open metapopulation. *Biometrics* **67**:577–587 DOI 10.1111/j.1541-0420.2010.01465.x.

Dittmar EM, Cimprich DA, Sperry JH, Weatherhead PJ. 2014. Habitat selection by juvenile black-capped vireos following independence from parental care. *Journal of Wildlife Management* **78**:1005–1011 DOI 10.1002/jwmg.738.

Dom´ıguez-Vega H, Monroy-Vilchis O, Balderas-Valdivia CJ, Gienger CM, Ariano-Sanchez´ D. 2012. Predicting the potential distribution of the beaded lizard and identification of priority areas for conservation. *Journal for Nature Conservation* **20**:247–253 DOI 10.1016/j.jnc.2012.04.003.

Elith J, Kearney M, Phillips S. 2010. The art of modelling range-shifting species. *Methods in Ecology and Evolution* **1**:330–342 DOI 10.1111/j.2041-210X.2010.00036.x.

Ficetola GF, Pennati R, Manenti R. 2012. Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. *Amphibia-Reptilia* **33**:251–259 DOI 10.1163/156853812X638536.

Ficetola GF, Pennati R, Manenti R. 2013. Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? *Population Ecology* **55**:217–226 DOI 10.1007/s10144-012-0350-5.

Fiske IJ, Chandler RB. 2011. Unmarked: an R package for fitting hierarchical models of wildlife occurrence and abundance. *Journal of Statistical Software* **43**:1–23.

Fox J. 2002. *An R and S-PLUS companion to applied regression*. Thousand Oaks: SAGE Publications.

Fredericksen TS. 2014. Thermal regulation and habitat use of the eastern box turtle in southwestern Virginia. *Northeastern Naturalist* **21**:554–564 DOI 10.1656/045.021.0406.

Godsoe W. 2010. I can't define the niche but I know it when I see it: a formal link between statistical theory and the ecological niche. *Oikos* **119**:53–60 DOI 10.1111/j.1600-0706.2009.17630.x.

Guisan A, Thuiller W. 2005. Predicting species distribution: offering more than simple habitat models. *Ecology Letters* **8**:993–1009 DOI 10.1111/j.1461-0248.2005.00792.x.

Jung RE, Droege S, Sauer JR, Landy RB. 2000. Evaluation of terrestrial and streamside salamander monitoring techniques at Shenandoh National Park. *Environmental Monitoring and Assessment* **63**:65–79 DOI 10.1023/A:1006413603057.

Kearney M, Porter W. 2009. Mechanistic niche modelling: combining physiological and spatial data to predict species ranges. *Ecology Letters* **12**:334–350 DOI 10.1111/j.1461-0248.2008.01277.x.

Kearney MR, Simpson SJ, Raubenheimer D, Kooijman SALM. 2013. Balancing heat, water and nutrients under environmental change: a thermodynamic niche framework. *Functional Ecology* **27**:950–965 DOI 10.1111/1365-2435.12020.

Lanza B, Pastorelli C, Laghi P, Cimmaruta R. 2006. A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). *Atti del Museo Civico di Storia Naturale di Trieste* **52**:5–135.

Lunghi E, Manenti R, Ficetola GF. 2014. Do cave features affect underground habitat exploitation by non-troglobite species? *Acta Oecologica* **55**:29–35 DOI 10.1016/j.actao.2013.11.003.

Lunghi E, Manenti R, Manca S, Mulargia M, Pennati R, Ficetola GF. 2014. Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments. *Salamandra* **50**:105–109.

MacKenzie DI, Kendall WL. 2002. How should detection probability be incorporated into estimates of relative abundance? *Ecology* **83**:3532–3532.

MacKenzie DI, Nichols JD, Royle JA, Pollock KH, Bailey LL, Hines JE. 2006. Occupancy estimation and modeling. In: *Inferring patterns and dynamics of species occurrence.* Waltham: Academic Press. p 324.

Manenti R, Lunghi E, Ficetola GF. The distribution of cave twilight-zone spiders depends on microclimatic features and trophic supply. *Invertebrate Biology* In Press.

Nogues´-Bravo D. 2009. Predicting the past distribution of species climatic niches. *Global Ecology and Biogeography* **18**:521–531 DOI 10.1111/j.1466-8238.2009.00476.x.

Peterson AT, Soberon´ J, Pearson RG, Anderson RP, Mart´ınez-Meyer E, Nakamura M, Araujo´ MB. 2011. *Ecological niches and geographic distributions*. Princeton: Princeton University Press.

Randin CF, Dirnbock T, Dullinger S, Zimmermann NE, Zappa M, Guisan A. 2006. Are niche-based species distribution models transferable in space? *Journal of Biogeography* **33**:1689–1703 DOI 10.1111/j.1365-2699.2006.01466.x.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* **43**:223–225 DOI 10.2307/2409177.

Richards SA, Whittingham MJ, Stephens PA. 2011. Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. *Behavioral Ecology and Sociobiology* **65**:77–89 DOI 10.1007/s00265-010-1035-8.

Romero A. 2009. *Cave biology*. New York: Cambridge University Press.

Romero A. 2012. Caves as biological space. *Polymath: An Interdisciplinary Arts and Sciences Journal* **2**:1–15.

Salvidio S, Lattes A, Tavano M, Melodia F, Pastorino MV. 1994. Ecology of a *Speleomantes ambrosii* population inhabiting an artificial tunnel. *Amphibia-Reptilia* **15**:35–45 DOI 10.1163/156853894X00533.

Saupe EE, Hendricks JR, Portell RW, Dowsett HJ, Haywood A, Hunter SJ, Lieberman BS. 2014. Macroevolutionary consequences of profound climate change on niche evolution in marine molluscs over the past three million years. *Proceeding of The Royal Society of London Series B* **281**:1–9 DOI 10.1098/rspb.2014.1995.

Seebacher F, Alford RA. 1999. Movement and microhabitat use of a terrestrial amphibian (*Bufo marinus*) on a tropical island: seasonal variation and environmental correlates. *Journal of Herpetology* **33**:208–214 DOI 10.2307/1565716.

Sewell D, Beebee TJ, Griffiths RA. 2010. Optimising biodiversity assessments by volunteers: the application of occupancy modelling to large-scale amphibian surveys. *Biological Conservation* **143**:2102–2110 DOI 10.1016/j.biocon.2010.05.019.

Soberon J, Nakamura M. 2009. Niches and distributional areas: concepts, methods, and assumptions. *Proceedings of the National Academy of Sciences of the United States of America PNAS* **106**:19644–19650 DOI 10.1073/pnas.0901637106.

Spotila JR. 1972. Role of temperature and water in the ecology of lungless salamanders. *Ecological Monographs* **42**:95–125 DOI 10.2307/1942232.

Stein A, Gerstner K, Kreft H. 2014. Environmental heterogeneity as a universal driver of species richness across taxa, biomes and spatial scales. *Ecology Letters* **17**:866–880 DOI 10.1111/ele.12277.

Stephens PA, Buskirk SW, Hayward GD, Del Rio CM. 2007. A call for statistical pluralism answered. *Journal of Applied Ecology* **44**:461–463 DOI 10.1111/j.1365-2664.2007.01302.x.

Stigall AL. 2012. Using ecological niche modelling to evaluate niche stability in deep time. *Journal of Biogeography* **39**:772–781 DOI 10.1111/j.1365-2699.2011.02651.x.

Vignoli L, Caldera F, Bologna MA. 2006. Trophic niche of cave populations of *Speleomantes italicus*. *Journal of Natural History* **40**:1841–1850 DOI 10.1080/00222930600973598.

Vignoli L, Caldera F, Bologna MA. 2008. Spatial niche of the Italian cave salamander, *Speleomantes italicus* (Dunn, 1923) (Plethodontidae, Amphibia), in a subterranean system of Central Italy. *Italian Journal of Zoology* **75**:59–65 DOI 10.1080/11250000701692729.

Vlachos CG, Bakaloudis DE, Kitikidou K, Goutner V, Bontzorlos V, Papakosta MA, Chatzinikos E. 2014. Home range and foraging habitat selection by breeding lesser kestrels (Falco naumanni) in Greece. *Journal of Natural History* **49**:371–381 DOI 10.1080/00222933.2013.825022.

Warren DL. 2012. In defense of 'niche modeling'. *Trends in Ecology and Evolution* **27**:497–500 DOI 10.1016/j.tree.2012.03.010.

Warren DL, Glor RE, Turelli M. 2008. Environmental niche equivalency vs. conservatism: quantitative approaches to niche evolution. *Evolution* **62**:2868–2883 DOI 10.1111/j.1558-5646.2008.00482.x.

Webb LE, Engel B, Berends H, Van Reenena CG, Gerrits WJJ, De Boer IJM, Bokkers EAM. 2014. What do calves choose to eat and how do preferences affect behaviour? *Applied Animal Behaviour Science* **161**:7–19 DOI 10.1016/j.applanim.2014.09.016.

Wong JWY, Kolliker¨ M. 2014. Effects of food restriction across stages of juvenile and early adult development on body weight, survival and adult life history. *Journal of Evolutionary Biology* **27**:2420–2430 DOI 10.1111/jeb.12484.

Zurell D, Elith J, Schroder B. 2012. Predicting to new environments: tools for visualizing model behaviour and impacts on mapped distributions. *Diversity and Distributions* **18**:628–634 DOI 10.1111/j.1472-4642.2012.00887.x.

Chapter II

Journal of Thermal Biology 60 (2016) 79–85

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders

Enrico Lunghi [a,b,c,n], Raoul Manenti [d], Giancarlo Canciani [e], Giorgio Scarì [d], Roberta Pennati [d], Gentile Francesco Ficetola [f,g]

[a] Universität Trier Fachbereich VI Raum-und Umweltwissenschaften Biogeographie, Campus I, Gebäude N Universitätsring 15, 54286 Trier, Germany
[b] Natural Oasis, Via di Galceti 141, 59100 Prato, Italy
[c] Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy
[d] Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy
[e] Via Novara 147 P, 28047 Oleggio, Italy
[f] Laboratoire d'Ecologie Alpine (LECA), Université Grenoble-Alpes, F-38000 Grenoble, France
[g] Centre National de la Recherche Scientifique (CNRS), LECA, F-38000 Grenoble, France

article info

Article history:
Received 6 March 2016
Accepted 13 June 2016
Available online 15 June 2016

Keywords:
Infrared termoghraphy
Speleomantes
Hydromantes
Biospeleology
Thermal ecology
Head–body temperature differences

abstract

Information on species thermal physiology is extremely important to understand species responses to environmental heterogeneity and changes. Thermography is an emerging technology that allows high resolution and accurate measurement of body temperature, but until now it has not been used to study thermal physiology of amphibians in the wild. Hydromantes terrestrial salamanders are strongly de-pending on ambient temperature for their activity and gas exchanges, but information on their body temperature is extremely limited. In this study we tested if Hydromantes salamanders are thermocon-form, we assessed whether there are temperature differences among body regions, and evaluated the time required to reach the thermal equilibrium. During summers of 2014 and 2015 we analysed 56 salamanders (Hydromantes ambrosii and Hydromantes italicus) using infrared thermocamera. We pho-tographed salamanders at the moment in which we found them and 1, 2, 3, 4, 5 and 15 min after having kept them in the hands. Body temperature was equal to air temperature; salamanders attained the equilibrium with air temperature in about 8 min, the time required to reach equilibrium was longer in individuals with large body size. We detected small temperature differences between body parts, the head being slightly warmer than the body and the tail (mean difference: 0.05 °C). These salamanders quickly reach the equilibrium with the environment, thus microhabitat measurement allows obtaining accurate information on their tolerance limits.

1. Introduction

In animals, body temperature is a fundamental trait linked to the execution of all physiological activities, such as locomotion, immune resistance, foraging and growth (Angilletta Jr. et al., 2002). Each species has its own optimal temperature, which is the best temperature at which the organism could realize its functions (Raske et al., 2012). Endothermic species use their metabolism to regulate their own temperature and maintain optimal tempera-ture during the time (Macdonald, 2010). On the other hand, ec-totherms often use surrounding environments to maintain their body temperature into a specific range, that define conditions in which biological functions can be carried out (Angilletta Jr. et al., 2002; Gunderson and Leal, 2016; Navas, 1996; Navas et al., 2008). Due to the spatial and temporal heterogeneity of environments, many ectotherms adopt thermal behaviour to maintain body temperature close to their preferred one (Feder, 1982; Navas, 1997; Raske et al., 2012). Reptiles have a semi-impermeable skin that prevents water loss, so they quite easily use solar radiation for reach their favourite temperature (Kaufmann and Bennett, 1989). On the other hand, amphibians present some hurdles related to thermoregulation because they have to balance the intake of en-ergy with evaporation of water through their skin (Hutchinson and Dupreé, 1992; Seebacher and Alford, 2002; Spotila, 1972; Tracy et al., 2007). In some cases, such as anurans living in ponds exposed to the sun, individuals can thermoregulate avoiding over-heating and dehydration as they continuously absorb water through their ventral surface, thus also balancing the incoming heat (Lillywhite, 1970). Furthermore, some frogs living in arid

---

[n] Corrsesponding author at: Universität Trier Fachbereich VI Raum-und Umweltwissenschaften Biogeographie, Campus I, Gebäude N Universitätsring 15, 54286 Trier, Germany.
E-mail address: enrico.arti@gmail.com (E. Lunghi).

http://dx.doi.org/10.1016/j.jtherbio.2016.06.010 0306-4565/

environments with extreme temperature developed a specialized morphology to minimize heat gain and maximize heat loss while avoiding water loss (Kobelt and Linsenmair, 1995), and some newts can thermoregulate by selecting the portions of the water column which hold the optimal temperature (Balogová and Gvoždík, 2015).

Information on species thermal physiology is increasingly used to understand species responses to environmental changes. For instance, ecophysiological analyses can use information on ther-mal tolerance of species to identify areas where climatic condi-tions are suitable, and to estimate potential impacts of environ-mental changes (Kearney and Porter, 2009). Studies on species thermal tolerance often use air temperature (T_a) nearby active individuals as a proxy of the thermal environment: this informa-tion is easily available, but in some cases may be a poor measure of the conditions actually experienced by individuals (Sunday et al., 2014). Actually, body temperature (T_b) of ectotherms may be strongly different from T_a because it is affected by behaviour, solar radiation, metabolism, conduction and evaporation (e.g. Bakken, 1992; Kearney and Porter, 2009; Pough et al., 2013; Tracy, 1976). Understanding whether ectotherms are at thermal equilibrium with their environment (thermoconformity) is extremely im-portant, as this information is needed to understand species re-sponses to environmental variations, and to predict potential im-pacts of climate changes (Balogová and Gvoždík, 2015). In thermal physiology studies, body temperature is traditionally measured with thermometric probes, but this limits the number of body regions for which temperature may be recorded, and requires manipulation of individuals, with the risk of influencing behaviour and body temperature (Lillywhite, 2010). Infrared thermocameras are an emerging approach for the study of thermal physiology in ectotherms. They provide instantaneous, high resolution images of surface temperature without the need of handling individuals, allow to identify thermal heterogeneity within individuals and, for small animals, their results are consistent with more traditional techniques, such as cloacal thermometers (Luna and Font, 2013; Sannolo et al., 2014; Tattersall and Cadena, 2010). Thermocameras have been successfully used to study thermoregulation in reptiles, but as far as we know they have been applied to amphibians only once under controlled conditions (Đikić et al., 2011).

Within amphibians, Plethodontid salamanders are a very in-teresting taxon for studies on thermal ecology. Plethodontids re-present about 66% of currently described caudate amphibians (AmphibiaWeb, 2016), and are among the tetrapods with the lowest metabolic rate (Chong and Mueller, 2012). This family is characterized by absence of lungs, so their respiration mainly oc-curs through the skin (Spotila, 1972). Gas exchanges require a constantly moist skin, thereby imposing limits on their habitat selection and thermoregulation (Feder, 1983; Huey, 1991; Peter-man and Semlitsch, 2014). Several plethodontids are often asso-ciated with underground environments, in which humidity is very high but the heat sources are very limited (Camp et al., 2014). Early studies suggested that plethodontids generally are thermocon-forms (Brattstrom, 1963), but it is possible that individuals regulate temperatures by selecting specific microhabitats (Spotila, 1972), or that the evaporative water loss reduces T_b at values significantly lower than ambient temperature (Bressin and Willmer, 2000).

European plethodontids (genus Hydromantes) often exploit underground environments, in which microclimatic features are suitable for their physiological needs (Lanza et al., 2006). Under-ground environments are dynamic systems in which few heat sources (e.g. rocks and external heat) seasonally interact with air flow and high moisture in determining complex thermal land-scapes (De Freitas, 1982, 1987; Lunghi et al., 2015), and this affects the physiology and distribution of cave dwelling species (Sunday et al., 2014).

In this study we used thermocamera images to study the thermal ecology of Hydromantes salamanders. First, we assessed whether body temperature of salamanders is equal to air tem-perature (i.e. whether salamanders are at the thermal equilibrium with the environment). Air temperature is a quick and easy ap-proach to the characterization of microhabitat for these sala-manders and, if T_b ¼T_a, air temperature can be a good proxy of operative conditions actually experienced by individuals (Kearney and Hewitt, 2009; Sunday et al., 2014). Second, we evaluated whether there are temperature differences among body regions. Finally, we manipulated animals to evaluate the time required to reach the thermal equilibrium, and to assess whether body size confers a higher thermal inertia, thus increasing the time needed to reach equilibrium.

2. Methods

2.1. Study system

We used a Fluke Ti32 infrared thermal imager (thermal sensitivity o0.045 °C, spatial resolution 1.25 mRad) to photograph salamanders and measure their body temperature T_b (emissivity 0.98). Overall, we analysed 31 H. ambrosii from two nearby caves (Cave A1: 44.18°N, 9.72°E 190 sl.m., max. depth 200 m, external opening: 4 3.7 m; Cave A2, 44.12°N and 9.77°E 330 sl.m., max. depth 20 m, external opening: 1.8 2.5 m) and 25 H. italicus from two nearby caves (Cave I1: 44.04°N, 10.25°E 912 sl.m., max. depth 15 m, external opening: 1.2 2 m; and Cave I2: 44.04°N and 10.26°E 890 sl.m., max. depth 20 m, external opening: 2 4 m). Cavities (A1, A2 and I1) were natural, while the Cavity I2 was a test mine. Caves examined for H. ambrosii were located in a karstic area while caves for H. italicus were opened in schist rocks. Individuals were photographed, without manipulation, at a distance of 35 cm, on the cave wall where they have been observed to be naturally active. For each individual, we calculated the temperature of pixels on head, trunk and tail (average7SE: 57071 pixels per individual measured) without counting limbs. Body temperature T_b was the mean temperature of pixels on head, trunk and tail.

2.2. Does air temperature represent body temperature of individuals?

Caves were divided in 3-m longitudinal intervals (hereafter: sectors), covering the whole cave or until the position of the last salamander; the size of sectors approximately corresponds to home range size (Lanza et al., 2006; Salvidio et al., 1994). Starting from cave entrance, every 3 m (e.g. at the end of each sector) we measured air temperature (°C) using a Lafayette TDP92 digital thermometer (accuracy: 0.1 °C). In each sector we used visual encounter surveys to detect the presence of active salamanders. We then photographed active salamanders using the infrared thermal imager to measure T_b, and calculated the average differ-ence between the air temperature T_a of the sector and T_b. Subse-quently, we used linear mixed models to assess the relationship between T_a and T_b. All mixed models considered sector, cave and species identity as random factors; this analysis was performed on 29 individuals for which data on body temperature and air tem-perature at the beginning of the experiment were available. Sample size was not homogeneous among groups, therefore in mixed models degrees of freedom were approximated and in some cases were not integer (Satterthwaite, 1946); the overall amount of variation explained by mixed models was assessed using condi-tional R^2 (Nakagawa and Schielzeth, 2013). Mixed models were also used to test whether temperature was significantly different between head, trunk and tail within the individuals (regional differences in temperature). Analyses were performed using the lme4 and lmerTest packages in R (Bates et al., 2014; Kuznetsova et al., 2015).

2.3. Test of equilibrium of body temperature

To confirm that in our study system salamanders' body tem-perature is at equilibrium, and to test the time required to reach thermal equilibrium, we manipulated 56 individuals; manipula-tion of salamanders was made using latex gloves. Individuals were captured within their habitat, weighed (accuracy: 0.1 g), kept in the hands of an observer, and then released at the collection point. Salamanders were placed on the hand palm and the hand was closed over them for 30 s, touching the whole body. The person performing the manipulation was always the same, the mean su-perficial temperature of the hand palm was 32.4 °C (SE¼0.4). In-dividuals were photographed using the infrared thermal imager to measure body temperature at the release and 1, 2, 3, 4, 5 and 15 min after release. Due to the difficult field conditions, some individuals were not photographed at all the time occasions (mean sample size7SD: 38.478.0 individuals per time occasion).

We then used non-linear mixed models (nlmm) (Pinheiro et al., 2014) to evaluate how and how fast body temperature goes at equilibrium. We considered two potential models:

1) Exponential loss of temperature $\Delta T°=k+e^{a\times t+b}$
2) Loss of temperature following an inverse power law $\Delta T°=k+a\times t^{b}$

where T° is T_b–T_a, t is the time after release, and k, a and b are the parameters to be estimated by the models. The fit of the two models was compared using Akaike's information criterion (AIC), and we then estimate model parameters, their significance, and the time required to achieve body equilibrium (defined as T ° r0.1 °C).

We also tested whether the velocity at which body temperature goes at equilibrium was slower in large individuals. Unfortunately, if we put both time after release and body size as independent variables in the nlmm, the model showed convergence problems. We therefore used standard mixed models to analyse the re-lationship between T° and body mass at the six intervals after the release (1, 2, 3, 4, 5 and 15 min).

3. Results

3.1. Relation between T_a and T_b

The infrared camera provided clear pictures of salamanders' body, with a spatial resolution sufficiently fine to measure the temperature of different body regions (Fig. 1). Before any manip-ulation, body temperature ranged between 8.17 and 15.89 °C. Salamanders were at thermal equilibrium with the air: the average difference between T_a and T_b was small (mean difference¼ 0.129 °C; 95% CI¼ 0.541/0.282), and T_b was strongly related to T_a (mixed model: $F_{1, 22.6}$ ¼18.8, P¼0.0002; conditional R^2 ¼0.98).

3.2. Temperature differences among body parts

We detected small but significant differences between head, body and tail ($F_{2, 107.8}$ ¼9.86, P¼0.0001, Fig. 1, Fig. 2). Specifically, within individuals, head was slightly warmer than both the body (Tukey's post hoc: mean difference7SE: 0.0570.02 °C, P ¼0.02) and the tail (mean difference: 0.0770.02 °C, P o0.0001), while the difference between body and tail temperature was not sig-nificant (mean difference: 0.0170.02 °C, P ¼0.70).

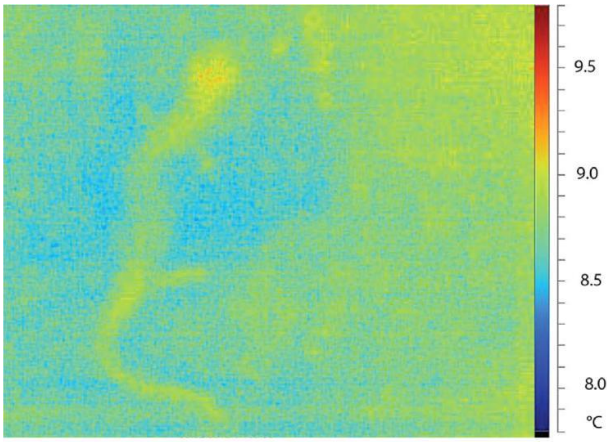


Fig. 1. Example of a picture obtained from the infrared thermal camera on a sal-amander, before capture.

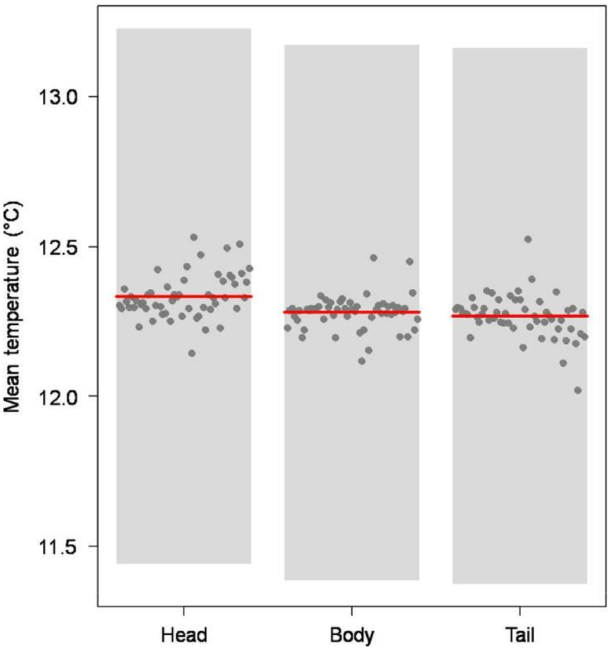


Fig. 2. Partial regression plots, showing temperature differences between body parts. The shaded areas represent the 95% confidence bands.

3.3. Equilibrium T_b T_a

Keeping individuals in hand for 30 s determined an increase of T_b of 6–10 °C. When animals were released, the difference between body temperature and air temperature (T°) quickly decreased with time; after 15 min T° was essentially zero (Fig. 3). The mixed model assuming exponential decrease showed much better fit than the one following an inverse power law (exponential model: AIC¼766.0; inverse power law model: AIC¼1044.9).

In the exponential model $\Delta T° = k + e^{a\times t+b}$ the parameter k was not significantly different from zero (Table 1), confirming that T°

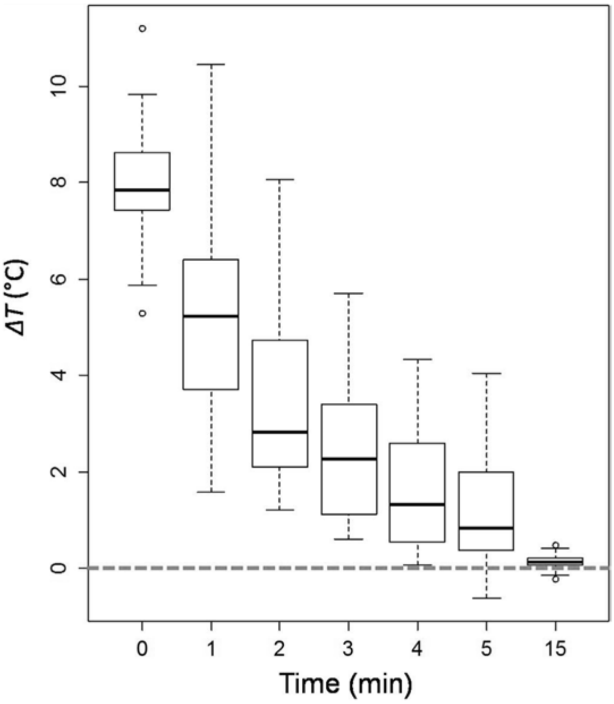


Fig. 3. Difference between body temperature and air temperature (T) in sala-manders released in the environment, after being kept in hand for 30 s. Bold lines are the medians, boxes hold the 2nd and the 3rd quartiles, while wiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range. Outliers beyond 1.5 times the interquartile range are shown as open dots.

Table 1

Results of the exponential non-linear mixed effect model $\Delta T° = k + e^{a \times t + b}$, evalu-ating the decrease of difference between body temperature and air temperature with time.

Parameter	B	SE	F	d.f.	P
a	0.48	0.04	301.8	1,215	o0.0001
b	2.11	0.02	19604.3	1,215	o0.0001
k	0.06	0.10	0.3	1,215	0.561

Table 2

Relationships between salamander weight and T°, at specific moments after re-lease. Sample size was not identical in the six test periods.

Time	B	SE	F	d.f.	P
At release	0.05	0.20	0.1	1,23	0.810
1 min after release	0.52	0.17	9.4	1,47	0.004
2 min after release	0.81	0.12	42.4	1,44	o0.001
3 min after release	0.77	0.12	40.0	1,33	o0.001
4 min after release	0.55	0.12	22.8	1,33	o0.001
5 min after release	0.44	0.11	15.7	1,41	o0.001
15 min after release	0.04	0.02	5.7	1,36	0.022

quickly approaches zero. According to this model, T° r0.1 °C after 8.2 min, i.e. body temperature reaches the equilibrium very quickly.

Salamanders with large body size required more time to reach thermal equilibrium. At the release, T° was unrelated to the weight of salamanders, but T° decreased more quickly with time in small than in large salamanders. As a consequence, for a given time after release, T° remained larger in the heaviest salamanders (Table 2, Fig. 4). For instance, 5 min after release small salamanders (weight o1 g) were essentially at the thermal equilibrium, while the largest salamanders showed a T° of 2–3 °C (Fig. 4b).

4. Discussion

4.1. Thermoconformity of Hydromantes

Our results show that, in Hydromantes salamanders, body temperature is at equilibrium with environment temperature. Even though the thermal environment within caves may be het-erogeneous, salamanders have limited movements and may re-main in the same cave sector for days (Lanza et al., 2006). If body temperature T_b is at equilibrium, T_b corresponds to the operative temperature T_e of individuals (Bakken, 1992), which is the steady-state temperature organism would attain if placed indefinitely in a given environment (Kearney and Porter, 2009). As a consequence, in these salamanders important ecophysiological parameters, such as T_b and T_e, can be easily estimated from air temperature, in-dicating that, at least for temperature, microhabitat features can be a good representation of operative conditions of individuals.

In several salamanders, it has been proposed that individuals can move within the thermal landscape to keep their body close to their preferred temperatures (Balogová and Gvoždík, 2015; Heath, 1975; Spotila, 1972). Hydromantes salamanders are not strictly cave-dwelling species, and can be active at the surface during cold, humid seasons (i.e. autumn and spring), but they have to move underground when outdoor conditions are too warm and dry, such as during Mediterranean summer. In summer, caves constitute a relatively continuous thermal gradient: cave sectors close to the surface have higher temperature, and temperature decreases in the deepest sectors (Culver and White, 2005; Lunghi et al., 2015). On the one hand, food is more abundant close to the surface (Fi-cetola et al., 2013), thus salamanders are restricted to a few tens of meters from the surface. On the other hand, given that sala-manders are at thermal equilibrium (Fig. 3), they must remain in relatively deep sectors, where conditions are within the pre-ferences of the species. The trade-off between these and other factors (e.g. humidity, not investigated by the present study) limits the distribution of salamanders to a narrow region of the underground space. The time needed to reach thermal equilibrium was slightly longer in salamanders with large body size (Fig. 4). These individuals might have slightly better opportunities to move to-ward unsuitable places for short time, and then coming back to more suitable areas. Large individuals can also have additional advantages, such as a better resistance to desiccation and to food shortage, and higher mobility. Actually, in the closely related H. strinatii, juvenile salamanders are restricted to a very narrow re-gion (5–15 m from the surface), while adults can exploit a rela-tively broader region of the underground space, 5–30 m from the surface (Ficetola et al., 2013; Salvidio and Pastorino, 2002), sug-gesting that the improved tolerance of large individuals may allow them to exploit broader niches. In our study, salamanders were exposed to temperatures 430 °C, which are higher than the conditions naturally occurring inside caves. Nevertheless, the short manipulation time (30 s) only increased body temperature of ap-prox. 8 °C (Fig. 1). Inside caves inhabited by salamanders, tem-perature differences of 8 °C between superficial and deep sectors naturally occur (Lunghi et al., 2015), therefore our analyses may approximate the thermal variation experienced by individuals moving from the surface to deep sectors.

Body temperature showed regional differences along sala-manders body, the head being slightly but significantly warmer (Figs. 1 and 2). Until now, very limited information was available

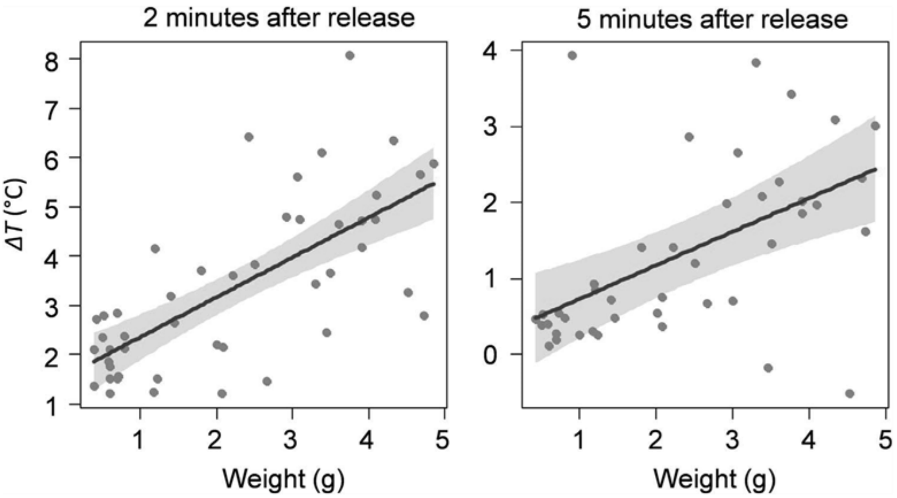


Fig. 4. Difference between body temperature and air temperature (T) in salamanders with different body size, two and five minutes after release. Salamanders were released in the environment after being kept in hand for 30 s. The shaded areas represent the 95% confidence bands.

on regional differences of body temperature in salamanders. The high thermal resolution of thermocamera allowed to clearly detect the very small (about 0.05 °C) temperature differences between the head and the rest of the body (Figs. 1 and 2). Plethodontid salamanders are sit-and-wait predators with a very low metabo-lism (Lanza et al., 2006). Even though they perform very limited movements, sensorial organs, such as eyes, Jacobson's organ and buccal mucous are grouped in the head area (Lanza et al., 2006), and the activities of metabolism and sensory system probably adsorb most of the metabolism of these salamanders, which in turn likely produces a small amount of metabolic heat.

Very few studies used thermography to analyse variation of temperature in amphibians. Đikić et al. (2011) analysed acclima-tion in marsh frogs in controlled conditions. They simulated in the laboratory the exit from hibernation sites, and found that the heat gain of frogs is slow, and even after more than 2 h, frogs are not at equilibrium with the surrounding environment. This might be caused by several reasons, for instance the larger body size of frogs. Regional differences in body temperature were also ob-served by Đikić et al. (2011). They identified a circumscribed area in multiple regions of the body and measured temperature of such areas. The heating rate was generally similar among body regions, but there were slight differences between their temperature: the eyes tended to be colder than the body, while limbs were slightly warmer. However, frogs never reached thermal equilibrium, therefore it is not clear whether in marsh frogs regional differ-ences persist at equilibrium. In our study salamanders were first photographed in their habitat without any manipulation to un-derstand if under natural conditions these salamanders showed differences in temperature between body regions. The existence of such differences in an ectotherm species is very interesting as thermoconform animals are often supposed to have the whole temperature of its body conformed to the surrounding one.

5. Conclusions

Thermocamera offers a valuable tool for the study of thermal ecology of ectotherms. On the one hand, it allows to measure small temperature differences between body parts without manipulat-ing individuals, thus enabling us to obtain new insights on the complexity of thermal properties of ectotherms. Furthermore, it is possible to quickly measure the major ecophysiological parameters of individuals in the wild, and to obtain data that can be used to better identify the thermal requirements of species, thus helping the characterization of species niches and improving our understanding of species responses to environmental variation.

Acknowledgments

GFF is a member of the LECA, which is part of Labex OSUG@2020 (Investissements d'avenir – ANR10 LABX56). Animal manipulation was allowed by Italian Ministero dell'Ambiente e della Tutela del Territorio e del Mare, permission no. 004002 3rd July 2013; 0013924 8 July 2014; 0021786 28 October 2014, 0009384 12 May 2015.

References

AmphibiaWeb, 2016. AmphibiaWeb: Information on Amphibian Biology and Con-servation. University of California, Berkeley, California.
Angilletta Jr., M.J., Niewiarowski, P.H., Navas, C.A., 2002. The evolution of thermal physiology in ectotherms. J. Therm. Biol., 249–268.
Bakken, G.S., 1992. Measurement and application of operative and standard op-erative temperatures in ecology. Am. Zool. 32, 194–216.
Balogová, M., Gvoždík, L., 2015. Can Newts cope with the heat? Disparate ther-moregulatory strategies of two sympatric species in water. PLoS One 10, e0128155.
Bates, D., Maechler, M., Bolker, B.M., Walker, S., 2014. lme4: linear mixed-effects models using S4 classes. R package version 1.0-6. ⟨www.r-project.org⟩.
Brattstrom, B.H., 1963. A preliminary review of the thermal requirements of am-phibians. Ecology 44, 238–255.
Bressin, S., Willmer, P.G., 2000. Estimation of thermal constants: the importance of using equilibrium temperature rather than ambient temperature demonstrated with hoverflies (diptera, syrphidae, genus Eristalis). J. Exp. Biol. 203, 2511–2517.
Camp, C.D., Wooten, J.A., Jensen, J.B., Bartek, D.F., 2014. Role of temperature in determining relative abundance in cave twilight zones by two species of lungless salamander (family Plethodontidae). Can. J. Zool. 92, 119–127.
Chong, R.A., Mueller, R.L., 2012. Low metabolic rates in salamanders are correlated with weak selective constraints on mitochondrial genes. Evolution 67, 894–899.
Culver, D.C., White, W.B., 2005. Encyclopedia of Caves. Elsevier Academic Press, Burlington; San Diego; London.
De Freitas, C.R., 1982. Cave climate: assessment of airflow and ventilation. J. Cli-matol. 2, 383–397.
De Freitas, C.R., 1987. Cave climate: assessment of heat and moisture exchange. J. Climatol. 7, 533–569.
Đikić, D., Kolarić, D., Lisičić, D., Benković, V., Horvat-Knežević, A., Školnik Gadanac, K., Tadić, Z., Oršolić, N., 2011. Digital thermography in analysis of temperature changes in Pelophylax ridibundus frog. Period. Biol. 114, 453–456.

Feder, M.E., 1982. Effects of latitude, season, elevation, and microhabitat on field body temperatures of neotropical and temperate zone salamanders. Ecology 63, 1657–1664.

Feder, M.E., 1983. Integrating the ecology and physiology of plethodontid salamanders. Herpetologica 39, 291–310.

Ficetola, G.F., Pennati, R., Manenti, R., 2013. Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? Popul. Ecol. 55, 217–226.

Gunderson, A.R., Leal, M., 2016. A conceptual framework for understanding thermal constraints on ectotherm activity with implications for predicting responses to global change. Ecol. Lett. 19, 111–120.

Heath, A.G., 1975. Behavioral thermoregulation in high altitude tiger salamanders Ambystoma tigrinum. Herpetologica 31, 84–93.

Huey, R.B., 1991. Physiological consequences of habitat selection. Am. Nat. 137, S91–S115.

Hutchinson, V.H., Dupreé, R.K., 1992. Thermoregulation. In: Feder, M.E., Burggren, W.W. (Eds.), Environmental Physiology of the Amphibians. The University of Chicago Press, Chicago, IL, pp. 206–249.

Kaufmann, J.S., Bennett, A.F., 1989. The effect of temperature and thermal acclimation on locomotor performance in Xantusia vigilis, the desert night lizard. Physiol. Zool. 62, 1047–1058.

Kearney, M., Hewitt, G.M., 2009. Stasipatric speciation: resurrecting a system to bury a hypothesis? Mol. Ecol. 18, 3331–3333.

Kearney, M., Porter, W., 2009. Mechanistic niche modelling: combining physiological and spatial data to predict species ranges. Ecol. Lett. 12, 334–350.

Kobelt, F., Linsenmair, K.E., 1995. Adaptations of the reed frog Hyperolius viridiflavus (Amphibia, Anura,Hyperoliidae) to its arid environment. VII. The heat-budget of Hyperolius viridiflavus nitidulus and the evolution of an optimized body shape. J. Comp. Physiol. B 165, 110–124.

Kuznetsova, A., Brockhoff, B., Christensen, H.B., 2015. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-29. ⟨www.r-project.org⟩.

Lanza, B., Pastorelli, C., Laghi, P., Cimmaruta, R., 2006. A review of systematics, taxonomy, genetics, biogeography and natural history of the genus Speleomantes Dubois, 1984 (Amphibia Caudata Plethodontidae). Atti del Museo Civico di Storia Naturale di Trieste, 52, pp. 5–135.

Lillywhite, H.B., 1970. Behavioral temperature regulation in the Bullfrog Rana catesbeiana. Copeia 1970, 158–168.

Lillywhite, H.B., 2010. Physiological ecology: field methods and perspective. In: Dood, C.K.J. (Ed.), Amphibian Ecology and Conservation. Oxford University Press, New York, pp. 363–382.

Luna, S., Font, E., 2013. Use of an infrared thermographic camera to measure field body temperatures of small lacertid lizards. Herpetol. Rev. 44, 59–62.

Lunghi, E., Manenti, R., Ficetola, G.F., 2015. Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? PeerJ 3, e1122.

Macdonald, D.W., 2010. The Encyclopedia of Mammals. Oxford University Press., Oxford.

Nakagawa, S., Schielzeth, H., 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol. Evol. 4, 133–142.

Navas, C.A., 1996. Metabolic physiology, locomotor performance, and thermal niche breadth in neotropical anurans. Physiol. Zool. 69, 1481–1501.

Navas, C.A., 1997. Thermal extremes at high elevations in the Andes: physiological ecology of frogs. J. Therm. Biol. 22, 467–471.

Navas, C.A., Gomes, F.R., Carvalho, J.E., 2008. Thermal relationships and exercise physiology in anuran amphibians: integration and evolutionary implications. Comp. Biochem. Physiol. Part A 151, 344–362.

Peterman, W.E., Semlitsch, R.D., 2014. Spatial variation in water loss predicts terrestrial salamander distribution and population dynamics. Oecologia 176, 357–369.

Pinheiro, P., Bates, D., DebRoy, S., Sarkar, D., 2014. Linear and Nonlinear Mixed Effects Models. R package version 3.1, 117. ⟨http://cran.r-project.org/web/packages/nlme⟩.

Pough, F.H., Janis, C.M., Heiser, J.B., 2013. Verterbate Life. Pearson, Boston.

Raske, M., Lewbart, G.A., Dombrowski, D.S., Hale, P., Correa, M., Christian, L.S., 2012. Body temperatures of selected amphibian and reptile species. J. Zoo Wildl. Med. 43, 517–521.

Salvidio, S., Latters, A., Tavano, M., Melodia, F., Pastorino, M.V., 1994. Ecology of a Speleomantes ambrosii population inhabiting an artificial tunnel. Amphib. Reptil. 15, 35–45.

Salvidio, S., Pastorino, M.V., 2002. Spatial segregation in the European plethodontid Speleomantes strinatii in relation to age and sex. Amphib. Reptil. 23, 505–510.

Sannolo, M., Mangiacotti, M., Sacchi, R., Scali, S., 2014. Keeping a cool mind: head– body temperature differences in the common wall lizard. J. Zool. 293, 71–79.

Satterthwaite, F.E., 1946. An approximate distribution of estimates of variance components. Biometrics 2, 110–114.

Seebacher, F., Alford, R.A., 2002. Shelter microhabitats determine body temperature and dehydration rates of a terrestrial amphibian (Bufo marinus). J. Herpetol. 36, 69–75.

Spotila, J.R., 1972. Role of temperature and water in the ecology of lungless salamanders. Ecol. Monogr. 42, 95–125.

Sunday, J.M., Bates, A.E., Kearney, M.R., Colwell, R.K., Dulvy, N.K., Longino, J.T., Huey, R.B., 2014. Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. Proc. Natl. Acad. Sci. USA 111, 5610–5615.

Tattersall, G.J., Cadena, V., 2010. Insights into animal temperature adaptations revealed through thermal imaging. Imaging Sci. J. 58, 261–268.

Tracy, C.R., 1976. A model of the dynamic exchanges of water and energy between a terrestrial amphibian and its environment. Ecol. Monogr. 46, 293–326.

Tracy, C.R., Betts, G., Tracy, C.R., Christian, K.A., 2007. Plaster models to measure operative temperature and evaporative water loss of amphibians. J. Herpetol. 41, 597–603.

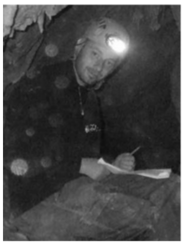
Enrico Lunghi He got Bachelor degree in Natural Science (2012) at University of Milan, and Master degree in Science of Nature and Humans – Curriculum Conservation and Management of Wildlife (2014) at University of Florence. Since 2015 he is studying as Ph.D. student at Trier University, Biogeography department, with a thesis project focused on Hydromantes species. Since 2015 he is a collaborator of Natural History Museum of University of Florence, section of zoology "La Specola". His researches are mainly focused on biospeleology and European cave salamanders.

Raoul Manenti Graduated in natural sciences and Ph.D. in animal biology, he performs researches in different fields of the zoology, from developmental biology to herpetology, biospeleology and conservation. He currently coordinates the Conservation Committee of the Societas Herpetologica Italica, he is member of the board of the Conservation Committee of the Societas Europaea Herpetolgica and member of the European Amphibian Red List Authority Working Group of the IUCN Species Survival Commission (SSC) Amphibian Red List Authority (RLA).

Giancarlo Canciani Graduated in Chemistry in 1976, he has been functionary and manager for the Liguria Region. He has been in charge of atmospheric pollution issues, pollution spreading modelling and Geographic Information Systems (GIS) devoted to environmental tools. At a later time he managed the Savona department and the scientific specialist systems unit of the Regional Environmental Agency of Liguria (ARPAL) coordinating especially controls, surveys, analyses, modelling and statistics of the remediation of the "ACNA" and "Stoppani" pollute sites. Retired from 2009, he's currently developing and applying thermography measures at several areas of interest.

Giorgio Scarì Graduated in Biology at the University of Milan he is Ph.D. in ecology and evolutionary biology. Since 1992 he collaborates to the didactics of the Università degli Studi di Milano with several practical courses; since 2001 till 2009 he was tenured of the course "Animal Biology" for the graduation on Bio-technology while at present he is tenured of the course "Animal Biology" and "Vertebrate Biology" for the graduations on Agricultural Sciences and Natural Sciences. Its research activity is mainly performed in the frame of Cell Biology, and it's focused to the settling of synthesis methodologies, treatments and structural/functional characterizations of Cells using nanocomposites and nanoparticles. He is co-author of more than 30 papers published in peer-reviewed journals and participated at several International Conferences and Workshop as also invited speaker.

Roberta Pennati Graduated in Natural Sciences in 1993 at the University of Milan. She awards a Ph.D. Degree in Natural and Environmental Sciences in 1998 at the University of Milano. Since 2002, she is research associate in Zoology at the Department of Biosciences, University of Milano. Her scientific activity concerns mainly developmental neurobiology of vertebrates and invertebrates with particular regard to amphibians and tunicates. Recent research interest regards the biology and ecology of amphibian species of the genus Hydromantes.



Gentile Francesco Ficetola He is a research scientist at the Alpine Ecology lab in France, with a multidisciplinary background in both modelling and genetics. He combines these two approaches to better understand how environmental systems change and which factors determine their modifications. Current research activities include: 1) Studying the factors that determine the distribution of amphibians and reptiles 2) Evaluating how evolutionary processes can be integrated into macro-ecological analyses; 3) Improving our understanding of biological invasions 4) Identifying strategies for amphibian conservation and 5) Using environmental DNA for the study of impact of human activities on biodiversity.

Chapter III

Unpublished manuscript

Microhabitat analyses challenge broad-scale patterns of niche evolution in terrestrial salamanders

Gentile Francesco Ficetola[1,2,3]*, Enrico Lunghi[4,5,6], Claudia Canedoli[2], Emilio Padoa-Schioppa[2], Roberta Pennati[3], Raoul Manenti[3]

1 Univ. Grenoble Alpes, Laboratoire d'Écologie Alpine (LECA), F-38000 Grenoble, France ; CNRS, Laboratoire d'Écologie Alpine (LECA), F-38000 Grenoble, France

2 Dipartimento di Scienze dell'Ambiente e del Territorio e di Scienze della Terra, Università degli Studi di Milano-Bicocca. Piazza della Scienza 1, 20126 Milano Italy

3 Department of Environmental Science and Policy, Università degli Studi di Milano. Via Celoria 26, 20133 Milano Italy

4 Universität Trier Fachbereich VI, Campus I, Gebäude N Universitätsring 15, 54286 Trier, Germany

5 Natural Oasis, Via di Galceti 141, 59100 Prato, Italy

6 Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy

Corresponding author: francesco.ficetola@gmail.com

Abstract

The extent to which closely related species share similar niches remains highly debated. Ecological niches are increasingly analyzed by combining distribution records with broad-scale climatic variables, but interactions between species and their environment often occur at fine scales. The idea that macroscale analyses correctly represents fine-scale processes relies on the assumption that average climatic variables are meaningful predictors of processes determining species persistence, but tests of this hypothesis are scarce. We compared broad- and fine-scale (microhabitat) approaches by analyzing the niches of European plethodontid salamanders. Macroecological niches were characterized by combining species presences with bioclimatic variables, while fine-scale analyses relied on presence records and microhabitat measurements obtained in the field. Both the microhabitat and the macroecological approaches identified niche differences among species, but the correspondence between micro- and macroecological niches was weak. Strikingly, species identified as dry-tolerant by microhabitat analyses were associated with the wettest climates in macroecological analyses. When exploring niche evolution, the macroecological approach suggested a close relationship between niche and phylogenetic history, but this relationship did not emerge in fine-scale analyses. The apparent pattern of niche evolution emerging in broad-scale analyses likely was the byproduct of related species having closely adjacent ranges. The environment actually experienced by most of animals is more heterogeneous than what is apparent from macro-scale predictors, and a better combination between macroecological and fine-grained data may be a key to obtain robust ecological generalizations.

Introduction

The idea that phylogenetically related species also tend to be ecologically similar has intrigued researchers since Darwin’s Origin of Species (Losos 2008). Phylogenetic signal is the tendency of closely related species to be more similar than expected under randomness (Blomberg and Garland 2002, Losos 2008). Phylogenetic signal is often observed for morphological and life history traits (e.g. Blomberg and Garland 2002, Losos 2008, Enriquez-Urzelai et al. 2015, Mesquita et al. 2016), and has also been detected for traits representing species niche, such as eco-physiological features, climatic niche, diet and habitat (Losos 2008, Peterson 2011). Nevertheless, signal for niche traits is not ubiquitous, as many studies have actually found a high evolutionary lability of niches (Losos 2008, Peterson 2011). There is thus a growing interest in the study of phylogenetic signal of niches, and of the conditions under which effects of phylogenetic signal on niche are stronger or can be better detected (Losos 2008, Peterson 2011).

The evolution of niches is often analyzed through a broad-scale (bioclimatic) approach, i.e. by combining species distribution data with coarse, 'scenopoetic' variables (Peterson 2011). These macroecological approaches have had increasing appeal given the availability of broad-scale information (e.g. species distribution data, climatic information, environmental data from remote sensing, phylogenies), and the impressive progress of ecological informatics (Beck et al. 2012). The broad geographical scale of these studies is both a strength and limitation. Working over macro-scales allows drawing general patterns that are hardly recovered using local analyses, but the data available over broad scales generally have a coarse resolution. For instance, most of analyses of relationships between animals and climate are performed at scales that are ~10,000 times larger than the study organisms (Beck et al. 2012, Potter et al. 2013). However, it is widely recognized that species distributions are the product of multi-scalar processes, and many interactions between species and the environment occur at fine scales (Soberon 2007, Fraterrigo et al. 2014). Thus abiotic conditions actually experienced by individuals do not necessarily correspond to such macro-predictors (Kearney et al. 2014, Scheffers et al. 2014a, Sunday et al. 2014), and bioclimatic predictors often are just surrogates of the fine-scale environmental features actually experienced by individuals (Kearney et al. 2014).

Until now, many studies have implicitly assumed that broad-scale variables are meaningful predictors of the parameters influencing species (mean field approximation; Bennie et al. 2014), without comparing the effects of micro- and macro-scale conditions. In order to assess how climate determines the distribution of species we need testing the appropriateness of the mean field approximation, and thus comparing the outcome of micro- and microclimate analyses (Bennie et al.

2014). Such comparison can be performed using statistical downscaling (Fridley 2009, Bucklin et al. 2013, Kearney et al. 2014) or explicit modelling of microclimate (Sears et al. 2011), but these approaches suffer some limitations (Bucklin et al. 2013), and do not empirically assess the actual microclimates used by organisms.

Alternatively, the comparison can be performed using microclimate data from real observations (Scheffers et al. 2014a). Microhabitat selection and thermoregulation through behavior are major processes allowing animals to maintain body conditions within their physiological limits, i.e. within the range of conditions imposed by the fundamental niche of the species (Sunday et al. 2014). Microhabitat selection by species in the wild can provide accurate data on species requirements, thus allowing us to draw measures of species niche, with a rationale analogous to analyses of operative temperature (Sunday et al. 2014) or to habitat preference experiments in which organisms are exposed to a variety of environmental conditions and can select those within their suitability range (Fig. 1; see e.g. Freidenburg and Skelly 2004). Hierarchical approaches, integrating analyses at multiple levels, can greatly enhance understanding of niches and help to evaluate under which conditions the different approaches are most appropriate (Gallien et al. 2012, Searcy and Shaffer 2016), but there are few comprehensive multi-scalar analyses (see Fraterrigo et al. 2014, Searcy and Shaffer 2016 for examples).

Terrestrial salamanders have been a frequent focus of analyses of bioclimatic niche. Niche analyses have been used to infer distribution changes and declines caused by climate change, to identify broad-scale drivers of biodiversity patterns, to analyze niche evolution in a phylogenetic context and even as a tool to describe new species (e.g. Kozak and Wiens 2007, Rissler and Apodaca 2007, Fisher-Reid et al. 2012, Kozak and Wiens 2016, Searcy and Shaffer 2016). In this study we analyzed niches of eight species of terrestrial salamanders [genus *Hydromantes*, subgenera *Speleomantes* and *Atylodes*; see Wake (2013)] using the microhabitat selection and bioclimatic approaches, and assessed the phylogenetic signal of niches with the two approaches. Despite being sometimes named "cave salamanders", these are not true cave-dwelling organisms: underground environments just are the habitats where salamander detection is easiest (Lanza et al. 2006).

European terrestrial salamanders are an interesting group for niche analyses. First, salamanders have superficial activity during cool and wet periods (from autumn to spring), but move to underground environments during summer, when external conditions would be too harsh (e.g., dry, hot). In these environments, they select sectors having microclimatic features within their physiological limits (Fig. 1; see Methods). Their microhabitat selection is similar to what is done in habitat preference experiments, in which organisms are placed in a gradient where they select environmental conditions within their suitability range (Freidenburg and Skelly 2004), and is thus particularly appropriate to identify the tolerance of species. Actually, previous analyses have shown that microhabitat selection provides reliable information on the operative conditions of individuals, thus allowing a good characterization of species biophysical niches (Lunghi et al. 2016). Second, the features of underground habitats are different but strongly dependent on conditions outside the cave (epigean). For instance, far from the surface, the mean temperature approximately corresponds to the average local temperature of the atmosphere, and underground conditions are heavily influenced by epigean variation of temperature and precipitation (Badino 2004, Lunghi et al. 2015). Underground environments are not a unique case, as there are many environments in which microclimate might be imperfectly modeled by macroclimate, such as streams, ponds, forests with dense understory and topographically complex landscapes (Fridley 2009, Sears et al. 2011, De Frenne et al. 2013, Scheffers et al. 2014a, Scheffers et al. 2014b), thus insights of our analyses can be relevant for a wide range of species and habitats. Finally, the fauna living underground and in the soil is rarely investigated by macroecological studies (Beck et al. 2012), even though it includes a major proportion of terrestrial biodiversity.

We analyzed the niche of salamander species using both a fine-grained (microhabitat, representing the operative conditions actually experienced by individuals) and a broad-scale perspective (i.e. combining presence localities with broad-scale bioclimatic variables). We tested to what extent information on niche features and evolution is conserved between these two scales of analysis, and identified the geographical and evolutionary factors determining the mismatch between fine-grained and coarse-grained analyses of niche evolution.

Methods

Study system

In summer, underground environments show a continuous microclimatic gradient: the superficial sectors have conditions similar to the epigean ones (light, high temperature, low humidity). However, far from the surface the microhabitat becomes wetter, colder and dark (Fig. 1). Salamanders move underground because they must reach the sectors where conditions are within the tolerance limits of the species (Sunday et al. 2014) but, as food is more abundant in superficial sectors (Ficetola et al. 2013, Lunghi et al. 2015, Lunghi et al. 2017), they are restricted to a few tens of meters from the surface. Generally, the realized niche does not correspond to the fundamental niche because of dispersal limitations and biotic interactions (Soberon and Nakamura 2009). These issues exist for all the environments (Peterson et al. 2011), but within this system, they are alleviated because the full environmental gradient exist within a few meters, well within the dispersal ability of individuals, and because of the lack of predators and competitors within these environments (*Hydromantes* species are allopatric, Fig. S1, no other terrestrial salamanders are present, and they are apex predators in these environments) (Lunghi et al. 2014). Movements are limited and home ranges small (6-22 m^2) (Lanza et al. 2006), therefore observations are unlikely to represent transient individuals. The study system thus can be viewed as a natural habitat selection experiment, in which individuals are exposed to continuous environmental gradients, within which they select the favorable conditions (i.e., the conditions within their fundamental niche). Furthermore, previous studies showed that the microhabitat conditions selected by salamanders are consistent through the year, and niche estimates from summer surveys are generally similar to estimates for the other seasons (Lunghi et al. 2015). Summer is the period in which salamander detection is easiest, thus analyses performed on summer observation allow an appropriate characterization of species niche. Finally, terrestrial salamanders are generally at equilibrium with their environment for temperature and water and, in the field, the average temperature difference between air and body temperature is < 0.5°C (Spotila 1972, Lunghi et al. 2016). Thus air conditions are an excellent proxy of operative conditions of individuals (Sunday et al. 2014, Lunghi et al. 2016).

Surveys and data collection

To measure species distribution and habitat at fine spatial scale (microhabitat) we surveyed caves in Mediterranean Italy and France, widely covering the range of all European *Hydromantes* species (Fig. S1a). We excluded caves from the narrow hybrid zone between *H. ambrosii* and *H. italicus* (Ruggi 2007). Surveys were performed in early summer (June-July 2011-2014; 270 caves and 1180 cave sectors surveyed), when the conditions outside the cave are unfavorable and underground detectability is highest (Lunghi et al. 2015). All surveys were performed during the central hours of sunny and dry days. Each cave was subdivided in 3-m longitudinal intervals (hereafter: sectors); the size of sectors approximately corresponds to home ranges size (Lanza et al. 2006, Ficetola et al. 2013), covering the whole cave or until the first empty sector after the last salamander. In each sector we used visual encounter surveys to detect the presence of active salamanders, and measured four abiotic variables known to influence salamander distribution: air temperature (°C; accuracy: 0.1°C) and relative humidity (%; accuracy: 0.1%) were recorded with a EM882 multi-function thermo-hygrometer, waiting until the measurement was stable (variation <0.1°C or <0.1% for > 60 seconds). Minimum and maximum incident light (illuminance, measured in lux, accuracy 0.01 lux) were recorded by performing at least 10 measures of illuminance in the portions of the sector receiving more and less light, respectively. Furthermore, as a biotic parameter, we counted the number of adult large *Meta* spiders (*M. menardi* or *M. bourneti*). These spiders are the major predators of arthropods in the study caves, and have been proposed as indicators of prey availability for salamanders (Ficetola et al. 2013, Manenti et al. 2015).

To analyse the bioclimatic niche, we obtained distribution records covering the whole range of all the *Hydromantes* species from the present study and from the literature (Lanza et al. 1995, Lanza et al. 2006, Ruggi 2007, Fiacchini et al. 2008, Lanza et al. 2009, Chiari et al. 2013, Pasmans et al. 2013). We only considered localities with accuracy of 1-km or better; localities within the hybrid zone between *H. ambrosii* and *H. italicus* were excluded (Ruggi 2007). To match the number of microhabitat predictors, we considered five bioclimatic parameters: mean temperature and summed precipitation during the period in which salamanders are active outside the cave (from September to May), temperature seasonality, precipitation seasonality and normalized digitizing vegetation index (NDVI) extracted at the 30 arc-second resolution from Worldclim (Hijmans et al. 2005) and from the ESA Land Cover CCI (http://maps.elie.ucl.ac.be/CCI/viewer/download.php). Tolerance to these parameters is assumed to directly influence animals, particularly during the periods in which they perform outdoor activity. To assess the robustness of our conclusions to the selection of parameters, we also repeated analyses using annual climatic features.

Microhabitat preferences of species

We used generalized linear mixed models (GLMMs) with binomial error to assess the within-cave relationships between each species and the features of cave sectors. In GLMMs, cave identity was included as random effect, salamander presence as independent, and the five microhabitat variables were the predictors. First, for each species we built the univariate models relating salamander presence to the five microhabitat variables. We tested both linear and quadratic relationships; quadratic terms were retained if they significantly improved fit. We then used the Akaike's Information Criterion (AIC) to build the minimum adequate models, best describing the occurrence pattern of each species on the basis of multiple predictors (Burnham and Anderson 2002). We built models considering all possible combinations of microhabitat variables, and ranked them using AIC. Some microhabitat variables were strongly correlated: minimum illuminance was related to maximum illuminance, while temperature data were negatively correlated with humidity (in the datasets of most species, $|r| > 0.7$). Models including highly correlated variables were excluded from the candidate models. The lowest-AIC model, i.e. the one explaining more variation with fewer predictors, was considered as the minimum adequate model for each species (Burnham and Anderson 2002).

A species is certainly present where it is detected, while non-detection may represent either real absences or failure of detecting the present species; not taking into account misdetection can influence regression results (MacKenzie et al. 2006). Previous analyses on a subset of species showed that, with our sampling protocol, detection probability is high but imperfect (approx. 0.75 per visit) (Ficetola et al. 2012, Lunghi et al. 2015). Therefore, in our models we weighted absences with a weight of 0.75 (following Gómez-Rodríguez et al. 2012). We calculated significance of variables using likelihood-ratio tests. For all species the residual deviance was similar or lower than the residual degrees of freedom (variance inflation factor of best-AIC models always $\leq$ 1.06), therefore overdispersion was not an issue. Before running analyses, illuminance was log-transformed, while humidity % was transformed using square-root-arcsine to improve normality and reduce skewness.

Niche overlap and equivalency among species

We used an approach based on Principal Component Analyses of environmental variables (PCA-env) to perform multivariate comparisons of niche overlap between pairs of species (Broennimann et al. 2012). PCA-env measures niche overlap between pairs of species or populations on the basis of occurrence and environmental data, is among the most reliable techniques for niche comparisons, and shows better performance than approaches based on species distribution modelling (Broennimann et al. 2012). PCA-env uses a kernel density function to compute the density of occurrences in the multivariate PCA space, in order to take potential bias into account that stems from unequal sampling effort. We calculated niche overlap and equivalency using the Schoener´s *D* metric (Warren et al. 2008). Schoener's *D* ranges between 0 (lack of overlap) and 1 (complete overlap), and is among the most widespread metrics of niche overlap in ecological, evolutionary and biogeographical studies (e.g. Warren et al. 2008, Rödder and Engler 2011). For the niche comparison of a species pair, PCA-env performs a principal component analysis (PCA) on the environmental spaces available to the two species (Broennimann et al. 2012). In the micro-habitat analysis, the "available space" was the microhabitat of all the sectors of caves within the species range. In the bioclimatic analysis the available space corresponded to the grid cells within 150 km from known presence points. This distance is three times the largest gap within a species range, thus likely includes all the areas potentially available to species dispersal (see Godsoe 2010).

Species distribution data and bioclimatic variables often show strong spatial autocorrelation, and this can influence the outcome of ecological analyses, but no formal approaches are currently available to incorporate autocorrelation into PCA-env. To assess the robustness of PCA-env to spatial autocorrelation, we repeated the bioclimatic analysis including an additional predictor representing spatial autocorrelation. For each species, we first built a spatial generalized additive model (GAM) with binomial error, using species presence / absence as dependent variable, and incorporating geographic coordinates of sites as tensor product smooth terms, using thin plate regression splines (Beale et al. 2010). We then used the spatial predictions of GAMs as an additional covariate in PCA-env. Even though the incorporation of spatial predictions as covariates is not a perfect approach to deal with autocorrelation, simulations showed that this implementation of GAMs helps to correctly estimate relationships in spatially structured datasets with relatively good performance (Beale et al. 2010). For both the microhabitat and bioclimatic analyses, significance of niche differences between species was assessed using the niche equivalency tests through 1999 permutations.

Relationships between microhabitat, bioclimatic niche and evolutionary history

Genetic distance between species pairs was calculated on the basis of three mitochondrial (12S, 16S and cyt-*b*) and two nuclear (RAG-1 and BNDF) genes, amplified by van der Meijden *et al.* (2009). We considered the 49 individuals for which data from all five genes were available (2-15 individuals per species). The concatenated genetic dataset contained 3494 base pairs (van der Meijden et al. 2009). The Tamura-Nei distance was calculated for each species pair, using the between group mean distance function in Mega 6. To calculate the geographical distances among species, we generated the polygon of the range of each species on the basis of presence records using α-hulls (Ficetola et al. 2014), and then calculated the Euclidean distances between the centroids of the ranges.

Microhabitat and bioclimatic niche distances between species were calculated as 1 - Shoener's *D*. We then evaluated the relationships between microhabitat, bioclimatic and genetic distances. First, we used non-metric multidimensional scaling (NMDS) for the graphical representation of niche distances among species (Legendre and Legendre 2012). For the graphical representation of among-species differences in habitat relationships, we calculated the mean values of environmental variables in the presence localities, and then fitted them to the NMDS space using vector fitting (Borcard et al. 2011). Vector fitting returned essentially the same niche differences between species obtained with PCA-env (Figs S2-S3), with the advantage of synthetically illustrating the relationships between all the species pairs in one single plot. Relationships between niche dissimilarity at micro- and macroecological level and genetic differentiation were analyzed with Mantel's test for ranked data (bivariate analyses) or with rank multiple regressions on distance matrices (MRDM; multivariate analyses) (Legendre and Legendre 2012), using 9999 permutations to assess significance. Previous studies have shown that the Mantel test and other metrics of phylogenetic signal (e.g. Abouheif index, Bolmberg's K) are closely related to each other because they are all based on a cross-product statistic, and the Mantel test is thus appropriate to assess phylogenetic signal for dissimilarity matrices (Hardy and Pavoine 2012, Pavoine and Ricotta 2013, Pavoine et al. 2014). After MRDM, we used commonality analysis to assess the unique and common contribution of intercorrelated independent variables (Prunier et al. 2015). Statistical analyses were run using the packages lme4, MuMIn, raster, vegan, ecodist and hyat in R 3.1 (www.r-project.org).

Results

In field surveys, we detected >2700 salamanders in 524 out of the 1180 cave sectors; the number of sectors in which we detected salamanders was heterogeneous among species (Table S1, Fig. S1).

Niche analyses at the microhabitat level

Relationships between species presence and abiotic variables were similar across the eight salamander species. All species were significantly associated with the sectors having highest humidity, lowest temperature, and lack of light. Relationships with spiders were generally weak (Fig. 2, Table 1). The relationship between humidity and two species (*H. flavus* and *H. italicus*) was non-linear, as probability of presence quickly decreased when humidity was <80% (Fig. S2). Furthermore, a non-linear relationship between temperature and *H. strinatii* indicated a sharp drop of suitability above 20°C (Fig. S2). Multiple regression models confirmed the univariate analyses: all species were associated with dark sectors characterized by high humidity and/or low temperature (Table S2).

Nevertheless, similarity tests showed significant niche differences for nearly all the species pairs. Niche overlap ranged between 0.165 and 0.799. Niche equivalency was rejected in 21/26 pairwise tests, and remained significant after sequential Bonferroni's correction in 19/26 tests (Table S3a). The majority of non-significant comparisons involved the species with most restricted range and smallest sample size (*H. sarrabusensis*). According to the microhabitat analyses, *H. ambrosii* and *H. strinatii* were the species most tolerant to light and to dry conditions, *H. sarrabusensis* was the species associated with warmest temperatures, while *H. genei*, *H. italicus* and *H. supramontis* were restricted to the darkest, wettest and coldest sectors (Figs. 2 and 3, Fig. S3).

Bioclimatic analysis

We obtained 556 presence localities, widely covering the range of all the species (5-179 records per species; Table S1, Fig. S1). Niche overlap measured at the bioclimatic level was generally limited (range: 0.001 - 0.504), and was lower than the overlap measured at the microhabitat level (paired samples *t*-test for unequal variances: $t_{54} = -6.1$, $p < 0.0001$). Niche equivalency was rejected in 25 out of 26 pairwise tests (Table S3b), and the single non-significant test involved the two species with smallest sample size (*H. sarrabusensis* and *H. supramontis*). According to the bioclimatic analyses, *H. ambrosii* and *H. strinatii* were associated with the coldest and wettest climates, while *H. sarrabusensis*, *H. supramontis*, *H. genei* and *H. flavus* were associated with warm and dry conditions (Fig. 3 and Fig. S3).

Microhabitat, bioclimatic niche and phylogenetic relationships

The correspondence between microhabitat and bioclimatic niches was weak. For instance, the microhabitat analysis identified *H. strinatii* and *H. ambrosii* among the species with the highest tolerance to dry sectors, while in the bioclimatic analyses they were associated with the wettest climates. Similarly, in the microhabitat analysis *H. genei* was associated with the coldest sectors, while it was among the species living in the warmest climates (Fig. 3). Overall, we found no relationship between niche dissimilarities calculated using the fine- and the coarse-scale approaches (Mantel's test: $r = -0.17$, $p = 0.36$, Fig. 4a).

Microhabitat distances were unrelated to evolutionary distances ($r = -0.06$, $p = 0.95$, Fig. 4b), while phylogenetically distant species showed the largest bioclimatic distances ($r = 0.53$, $p = 0.001$, Fig. 4c). However, the relationship between bioclimatic distance and evolutionary history was complicated by the fact that species phylogenetically distant also live in distant geographical areas ($r = 0.47$, $p = 0.013$), and bioclimatic distance was positively related to geographical distance between species ranges ($r = 0.52$, $p = 0.01$). Altogether, geographical and evolutionary distances explained bioclimatic distance well (MRDM: $R^2 = 0.39$, $p < 0.003$), but disentangling their relative role was difficult. In a commonality analysis, both variables showed a limited unique effect (phylogenetic distance: unique effect = 0.12; geographical distance: unique effect = 0.11), while more explanatory power was shared between these two parameters (Table S4). These results were robust to different approaches to the calculation of niches at both the microhabitat and bioclimatic level, and to the incorporation of parameters representing spatial autocorrelation (Supplementary Results).

Discussion

Both microhabitat and bioclimatic analyses identified clear niche differences between species, but the outcome of the two approaches showed striking dissimilarities. The bioclimatic analyses suggested a close relationship between niche and evolutionary divergence, i.e. a strong phylogenetic signal of niches, while the microhabitat divergence was unrelated to either phylogeny or to the bioclimatic pattern.

Theory clearly acknowledges the multi-scalar nature of niches, and several studies have shown that species distribution is the product of processes acting at both broad and fine scale (reviewed in Peterson et al. 2011). An increasing number of studies has tested whether ecological niches retain a signal of phylogenetic history, and many of them have used a bioclimatic approach for niche definition (Peterson 2011). However, the geographical distribution of organisms is strongly related to their evolutionary history, and recent work suggests that complex interplay between present-day distribution, evolutionary history, and the spatial autocorrelation of bioclimatic variables may complicate the reconstruction of niche evolution (Warren et al. 2014). Warren et al. (2014) proposed a conceptual framework, in which diversification mostly occurs through allopatric speciation. Sister-species are thus generally allopatric, and only phylogenetically distant species may have overlapping ranges, because they have limited competition. Under this framework, closely related clades may show the strongest apparent niche divergence, even if the opposite may be true (e.g., unrelated species exist in sympatry because of limited competition, i.e. small niche overlap) (Gutiérrez et al. 2014). Our study shows that a similar interplay between evolutionary history and geography may even determine the opposite pattern. Allopatric speciation was the most likely driver of the differentiation between terrestrial salamander species (Carranza et al. 2008, Chiari et al. 2013), but strong interspecific competition (Cimmaruta et al. 1999) and barriers likely cause the absence of sympatry between species (Fig. S1), while poor dispersal limits their geographical spread. Under these conditions, closely related species often have proximate ranges, and this may cause a pattern with closely related species sharing similar niches (Fig. 4c), even in absence of a true phylogenetic effect. As a consequence, niche comparisons only on the basis of bioclimatic data can miss the full history: the geography of speciation might be the actual driver of most observed patterns on niche evolution, instead of the inferred ecological processes (Warren et al. 2014). The niche comparison method used here (Broennimann et al. 2012) is considered to be able to correct at least in part for the similarity determined by spatial autocorrelation (Warren et al. 2014), yet, a clear effect of geographical distance on bioclimatic niche differentiation remained evident. Actually, it was hard to tell whether the niche similarity between closely related species was the result of niche conservatism, or whether it was just the byproduct of related species having nearby ranges (Table S4).

Two complementary approaches may help to improve analyses of niches. On the one hand, measures more closely related to the fundamental niche (e.g. performance, microhabitat selection, tolerance limits, operational conditions), if available, can be used to test the reliability of bioclimatic analyses (Grandcolas et al. 2011). For instance, in terrestrial salamanders, the average operational temperature measured at the microhabitat level was unrelated to the average air temperature during the activity season, obtained from global gridded data (Fig. S6), and such discrepancy casts doubts on the reliability of the bioclimatic results alone. On the other hand, the growing availability of spatial datasets and analytical tools allows quickly extracting information that would be much harder to obtain at the microhabitat level, and this has likely helped the fast progress of macroecological studies. Joint availability of broad-scale and fine-grained data is limited (Beck et al. 2012), and researchers need to assess the validity of macroecological analyses, even in the absence of information on performance at the small-scale. If the relationship between niche and history abruptly changes when taking into account geography, or if we cannot tease apart their relative role, then Warren's (2014) hypothesis that we are mistaking geography for biology is a likely explanation. Spatial patterns are inherently linked to ecological processes, thus researchers must utilize approaches that allow explicitly take into account the spatial structure of their data. For instance, the simple effect of geographical distance may be considered as a null-model, over which the phylogenetic history can be compared (McIntire and Fajardo 2009), even though the spatial effect of past geography, topographical and ecological barriers may be complex, and it is not so easy to explicitly take them into account.

Microhabitat and macroecological analyses certainly characterize non-identical aspects of the niche, still parameters such as thermal preferences have relevant implications on broad scale species distribution (Kearney and Porter 2009), and thus we expected some relationships between them. We suggest that in our study system the microhabitat approach may better represents species niches because *i*) at least for some parameters (e.g. temperature), microhabitat is an excellent proxy of operative eco-physiological conditions of salamanders (Lunghi et al. 2016), which are a major approach to the measurement of fundamental niches (Kearney and Porter 2009); *ii*) the microhabitat approach is not biased by dispersal limitations or biotic interactions and *iii*) within each cave, a full range of conditions generally exists, from the harshest to the most suitable, enabling a parallel with habitat preference experiments (Fig. 1).

Niche analyses are increasingly used to answer multiple ecological and evolutionary questions, such as predictions of species' responses to climate change, analyses of biodiversity drivers and even to analyze local adaptations and identify species. Studies combining distribution data with macroecological predictors can be extremely effective, and some of them have been able to analyze thousands of species at the continental or even global scale. Such broad scale analyses are based on the assumption that grid-cell average climatic conditions provide a good prediction of the probability of species persistence in a site (Bennie et al. 2014), but in most of cases, this assumption is untested. A few studies have evaluated whether species fitness can be actually predicted by broad-scale analyses (e.g. Brambilla and Ficetola 2012, Searcy and Shaffer 2016), and found mixed results. For instance, Searcy and Shaffer (2016) tested whether climatic variables important in broad-scale species distribution models are also related to salamander recruitment, and observed some match between the two approaches. However, the strength of the match was strongly dependent on metrics and methods used to develop the distribution models, and different approaches yielded non-identical predictions of species responses to climate change (Searcy and Shaffer 2016).

Differences between microhabitat and macrohabitat approaches can be particularly relevant for animals living in complex landscapes and specific microhabitats (e.g. soil, vegetation, freshwater…) where conditions are very different from the commonly used measures of climate, such as mean air temperature (Fridley 2009, Sears et al. 2011, De Frenne et al. 2013, Scheffers et al. 2014a, Scheffers et al. 2014b). Actually, such organisms include many amphibians, insects (Sunday et al. 2014) and likely other terrestrial invertebrates. These taxa are not those most studied in macroecology (Beck et al. 2012), but comprise the majority of terrestrial animals, thus the discrepancy between microhabitat and bioclimatic analyses may be present for many organisms.

Nevertheless, there are also systems in which this pattern was not observed, as some studies on surface-living salamanders found concordance between fine-scale (microclimate, body temperature) and bioclimatic data (Kozak and Wiens 2007, Fisher-Reid et al. 2013). Still some of these studies analyzed a small number of taxa, or only considered a limited range of microhabitat parameters, therefore assessing the correspondence between our conclusion and these previous studies is not easy. It might also be argued that animals associated with underground environments are special cases, if they shelter in microhabitats that are independent from macrohabitat conditions. However, this is not the case, given that underground temperature and water availability are tightly linked to epigean temperature and precipitation (Badino 2004, Lunghi et al. 2015). Actually, underground environments receive a much lower interest in the macroecological / biogeographical literature than more visible aboveground habitats, but host a major portion of Earth biodiversity (Brandmayr et al. 2013).

Macroecology has allowed us to move from reductionist, small scale ecology to a much broader approach with great potential for generalization, which can provide key responses to the global biodiversity crisis (Brown 1995, Kerr et al. 2007). Nevertheless, when laying the foundations of macroecology, Brown (1995) described himself as an oddball that continues combining reductionist and holistic approaches. The integration of multiple approaches certainly requires more time and investments, but the urgency to obtain answers should not preclude the need of robust, biologically sound data (Bernardo 2014). The integration of studies at multiple scales allows to take into account a broader spectrum of processes influencing populations, thus providing more accurate inference on niche evolution (Searcy and Shaffer 2016). A better combination between bioclimatic and fine-grained data (Sandel 2015), and also considering additional niche components such as diet and other biotic interactions, may be a key to obtain robust generalizations that can help us to address the consequences of global changes.

Aknowledgements We are grateful to speleologists helping us during fieldwork. The study was authorized by the Sardinia Region and by the Italian Ministry of the Environment (prot. 0040002). This study was funded by Univ. Milano-Bicocca, by Labex OSUG@2020 (Investissements d'avenir – ANR10 LABX56) and by Mohamed bin Zayed Species Conservation Fund (150511222).

References

Badino, G. 2004. Cave temperatures and global climatic change. — Int. J. Speleol. 33: 103-114.
Beale, C. M. et al. 2010. Regression analysis of spatial data. — Ecol. Lett. 13: 246-264.
Beck, J. et al. 2012. What's on the horizon for macroecology? — Ecography 35: 673-683.
Bennie, J. et al. 2014. Seeing the woods for the trees – when is microclimate important in species distribution models? — Global Change Biol. 20: 2699-2700.
Bernardo, J. 2014. Biologically grounded predictions of species resistance and resilience to climate change. — Proc. Natl. Acad. Sci. USA 111: 5450-5451.
Blomberg, S. P. and Garland, T. 2002. Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods. — J. Evol. Biol. 15: 899-910.
Borcard, D. et al. 2011. Numerical Ecology with R. — Springer.
Brambilla, M. and Ficetola, G. F. 2012. Species distribution models as a tool to estimate reproductive parameters: a case study with a passerine bird species. — J. Anim. Ecol. 81: 781–787.
Brandmayr, P. et al. 2013. Hypogean carabid beetles as indicators of global warming? — Environ. Res. Lett. 8: 11.
Broennimann, O. et al. 2012. Measuring ecological niche overlap from occurrence and spatial environmental data. — Global Ecol. Biogeogr. 21: 841-897.

Brown, J. H. 1995. Macroecology. — The University of Chicago Press.

Bucklin, D. N. et al. 2013. Climate downscaling effects on predictive ecological models: a case study for threatened and endangered vertebrates in the southeastern United States. — Reg. Envir. Chang. 13: S57-S68.

Burnham, K. P. and Anderson, D. R. 2002. Model selection and multimodel inference: a practical information-theoretic approach. — Springer Verlag.

Carranza, S. et al. 2008. Biogeography and evolution of European cave salamanders, *Hydromantes* (Urodela : Plethodontidae), inferred from mtDNA sequences. — J. Biogeogr. 35: 724-738.

Chiari, Y. et al. 2013. Phylogeography of Sardinian cave salamanders (genus *Hydromantes*) is mainly determined by geomorphology. — Plos One 7: e32332.

Cimmaruta, R. et al. 1999. Spatial distribution and competition in two parapatric sibling species of European plethodontid salamanders. — Ethol. Ecol. Evol. 11: 383-398.

De Frenne, P. et al. 2013. Microclimate moderates plant responses to macroclimate warming. — Proc. Natl. Acad. Sci. USA 110: 18561-18565.

Enriquez-Urzelai, U. et al. 2015. Locomotor Mode and the Evolution of the Hindlimb in Western Mediterranean Anurans. — Evol. Biol. 42: 199-209.

Fiacchini, D. et al. (eds.) 2008. Biospeleologia nell'Appennino. Studi e ricerche su Anfibi e Invertebrati, con particolare riferimento all'Appennino Umbro-Marchigiano. — Tecnostampa Edizioni.

Ficetola, G. F. et al. 2012. Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. — Amphibia-Reptilia 33: 251-259.

Ficetola, G. F. et al. 2013. Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? — Popul. Ecol. 55: 217-226.

Ficetola, G. F. et al. 2014. An evaluation of the robustness of global amphibian range maps. — J. Biogeogr. 41: 211–221.

Fisher-Reid, M. C. et al. 2013. Parapatric divergence of sympatric morphs in a salamander: incipient speciation on Long Island? — Mol. Ecol. 22: 4681-4694.

Fisher-Reid, M. C. et al. 2012. How is the rate of climatic-niche evolution related to climatic-niche breadth? — Evolution 66: 3836-3851.

Fraterrigo, J. M. et al. 2014. Local-scale biotic interactions embedded in macroscale climate drivers suggest Eltonian noise hypothesis distribution patterns for an invasive grass. — Ecol. Lett. 17: 1447-1454.

Freidenburg, L. K. and Skelly, D. K. 2004. Microgeographical variation in thermal preference by an amphibian. — Ecol. Lett. 7: 369-373.

Fridley, J. D. 2009. Downscaling Climate over Complex Terrain: High Finescale (< 1000 m) Spatial Variation of Near-Ground Temperatures in a Montane Forested Landscape (Great Smoky Mountains). — J. Appl. Meteorol. Climatol. 48: 1033-1049.

Gallien, L. et al. 2012. Invasive species distribution models – how violating the equilibrium assumption can create new insights. — Global Ecol. Biogeogr. 21: 1126-1136.

Godsoe, W. 2010. I can't define the niche but I know it when I see it: a formal link between statistical theory and the ecological niche. — Oikos 119: 53-60.

Gómez-Rodríguez, C. et al. 2012. Integrating detection probabilities in species distribution models of amphibians breeding in Mediterranean temporary ponds. — Divers. Distrib. 18: 260-272.

Grandcolas, P. et al. 2011. Mapping extrinsic traits such as extinction risks or modelled bioclimatic niches on phylogenies: does it make sense at all? — Cladistics 27: 181-185.

Gutiérrez, E. E. et al. 2014. Can biotic interactions cause allopatry? Niche models, competition, and distributions of South American mouse opossums. — Ecography 37: 741-753.

Hardy, O. J. and Pavoine, S. 2012. Assessing phylogenetic signal with measurement error: a comparison of Mantel tests, Blomberg et al.'s K, and phylogenetic distograms. — Evolution 66: 2614-2621.

Hijmans, R. J. et al. 2005. High resolution interpolated climate surfaces for global land areas. — Int. J. Climatol. 25: 1965-1978.

Kearney, M. and Porter, W. 2009. Mechanistic niche modelling: combining physiological and spatial data to predict species' ranges. — Ecol. Lett. 12: 334-350.

Kearney, M. R. et al. 2014. Microclimate modelling at macro scales: a test of a general microclimate model integrated with gridded continental-scale soil and weather data. — Methods Ecol. Evol. 5: 273-286.

Kerr, J. T. et al. 2007. The macroecological contribution to global change solutions. — Science 316: 1581-1584.

Kozak, K. H. and Wiens, J. J. 2007. Climatic zonation drives latitudinal variation in speciation mechanisms. — Proc. R. Soc. B 274: 2995-3003.

Kozak, K. H. and Wiens, J. J. 2016. What explains patterns of species richness? The relative importance of climatic-niche evolution, morphological evolution, and ecological limits in salamanders. — Ecool. Evol. 6: 5940-5949.

Lanza, B. et al. 2009. Vertebral numbers in the Caudata of the Western Palaeartic (Amphibia). — Atti Mus. Civ. St. Nat. Trieste 54: 3-114.

Lanza, B. et al. 1995. Morphologic and genetic studies of the European plethodontid salamanders: taxonomic inferences (genus *Hydromantes*). — Monografie XVI. Museo Regionale di Scienze Naturali.

Lanza, B. et al. 2006. A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). — Atti Mus. Civ. St. Nat. Trieste 52 (Suppl.): 5-135.

Legendre, P. and Legendre, L. 2012. Numerical Ecology. — Elsevier.

Losos, J. B. 2008. Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species. — Ecol. Lett. 11: 995-1003.

Lunghi, E. et al. 2016. Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. — J. Therm. Biol. 60: 79–85.

Lunghi, E. et al. 2014. Do cave features affect underground habitat exploitation by non-troglobite species? — Acta Oecol. 55: 29-35.

Lunghi, E. et al. 2015. Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? — PeerJ 3: e1122.

Lunghi, E. et al. 2017. Cave features, seasonality and subterranean distribution of non-obligate cave dwellers. — PeerJ e3169.

MacKenzie, D. I. et al. 2006. Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence. — Academic Press.

Manenti, R. et al. 2015. Distribution of spiders in cave twilight zone depends on microclimatic features and trophic supply. — Invertebr. Biol. 134: 242-251.

McIntire, E. J. B. and Fajardo, A. 2009. Beyond description: the active and effective way to infer processes from spatial patterns. — Ecology 90: 46-56.

Mesquita, D. O. et al. 2016. Life-History Patterns of Lizards of the World. — Am. Nat. 187: 689-705.

Pasmans, F. et al. 2013. Resistance to Chytridiomycosis in European Plethodontid Salamanders of the Genus *Speleomantes*. — Plos One 8: e63639.

Pavoine, S. et al. 2014. Life history traits, but not phylogeny, drive compositional patterns in a butterfly metacommunity. — Ecology 95: 3304-3313.

Pavoine, S. and Ricotta, C. 2013. Testing for phylogenetic signal in biological traits: the ubiquity of cross-product statistics. — Evolution 67: 828-840.

Peterson, A. T. 2011. Ecological niche conservatism: a time-structured review of evidence. — J. Biogeogr. 38: 817-827.

Peterson, A. T. et al. 2011. Ecological niches and geographic distributions. — Princeton University Press.

Potter, K. A. et al. 2013. Microclimatic challenges in global change biology. — Global Change Biol. 19: 2932-2939.

Prunier, J. G. et al. 2015. Multicollinearity in spatial genetics: separating the wheat from the chaff using commonality analyses. — Mol. Ecol. 24: 263-283.

Rissler, L. J. and Apodaca, J. J. 2007. Adding more ecology into species delimitation: Ecological niche models and phylogeography help define cryptic species in the black salamander (*Aneides flavipunctatus*). — Syst. Biol. 56: 924-942.

Rödder, D. and Engler, J. O. 2011. Quantitative metrics of overlaps in Grinnellian niches: advances and possible drawbacks. — Global Ecol. Biogeogr. 20: 915-927.

Ruggi, A. 2007. Descrizione di una zona di contatto e ibridazione tra *Speleomantes italicus* e *S. ambrosii bianchii* (Amphibia: Plethodontidae) sulle Alpi Apuane, mediante marcatori nucleari e mitocondriali. — Università degli Studi della Tuscia di Viterbo. http://dspace.unitus.it/handle/2067/240.

Sandel, B. 2015. Towards a taxonomy of spatial scale-dependence. — Ecography 38: 358-369.

Scheffers, B. R. et al. 2014a. Microhabitats reduce animal's exposure to climate extremes. — Global Change Biol. 20: 495-503.

Scheffers, B. R. et al. 2014b. Microhabitats in the tropics buffer temperature in a globally coherent manner. — Biol. Lett. 10: 4.

Searcy, C. A. and Shaffer, H. B. 2016. Do Ecological Niche Models Accurately Identify Climatic Determinants of Species Ranges? — Am. Nat. 187: 423-435.

Sears, M. W. et al. 2011. The World Is not Flat: Defining Relevant Thermal Landscapes in the Context of Climate Change. — Integr. Comp. Biol. 51: 666-675.

Soberon, J. 2007. Grinnellian and Eltonian niches and geographic distributions of species. — Ecol. Lett. 10: 1115-1123.

Soberon, J. and Nakamura, M. 2009. Niches and distributional areas: Concepts, methods, and assumptions. — Proc. Natl. Acad. Sci. USA 106: 19644-19650.

Spotila, J. R. 1972. Role of temperature and water in the ecology of lungless salamanders. — Ecol. Monogr. 42: 95-125.

Sunday, J. M. et al. 2014. Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. — Proc. Natl. Acad. Sci. USA 111: 5610-5615.

van der Meijden, A. et al. 2009. Phylogenetic relationships of Sardinian cave salamanders, genus *Hydromantes*, based on mitochondrial and nuclear DNA sequence data. — Mol. Phylogenet. Evol. 51: 399-404.

Wake, D. B. 2013. The enigmatic history of the European, Asian and American plethodontid salamanders. — Amphibia-Reptilia 34: 323-336.

Warren, D. L. et al. 2014. Mistaking geography for biology: inferring processes from species distributions. — Trends Ecol. Evol. 29: 572-580.

Warren, D. L. et al. 2008. Environmental niche equivalency versus conservatism: Quantitative approaches to niche evolution. — Evolution 62: 2868-2883.

All data will be available from the Dryad Digital Repository:

Supplementary material:

Supplementary results: niche analyses repeated measuring niches using different approaches, and supplementary figures.

Table 1 Relationships between the occurrence of eight species of salamanders in underground sectors and microhabitat features. Results of univariate generalized linear mixed models taking into account imperfect detection. *B*: unstandardized regression coefficients. Q: quadratic relationships (see Fig. S2); all the other models are linear. Significant values are in bold. Degrees of freedom are 1 for linear models, and 2 for quadratic models.

Species	Humidity			Temperature			Min. Light			Max. Light			Spider presence		
	B	χ^2	P	*B*	χ^2	P	*B*	χ^2	P	*B*	χ^2	P	*B*	χ^2_1	p
H. ambrosii	3.5	6.4	**0.012**	-0.14	10.2	**0.001**	-0.6	12.1	**<0.001**	-0.4	15.5	**<0.001**	-0.29	0.2	0.644
H. flavus	Q	32.6	**<0.001**	-0.57	12.7	**<0.001**	-76.4	20.8	**<0.001**	-36.6	32.0	**<0.001**	0.20	0.1	0.741
H. genei	20.5	16.2	**<0.001**	-0.85	7.7	**0.006**	-1.1	8.7	**0.003**	-0.4	6.4	**0.012**	-0.85	1.7	0.192
H. imperialis	7.5	14.5	**<0.001**	-0.39	24.5	**<0.001**	-1.8	18.5	**<0.001**	-0.4	13.0	**<0.001**	0.55	0.3	0.598
H. italicus	Q	41.8	**<0.001**	-0.24	19.5	**<0.001**	-3.7	48.2	**<0.001**	-0.7	50.2	**<0.001**	-0.38	1.0	0.317
H. sarrabusensis	12.8	4.3	**0.037**	-0.57	4.1	**0.043**	-3.8	4.7	**0.030**	-1.5	6.0	**0.014**	2.84	2.5	0.115
H. strinatii	6.0	16.7	**<0.001**	Q	25.3	**<0.001**	-0.8	13.4	**<0.001**	-0.4	15.9	**<0.001**	0.27	0.4	0.527
H. supramontis	14.9	27.5	**<0.001**	-0.62	26.7	**<0.001**	-3.7	18.1	**<0.001**	-0.6	10.6	**0.001**	-0.33	0.1	0.705

Figure legends

Figure 1. How microhabitat selection can mirror habitat selection experiments. At increasing depths, temperature decreases and humidity increases: salamanders are only found when conditions are within the species range. The figure represents the microhabitat and salamander distribution actually observed in the cave "*Brecca su Fenugu"* (39°42'N, 9°25'E).

Figure 2. Microhabitat conditions in cave sectors where salamanders were detected (colored dots) or undetected (black dots). Dots represent the mean conditions of occupied / unoccupied sectors; error bars are twice the standard errors.

Figure 3. Niche differences among salamander species according to a) microhabitat and b) broad-scale bioclimatic analyses (multidimensional scaling plots). Dots represent the scores of species in the multivariate space; blue arrows are environmental variables added to plots using vector fitting.

Figure 4. Relationships between microhabitat, bioclimatic, and genetic distances between salamander species. Values on the plots are the results of Mantel's tests.

Figure 1

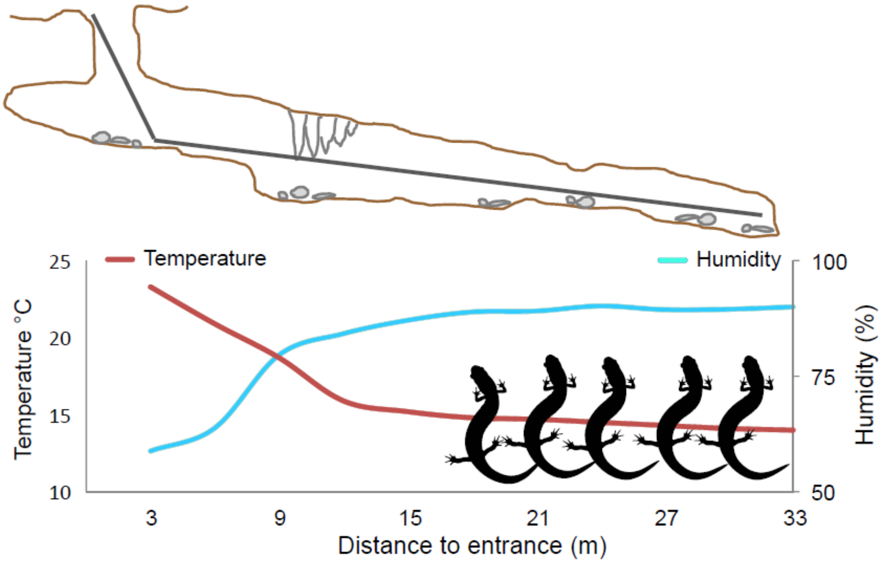


Figure 2

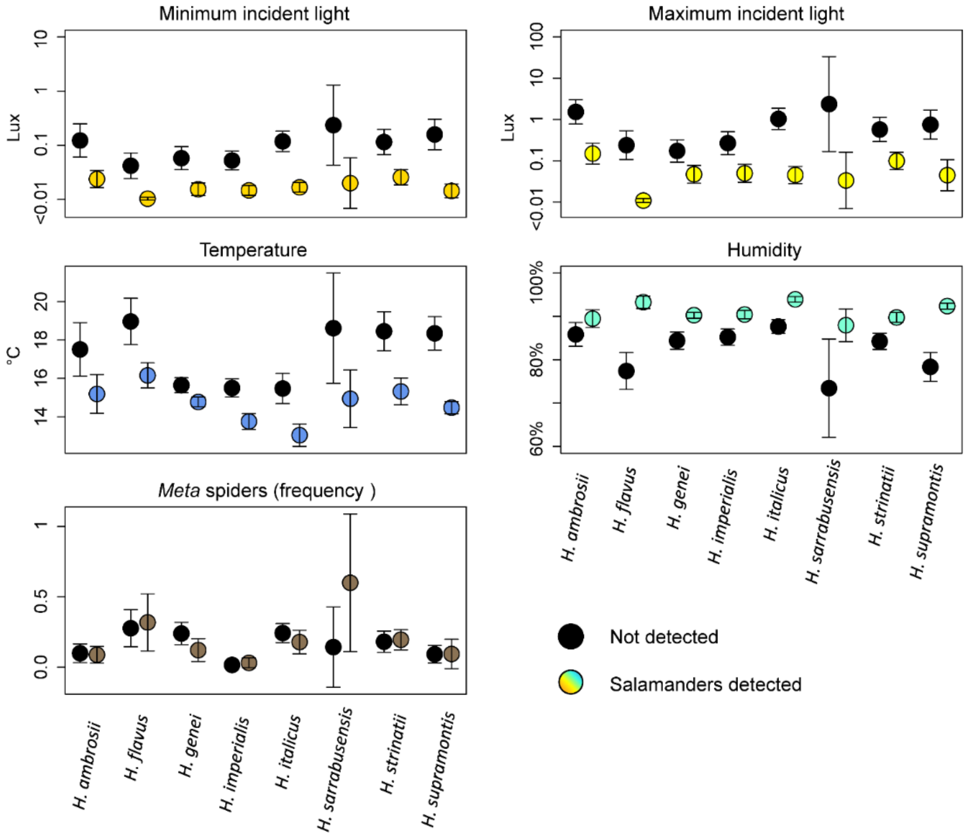


Figure 2

Figure 3

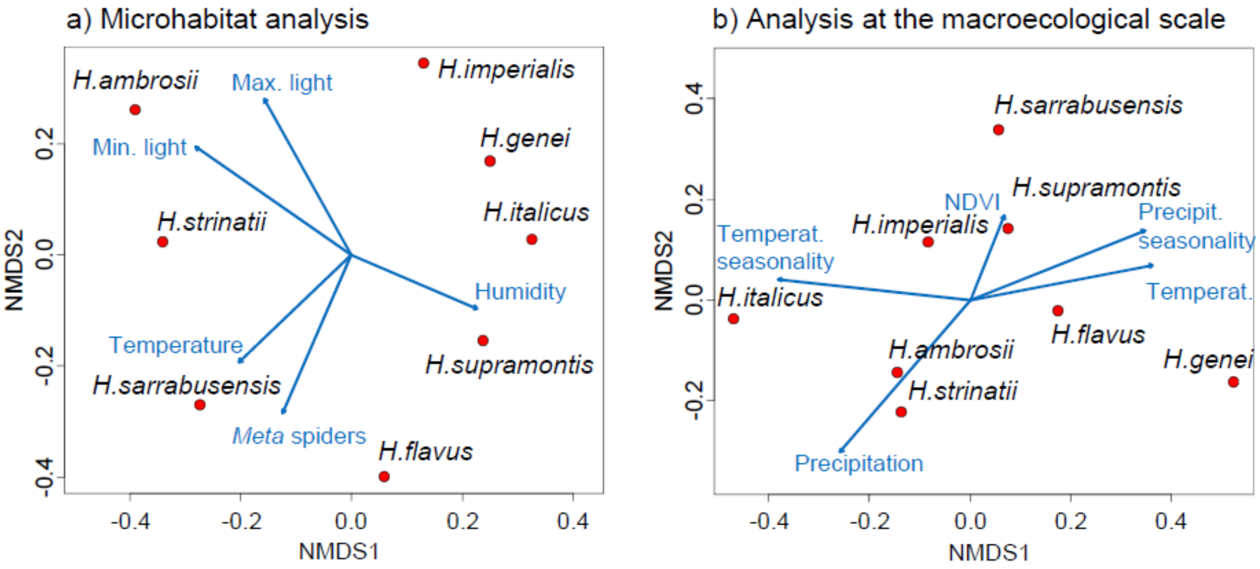
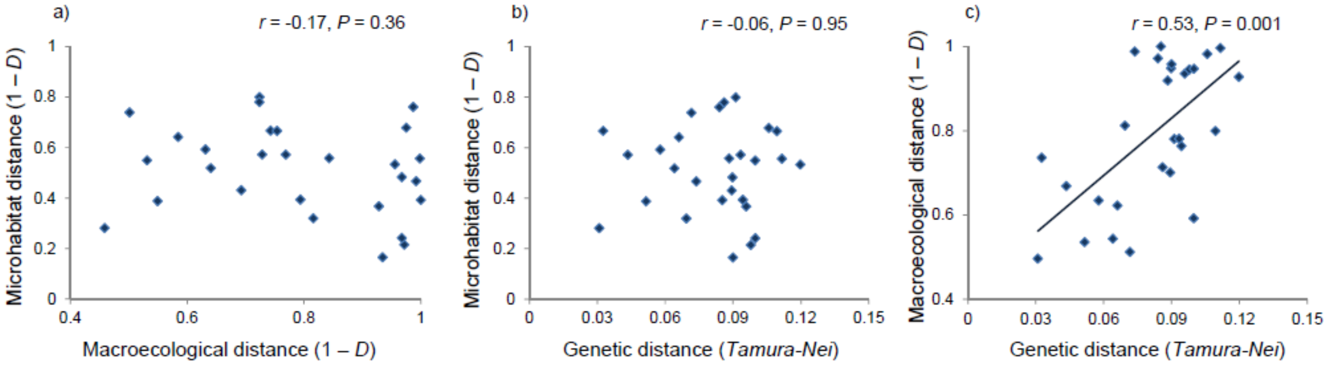


Figure 4



Chapter IV

Unpublished manuscript

Ecological niche studies give evidences for the adaptive role of introgression and hybrid zones occurrence

Raoul Manenti[1,*], Enrico Lunghi[2,3,4], Roberta Pennati[1], Gentile Francesco Ficetola[1,5]

[1]Dipartimento di Bioscienze, Università degli Studi di Milano. Via Celoria 26, 20133 Milano Italy

[2]Universität Trier Fachbereich VI Raum- und UmweltwissenschaftenBiogeographie, Campus I, Gebäude N Universitätsring 15, 54286 Trier, Germany

[3]Natural Oasis, Via di Galceti 141, 59100 Prato, Italy

[4]Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy

[5]Laboratoire d'Ecologie Alpine (LECA), Université Grenoble-Alpes, F-38000 Grenoble, France

*Corresponding author: raoulmanenti@gmail.com

Abstract

Quantification of niche similarity may be an important instrument to test whether hybrid populations have niches that are similar or intermediate to their parental species, or instead occupy transgressive niches. However, the evolution of niche is often studied using macroecological data with a coarse resolution. European cave salamanders (*Hydromantes ambrosii*, *H. italicus* and their hybrid populations) are an excellent experimental model to perform fine- scale assessment of niche differences between parental species and their hybrids of the species

In June-July 2012-2016, we surveyed 138 natural and artificial caves. We subdivided each cave in 3-m longitudinal sectors, in which we detected active salamanders and measured microhabitat features (humidity, air temperature and incident light). We then used the PCA-env approach to assess niche shifts between pure and introgressed salamander populations, we calculated niche overlap using the Schoener's *D* statistics and we used equivalency tests to assess the significance of niche differences between taxa.

Pure H. ambrosii populations were linked to dark and cold underground sectors, while pure *H. italicus* populations chose dark and wet sectors. *H ambrosii* introgressed populations showed a significant (P= 0.001) expansion toward warmer, drier and more luminous conditions compared to the non-introgressed populations. Introgressed *H. italicus* showed a significant niche shift (P = 0.006), with expansion toward conditions with lower humidity and more light compared to *H. italicus*.

Our results are consistent with the transgressive niche hypothesis: hybrids have a broader ecological niche than the parental species, allowing them to exploit environments with harsher conditions.

Keywords

Introgressive hybridisation, disruptive selection, adaptive radiation, salamander, salamandra, cave, biospeleology

Introduction

Many species of vertebrates are supposed to have attained a post-zygotic reproductive isolation generally only after millions of years since their original separation from a common ancestor (Fitzpatrick, 2004). The speciation events carrying reproductive isolation between two or more populations belonging to the same ancestor can proceed both through a gradual divergence (Seehausen, 2004), generally involving parapatry or allopatry (Seehausen, 2004), or may instead involve different genomic events which end in the establishment of novel evolutionary lineages. The factors that generally promote speciation events are mechanisms determining isolation that are rewarded by the differential action of selective pressures (Fitzpatrick, 2004; Garrick et al, 2014).

When species extend their geographical range, such as for example following a glacial period that determined isolation, they exploit new habitats and may interact with species previously unknown to them. If a species comes across another closely related species, the two may interbreed, eventually leading to offspring in a more or less narrow hybrid zone.

Since the Mayr's definition of species as 'interbreeding natural populations that are reproductively isolated from other such groups', the existence of hybrid zones between different species has been regarded as an element determining the collapse of reproductive isolation (Tanaka, 2007; De Hert et al, 2012). However, the gene flow between incipient species can also represent a source for potentially beneficial alleles (Ficetola & Stock, 2016; Bay & Ruegg, 2017). The occurrence of hybrid zones between closely related species is more frequent than expected and furnishes important case studies for the comprehension of the evolution of adaptive traits; this is an aspect that is gaining more and more attention in the research of biodiversity causes (Grant & Grant, 2016). Hybrid zones give to loci the opportunity to act on new genetic backgrounds and in different environmental conditions and induce positive or negative responses to new selective pressures. Over time hybrid zones may be more or less stable depending on the fitness strengths of the hybrids and on the occurring of ecological and reproductive barriers. The loss of reproductive isolation, prompting hybridization between divergently adapted populations is best known in conservation biology as a cause of extinction linked to anthropic actions like species translocations or dispersal barriers removal and bringing formerly allopatric species into secondary (Seehausen, 2004), when secondary contact occurs exchange of pre-zygotic barriers established during the different species evolution may yield to exclusively inviable or poorly infertile offspring, generating fitness disadvantages and compromising populations viability (Seehausen, 2004).

On the other hand, the gene flow between related species can also be a source for potentially beneficial alleles. For example, the different stages of introgression recorded between Darwin’s finch species that differ for the beaks size, have been hypothesized to play a strong adaptive role during long droughts (Grant & Grant, 2016). During drought seasons, mortality is extremely high with selective pressures acting on beak size and favouring hybrids by a strong increase of the gene flow levels between species (Grant & Grant, 2016). This aspect is confirmed by a recent study (Bay & Ruegg, 2017) analysing genetic mechanisms acting in hybrid zones, which revealed that the occurrence of hybrid zones, with contact between related species, can provide opportunities for the existence of adaptive introgression phenomena at the genomic level.

If the genetic aspects involved in the occurrence and maintenance of hybrid zones are currently being widely studied, much less attention has been paid to the characterization of the ecological niche of hybrid populations. Species niche differentiation is an important trait, that can enhance the comprehension of the factors determining the occurring differences between related species and explain the geographical separation that are observed among them.

Interspecific differences in ecological niche among species may be impressive, even between closely related species, but very few information is available on the difference that may occur from hybrid populations and parental species and how they may be related to introgression phenomena. The increasing availability of large data sets allowing the comparison between both species’ distributions patterns and environmental features, together with the strong improvement of powerful statistical tools, slows the identification of the species-specific ecological conditions, at least over broad geographical scales (‘realized Grinnelian niche’; Soberon & Nakamura, 2009). In particular, the quantification of niche similarity and overlap among different taxa, together with phylogenetic data, allows to better understand evolutionary processes like adaptation and speciation (Seehausen, 2004) and may furnish an important instrument to test whether hybrid populations have niches that are similar or intermediate to their parental species or instead occupy transgressive niches (Ficetola & Stock, 2016).

The intermediate niche theories predict that hybrids should have intermediate niche occupancy features compared to their parental species, that would be inferior competitors by respect to the hybrids’ performances that would be maximal when occupying intermediate niches (Moore, 1977). This condition has been reported for different vertebrates and occurs when the geographical and environmental partitioning between parental species is a consequence of a gradient of habitat features along which hybrid individuals can profit of intermediate conditions (Pagano et al, 2001; Otis et al, 2017). Conversely the transgressive niche hypothesis (Ficetola & Stock, 2016) supposes that introgressed populations show ecological niches that differ to those of both parental species meaning that hybrid populations can have advantages and exploit harsher habitat conditions. In a recent study on the realized ecological niches of five Palaearctic green toad species, (Ficetola & Stock, 2016) found evidences for transgressive niche in two hybrid species that are able to face harsher climatic condition than their parental lineages.

However, the study of ecological niche in hybrid zones is challenging as the niche evolution is often studied using macroecological data and based on a broad-scale (bioclimatic) approach, that even if may allow to draw general patterns hardly recoverable using local analyses, generally leads to a coarse resolution of the processes (Noriega et al, 2013). Hybrid zones often occur between the two parental species and have limited extensions (Garrick et al, 2014), increasing the risk of confounding features linked to geographical conditions with evolutionary processes that often occur at the microhabitat scale if only a broad scale approach is involved (Warren et al, 2014).

For this reason, the high potential to disentangle the processes leading hybridisation that is played by the study of ecological niche, is hard to be used as micro-scale studies approach cannot be applied to all the species or require experiments under controlled environmental conditions. A valid experimental model to perform micro-scale analyses to assess niche differences in hybrid zones may be furnished by European cave salamanders of the genus *Hydromantes* (Wake, 2013). *Hydromantes* salamanders have superficial activity during cool and wet seasons (from autumn to spring), while exploit different underground environments during dry and hot periods (e.g., late spring and summer); when underground, they select sectors having microclimatic features within their physiological constraints (Ficetola et al, 2012; Lunghi et al, 2016). Thus the microhabitat features at which these salamanders are recorded during summer periods approximate well the body conditions and the tolerance to the limiting factors that once surveyed may allow an efficient and refined comparison between hybrid and parental populations/species. In particular, the habitat selection of *Hydromantes* species during summer is analogous to a habitat selection experiment along a continuous gradient of light, humidity and temperature. In this study, we analysed the niches of two European cave salamanders species, *Hydromantes ambrosii* and *H. italicus* and their hybrid populations. *H. ambrosii* and *H. italicus* show a narrow hybrid zone derived from a secondary contact event following the last glacial stage (Ruggi et al, 2005); in this area both *H. ambrosii* populations introgressed with *H. italicus* genes and *H. italicus* populations introgressed with *H. ambrosii* genes occur. We performed surveys during the central hours of sunny and dry days in early summer, when the outdoor conditions are most unfavourable (hot and dry) and the salamanders underground activity is highest and tested if hybrid introgressed populations show intermediate environmental requirements (intermediate niche hypothesis) or exploit different habitat conditions (transgressive niche hypothesis) beside parental non-introgressed populations.

Materials and Methods

Study sites

To measure the microhabitat of pure and introgressed populations, we surveyed natural and artificial caves along the hybrids contact zone and within the range of the two salamander species (Fig. 1a). Surveys were performed in early summer (June-July 2012-2016), when conditions outside the caves are unfavorable and the detectability of salamanders active underground is highest (Lunghi et al, 2015). We performed all the surveys during the central hours of dry and sunny days. We subdivided each cave in 3-m longitudinal intervals (hereafter: sectors), covering the whole cave. The size of these sectors approximately corresponds to the size of salamander home ranges (Lanza et al, 2006; Ficetola et al, 2013). We stopped surveys when access was impossible without speleological equipment. In each sector we used visual encounter surveys (Crump & Scott, 1994) to detect active salamanders, and measured three abiotic variables known to influence salamander distribution: relative humidity (%), air temperature (°C) and maximum incident light (illuminance, measured in lux) (Ficetola et al, 2012; Lunghi et al, 2017). Air temperature and humidity were recorded using a LAFAYETTE TDP-92 thermo-hygrometer. Illuminance was recorded using a EM882 light-meter (PCE Instruments), by performing >10 measures in the portions of the sector receiving more light. See (Ficetola et al, 2012; Ficetola et al, 2013) for additional details on sampling methods. In very deep caves, we stopped measuring microhabitat 6 m after the deepest detected salamander.

Definition of the genetic status of study populations

The introgression between *H. ambrosii* and *H. italicus* was previously studied by Ruggi (2007), who analysed the genetic features of the study species, with a special focus on the contact zone. Ruggi analysed 646 individuals from 39 populations covering the whole range of both species; 24 of these populations (471 individuals) were located within the hybrid zone. Individuals were analysed using mitochondrial DNA and 24 allozyme loci. Seven of these allozyme loci were discriminant between *H. ambrosii* and *H. italicus*. These genetic data were used to identify pure populations, and to calculate the average frequency of introgressed genes in each population (see Fig. 14 and table 11 in Ruggi, 2007).

The sites analysed by the present study were assigned to four genetic groups: pure *H. ambrosii* (*H. ambrosii* sites with limited or no introgression of *H. italicus* genes); introgressed *H. ambrosii* (*H. ambrosii* sites in which the average frequency of *H. italicus* genes was > 10%); pure *H. italicus* (*H. italicus* sites with limited or no introgression of *H. ambrosii*); introgressed *H. italicus* (*H. italicus* in which the average frequency of *H. ambrosii* genes was > 10%). We assumed that each study site had the same genetic features of the nearest population with available genetic data (Ruggi, 2007). Within the hybrid zone, the distance between study populations and reference genetic data was always small (average ± SD: 746 ± 590m), suggesting that the introgression status can be reliably attributed.

Statistical analyses

First, we used generalized mixed models (GLMM; binomial error distribution) to confirm that the distribution of salamanders of each group is related to the recorded microhabitat features. In GLMM, detection/non detection was the dependent variable, microhabitat features (temperature, humidity and light) were the independent variables, and cave identity was included as random effect. Non-detection of salamanders may represent either real absences or failure of detecting them; not taking into account potential misdetections can influence regression results (MacKenzie et al, 2006). With our sampling protocol, detection probability is high but lower than one (approx. 0.75 per visit) (Ficetola et al, 2012;

Lunghi et al, 2015). Therefore, in our models absences received a weight of 0.75 (following Gómez-Rodríguez et al, 2012).

We built models with all the combinations of independent variables, and ranked them using the Akaike's Information Criterion (AIC) to identify the minimum adequate model for each species, i.e. the model explaining more variation with fewer variables (Burnham & Anderson, 2002). In caves, there was a strong negative correlation between air temperature and humidity ($r = -0.57$, $P < 0.0001$), and collinearity can bias the results of regression analyses. Models including both these correlated variables were excluded from the list of candidate models. For each candidate model, we also calculated the AIC weight (w) (Lukacs et al, 2007). Before analyses, light intensity was transformed using logarithms, while humidity (%) was square-root-arcsine transformed to improve normality and reduce skewness.

We then used the PCA-env approach (Broennimann et al, 2012; Petitpierre et al, 2012; Di Cola et al, 2017) to assess niche shifts between pure and introgressed salamander populations. First, we built univariate plots, to compare the frequency distribution of pure and introgressed populations under the range of available environmental conditions. Subsequently, we used Principal Component Analyses (PCA-env) to perform multivariate niche comparisons, using the three available microhabitat features (air temperature, relative humidity and incident light in the cave sector). Niche overlap was calculated using the Schoener's D statistics (Schoener, 1970; Warren et al, 2008), which ranges between 0 (lack of overlap) and 1 (complete overlap). Schoener´s D shows excellent performance compared to other overlap metrics (Warren et al, 2008; Rödder & Engler, 2011), and is among the most widespread metrics of niche overlap in ecological, evolutionary and biogeographical studies. The niche expansion of introgressed populations compared to the parental ones was the proportion of the occurrence density of introgressed populations that lay outside the conditions occupied in the native distribution (Di Cola et al, 2017).

We used equivalency tests (Warren et al, 2008; Broennimann et al, 2012) to assess the significance of niche differences between taxa (999 random permutations). Equivalency tests were performed using the ecospat niche equivalency test function with the R software ecospat (Di Cola et al, 2017).

Population were assigned to the different groups (introgressed vs. non-introgressed) on the basis of subjective thresholds. In the main analyses, we assigned populations with the frequency of introgressed genes >10% to the "introgressed" group, and populations with frequency of introgressed genes < 10% to the "pure" group. In a second analysis, we considered populations with frequency of introgressed genes > 20% as the introgressed ones (Endler, 1977), and repeated analyses to assess the robustness of our results to this subjective threshold. All analyses were performed using R 3.3.3 (R Development Core Team, 2017)

Results

Overall, we monitored 121 caves (694 cave sectors) and observed 1190 salamanders in 278 sectors (Fig. 1, Table 1). Sample size was larger for pure populations than for introgressed populations. For *H. ambrosii*, the frequency of introgressed genes was always <1% in pure populations, while in the sites of introgressed populations the frequency of *H. italicus* genes ranged between 20 and 43% (Table 1, Fig. S1). For *H. italicus*, the frequency of *H. ambrosii* genes was ≤8% in sites assigned to the "pure" group, while ranged between 13 and 36% for sites of the introgressed group (Table 1, Fig. S1).

Hydromantes ambrosii: introgressed vs. non-introgressed populations

For non-introgressed *H. ambrosii* populations, the best-AIC model suggested that salamanders were related to sectors with lack of light and cold temperature (Table 2a). The relationships between microhabitat and salamander presence were analogous for introgressed *H. ambrosii*. Also for introgressed populations the best-AIC model indicated a relationship with low temperature, but an alternative candidate model, with similar AIC value, also suggested association with the darkest sector.

Despite both groups being associated with similar microhabitat, the conditions of occupied sectors were not identical, as introgressed *H. ambrosii* were often observed in sectors with warmer temperature, lower humidity and more light, compared to the non-introgressed populations. For instance, 50% of cave sectors with introgressed *H. ambrosii* showed temperature >14.4°C, humidity below 91% and light above 0.13 lux (Fig. 3a-c). These conditions also existed in caves with pure *H. ambrosii* (Fig. 3), but were only rarely occupied. As a consequence, the median conditions of sectors with pure *H. ambrosii* were characterized by lower temperature, higher humidity and lower light (Table 2b).

Overall, PCA-env showed strong and significant niche shift (*P* = 0.001) of introgressed populations, with expansion toward harsher conditions (warmer, drier and more luminous sectors) compared to the non-introgressed populations (Fig. 3a).

Hydromantes italicus: introgressed vs. non-introgressed populations

In non-introgressed *H. italicus* populations, the best-AIC model suggested association with the darkest, most humid sectors, and the relationship between microhabitat and salamanders was very similar for introgressed populations (Table 2).

Despite both groups were associated to similar microhabitat, the conditions of occupied sectors were not identical, as introgressed *H. italicus* were also observed in sectors with slightly higher temperature, lower humidity and more light, compared to the non-introgressed populations.

For instance, introgressed populations were more often observed in cave sectors with humidity below 92% and light above 0.5 lux, compared to non-introgressed populations (Fig. 2e-f).

Overall, introgressed *H. italicus* showed a significant niche shift (P = 0.006), with expansion toward harsher conditions (with lower humidity and more light; Table 3, Fig. 3b). Results remained identical if only sites with proportion of introgressed genes ≥ 20% were defined to be the introgressed ones (niche similarity test: I = 0.636, P 0.022).

Equivalency tests did not detect significant differences between introgressed *H. ambrosii* and introgressed *H. italicus* (Table 3).

Discussion

Our surveys extensively covered the distribution range of *H. ambrosii* and *H. italicus* salamanders showing that both species during dry and hot periods do not randomly occur in caves but select underground sites with specific features. In particular, pure *H. ambrosii* populations are linked to dark and cold underground sectors and pure *H. italicus* populations choose dark and wet sectors. These results confirm the habitat choice of *H. italicus* as reported by previous studies (Lunghi et al, 2014; Lunghi et al, 2015) which report the correlation of the species also with sectors with high occurrence of troglophilous *Meta menardi* spiders that are indicator of higher trophic richness for caves (Manenti et al, 2015). The results provide a first assessment of microhabitats requirements for *H. ambrosii*., even if its occurrence in sites with lower humidity levels as already been recorded by (Cimmaruta et al, 1999). The fact that caves show heterogeneous environmental conditions affecting species presence / absence patterns is in agreement with results of studies performed on other species of the *Hydromantes* genus and on different North American plethodontid salamanders (Briggler & Prather, 2006; Camp & Jensen, 2007).

The most interesting result obtained by our analyses is that both *H. ambrosii* and *H. italicus* introgressed populations showed a significant niche shift, with a manifest expansion toward harsher environmental conditions than those experienced by the parental populations. The use of underground habitats by *Hydromantes* salamanders is linked to the necessity to find suitable conditions in terms of temperature and humidity to maintain viable physiological conditions (Lanza et al, 2006; Lunghi et al, 2015). Depending on these physiological constraints salamanders try to frequent the underground sectors closest to the surface, which usually offer richer trophic resources that the deeper ones. As these lungless salamanders are at equilibrium with the surrounding environment in terms of temperature (Lunghi et al, 2016), the recorded features of the exploited sectors, when underground, reflect the physiological limits that they are able to experience. Our findings underline that from an ecological point of view, the hybrid populations are able to survive at stricter conditions than the parental populations. Hybrid zones are often reported to occur along ecological gradients, as transitional environments may allow contact between species that exploit different ecological niches (Culumber et al., 2012). In such a situation, hybrids might be both less fit than parental in either niche or at the contrary better occupy intermediate niche. Our results support from an ecological point of view the recent evidences that the occurrence of hybrid zones may also provide opportunities for the existence of adaptive introgression phenomena at the genomic level (Grant & Grant, 2016; Bay & Ruegg, 2017). In our case the introgressed populations of both parental species, during dry periods, may exploit harsher underground spaces than pure populations and likely access underground spaces richer of food resources.

Our results are consistent with the transgressive niche hypothesis (Ficetola & Stock, 2016) hybridisation allowed the salamanders in the hybrid zone to have an ecological niche that reach beyond those of the parental species. The occurrence of hybrid zones is quite spread worldwide and involves plants, invertebrate and vertebrate species. In the scientific literature there is a huge amount of studies that are investigating the role of hybrid zones (Seehausen, 2004) trying to disentangle both extrinsic and intrinsic factors that s can affect hybrid zones occurrence and maintenance along time. While several studies have evaluated the genetic mechanisms favouring hybridisation, the past and current biogeographical, processes determining the existence of contact zones between similar species, the fitness of hybrids and its consequences (Barton & Hewitt, 1985; Arnold & Hodges, 1995), few attempts exist in the comparison of ecological niche between hybrids and parental species (Ficetola & Stock, 2016).

The comparison of niche similarity among pure and introgressed populations, together with phylogenetic data, allows to better understand evolutionary processes like adaptation and speciation. Moreover, the papers that deal with niches, usually use macroecological data and compare the realized niche (i.e. the environmental conditions under which individuals are effectively found) instead of the fundamental niche. However, determining fundamental niche is challenging, as it generally requires assessing individual performance and fitness under controlled conditions, and this approach cannot be realistic to all the species. In our case, the niche analysis of pure and introgressed *Hydromantes* salamanders provide the first approach based on environmental conditions reflecting the fundamental niche in a hybrid zone. The genetic surveys of the population outside and inside the hybrid zone carried out with allozyme electrophoresis evidence that *H italicus* and *H ambrosii* have secondarily met and hybridized in the narrow area constituted by Apuan Alps (North-West Tuscany). The population in the hybrid zone have alleles typical of both species with various frequencies. Ex situ experiments of artificial sintopy between *H. italicus* and *H ambrosii* have shown that the two species hybridize forming viable and fertile hybrids confirming the relationship occurring between the two species likely linked in the past before the glacial events (Ruggi et al, 2005). On the whole our study provide new insights on the relationship that may occur between hybridisation and niche evolution; in particular our study shows that the natural fusion of two different species is possible in taxa like may amphibians and urodeles in particular in which populations are generally small, they occupy confining habitats such as in the case of cave salamander single or limited karst mountains and the environment is naturally perturbed by the recurring of intense dry periods. Our research poses also the bases to identify genetic adaptive traits in populations of endangered amphibians species that are exposed to the ongoing climate change.

References

Arnold, M.L. & Hodges, S.A. (1995) Are natural hybrids fit or unfit relative to their parents? *Trends Ecol. Evol.*, 10, 67 - 71.

Barton, N.H. & Hewitt, G.M. (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, 16, 113–148.

Bay, R.A. & Ruegg, K. (2017) Genomic islands of divergence or opportunities for introgression? *Proc R Soc Lond [Biol]*, 284, 20162414.

Briggler, J.T. & Prather, J.W. (2006) Seasonal use and selection of caves by plethodontid salamanders in a Karst area of Arkansas. *Am. Midl. Nat.*, 155, 136-148.

Broennimann, O., Fitzpatrick, M.C., Pearman, P.B., Petitpierre, B., Pellissier, L., Yoccoz, N.G., Thuiller, W., Fortin, M.-J., Randin, C., Zimmermann, N.E., Graham, C.H. & Guisan, A. (2012) Measuring ecological niche overlap from occurrence and spatial environmental data. *Global Ecol. Biogeogr.*, 21, 841-897.

Burnham, K.P. & Anderson, D.R. (2002) *Model selection and multimodel inference: a practical information-theoretic approach* Springer Verlag, New York.

Camp, C.D. & Jensen, J.B. (2007) Use of twilight zones of caves by plethodontid salamanders. *Copeia*, 2007, 594-604.

Cimmaruta, R., Forti, G., Nascetti, G. & Bullini, L. (1999) Spatial distribution and competition in two parapatric sibling species of European plethodontid salamanders. *Ethology Ecology & Evolution*, 11, 383-398.

Crump, M.L. & Scott, J., N. J. (1994) Visual Encounter Surveys. In: Heyer, W.R., Donnelly, M.A., McDiarmid, R.W., Hayek, L.C., Foster, M.S., Eds. *Measuring and monitoring biological diversity: standard methods for Amphibians*, pp. 84-92. Smithsonian Institution Press, Washington.

Culumber, Z.W., Shepard, D.B., Coleman, S.W., Rosenthal, G.G., Tobler, M., 2012. Physiological adaptation along environmental gradients and replicated hybrid zone structure in swordtails (Teleostei: *Xiphophorus). Journal of Evolutionary Biology* 25, 1800-1814.

De Hert, K., Jacquemyn, H., Van Glabeke, S., Roldan-Ruiz, I., Vandepitte, K., Leus, L. & Honnay, O. (2012) Reproductive isolation and hybridization in sympatric populations of three Dactylorhiza species (Orchidaceae) with different ploidy levels. *Annals of Botany*, 109, 709-720.

Di Cola, V., Broennimann, O., Petitpierre, B., Breiner, F.T., D'Amen, M., Randin, C., Engler, R., Pottier, J., Pio, D., Dubuis, A., Pellissier, L., Mateo, R.G., Hordijk, W., Salamin, N. & Guisan, A. (2017) ecospat: An R package to support spatial analyses and modeling of species niches and distributions. *Ecography*, n/a-n/a.

Endler, J.A. (1977) *Geographic variation, speciation and clines* Princeton University Press., Princeton.

Ficetola, G.F., Pennati, R. & Manenti, R. (2012) Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. *Amphibia-Reptilia*, 33, 251-259.

Ficetola, G.F., Pennati, R. & Manenti, R. (2013) Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? *Population Ecology*, 55, 217-226.

Ficetola, G.F. & Stock, M. (2016) Do hybrid-origin polyploid amphibians occupy transgressive or intermediate ecological niches compared to their diploid ancestors? *Journal of Biogeography*, 43, 703–715.

Fitzpatrick, B.M. (2004) Rates of evolution of hybrid inviability in birds and mammals. *Evolution*, 58, 1865-1870.

Garrick, R.C., Benavides, E., Russello, M.A., Hyseni, C., Edwards, D.L., Gibbs, J.P., Tapia, W., Ciofi, C. & Caccone, A. (2014) Lineage fusion in Galapagos giant tortoises. *Mol. Ecol.*, 23, 5276-5290.

Gómez-Rodríguez, C., Bustamante, J., Díaz-Paniagua, C. & Guisan, A. (2012) Integrating detection probabilities in species distribution models of amphibians breeding in Mediterranean temporary ponds. *Divers. Distrib.*, 18, 260-272.

Grant, P.R. & Grant, R. (2016) Introgressive hybridization and natural selection in Darwin’s finches. *Biological Journal of the Linnean Society*, 117, 812-822.

Lanza, B., Pastorelli, C., Laghi, P. & Cimmaruta, R. (2006) A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). *Atti del Museo Civico di Storia Naturale di Trieste*, 52.

Lukacs, P.M., Thompson, W.L., Kendall, W.L., Gould, W.R., Doherty, P.F., Burnham, K.P. & Anderson, D.R. (2007) Concerns regarding a call for pluralism of information theory and hypothesis testing. *J. Appl. Ecol.*, 44, 456-460.

Lunghi, E., Manenti, R. & Ficetola, G.F. (2014) Do cave features affect underground habitat exploitation by non-troglobite species? *Acta Oecol*, 55, 29 - 35.

Lunghi, E., Manenti, R. & Ficetola, G.F. (2015) Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? *PeerJ*, 3, e1122.

Lunghi, E., Manenti, R., Canciani, G., Scari, G., Pennati, R. & Ficetola, G.F. (2016) Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. *Journal of Thermal Biology*, 60, 79-85.

Lunghi, E., Manenti, R. & Ficetola, G.F. (2017) Cave features, seasonality and subterranean distribution of non-obligate cave dwellers. *PeerJ*, 5, e3169.

MacKenzie, D.I., Nichols, J.D., Royle, J.A., Pollock, K.H., Bailey, L.A. & Hines, J.E. (2006) *Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence* Academic Press, Burlington, MA.

Manenti, R., Lunghi, E. & Ficetola, G.F. (2015) Distribution of spiders in cave twilight zone depends on microclimatic features and trophic supply. *Invertebrate Biology*, 134, 242 - 251.

Moore, W.S. (1977) An evaluation of narrow hybrid zones in vertebrates. *The Quarterly Review of Biology*, 52, 263-277.

Nakagawa, S. & Schielzeth, H. (2013) A general and simple method for obtaining R^2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, 4, 133-142.

Noriega, A.L., Sobéron, J. & Miller, C.P. (2013) Process-based and correlative modeling of desert mistletoe distribution: a multiscalar approach. *Ecosphere*, 4, 1–23

Otis, J., Thornton, D., Rutledge & Murray, D.L. (2017) Ecological niche differentiation across a wolf-coyote hybrid zone in eastern North America. *Div. Distrib.*, 23 529-539.

Pagano, A., Crochet, P.A., Joly, P. & Lodé, T. (2001) Distribution and habitat use of water frog hybrid complexes in France. *Glob. Ecol. Biogeogr.*, 10, 433–441.

Petitpierre, B., Kueffer, C., Broennimann, O., Randin, C., Daehler, C. & Guisan, A. (2012) Climatic niche shifts are rare among terrestrial plant invaders. *Science*, 335, 1344-1348.

R Development Core Team (2017) *R: A language and environment for statistical computing* R Foundation for Statistical Computing, Vienna.

Richards, S.A. (2005) Testing ecological theory using the information-theoretic approach: examples and cautionary results. *Ecology*, 86, 2805-2814.

Rödder, D. & Engler, J.O. (2011) Quantitative metrics of overlaps in Grinnellian niches: advances and possible drawbacks. *Global Ecol. Biogeogr.*, 20, 915-927.

Ruggi, A., Cimmaruta, R., forti, G. & Nascetti, G. (2005) Preliminary study iof a hybrid zone between *Speleomantes italicus* and *S. ambrosii* on the apunae Alps, using RFLP analysis. *Annali Museo Civico di Storia naturale Giacomo Doria di Genova*, 97, 135 - 144.

Ruggi, A. (2007) *Descrizione di una zona di contatto e ibridazione tra Speleomantes italicus e S. ambrosii bianchii (Amphibia: Plethodontidae) sulle Alpi Apuane, mediante marcatori nucleari e mitocondriali* Università degli Studi della Tuscia di Viterbo. http://dspace.unitus.it/handle/2067/240, Viterbo.

Schoener, T.W. (1970) Nonsynchronous spatial overlap of lizards in patchy habitats. *Ecology*, 51, 408-418.

Seehausen, O. (2004) Hybridization and adaptive radiation. *Trends Ecol. Evol.*, 19, 198 - 207.

Tanaka, Y. (2007) Introgressive hybridization as the breakdown of postzygotic isolation: A theoretical perspective. *Ecol. Res.*, 22, 929 - 939.

Wake, D.B. (2013) The enigmatic history of the European, Asian and American plethodontid salamanders. *Amphibia-Reptilia*, 34, 323-336.

Warren, D.L., Glor, R.E. & Turelli, M. (2008) Environmental niche equivalency versus conservatism: Quantitative approaches to niche evolution. *Evolution*, 62, 2868-2883.

Warren, D.L., Cardillo, M., Rosauer, D.F. & Bolnick, D.I. (2014) Mistaking geography for biology: inferring processes from species distributions. *Trends Ecol. Evol.*, 29, 572 - 580.

Table 1. number of analysed sites for the four groups, and average frequency of introgressed genes ± SD (estimated from Ruggi, 2007).

Group	*N* caves	*N* cave sectors	*N* sectors with presence	Frequency introgressed genes
Pure *H. ambrosii*	41	276	136	0 ± 0%
Introgressed *H. ambrosii*	10	37	16	37 ± 7%
Introgressed *H. italicus*	28	187	66	28 ± 9%
Pure *H. italicus*	42	194	60	2 ± 2%

Table 2. a) Relationships between the presence/absence of salamanders in cave sectors, and microhabitat features. For each group, we show the best AIC model and, if any, candidate models within four AIC units from the best model (Richards, 2005). For each model, we list the predictors and the regression coefficients. I: introgressed populations; *K*: number of parameters in the model; *w*: AIC weight; R^2_M: marginal R^2 (Nakagawa & Schielzeth, 2013).

a) Relationships between presence and habitat features

Group	Model	*K*	AIC	*w*	R^2_M
H. ambrosii	Light (-0.35); temperature (-0.32)	4	285.9	0.97	0.34
H. ambrosii (introgressed)	temperature (-0.47) Light (-0.46); temperature (-0.40)	3 4	39.8 40.0	0.49 0.43	0.41 0.44
H. italicus	Light (-0.56); humidity (14.0)	4	162.5	0.99	0.63
H. italicus (I)	Light (-0.54); humidity (5.7)	4	188.7	0.89	0.28

Table 3. Niche similarity (Shoener's *D*) and niche equivalency tests between pure and introgressed populations of salamanders. We also show the comparison between Introgressed *H. ambrosii* and introgressed *H. italicus*, and between pure *H. ambrosii* and pure *H. italicus.*

	D	*P*	Niche expansion
H. ambrosii vs. introgressed *H. ambrosii*	0.608	0.001	0.26
H. italicus vs. introgressed *H. italicus*	0.654	0.006	0.22
Introgressed *H. ambrosii* vs. introgressed *H. italicus*	0.609	0.078	
pure *H. ambrosii* vs. pure *H. italicus*	0.757	0.001	

Figure legends

Figure 1. Distribution of study localities. The blue and orange polygons are the range of *H. ambrosii* and *H. italicus*, respectively.

Figure 2. Density of occurrence of pure and introgressed salamander populations, in relation to environmental features (solid coloured areas). Grey areas represent the overlap between pure and introgressed populations; lines represent the environmental conditions available to each group of populations.

Figure 3. PCA-env performed on pure and introgressed salamander populations. Grey areas represent the overlap between pure and introgressed populations, while solid coloured areas are the environmental conditions only occupied by one group of populations. Lines represent the conditions available to each group of populations. The figure also show the correlation circles of principal component analyses performed on the data.

Figure 1

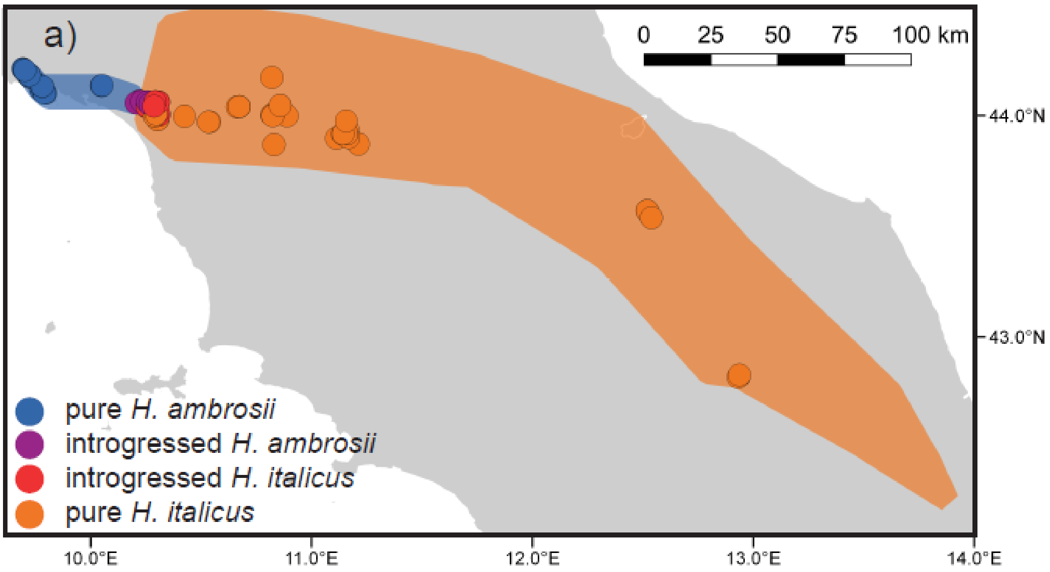


Figure 2

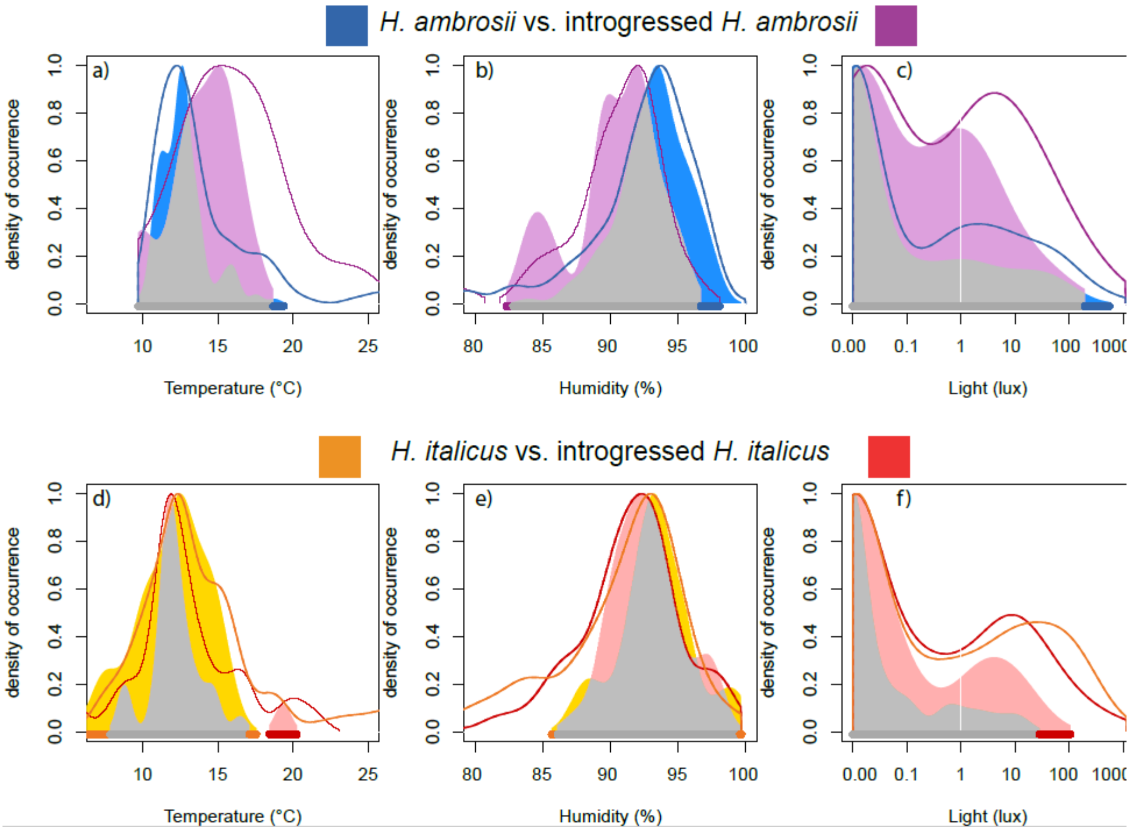
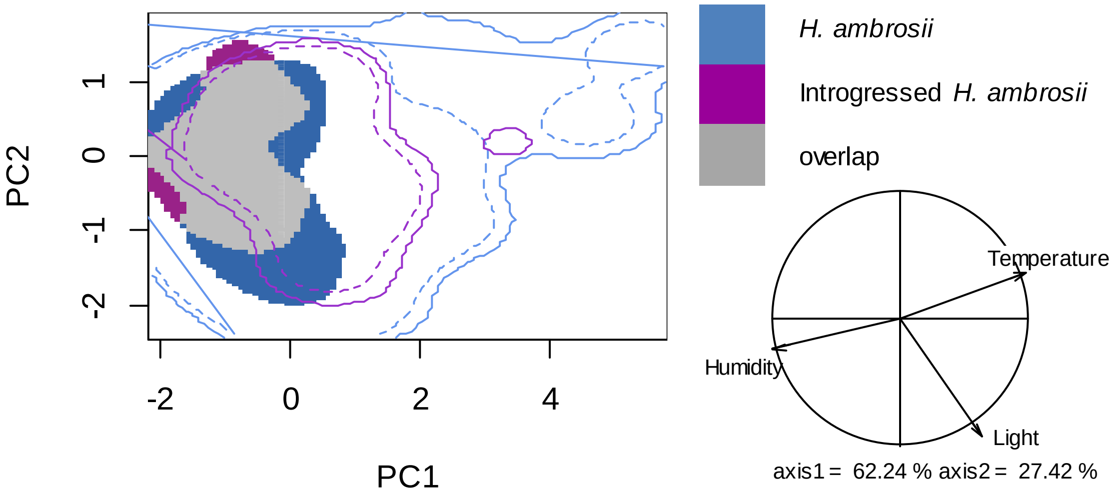
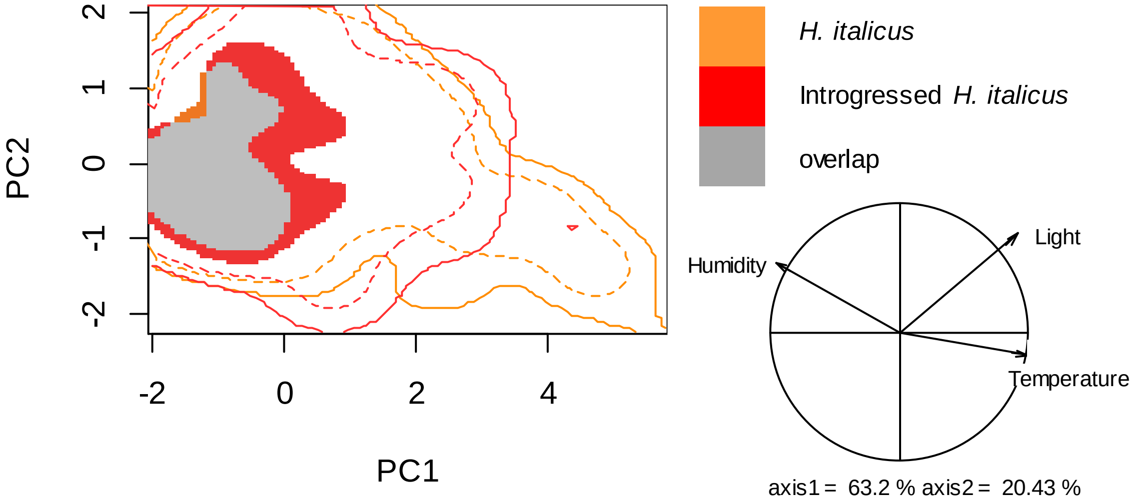


Figure 3

a) *H. ambrosii* vs. Introgressed *H. ambrosii*



b) *H. Italicus* vs. Introgressed *H. italicus*



Chapter V

Accepted in Amphibia-Reptilia, October 2017

The first ecological study on the oldest allochthonous population of European cave salamanders (*Hydromantes* sp.)

Enrico Lunghi[1,2,3,*], Olivier Guillaume[4], Pauline Blaimont[5], Raoul Manenti[6]

[1]Universität Trier Fachbereich VI Raum-und Umweltwissenschaften Biogeographie, Universitätsring 15, 54286 Trier, Germany

[2]Natural Oasis, Via di Galceti 141, 59100 Prato, Italy

[3]Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy

[4]Station d'Ecologie Expérimentale du CNRS 2, route du CNRS, 09200 Moulis France

[5]University of California, Santa Cruz 1156 High Street, Santa Cruz, CA 95060

[6]Dipartimento di Bioscienze, Università degli Studi di Milano, via Celoria 26, 20133 Milano Italy

[*]Corresponding author: enrico.arti@gmail.com

Type of manuscript: short note

Word count: abstract (135), manuscript (2,790)

Abstract

The introduction of allochthonous species often represents a serious problem for ecosystems and native species. Usually, these cases involve common widespread species that show high adaptability and strong competitiveness against local species. Within amphibians, the introduction of allochthonous species mainly deals with anurans, while cases involving caudata are few and poorly studied. We report the first assessment of an introduced population of European plethodontid salamanders. This population is located in the French Pyrenees and represents the oldest allochthonous *Hydromantes* population. We reconstructed the history of its introduction and collected data on the ecology and feeding habits of this population. Our results show that this population is stable and reproductive, showing strong similarities to Italian mainland species of *Hydromantes*. This study provided the base for further studies focused on this allochthonous population of European cave salamanders.

Keywords: Pyrenees, *Speleomantes,* alien, Plethodontidae, biospeleology, amphibian, hybrid, cave biology

The introduction of allochthonous species is often detrimental to the environment (Nori et al, 2011; Gürtler et al, 2017). This is particularly true for invasive species, which are leading causes of biodiversity loss worldwide (Doherty et al, 2016; Ward-Fear et al, 2016). Introductions may be deliberate (i.e., to create food stock, to introduce control agents for pests, to perform research), but they can also occur naively or accidentally (Sket, 1997; Franch et al, 2007; Dyer et al, 2017). Regarding amphibians, in a few cases species were introduced as food resources, while the pet trade is considered the main source of allochthonous species (Kraus, 2009). Among the most invasive amphibian species there are *Lithobates catesbeianus* and *Xenopus laevis*. The well-known literature relating to such introduced species report that they represent one of the major causes of biodiversity loss, directly affecting both native species and biotopes (Lillo et al, 2005; Ficetola et al, 2007; Ficetola et al, 2010). *L. catesbeianus* is often infected by the fungus *Batrachochytrium dendrobatidis,* so it may also represent one of the major vectors promoting the spread of chytridiomycosis (Garner et al, 2006). Furthermore, as in the case of the green frogs of the genus *Pelophylax*, introduced species can breed with native species, promoting the spread of hybrids and thus representing a serious threat to biodiversity (Holsbeek et al, 2008; Maletzky et al, 2008; Meilink et al, 2015).

Along with these numerous and well-known introductions of frog species, there are a few understudied cases related to the introduction of salamander species in Europe as in the case of information relating to the introduction of European plethodontid salamanders (genus *Hydromantes*; see Wake, 2013). European plethodontids are terrestrial salamanders which lack lungs and an aquatic stage (Lanza et al, 2006a). The distribution of these species is almost confined to Italy; only one has a small range in a portion of the French Provence (Lanza et al, 2006a). Three of eight species (*H. strinatii, H. ambrosii* and *H. italicus*) are distributed in mainland Italy, while the other five (*H. flavus, H. imperialis, H. supramontis, H. sarrabusensis* and *H. genei*) exclusively inhabit Sardinia island (Lanza et al, 2006b; Chiari et al, 2012). The biogeographic factors that determined such a distribution are unclear and still debated (Macey, 2005; Lanza et al, 2006a; Carranza et al, 2008; Wake, 2013). Despite their vernacular name, European cave salamanders are mainly epigeous species which exploit several types of environments, such as forests, bare rocks and even dry stone walls (Lanza et al, 2006a; Manenti, 2014; Costa, Crovetto and Salvidio, 2016). However, because of their specific physiology (Spotila, 1972), when external conditions become too harsh, they move underground looking for a suitable microclimate (Ficetola, Pennati and Manenti, 2012; Lunghi, Manenti and Ficetola, 2014a). In such environments, these salamanders tend to occupy areas in which adequate microclimatic conditions are realized (i.e. relatively cold temperature and high moisture) and where prey are likely to be more abundant (Salvidio et al, 1994; Ficetola, Pennati and Manenti, 2013; Lunghi, Manenti and Ficetola, 2014a; Lunghi, Manenti and Ficetola, 2017). Recent studies show that the microhabitat features at which *Hydromantes* salamanders are recorded during summer periods, approximate well the body conditions and the tolerance to the climatic limiting factors (Lunghi et al, 2016). Indeed, the high sensitivity of these salamanders forces them to select different parts of the subterranean environment according to seasonality (Lunghi, Manenti and Ficetola, 2015), as they quickly reach thermal equilibrium with their surrounding environment (Lunghi et al, 2016). *Hydromantes* carefully chose underground sites also because such environments represent safe places in which they can breed and escape most of their natural predators (Lanza et al, 2006a; Lunghi et al, 2014b; Salvidio et al, 2017).

Hydromantes species have been introduced six times beyond their natural range. An experiment of artificial ex situ syntopy between *H. italicus* and *H. ambrosii* was done in 1983 in an Italian cave located outside the range of both mentioned species (Forti et al, 2005; Lanza et al, 2006a). The species successfully persisted in the new location, giving birth to viable and fertile hybrids (Forti et al, 2005; Cimmaruta et al, 2013). There is no data on their spreading to surrounding sites available. A transplantation experiment of *H. ambrosii* individuals in a cave within the range of *H. strinatii* was performed during the summer of 1991 (Cimmaruta et al., 1999). The experiment established an allochthonous population of *H. ambrosii* in syntopy with *H. strinatii* (Cimmaruta et al., 1999). Another case deals with *H. italicus* in a population recently established in an artificial site in central Germany (Veith M., pers. comm.). Two cases deal with *H. strinatii*, a population was established in the Italian Karst (North-East Italy) and still persists (Lanza et al. 2006a) and another population was recently discovered in a natural cave of west-central France, 500 km far from the natural range of the species (Lucente et al, 2016). The last case represents the most ancient and interesting case of the *Hydromantes* salamander translocations. In 1970, at least 20 individuals belonging to a rearing established in the Subterranean Laboratory of the Scientific Station of Moulis (Fr) were released in a mine in the French Pyrenees (Pascal et al, 2003). Progenitors were caught from about fifteen localities in France and Italy (from Maritime Alps to Tuscan Apennines). At the time of collection, those *Hydromantes* were all considered to belong to the same species (*H. italicus*); however, individuals were probably sampled from all three of the mainland species (*H. strinatii, H. ambrosii and H. italicus*) (Guillaume and Durand, 2003).

Ecological studies on these allochthonous populations may produce new data reporting on how species adapt to a novel area. In this study, we report the first status assessment of the oldest introduced population of *Hydromantes* salamanders, with emphasis on their ecology and feeding habits.

The allochthonous population of *Hydromantes* inhabits a mine situated in Parc naturel régional des Pyrénées Ariégeoises, France. The mine has a total development of 39 m. The inner environment shows a general uniform morphology, having an average width (± SD) of 1.57 ± 0.04 m and average height of 2.04 ± 0.04 m. In the first 18 m of the mine, seepage of water created a pool which held several larvae of the fire salamander (*Salamandra salamandra*) and had the following physical characteristics: pH = 8, hardness = 15 °d, NO_2 = 0 and NO_3 = 10. Nearly no information is present in the literature about this population; the only available information being contained in the breeding books of the Subterranean Laboratory of the Scientific Station of Moulis. From 1966 to 1970 several individuals of the *Hydromantes* species were kept in captivity inside the laboratory to perform studies on their breeding behavior. In 1967 at least 90 adults were reared in the laboratory (Durand, 1967; Durand, 1970; Lanza et al, 2006a). It is not possible to quantify how many individuals died or were fixed in alcohol between 1966 and 1970 (books of subterranean laboratory report with security the fixing of 34 adults and 40 eggs). In 1970 the CNRS aborted the study program on *Hydromantes*, so that year likely represents when individuals were released inside the mine. The only available information reports the release of about 20 individuals (Guillaume and Durand, 2003); however, considering that in 1970 no further individuals were fixed in alcohol, we do not know if all the other remaining *Hydromantes* from the laboratory were also released in the mine.

The inner environment of the mine was divided into portions of 3 m-length (hereafter sector), such subdivision allows a reliable data collection of microclimatic features (Lunghi, Manenti and Ficetola, 2015) and roughly represents the known home range of mainland *Hydromantes* species (Salvidio et al, 1994). During the first half of August 2016 we visited the mine twice, in late morning, and in each sector we recorded data on temperature and humidity using a TDP92 thermo-hygrometer (accuracy: 0.1°C and 0.1%) adopting precautions to avoid influence on cave microclimate (Lopes Ferreira et al, 2015), while the average incident light of sectors was estimated using a PCE 170 light meter (minimum recordable light: 0.01 lux). In each sector we also recorded the abundance of *Meta menardi* spiders, because this species is considered to be a good proxy of prey availability (Manenti, Lunghi and Ficetola, 2015).

In each sector two surveyors dedicated 3.5 min each to assess the presence of *Hydromantes* by Visual Encounter Survey (Crump and Scott, 1994), an efficient method used to detect caudata species (Flint and Harris, 2005). For each individual, we recorded the position (distance from the mine entrance) and we took biometrics (SVL and weight). Individuals were divided into three groups: we considered juveniles all *Hydromantes* with SVL $\leq$ 40 mm, while within adults we separated males and females basing on the presence of secondary sexual characters of males (Lanza et al, 2006a).

During the first survey we randomly selected 26 individuals (3 juveniles, 11 males and 12 females; 65% of observed population, see Results) to perform stomach flushing to check for prey items stored in their stomachs (Salvidio, 1992). Stomach flushing was performed using a 5 ml syringe connected to a soft-plastic pipe with a 0.1 mm diameter. The plastic pipe was inserted into the mouth and 5 ml of water was gently injected into their stomach. Reflux was collected in a graduated pipette using a small funnel (Salvidio et al, 2012). Contents of the pipettes were examined with an optic microscope and all recognizable prey items were classified at least until order level. For each individual, we counted the number of recognizable items with the following equation:

$$\sum_{i=1}^{n} i = a + b + c$$

where: *a*) is the number of integer items; *b*) is the number of lone heads; *c*) is the difference between lone abdomens and lone heads (only when abdomens > heads) and *i* represents the number of taxonomic order.

European cave salamanders have a high detectability during their underground phase (Lunghi, Manenti and Ficetola, 2015); thus, giving that we observed most of the individuals present in the mine in that time, we used the program Past to identify age classes within adult *Hydromantes*.

We used Generalized Linear Mixed Models (GLMM) to identify if occurrence of cave salamanders was related to both environmental and biological features. As the dependent variable we used the salamanders presence observed in each sector. We used temperature, humidity and minimum light as environmental variables, while abundance of *Meta* spiders as a biological variable and sector identity as a random factor. All possible models were built and ranked following AICc while nested models and models with AICc higher than the simplest were not considered (Richards, Whittingham and Stephens, 2011).

Finally, we used Linear Mixed Models (LMM) to identify if age class or sex influenced the distribution of cave salamanders inside the mine. We used the respective abundance of sexes and age classes as dependent variables, while distance from mine entrance was used as an independent variable.

GLMM and LMM were performed in the R environment using packages lme4, nlme, MuMIn and MASS (Venables and Ripley, 2002; Bates et al, 2015; Bartoń, 2016; Pinheiro et al, 2016; R Core Team, 2016).

Hydromantes presence was positively related to sector temperature (Table 1), with salamanders tending to occupy areas in which temperatures were warmer ($B = 606.5$, $\chi^2 = 12.84$, $P < 0.001$). Occupied sectors showed a temperature range of 12.2 – 12.9 °C, while humidity fluctuated between 93.5 and 95.5 % and minimum light ranged from 0 to 8.47 lux. Within our surveys the maximum number of observed *Hydromantes* was 40 (9 juveniles, 14 males and 17 females). Based on SVL size, we identified two age classes for adult males and one for adult females and juveniles (Table 2). Juveniles significantly occupied sectors closest to the mine entrance ($F_{1,37}$ = 21.06, P< 0.001), while within adults, sex did not have any influence on their distribution ($F_{1,29}$ = 2.64, P = 0.161). Considering the 26 individuals which underwent stomach flushing, none had an empty stomach and only 6 of them (3 males and 3 females) regurgitated prey items which were not possible to identify due to their advanced state of digestion. Within the other sampled *Hydromantes* (77%) we identified 82 prey items belonging to 6 taxonomic orders: Diptera (92%), Araneae (3%), Coleoptera (2%), Isopoda (1%), Hemiptera (1%) and Trichoptera (1%).

The Pyrenean *Hydromantes* population showed a preference for relative warmer temperature during the study period (Table 1). The features of the occupied sectors matched those observed in Italian mainland species during the same period, where salamanders occupied sectors close to the cave entrance, but deep enough to show specific microclimate (Lunghi, Manenti and Ficetola, 2015). Even spatial distribution of individuals followed what was already observed in Italian species, with juveniles occupying sectors close to the main entrance, which are areas where prey is likely to be more abundant (Ficetola, Pennati and Manenti, 2013). The studied mine has a gate at the entrance which limits environmental influences from external surrounding areas, promoting suitable conditions for *Hydromantes* within the first few meters.

Based on our observations the current population is comprised of approximately 22% juveniles and 78% adults with a similar proportion of both sexes (Salvidio, 2008). Considering all adults, the difference in dimensions of the two sexes falls within the size gap which normally occurs in *Hydromantes* mainland species (Salvidio and Bruce, 2006). However, looking at the two different groups of males, we could clearly see that the size of the second group was definitively bigger (around 20%), so they probably represent an older group/generation. Release of *Hydromantes* in the French Pyrenees happened about 50 years ago (Guillaume and Durand, 2003) thus, considering the available information on *Hydromantes* life span, this population has likely reached at least the fourth/fifth generation (Lanza et al, 2006a). Evaluating all information obtained by this study, the Pyrenean *Hydromantes* population seems to be dynamic and reproductive.

Our study represents the premier data on the diet of an allochthonous population of *Hydromantes*. These salamanders show a generalist and opportunistic diet, hunting on any available prey nearby using their protrusible tongue (Deban and Richardson, 2011). Their diet includes several invertebrate orders, making them able to feed on a large number of different prey items (Salvidio, 1992; Vignoli, Caldera and Bologna, 2006; Salvidio et al, 2012). Observed stomach contents of this allochthonous population of *Hydromantes* allows us to hypothesize that its feeding behavior basically remained the same of that of the mainland Italian species (Salvidio, 1992; Vignoli, Caldera and Bologna, 2006). In fact, more than 90% of their summer diet was composed by dipterans (mostly *Limonia nubeculosa* adults), a taxon which often shows high abundances in subterranean environments during hot seasons (Salvidio et al, 1994; Manenti, Lunghi and Ficetola, 2015). The only Hemiptera found within prey items belong to the family Veliidae. Riffle bugs are generally found in bodies of water or on emergent vegetation; however, is it possible to find some individuals on plants away from water (Epler, 2006). Therefore, it is possible that *Hydromantes* preyed on this bug directly from the body of water present in the first few meters of the mine.

This study provides new insights on a poorly studied phenomenon, the introduction of a European salamander in areas outside of their native range. Currently, most of the existing reports on such phenomenon deal with the olm (*Proteus anguinus*), one of the most intriguing species which has been relocated on different occasions mainly for scientific purposes in some Italian, French and German localities; however, the studies of their impact on the native cave communities remain partial and confined in the grey literature (Dolce and Pichl, 1982).Our assessment of the allochthonous population of *Hydromantes* represents a first step to understanding if these salamanders may be detrimental for native communities or not. The data obtained from stomach content analyses revealed the capability to capture a wide range of native invertebrates that enter the mine during the summer and the predation pressure of this allochthonous salamander population may affect the dynamics of the autochthonous invertebrate community. The results clearly show that the studied population can persist at the site of release with abundances similar to those observed, for example, in *H. italicus* in its natural range (Lunghi, Manenti and Ficetola, 2014a; Lunghi, Manenti and Ficetola, 2015). Although such population is often considered to be composed only by *H. strinatii* individuals (Raffaëlli, 2007; Lucente et al, 2016), our study emphasizes the fact that founders of the Pyrenean *Hydromantes* population potentially belonged to all three mainland species, and therefore may be genetically unique. This study provided the first ecological data on this allochthonous *Hydromantes* population, representing the foundation for further studies which will deeply investigate the ecology, the genetic structure and the potential impact of these animals on local species.

References

Bartoń, K. (2016): MuMIn: Multi-Model Inference. R package version 1.15.6.

Bates, D., Maechler, M., Bolker, B., Walker, S. (2015): Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw.**67**: 1-48.

Carranza, S., Romano, A., Arnold, E.N., Sotgiu, G. (2008): Biogeography and evolution of European cave salamanders, *Hydromantes* (Urodela: Plethodontidae), inferred from mtDNA sequences. J. Biogeogr. **35**: 724-738.

Chiari, Y., van der Meijden, A., Mucedda, M., Lourenço, J.M., Hochkirch, A., Veith, M. (2012): Phylogeography of Sardinian cave salamanders (Genus *Hydromantes*) is mainly determined by geomorphology. PLoS ONE **7**: e32332.

Cimmaruta, R., Forti, G., Nascetti, G., Bullini, L. (1999): Spatial distribution and competition in two parapatric sibling species of European plethodontid salamanders. Ethol. Ecol. Evol. **11**: 383-398.

Cimmaruta, R., Forti, G., Lucente, D., Nascetti, G. (2013): Thirty years of artificial syntopy between *Hydromantes italicus* and *H. ambrosii ambrosii* (Amphibia, Plethodontidae). Amphibia-Reptilia **34**: 413-420.

Costa, A., Crovetto, F., Salvidio, S. (2016): European plethodontid salamanders on the forest floor: local abundance is related to fine-scale environmental factors. Herpetol. Conserv. Biol. **11**: 344-349.

Crump, M.L., Scott, N.J. (1994): Visual Encounter Surveys. In: Measuring and monitoring biological diversity: standard methods for Amphibians, p. 84-92. Heyer, W.R., Donnelly, M.A., McDiarmid, R.W., Hayek, L.C., Foster, M.S., Eds., Washington, Smithsonian Institution Press.

Deban, S.M., Richardson, J.C. (2011): Cold-Blooded snipers: thermal independence of ballistic tongue projection in the salamander *Hydromantes platycephalus*. J. Exp. Zool.**315**: 618-630.

Doherty, T.S., Glen, A.S., Nimmo, D.G., Ritchie, E.G., Dickman, C.R. (2016): Invasive predators and global biodiversity loss. Proc. Natl. Acad. Sci. U.S.A. **113**: 1-5.

Dolce, S., Pichl, E. (1982): Le attuali conoscenze sulla difusione di *Proteus anguinus* in territorio italiano. Atti Mus. Civ. Stor. Nat. Trieste **32**: 245-254.

Durand, J.P. (1967): Sur l’ontogenèse d’*Hydromantes italicus* Dunn (Urodèle, Plethodontidae). C. R. Acad. Sci.**65**: 1533-1535.

Durand, J.P. (1970): Fortpflanzung und Entwicklung von *Hydromantes*, dem Höhlenmolch. Aqua Terra**7**: 42-48.

Dyer, E.E., Cassey, P., Redding, D.W., Collen, B., Franks, V., Gaston, K.J., Jones, K.E., Kark, S., Orme, C.D.L., Blackburn, T.M. (2017): The global distribution and drivers of alien bird species richness. PLoS Biol.**15**: e2000942.

Epler, J.H. (2006): Identification manual for the aquatic and semi-aquatic Heteroptera of Florida Tallahassee, State of Florida. Department of Environmental Protection. Division of Water Resource Management.

Ficetola, G.F., Coïc, C., Detaint, M., Berroneau, M., Lorvelec, O., Miaud, C. (2007): Pattern of distribution of the American bullfrog *Rana catesbeiana* in Europe. Biol. Invasions**9**: 767-772.

Ficetola, G.F., Maiorano, L., Falcucci, A., Dendoncker, N., Boitani, L., Padoa-Schioppa, E., Miaud, C., Thuiller, W. (2010): Knowing the past to predict the future: land-use change and the distribution of invasive bullfrogs. Global Change Biol.**16**: 528-537.

Ficetola, G.F., Pennati, R., Manenti, R. (2012): Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. Amphibia-Reptilia **33**: 251-259.

Ficetola, G.F., Pennati, R., Manenti, R. (2013): Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? Popul. Ecol.**55**: 217-226.

Flint, W.D., Harris, R.N. (2005): The efficacy of Visual Encounter Surveys for population monitoring of *Plethodon punctatus* (Caudata: Plethodontidae). J. Herpetol. 39: 578-584.

Forti, G., Lanza, B., Cimmaruta, R., Nascetti, G. (2005): An experiment of artificial syntopy ex situ between *Speleomantes italicus* and *S. ambrosii ambrosii*. Ann. Mus. civ. stor. nat. Giacomo Doria **97**: 123-133.

Franch, M., Llorente, G.A., Montori, A., Richter-Boix, A., Carranza, S. (2007): Discovery of an introduced population of *Discoglossus pictus* beyond its known distributional range. Herp. Rev. **38**: 356-359.

Garner, T.W.J., Perkins, M.W., Govindarajulu, P., Seglie, D., Walker, S., Cunningham, A.A., Fisher, M.C. (2006): The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biol. Letters **2**: 455-459.

Guillaume, O., Durand, J.P. (2003): Les Spélerpès : genre *Speleomantes* Dubois, 1984. In: Évolution holocène de la faune de Vertébrés de France: invasions et disparitions. Rapport au Ministère de l'Écologie et du Développement Durable (Direction de la Nature et des Paysages), p. 143-144. Pascal, M., Lorvelec, O., Vigne, J.-D., Keith, P., Clergeau, P., Eds., Paris, France, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, Muséum National d'Histoire Naturelle.

Gürtler, R.E., Izquierdo, V.M., Gil, G., Cavicchia, M., Maranta, A. (2017): Coping with wild boar in a conservation area: impacts of a 10-year management control program in north-eastern Argentina. Biol. Invasions**19**: 11-24.

Holsbeek, G., Mergeay, J., Hotz, H., Plötner, J., Volckaert, F.A.M., De Meester, L. (2008): A cryptic invasion within an invasion and widespread introgression in the European water frog complex: consequences of uncontrolled commercial trade and weak international legislation. Mol. Ecol. **17**: 5023-5035.

Juberthie, C., Durand J. & Dupuy M. (1996) La reproduction des Protées (*Proteus anguinus*): bilan de 35 ans d'élevage dans les grottes-laboratoires de Moulis et d'Aulignac. Mém. Biospéol. **23**, 53-56.

Kraus, F. (2009): Alien Reptiles and Amphibians a scientific compendium and analysis Dordrecht, Springer.

Lanza, B., Pastorelli, C., Laghi, P., Cimmaruta, R. (2006a): A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). Atti Mus. Civ. Stor. Nat. Trieste**52**: 5-135.

Lanza, B., Andreone, F., Bologna, M.A., Corti, C., Razzetti, E. (2006b): Fauna d'Italia. Amphibia Bologna, Calderini.

Lillo, F., Marrone, A., Sicilia, A., Castelli, G., Zava, B. (2005): An invasive population of *Xenopus laevis* (DAUDIN, 1802) in Italy. Herpetozoa **18**: 63-64.

Lopes Ferreira, R., Mendes Martins, V., Arantes Paixão, E., Souza Silva, M. (2015): Spatial and temporal fluctuations of the abundance of Neotropical cave-dwelling moth *Hypena* sp. (Noctuidae, Lepidoptera) influenced by temperature and humidity. Subterr. Biol.**16**: 47-60.

Lucente, D., Renet, J., Gailledrat, M., Tillet, J., Nascetti, G., Cimmaruta, R. (2016): A new population of European cave salamanders (genus *Hydromantes*) from west-central France: relict or introduction? Herpetol. Bull. **138**: 21-23.

Lunghi, E., Manenti, R., Ficetola, G.F. (2014a): Do cave features affect underground habitat exploitation by non-troglobite species? Acta Oecol. **55**: 29-35.

Lunghi, E., Manenti, R., Manca, S., Mulargia, M., Pennati, R., Ficetola, G.F. (2014b):Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments. Salamandra **50**: 105-109.

Lunghi, E., Manenti, R., Ficetola, G.F. (2015): Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? PeerJ **3**: e1122.

Lunghi, E., Manenti, R., Canciani, G., Scarì, G., Pennati, R., Ficetola, G.F. (2016): Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. J. Therm. Biol. **60**: 79-85.

Lunghi, E., Manenti, R., Ficetola, G.F. (2017): Cave features, seasonality and subterranean distribution of non-obligate cave dwellers. PeerJ 5: e3169.

Macey, J.R. (2005): Plethodontid salamander mitochondrial genomics: a parsimony evaluation of character conflict and implications for historical biogeography. Cladistics **21**: 194-202.

Maletzky, A., Mikulíček, P., Franzen, M., Goldschmid, A., Gruber, H.-J., Horák, A., Kyek, M. (2008): Hybridization and introgression between two species of crested newts (*Triturus cristatus* and *T. carnifex*) along contact zones in Germany and Austria: morphological and molecular data. Herpetol. J. **18**: 1-15.

Manenti, R. (2014): Dry stone walls favour biodiversity: a case-study from the Appennines. Biodivers. Conserv. **23**: 1879-1893.

Manenti, R., Lunghi, E., Ficetola, G.F. (2015): Distribution of spiders in cave twilight zone depends on microclimatic features and trophic supply. Invertebr. Biol.**134**: 242-251.

Meilink, W.R.M., Arntzen, J.W., van Delft, J.J.C.W., Wielstra, B. (2015): Genetic pollution of a threatened native crested newt species through hybridization with an invasive congener in the Netherlands. Biol. Conserv. **184**: 145-153.

Nori, J., Akmentins, M.S., Ghirardi, R., Frutos, N., Leynaud, G.C. (2011): American bullfrog invasion in Argentina: where should we take urgent measures? Biodivers. Conserv. **20**: 1125-1132.

Pascal, M., Lorvelec, O., Vigne, J.-D., Keith, P., Clergeau, P. (2003): Évolution holocène de la faune de Vertébrés de France: invasions et disparitions. Rapport au Ministère de l'Écologie et du Développement Durable (Direction de la Nature et des Paysages) Paris, France, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, Muséum National d'Histoire Naturelle.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Team, R.C. (2016): nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-128.

R Core Team (2016): R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing.

Raffaëlli, J. (2007): Les Urodeles du monde Condé-sur-Noireau, Penclen Edition.

Richards, S.A., Whittingham, M.J., Stephens, P.A. (2011): Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. Behav. Ecol. Sociobiol.**65**: 77-89.

Salvidio, S. (1992): Diet and food utilization in the European plethodontid *Speleomantes ambrosii*. Vie Milieu **42**: 35-39.

Salvidio, S., Latters, A., Tavano, M., Melodia, F., Pastorino, M.V. (1994): Ecology of a *Speleomantes ambrosii* population inhabiting an artificial tunnel. Amphibia-Reptilia **15**: 35-45.

Salvidio, S., Bruce, R.C. (2006): Sexual dimorphism in two species of european plethodontid salamanders, genus *Speleomantes*. Herpetol. J. **16**: 9-14.

Salvidio, S. (2008): Temporal variation in adult sex ratio in a population of the terrestrial salamander *Speleomantes strinatii*. Herpetol. J. **18**: 66-68.

Salvidio, S., Romano, A., Oneto, F., Ottonello, D., Michelon, R. (2012): Different season, different strategies: feeding ecology of two syntopic forest-dwelling salamanders. Acta Oecol. **43**: 42-50.

Salvidio, S., Palumbi, G., Romano, A., Costa, A. (2017): Safe caves and dangerous forests? Predation risk may contribute to salamander colonization of subterranean habitats. Sci. Nat. **104**: 20.

Sket, B. (1997): Distribution of *Proteus* (Amphibia: Urodela: Proteidae) and its possible explanation. J. Biogeogr.**24**: 263-280.

Spotila, J.R. (1972): Role of temperature and water in the ecology of lungless salamanders. Ecol. Monogr. **42**: 95-125.

Venables, W.N., Ripley, B.D. (2002): Modern Applied Statistics with S. Fourth Edition New Yourk, Springer.

Vignoli, L., Caldera, F., Bologna, M.A. (2006): Trophic niche of cave populations of *Speleomantes italicus*. J. Nat. Hist.**40**: 1841-1850.

Wake, D.B. (2013): The enigmatic history of the European, Asian and American plethodontid salamanders. Amphibia-Reptilia **34**: 323-336.

Ward-Fear, G., Hayward, M.W., L'Hotellier, F., Herman, K., Kabat, A.P., Gibbons, J.P. (2016): The implications of biodiversity loss for the dynamics of wildlife in Australia. Anim. Conserv. **19**: 504-505.

Acknowledgments

We thank L. Winandy for the help on the field, S. Salvidio for his precious information relatively to stomach flushing and L. Kaven for have checked manuscript spelling.

Table 1: First best five AICc models relating *Hydromantes* distribution. Presence of species were considered as dependent variable, while Humidity, Illuminance (Lux), Temperature and *Meta* abundance were considered as independent variables.

Independent variables included into the model				df	AICc	Δ-AICc	weight
Humidity	Lux	*Meta*	Temp				
			712.3	3	9.4	0	0.802
-1285			579.4	4	14.7	5.24	0.058
	-298		724.1	4	14.7	5.24	0.058
		-421.20	716.8	4	14.7	5.24	0.058
				2	18.3	8.91	0.009

Table 2: Biometrics of *Hydromantes*. For each group of salamanders (adult Males, adult Females and Juveniles) we show average weight (± SD), maximum and minimum SVL and the average SVL (± SD) of identified age class.

	Weight ± SD (g)	SVL min (mm)	SVL max (mm)	Age class1 (proportion)	Average ± SD (mm)	Age class2 (proportion)	Average ± SD (mm)
Males	2.91 ± 0.44	50	65	0.57	52.62 ± 1.65	0.43	62.66 ± 2.06
Females	4.14 ± 0.95	45	75	1	66.59 ± 5.52	–	–
Juveniles	1.17 ± 0.32	30	45	1	36.2 ± 6.43	–	–

Chapter VI

Submitted to Scientific Reports, November 2017

Linking environmental suitability, abundance and population processes: which dynamics determine variation across salamander populations?

Enrico Lunghi[1,2,3,*], Raoul Manenti[4], Manuela Mulargia[5], Michael Veith[1], Claudia Corti[2], Gentile Francesco Ficetola[4,6]

[1]Trier University, Department of Biogeography, Trier, Germany

[2]Natural History Museum of University of Florence, Section Zoology "La Specola", Firenze, Italy

[3]Natural Oasis, Prato, Italy

[4]Department of Environmental Sciences and Policy, Università degli Studi di Milano, Milano, Italy

[5]Speleo Club Nuoro, Nuoro, Italy

[6] Univ. Grenoble Alpes, CNRS, Laboratoire d'Écologie Alpine (LECA), F-38000 Grenoble, France

*Corresponding author: enrico.arti@gmail.com

Abstract

Species can show strong variation of local abundance across their ranges. Recent analyses suggested that variation in abundance can be related to environmental suitability, as the highest abundances are often observed in populations living in the most suitable areas. However, there is limited information on the mechanisms through which variation in environmental suitability determines abundance. We analysed populations of the microendemic salamander *Hydromantes flavus*, and tested several hypotheses on potential relationships linking environmental suitability to population parameters. For multiple populations across the whole species range, we assessed suitability using species distribution models, and measured density, activity level, food intake and body condition index. In high-suitability sites, the density of salamanders was up to 30-times higher than in the least suitable ones. Variation in activity levels and population performance can explain such variation of abundance. In high-suitability sites, salamanders were active close to the surface, and showed a low frequency of empty stomachs. Furthermore, when taking into account seasonal variation, body condition was better in the most suitable sites. Our results show that the strong relationship between environmental suitability and population abundance can be mediated by the variation of parameters strongly linked to individual performance and fitness.

Introduction

Organisms show a strong diversity of distribution patterns: some species are limited to very narrow ranges such as mountaintops or islets, while others are widespread throughout entire continents. Multiple factors, both biotic and abiotic, interact to shape species ranges. Generally, a species occurs in areas where abiotic conditions are suitable (i.e., positive intrinsic growth rate), where biotic interactions allow the persistence of viable populations (i.e., positive total growth rate), and where dispersal and colonization are possible [1,2]. In the last decade, the combination of these concepts with species distribution models has boosted our understanding of processes affecting species distribution, allowing to assess the factors determining the limits of species ranges and to predict distributional changes in response to past and future environmental changes [3]. However, within the range of a species, there is often huge heterogeneity of key parameters (both biotic and abiotic), and such variability can have profound effects on populations. In the areas with highly-suitable environmental features, it is expected that individuals have a better performance. In the last years, there is a growing interest on relationships between the spatial variation of environmental suitability within species ranges, and key parameters of populations, such as fitness and demography [4-6], with many studies assessing correlations between environmental suitability (derived from correlative ecological niche models) and population abundance. A recent meta-analysis summarized the relationship between environmental suitability, derived from SDM, and the spatial variation of population abundance across multiple species of plants, invertebrates and vertebrates, and found consistently highest abundances in the most suitable sites [7].

Such positive relationship is probably the consequence of multiple processes acting at the population level [4]. For instance, in sites where the environment is highly favourable, individuals can focus on activities allowing resource acquisition (e.g., foraging) rather than on buffering negative environmental effects [4,8-11]. In this scenario, suitable conditions can promote survival and breeding success of individuals [5,12], leading to an increase of local abundance [4,13] and thus to the perceived correlation between abundance and environmental suitability [6,7,14] (Fig. 1). Alternative pathways that can explain the relationship between environmental suitability and local species abundance involve the variation of available resources. For instance, climatic or environmental variations can influence food availability, with cascade effects on the growth rate and fitness of individuals [15-17]. Such relationship can be particularly important for animals with highly specialized diet. Nevertheless, more work is needed to assess the multiple and complex relationships that can occur between environmental suitability and population parameters, given that until to date most of the studies just considered the most evident correlation (i.e., suitability vs. abundance), without trying to identify the population-level processes that can determine such relationship (Fig. 1).

In the present work we investigated the links between range-wide variation of environmental suitability and multiple population parameters: species abundance, activity pattern, feeding performance and body condition. We focused on the micro-endemic cave salamander, *Hydromantes flavus* (see Material and methods), which represents an excellent model species. *Hydromantes flavus* has a very narrow distribution range (Fig. 2), facilitating an adequate sampling. Collecting data across the entire range of a species is usually challenging [18], but is important to accurately describe responses to environmental gradients [19]. Furthermore, *H. flavus* is a generalist predator of small invertebrates [20,21], has few known predators, and there are no other terrestrial salamanders (i.e., main competitors) within its distribution range [22], thus biological interactions probably do not determine major biases to our study.

We predicted that spatial variation of environmental suitability can have multiple consequences on population features, and we explored multiple potential pathways (Fig. 1). *H1*) The activity hypothesis predicts that a species is more active when environmental conditions are suitable [9]. When they are active, salamanders exit their underground refuges (e.g., deep areas of caves) to reach the surface, were prey abundance is highest and most salamander activity occurs [23-25]. If the activity hypothesis is correct we expect that, in areas with higher environmental suitability, salamanders are more often found close to the surface (i.e., they are active outside their underground refuges; see Material and Methods). *H2*) The foraging hypothesis predicts that in highly suitable areas individuals can devote more time to forage and/or can find higher food availability, thus we expect better foraging performance. *H3*) The body condition hypothesis predicts that longer activity and better foraging allows improving body condition (i.e., more muscles, more energy stored) [26], which is a fitness-related trait [27]. *H4*) Positive relationships between environmental suitability and activity pattern, feeding and body condition are expected to improve fitness, with potential effects on abundance [7]. Therefore, we finally predict a positive relationship between the spatial variation of suitability and local abundance across the whole species range.

Results

Environmental suitability. Overall, records of *H. flavus* were available from 25 grid cells (Fig. 2). An ensemble species distribution model (SDM) showed an excellent performance, with an overall true skill statistic = 0.879 (sensitivity: 100%; specificity: 87.7%). The SDM suggested that environmental suitability increased in the highest elevation areas of the Monte Albo, where mean temperature was low and annual precipitation was high (Fig. 2).

Distribution of salamanders. We measured the distance from the surface (depth) of 173 salamanders from eight populations (Table 1). The average depth was highly variable among populations (Table 1), ranging from 8.5 to ~150 m. The average depth was significantly higher in sites with low environmental suitability (ES) (r = -0.87, P = 0.011; Fig. 3a).

Stomach contents. We collected stomach contents from 212 salamanders in three populations (Table 1). One population live in an area with limited suitability (ES = 0.431), while two were found in areas with high suitability (ES >0.53). Thirty-seven stomach contents showed only unidentifiable material and were therefore discarded from the analyses (13 for Site 1, 21 for Site 5 and 3 for Site 7). Twenty-three salamanders had empty stomach, while in 152 cases we recognized at least one prey item. The frequency of salamanders with empty stomach differed strongly among populations ($x^2 = 14.52$, df = 2, $P < 0.001$); empty stomachs were more frequent in salamanders living in less suitable sites (Fig. 3b). The frequency of empty stomachs was unrelated to the survey period ($x^2 = 1.39$, df= 1, $P = 0.239$). Bayesian CIs confirmed that the salamanders of the two sites with the highest ES had a similarly low frequency of empty stomachs, as their 95% CIs showed wide overlap, while the pattern was clearly different in the least suitable site (Fig. 3b).

Body condition index (BCI). Within the eleven sites, we measured and weighted 313 salamanders (141 females, 104 males and 68 juveniles). The BCI did not show correlation with salamanders length ($r = 0.071$, df = 311, $P = 0.213$). Average BCI was significantly higher in populations living in areas with high environmental suitability ($F_{1,\ 7.78}= 10.08$, $P = 0.013$) (Fig. 4a), and showed significant variability among survey month ($F_{4,\,285.17} = 4.73$, $P = 0.001$), being higher in late spring-early summer (Fig. 4b). Furthermore, we detected significant variation among age/sex groups ($F_{2,\,302.32} = 4.62$, $P = 0.01$). Orthogonal contrast showed that differences between adults and juveniles were not significant ($F_{1,\,304.23} = 3.3$, $P = 0.07$) while, within adults, males showed significantly lower BCI than females ($F_{1,\,300.26} = 6.56$, $P = 0.011$) (Fig. 4c).

Abundance and density of populations. Population abundance was estimated using a *N*-mixture model with Poisson error, as it showed lower AIC than zero-inflated models (Poisson model: AIC =

555.74, zero-inflated Poisson: AIC = 557.74). *N*-mixture models indicated a high detection probability of individuals (mean ± SE: 0.504 ± 0.029). The estimated number of salamanders was highly variable among sites, ranging from five to 103 individuals per site (Table 1); the average of density estimates across all sites was 0.06 ± 0.03 individuals/m^2. Salamander density strongly differs among sites, being significantly higher in areas showing the highest environmental suitability (r = 0.893, N = 7, P = 0.007; Table 1 and Fig. 3c).

Discussion

Analysing multiple population parameters is essential to unravel the complex processes linking environmental suitability and individual fitness, and to understand the factors determining spatial variation of species abundance (Fig. 1). In this study, intensive sampling on multiple populations provided data on several key features and allowed to test multiple hypotheses that can affect the ES-abundance relationship of a species. Even though we did not consider all the potential processes and population features (e.g., no data on breeding success or survival are available, Fig. 1), our study provides a first insight on how the interplay of multiple processes can determine the variability of abundance that can be observed across a species' range.

Modelling the ES of *Hydromantes flavus* allowed us to identify strong suitability heterogeneity within the Monte Albo, with suitability variation at least in part related to altitude. At the mountain base, the microclimate is drier and warmer compared to the top, and the humid conditions at the top of the mountain probably increases suitability for *Hydromantes* salamanders. *Hydromantes* are lungless salamanders that require high environmental humidity for breathing they have a narrow thermal niche, and their activity at the surface is only possible during wet, fresh periods [22]. In higher ES areas, local climate shows a prolonged suitability for *Hydromantes*, a condition that likely reduces their inactivity period [23]. Indeed, when external climate is cool and moist, salamanders exit from their refuges to prey in environments with high prey abundance [28]. As a consequence, SDM identified a suitability gradient from the lowest to the highest altitudes of the Monte Albo.

The activity hypothesis predicts that a species is more active when environmental conditions are suitable [9]. *Hydromantes* are epigean terrestrial salamanders that can spend long periods in underground environments to escape unsuitable environmental conditions, particularly during dry and hot seasons [22]. However, underground environments are not homogeneous, and are strongly influenced by external environmental conditions [29]. The activity of salamanders, and their relative exploitation of microhabitats, is strongly affected by a trade-off between their physiological constraints and the need of food. On the one hand, microclimatic conditions of shallow cave sectors are similar to the ones found outdoor, and these sectors can be highly unsuitable when external conditions are harsh (dry and hot) [23]. On the other hand, food availability is generally higher close to the surface, therefore the exploitation of these sectors is important, particularly when the need of resources for growth or reproduction is particularly high [25]. Salamander populations inhabiting the areas with most suitable climate (i.e., wet and cool climate) were more frequently found close to the surface than the ones inhabiting the least suitable sites, probably because the outdoor climatic conditions have a direct impact on the microclimate of sites where salamanders live. Therefore, high climatic suitability favours a prolonged activity and the exploitation of cave sectors where more resources are available [25].

The foraging hypothesis predicts better foraging performance in highly suitable sites, and the high frequency of empty stomachs that we recorded in the less suitable sites confirms that foraging events occurring in these populations may do not have the same frequency compared to high ES sites. Sampling was performed during spring and autumn, which likely are the periods of highest activity [22]. During these seasons, salamanders probably intensify their foraging activity before aestivation and the winter diapause. Several processes can determine differences in foraging success between populations that inhabit different sites. In high ES areas salamanders can devote more time to foraging, while in low ES areas prolonged unsuitable conditions force individuals to remain in shelters [23,25]. Furthermore, it is also possible that resource availability shows spatial heterogeneity, and more resources can be available in the best sites, thus favouring preying activity [30]. Distinguishing between these non-exclusive processes is not easy; moreover, we did not measure variation of prey abundance. However, *Hydromantes* salamanders are generalist predators consuming a wide range of prey, and are able to feed on most of the underground and outdoor invertebrates [20,21]; thus we expect that preys are generally present throughout the whole species range. Nevertheless, detailed analyses are needed to assess whether the spatial variation of prey availability determines the differences in feeding performance across populations.

The body condition hypothesis predicts that longer activity period and/or better foraging allow improving the conditions of individuals. Body condition of salamanders showed strong variation among populations, being significantly higher in high ES areas (Fig. 4a). The higher activity and better foraging are likely involved in the difference in body conditions among salamanders from different areas. However, ES was not the only factor determining BCI variation. In our analyses, body condition was measured using the Residual Index. This index has an excellent performance in limiting the effect of body size on BCI [31], thus it is not surprising that BCI differences between adults and juveniles were small. Within adults, body condition of females was significantly higher than in males (Fig. 4c), and this probably happens because females accumulate reserves for the breeding activities, which can last several months [32]. Body condition also showed a strong seasonal variation, with better body condition in June (Fig. 4b). Starting from June, outdoor conditions become extremely unsuitable for cave salamanders who move in deep underground shelters where food availability is low [23]. Therefore, *Hydromantes* likely intensively forages during the previous months in order to store more energy for aestivation [22], and this explains the good body condition observed in June.

Positive relationships between suitability and activity pattern, feeding and/or body condition, are expected to improve fitness, with potential effects on abundance. Given that the previous hypotheses were confirmed, we expected a positive relationship between the spatial variation and local abundance across the whole species range. The density of salamanders showed a 30-fold variation among sites and, following our prediction, populations living in high ES areas showed the highest densities (Fig. 3c). We hypothesize that such high density is related to the co-action of multiple processes, such as prolonged activity and better feeding, which in turn improve body condition (Fig. 1). Individual body condition is a key factor which increases individual fitness and, indirectly, can affect species local abundance through different pathways [27]. First, a better body condition makes individuals able to better withstand stressing events (e.g., starvation, adverse environmental conditions), thus improving survival [33,34]. Furthermore, individuals showing better body conditions may devote more resources to breeding and parental care, improving the number and survival of offspring [35]. These two paths are not mutually exclusive and can work synergistically. Both paths can promote population growth, especially if biological interactions do not represent a major limitation [1]. In the study species, measuring survival and breeding success is challenging, as these amphibians show an elusive behaviour, and monitoring breeding activity in nature is rarely possible. For instance, in 40 years of studies on *H. flavus*, only one egg clutch was observed during a speleological exploration [22,36]. Nevertheless, given that the detection probability of these salamanders in caves is very high, in future studies capture-mark-recapture might provide better information on the differences of survival and individual growth rate across populations [37].

Identifying the processes determining correlations between ES and local species abundance is not easy, as many factors may affect the final outcome [7]. Indeed, given that the relationship between ES and species abundance is not always positive [4], in some circumstances analysing the multiple processes that interact in determining population performance may allow to unravel the complex dynamics acting at a local scale; nonetheless, they may also help in linking local-scale population processes to processes acting at broader scales, such as range-wide variation of suitability. Measuring multiple population parameters represents a key tool to understand the actual effects of environmental variation on populations. Such approach will allow to move beyond the mere measure of population abundance, to improve our understanding of the variation of fitness and population dynamics across species ranges, and also provide key data to inform explicit and mechanistic modelling of populations [3].

Material and methods

Ethic statement. All studies were authorized by the Italian Ministry of Environment (9384/PNM of 12/05/2015).

Study species and area. *Hydromantes flavus* is one of eight European plethodontid salamanders [38]. It has a small distribution range (< 90 km^2) and is endemic to the Monte Albo in north-eastern Sardinia [22], Italy (Fig. 2). Plethodontid salamanders are lungless and breathe mostly through the skin [22], thus they have a narrow ecophysiological niche, requiring high moisture and relatively cold temperature [22,39]. Such microclimatic requirements are generally found in underground environments (e.g., caves), where the species can be observed throughout the year [23], especially when outdoor conditions became unsuitable [40,41]. However, *H. flavus* is not an obligate cave dweller: during suitable seasons it is active outdoor, preying on invertebrates [28]. When underground, these salamanders usually occupy sectors not far from the surface, to be closer to food resources [25]. Underground shallow areas are strongly influenced by external climatic condition, thus external climate influence salamanders even when they are underground [23].

Suitability modelling. Correlative species distribution models were used to assess relationships between salamander distribution and major bioclimatic variables, and to obtain measures of broad-scale environmental suitability (ES). We considered four bioclimatic variables: annual mean temperature, temperature seasonality, annual total precipitation and precipitation seasonality; these variables were extracted from the Worldclim dataset at the 30 arc-seconds resolution (approx. 920×700m within the study area) [42]. These variables represent average conditions and their variability across the year, and are major determinants of vertebrate distribution [43]. Furthermore, these variables are enough to explain most of the climatic variation [44], and other important variables (e.g., winter and summer temperatures) are strongly related to linear combinations of the four variables considered. We did not include variables representing the biotic habitat [7], because these salamanders are mostly related to fine-scale microhabitat variables that are not captured by remote sensing or broad-scale habitat maps [25]. To calibrate models, we used all published presence records of the species (reviewed in [22,45]), updated with records from our own surveys (Fig. 2). SDM were built using five modelling approaches: Generalized Additive Models, Boosting Regression Trees, Classification Trees, Multiple Adaptive Regression Splines and Random Forests. Models were calibrated using a 67% random sample of the presence data and evaluated against the remaining 33% data using the True Skill Statistic [46]. This analysis was repeated five times, thus providing a fivefold cross-validation; models were run using biomod2 [47] in R. Given that alternative SDM can provide variable outputs, ensemble forecasting of the different SDM [48] was then used to obtain an overall suitability map following ref. [49].

Surveys. From June 2013 to May 2017, we performed multiple detailed surveys in a total of 14 underground sites (caves) where *Hydromantes flavus* was known to be present, covering the whole range of the species [22] (Table 1, Fig. 2). Surveys were performed from April to October, the period in which *Hydromantes* salamanders are more active and show the highest abundance and detectability in underground environments [23,50]. During the first survey, we divided each cave in 3-m longitudinal sectors, and measured the maximum height of the ceiling and the maximum width of each sector [41]. These measures were then used to estimate the explored cave surface. Subsequently, we measured multiple features of the populations living in these caves: distribution of salamanders in these caves, stomach content, body condition and abundance. Some of these population features required sampling during specific months and/or capturing a minimum number of individuals (see below for details). Due to sampling constraints, it was impossible to measure all the population features for all the caves, nevertheless for all the features we gathered data covering the whole range of the species (Table 1).

Distribution of salamanders within caves. To measure salamander's distribution, we performed surveys in May-June, as in this period the exploitation of cave environments is the highest [23]. Since several caves received multiple surveys (11 caves surveyed; average: 4.82 surveys per cave; Table 1), for each cave we considered the survey with the highest number of observations. Only caves with ≥ 5 individuals detected in at least one survey were considered. We measured the depth (distance from the cave entrance) of all salamanders detected inside the caves, using a 30-m fibre glass tape meter and a laser-meter (accuracy 2 mm). We used the correlation between the average depth of salamanders (log-transformed) and environmental suitability to test the activity hypothesis.

Stomach content. In three populations (Table 1), we performed stomach flushing, which is an unharming technique widely used to check stomach contents of amphibians [51]. For each population, we performed two capture sessions in spring and two in autumn (period: autumn 2015-spring 2017), obtaining in total four samples per site. Stomach flushing was performed using a 5 ml syringe connected to a 1 mm ⌀ plastic pipe; the far end of the pipe was introduced in the oral cavity of the salamander and 5 ml of water was gently injected in the stomach. Reflux was canalized by a funnel into a plastic jar. The obtained stomach contents were preserved in 75% ethanol and then identified using an optical microscope. Stomach contents were separated in two groups: empty (no items detected) and full (at least one food item was observed).

To assess if the frequency of empty stomachs differs among caves (i.e., to test the foraging hypothesis), we run the binomial Generalized Linear Models (GLMM, R packages lme4 and lmerTest; [52,53]) using the stomach condition (empty/full) as dependent variable, while season (autumn/spring) and cave identity were used as independent variables. We also used the Bayesian equal-tailed Jeffreys intervals (package MKmisc; [54]) to estimate 95% CI of the frequency of empty stomachs across populations, as Jeffreys intervals are a robust approach for the estimation of binomial CI [55].

Body condition index. Captured salamanders were weighted (using an electronic scale; precision: 0.01 g) and measured (total length; using a plastic ruler). To consider the maximum number of sites in this analysis, we included also the cave in which only one salamander was measured (Site 6, Table 1); however, excluding this cave the results did not change. For each individual we calculated the Residual Index, which is the differences between the observed and the predicted body mass of animals and is considered among the most reliable body condition indexes [31,56]. To calculate the residual index, we performed a regression analysis using log-transformed weight and total length of salamanders, as this improves the linearity of the relationship, and extracted the residuals for each individual [31,56]. We considered the total length because these animals often store fat also in the tail [57]. We identified age classes and sex on the basis of secondary sexual characters and body size. Salamanders with male sexual characters (mental glands and premaxillary teeth) were considered adult males; individuals without male characters but ≥80 mm were considered adult females (80 mm is the size of the smallest observed adult males); individuals <80 mm were considered juveniles [22].

Populations were surveyed in different periods of the year (from October 2015 to April 2016), and salamander body features were measured in 20 surveys performed in 11 populations (average: 1.8 surveys per site). To avoid pseudoreplication, for each population we considered only one survey per month, selecting the one with the highest number of measured salamanders. Body weight can show seasonal variation. To test the body condition hypothesis, we run a LMM (package lme4; [52]) considering BCI of salamanders as dependent variable, ES, month of survey and group (m/f/j) as independent variables, and population identity as random factor. Sample size was not homogeneous among populations, thus residual degrees of freedom were approximated following Satterthwaite [58]. Given that we detected significant BCI differences between the tree groups, we used orthogonal contrast to test whether there are differences between juvenile and adults and, within adults, and between males and females.

Population abundance and density. In seven caves, we performed repeated visual encounter surveys in a short period, to estimate salamanders abundance. During each survey, the same person dedicated 7.5 min. of observation to each 3-m long longitudinal sectors counting the active salamanders [50]. Each cave was surveyed five times in 2016, from May 4th to June 27th. In this period, cave occupancy and detection probability of salamanders is the highest, immigration/emigration to/from the cave is minimum, and no hatches are known to occur [23,36], thus allowing to meet the closed population assumptions of *N*-mixture models [59].

We used *N*-mixture models to estimate population size on the basis of repeated counts [60]. This approach provides accurate estimates of actual population size, particularly in species with high detection probability such as cave salamanders [61]. We used Akaike's Information Criterion (AIC) to select the most appropriate error distribution (Poisson or zero-inflated Poisson); negative binomial models were not considered as they can produce infinite abundance estimates [62]. We used Empirical Bayes methods to estimate the posterior distribution of the abundance (mean and 95% Bayesian confidence intervals, CI) [63]. We calculated population density on the basis of abundance estimates and the surveyed surface of caves, and then assessed the correlation between population density (square-root transformed) and ES. *N*-mixture models were run using the unmarked package in R [64].

References

1 Soberón, J. Grinnellian and Eltonian niches and geographic distributions of species. *Ecology Letters* **10**, 1115-1123 (2007).

2 Soberón, J. & Nakamura, M. Niches and distributional areas: concepts, methods, and assumptions. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 19644-19650, doi:10.1073/pnas.0901637106 (2009).

3 Kearney, M. & Porter, W. Mechanistic niche modelling: combining physiological and spatial data to predict species ranges. *Ecology Letters* **12**, 334-350, doi:10.1111/j.1461-0248.2008.01277.x (2009).

4 Thuiller, W. *et al.* Does probability of occurrence relate to population dynamics? *Ecography* **37**, 1155-1166, doi:10.1111/ecog.00836 (2014).

5 Brambilla, M. & Ficetola, G. F. Species distribution models as a tool to estimate reproductive parameters: a case study with a passerine bird species. *Journal of Animal Ecology* **81**, 781-787, doi:10.1111/j.1365-2656.2012.01970.x (2012).

6 VanDerWal, J., Shoo, L. P., Johnson, C. N. & Williams, S. E. Abundance and the environmental niche: environmental suitability estimated from niche models predicts the upper limit of local abundance. *The American Naturalist* **174**, doi:10.1086/600087 (2009).

7 Weber, M. M., Stevens, R. D., Diniz-Filho, J. A. F. & Grelle, C. E. V. Is there a correlation between abundance and environmental suitability derived from ecological niche modelling? A meta-analysis. *Ecography* **40**, 817-828, doi:10.1111/ecog.02125 (2017).

8 Corbet, S. A. *et al.* Temperature and the pollinating activity of social bees. *Ecol. Entomol.* **18**, 17-30 (1993).

9 Hetema, R. S. *et al.* Activity re-assignment and microclimate selection of free-living Arabian oryx: responses that could minimise the effects of climate change on homeostasis? *Zoology* **115**, 411-416, doi:10.1016/j.zool.2012.04.005 (2012).

10 Bachofen, C., Moser, B., Hoch, G., Ghazoul, J. & Wohlgemuth, T. No carbon "bet hedging" in pine seedlings under prolonged summer drought and elevated CO2. *Journal of Ecology*, 1-16, doi:10.1111/1365-2745.12822 (2017).

11 Parisi, M. G., Mauro, M., Sarà, G. & Cammarata, M. Temperature increases, hypoxia, and changes in food availability affect immunological biomarkers in the marine mussel *Mytilus galloprovincialis*. *Journal of Comparative Physiology B*, doi:10.1007/s00360-017-1089-2 (2017).

12 Fitzgerald-Dehoog, L., Browning, J. & Allen, B. J. Food and heat stress in the California mussel: evidence for an energetic trade-off between survival and Growth. *Biol. Bull.* **223**, 205-216 (2012).

13 Weber, M. M. & Viveiros Grelle, C. E. Does environmental suitability explain the relative abundance of the Tailed tailless bat, *Anoura caudifer*? *Natureza & Conservação* **10**, 221-227, doi:http://dx.doi.org/10.4322/natcon.2012.035 (2016).

14 Martínez-Meyer, E., Díaz-Porras, D., Peterson, A. T. & Yáñez-Arenas, C. Ecological niche structure and rangewide abundance patterns of species. *Biol. Letters* **9**, 20120637, doi:http://dx.doi.org/10.1098/rsbl.2012.0637 (2013).

15 Ortega, J., López, P. & Martin, J. Environmental drivers of growth rates in Guadarrama wall lizards: a reciprocal transplant experiment. *Biological Journal of the Linnean Society* **XX**, 1-11, doi:https://doi.org/10.1093/biolinnean/blx068 (2017).

16 Hsu, B.-Y., Dijkstra, C. & Groothuis, T. G. G. Organizing effects of adverse early-life condition on body mass, compensatory growth and reproduction: experimental studies in rock pigeons. *Journal of Avian Biology* **48**, 1-11, doi:10.1111/jav.01172 (2017).

17 Wong, J. W. Y. & Kölliker, M. Effects of food restriction across stages of juvenile and early adult development on body weight, survival and adult life history. *Journal of Evolutionary Biology* **27**, 2420-2030, doi:10.1111/jeb.12484 (2014).

18 Jiménez-Valverde, A., Lobo, J. M. & Hortal, J. Not as good as they seem: the importance of concepts in species distribution modelling. *Diversity and Distributions* **14**, 885-890, doi:10.1111/j.1472-4642.2008.00496.x (2008).

19 Thuiller, W., Brotons, L., Araújo, M. B. & Lavorel, S. Effects of restricting environmental range of data to project current and future species distributions. *Ecography* **27**, 165-172, doi:10.1111/j.0906-7590.2004.03673.x (2004).

20 Vignoli, L., Caldera, F. & Bologna, M. A. Trophic niche of cave populations of *Speleomantes italicus*. *J. Nat. Hist.* **40**, 1841-1850 (2006).

21 Salvidio, S., Oneto, F., Ottonello, D., Costa, A. & Romano, A. Trophic specialization at the individual level in a terrestrial generalist salamander. *Canadian Journal of Zoology* **93**, 79-83, doi:dx.doi.org/10.1139/cjz-2014-0204 (2015).

22 Lanza, B., Pastorelli, C., Laghi, P. & Cimmaruta, R. A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). *Atti Mus. Civ. Stor. Nat. Trieste* **52**, 5-135 (2006).

23 Lunghi, E., Manenti, R. & Ficetola, G. F. Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? *PeerJ* **3**, e1122, doi:10.7717/peerj.1122 (2015).

24 Lunghi, E., Manenti, R. & Ficetola, G. F. Cave features, seasonality and subterranean distribution of non-obligate cave dwellers *PeerJ* **5**, e3169, doi:10.7717/peerj.3169 (2017).

25 Ficetola, G. F., Pennati, R. & Manenti, R. Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? *Popul. Ecol.* **55**, 217-226 (2013).

26 Díaz-López, G. *et al.* Relationship between body weight and body condition score with energy content in the carcass of Pelibuey ewes. *Austral Journal of Veterinary Sciences* **49**, 77-81 (2017).

27 Jakob, E. M., Marshall, S. D. & Uetz, G. W. Estimating fitness: a comparison of body condition indices. *Oikos* **77**, 61-77 (1996).

28 Salvidio, S., Romano, A., Oneto, F., Ottonello, D. & Michelon, R. Different season, different strategies: feeding ecology of two syntopic forest-dwelling salamanders. *Acta Oecol.* **43**, 42-50 (2012).

29 Culver, D. C. & Pipan, T. *The biology of caves and other subterranean habitats*. (Oxford Unuiversity Press, 2009).

30 de la Vega, C. *et al.* Seasonal variation of Harbor Seal's diet from the Wadden Sea in relation to prey availability. *PloS ONE* **11**, e0155727, doi:journal.pone.0155727 (2016).

31 Băncilă, R. I., Hartel, T., R., P., Smets, J. & Cogălniceanu, D. Comparing three body condition indices in amphibians: a case study of yellow-bellied toad *Bombina variegata*. *Amphibia-Reptilia* **31**, 558-562, doi:10.1163/017353710X518405 (2010).

32 Lunghi, E. *et al.* Comparative reproductive biology of European cave salamanders (genus Hydromantes): nesting selection and multiple annual breeding. *Salamandra* (in press).

33 Wit, J., Loeschcke, V. & Kellermann, V. Life span variation in 13 *Drosophila* species: a comparative study on life span, environmental variables and stress resistance. *Journal of Evolutionary Biology* **28**, 1892-1900, doi:10.1111/jeb.12706 (2015).

34 Javier Sacristán, H. *et al.* Energy reserves mobilization: Strategies of three decapod species. *PloS ONE* **12**, e0184060, doi:https://doi.org/10.1371/journal.pone.0184060 (2017).

35 Milenkaya, O., Catlin, D. H., Legge, S. & Walters, J. R. Body condition indices predict reproductive success but not survival in a sedentary, tropical bird. *PLoS ONE* **10**, e0136582, doi:10.1371/journal.pone.0136582 (2015).

36 Lunghi, E. *et al.* Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments. *Salamandra* **50**, 105-109 (2014).

37 Lindström, J., Reeve, R. & Salvidio, S. Bayesian salamanders: analysing the demography of an underground population of the European plethodontid *Speleomantes strinatii* with statespace modelling. *BMC Evolutionary Biology* **10**, 4, doi:http://www.biomedcentral.com/1472-6785/10/4 (2010).

38 Sillero, N. *et al.* Updated distribution and biogeography of amphibians and reptiles of Europe. *Amphibia-Reptilia* **35**, 1-31 (2014).

39 Lunghi, E. *et al.* Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. *J. Therm. Biol.* **60**, 79-85, doi:http://dx.doi.org/10.1016/j.jtherbio.2016.06.010 (2016).

40 Ficetola, G. F., Pennati, R. & Manenti, R. Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. *Amphibia-Reptilia* **33**, 251-259 (2012).

41 Lunghi, E., Manenti, R. & Ficetola, G. F. Do cave features affect underground habitat exploitation by non-troglobite species? *Acta Oecol.* **55**, 29-35, doi:http://dx.doi.org/10.1016/j.actao.2013.11.003 (2014).

42 Hijmans, R. J., Cameron, S. E., Parra, J. L., Jonesc, P. G. & Jarvisc, A. Very high resolution interpolated climate surfaces for global land areas. *International Journal Of Climatology* **25**, 1965-1978, doi:10.1002/joc.1276 (2005).

43 Boucher-Lalonde, V., Morin, A. & Currie, D. J. A consistent occupancy – climate relationship across birds and mammals of the Americas. *Oikos* **123**, 1029-1036, doi:10.1111/oik.01277 (2014).

44 Mazel, F. *et al.* Global patterns of β-diversity along the phylogenetic timescale: the role of climate and plate tectonics. *Global Ecology and Biogeography* **26**, 1211-1221, doi:10.1111/geb.12632 (2017).

45 Lanza, B., Caputo, V., Nascetti, G. & Bullini, L. Morphologic and genetic studies of the European plethodontid salamanders: taxonomic inferences (genus *Hydromantes*). *Monografie del Museo Regionale di Scienze Naturali, Torino* **16**, 1-366 (1995).

46 Allouche, O., Tsoar, A. & Kadmon, R. Assessing the accuracy of species distribution models: prevalence, kappa and the true skill statistic (TSS). *J. Appl. Ecol.* **43**, 1223-1232, doi:10.1111/j.1365-2664.2006.01214.x (2006).

47 Thuiller, W., Lafourcade, B., Engler, R. & Araújo, M. B. BIOMOD – A platform for ensemble forecasting of species distributions. *Ecography* **32**, 369-373, doi:10.1111/j.1600-0587.2008.05742.x (2009).

48 Araújo, M. B. & New, M. Ensemble forecasting of species distributions. *Trends in Ecology and Evolution* **22**, 42-47, doi:10.1016/j.tree.2006.09.010 (2007).

49 Thuiller, W. *et al.* The European functional tree of bird life in the face of global change. *Nature Communications* **5**, 3118, doi:10.1038/ncomms4118 (2014).

50 Lunghi, E., Manenti, R. & Ficetola, G. F. in *Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali* (eds F. Stoch & P. Genovesi) 212-215 (2016).

51 Costa, A. *et al.* What goes in does not come out: different non-lethal dietary methods give contradictory interpretation of prey selectivity in amphibians. *Amphibia-Reptilia* **35**, 255-262, doi:10.1163/15685381-00002944 (2014).

52 Douglas, B., Maechler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**, 1-48, doi:10.18637/jss.v067.i01 (2015).

53 Kuznetsova, A., Brockhoff, B. & Christensen, H. B. lmerTest: Tests in Linear Mixed Effects Models. *R package version 2.0-29. www.r-project.org* (2016).

54 Kohl, M. MKmisc: Miscellaneous functions from M. Kohl. *R package version 0.993. http://www.stamats.de* (2016).

55 Brown, L. D., Cai, T. T. & DasGupta, A. Interval estimation for a binomial proportion. *Statistical Science* **16**, 101-117 (2001).

56 Labocha, M. K., Schutz, H. & Hayes, J. P. Which body condition index is best? *Oikos* **123**, 111-119, doi:10.1111/j.1600-0706.2013.00755.x (2014).

57 Scott, D. E., Casey, E. D., Donovan, M. F. & Lynch, T. K. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. *Oecologia* **153**, 521-532, doi:10.1007/s00442-007-0755-6 (2007).

58 Satterthwaite, F. E. An approximate distribution of estimates of variance components. *Biometrics Bulletin* **2**, 110-114 (1946).

59 MacKenzie, D. I. *et al. Occupancy estimation and modeling. Inferring patterns and dynamics of species occurrence*. 344 (Academic Press, 2006).

60 Royle, J. A. *N*-mixture models for estimating population size from spatially replicated counts. *Biometrics* **60**, 108-115, doi:10.1111/j.0006-341X.2004.00142.x (2004).

61 Ficetola, G. F., Romano, A., Salvidio, S. & Sindaco, R. Optimizing monitoring schemes to detect trends in abundance over broad scales. *Anim. Conserv.*, doi:10.1111/acv.12356 (2017).

62 Dennis, E. B., Morgan, B. J. T. & Ridout, M. S. Computational aspects of N-mixture models. *Biometrics* **71**, 237-246, doi:10.1111/biom.12246 (2015).

63 Royle, J. A. & Dorazione, R. M. (Academic Press, 2008).

64 Fiske, I. & Chandler, R. unmarked: an R package for fitting hierarchical models of wildlife occurrence and abundance. *J. Stat. Softw.* **43**, 1-23, doi:http://www.jstatsoft.org/v43/i10/ (2011).

Acknowledgements

We thank all speleologists which were of great help during field work. E. L. was supported by the National Speleological Society and Instrumentl.

Author contributions

E.L. and G.F.F. conceived and designed the experiments, performed analyses, wrote the first draft of the paper and prepared Table and Figures; E.L. and M.M. performed most of field activities. E.L., R.M., C.C., M.V. and G.F.F. contributed in reviewing the manuscript.

Table

Table 1. Monitored sites (caves). For each site we report: geographic coordinates; elevation (m a.s.l); environmental suitability (ES); total number of performed surveys; average depth of observed salamanders (± SE); number of empty and sampled stomachs; body condition index (residual index ± SE; in parenthesis the *N* of salamanders); estimated population size (mean and 95% CI); estimated density (salamanders/m^2).

Site	Lat	Long	Elevation	ES	Surveys	Salamander depth	Empty stomach	Residual Index	Estimated population size	Estimated density
Site 1	40.49	9.59	267	0.431	9	41.75 ± 6.14	8/24	(51) -0.140 ± 0.02	10.06 (8-13)	0.008
Site 2	40.51	9.61	116	0.470	7		-	(10) -0.030 ± 0.04	-	-
Site 3	40.51	9.61	116	0.470	7	10.3 ± 0.46	-	(5) -0.149 ± 0.06	6.96 (5-9)	0.051
Site 4	40.56	9.64	777	0.701	5		-	(3) 0.241 ± 0.06	5.50 (4-8)	0.231
Site 5	40.46	9.52	1029	0.647	10	12.36 ± 0.58	15/166	(158) -0.001 ± 0.01	103.59 (98-109)	0.077
Site 6	40.51	9.61	107	0.343	6		-	(1) -0.075	-	-
Site 7	40.47	9.53	679	0.531	5	8.5 ± 3.32	0/22	(29)-0.010 ± 0.03	9.7 (8-12)	0.031
Site 8	40.54	9.65	265	0.476	5	14.9 ± 0.94	-	-	5.69 (5-8)	0.006
Site 9	40.58	9.68	94	0.425	16	19.71 ± 2.33	-	(6) -0.027 ± 0.05	9.69 (7-13)	0.014
Site 10	40.56	9.68	116	0.420	1		-	(6) 0.040 ± 0.03	-	-
Site 11	40.57	9.64	954	0.726	1		-	(21) 0.060 ± 0.05	-	-
Site 12	40.55	9.62	902	0.786	1		-	(23) 0.180 ± 0.04	-	-
Site 13	40.58	9.69	50	0.339	1	148.27 ± 1.72	-	-	-	-
Site 14	40.49	9.58	349	0.431	1	21.43 ± 2.76	-	-	-	-

Figures

Figure 1: Potential relationships between climatic suitability, population parameters and species abundance. In blue the proposed pathways, in orange pathways for which no data are available. Filled arrows represent hypotheses tested in the present study.

Figure 2: Distribution area of *Hydromantes flavus* showing the relationship between climatic variables and Environmental Suitability (ES). Orange circles indicate all sites used in SDM analysis to estimate ES for the species; red squares represent surveyed site in the present study.

Figure 3: Relationship between features of salamander populations and environmental suitability. a) Activity pattern (distance from cave entrance); b) Feeding performance (frequency of empty stomachs); c) Estimated density. Bars represent standard errors.

Figure 4: Variables related to the variation of body condition index (BCI) of salamanders. Plots represent relationships between BCI and a) environmental suitability; b) month of survey; c) age class and sex. Horizontal line represents median values, while shaded box are 95% CI.

Figure 1

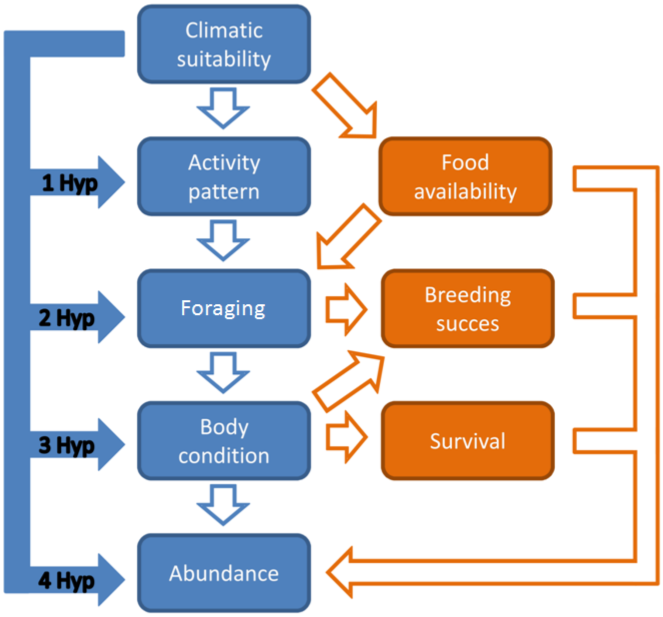


Figure 2

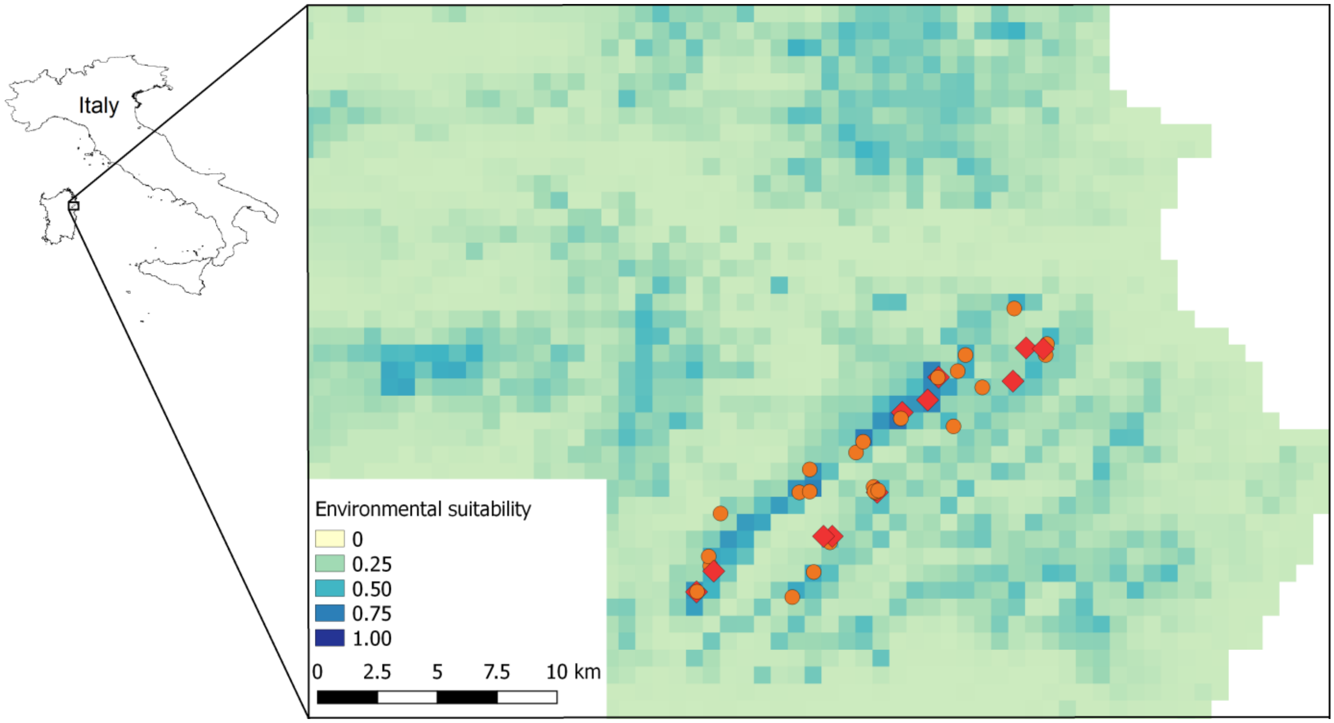


Figure 3

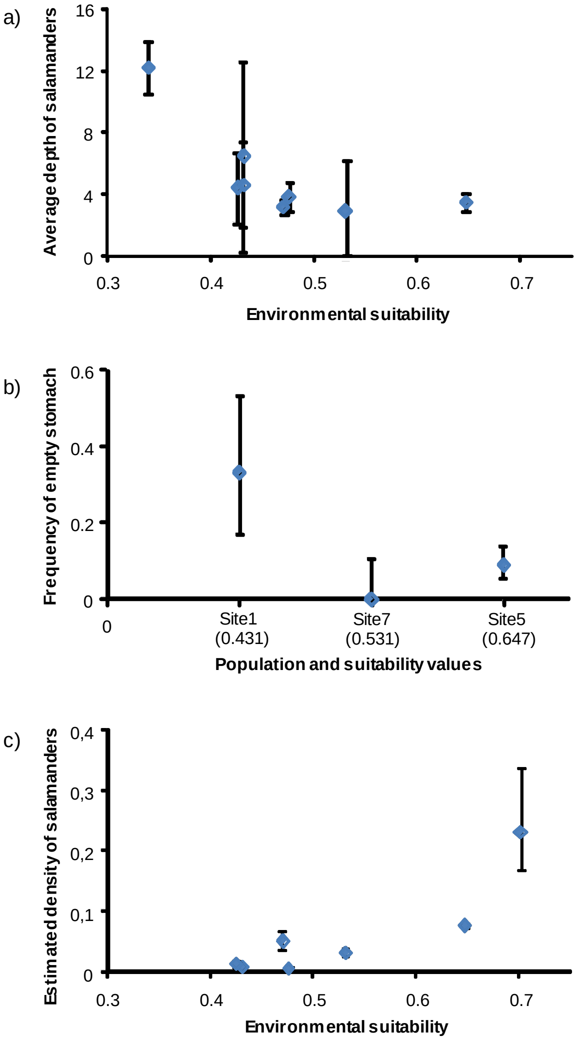
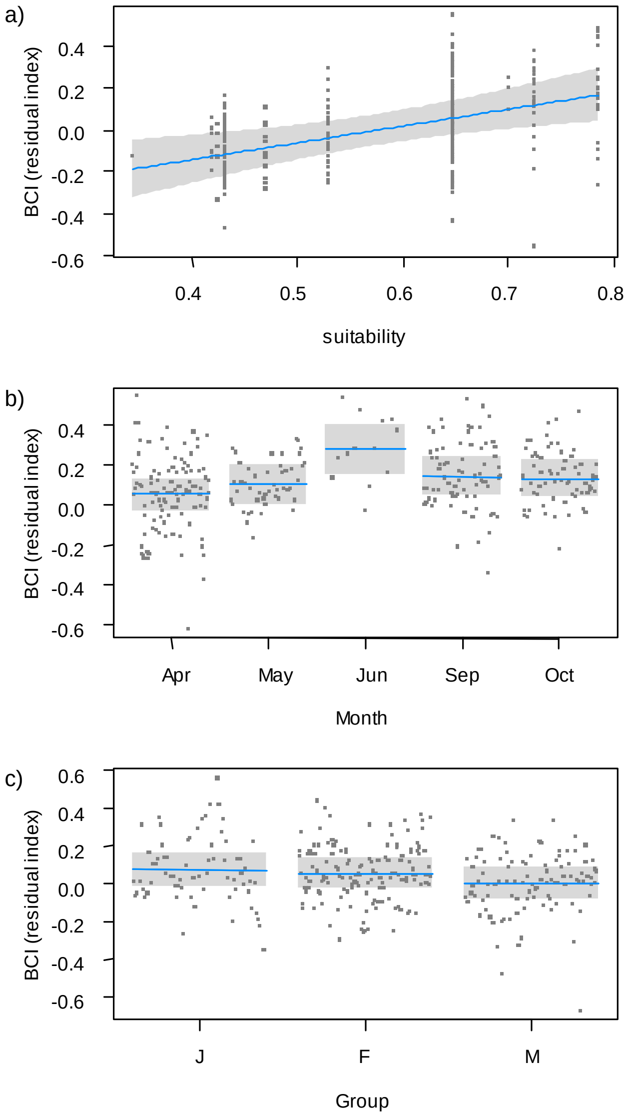


Figure 4



Chapter VII

Submitted to International Journal for Parasitology: Parasites and Wildlife,

November 2017

Batracobdella leeches, environmental features and terrestrial salamanders

Enrico Lunghi[1,2,3,*], Gentile Francesco Ficetola[4,5], Manuela Mulargia[6], Roberto Cogoni[7], Michael Veith[1], Claudia Corti[2], Raoul Manenti[4]

1) Universität Trier Fachbereich VI Raum-und Umweltwissenschaften Biogeographie, Universitätsring 15, 54286 Trier, Germany

2) Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy

3) Natural Oasis, Via di Galceti 141, 59100 Prato, Italy

4) Department of Environmental Science and Policy, University of Milan, via Celoria 26, Milano, Italy

5) Laboratoire d’Ecologie Alpine (LECA), CNRS, Université Grenoble Alpes, Grenoble,

France
6) Via Isalle 4, 08029 Siniscola, Italy

7) Unione Speleologica Cagliaritana, Quartu Sant'Elena (CA), Italy

*Corresponding author, mail: Universität Trier Fachbereich VI Raum-und Umweltwissenschaften Biogeographie, Universitätsring 15, 54286 Trier, Germany; email address: enrico.arti@gmail.com; telephone: +39 3391604627

Declarations of interest: none

Abstract

Leeches can parasitize many vertebrate taxa. In amphibians, leech parasitism often has potential detrimental effects, being one of the causes of local decline of populations. Most of the information on host-parasite interactions involving leeches and amphibians is related to freshwater environments, while they are extremely limited for terrestrial amphibians. In this work, we studied the relationship between the leech *Batracobdella algira* and the European terrestrial salamanders of the genus *Hydromantes*, identifying, for the first time, environmental features related to the presence of the leeches and possible effects on these hosts. We performed our observation throughout Sardinia (Italy), covering the distribution area of all *Hydromantes* species endemic to this island. From September 2015 to May 2017, we performed >150 surveys in 26 underground environments, collecting data on 2,629 salamanders and 131 leeches. Water hardness was the only environmental feature related to the presence of *B. algira*, linking this leech to active karstic systems. Leeches were more frequently parasitizing salamanders with large body size and better Body Condition Index. Our study shows the importance of abiotic environmental features for host-parasite interactions, and poses new questions on complex interspecific interactions between this ectoparasite and amphibians.

Keywords: parasitism, cave, interaction, leech, *Speleomantes,* BCI.

1. Introduction

Leeches, with around 700 different species, represent a specialized group of annelids widespread across the world (Sket and Trontelj, 2008). One particular group, the order Rhynchobdellida, includes leeches lacking jaws and teeth, which are commonly feeding on vertebrate blood (Sawyer, 1981; Moser et al., 2009). Because of their particular feeding strategy, hematophagous leeches arouse a special interest in researchers, as they may cause both negative and positive effects on hosts' health (Nehili et al., 1994; Wells, 2007; Al-Khleif et al., 2011; Elliot and Kutschera, 2011; Brisola Marcondes et al., 2017). Hematophagous leeches are able to parasitize on a wide range of vertebrates and, in some circumstances, they may be specialized on specific taxa (Sawyer, 1981). After having stuck to their host, leeches induce a chemical reaction aimed to attenuate the host immune response (Salzet et al., 2000; DuRant et al., 2015). This particular feeding strategy, may also promote hosts vulnerability to further potential infections (Rigbi et al., 1987; Daszak et al., 1999). However, despite these notable effects, the biology of many leech species, and the effects of their interspecific interactions, remain poorly studied.

Interactions between leeches and amphibians have been described several times (Trauth and Neal, 2004; Beukema and de Pous, 2010; Tiberti and Gentilli, 2010). Leeches feed on Amphibian larvae, adults and, most likely, on their eggs as well (Howard, 1978; Mock and Gill, 1984; Veith and Viertel, 1993; Romano and Di Cerbo, 2007; Wells, 2007), sometimes being considered an additional cause of species decline (Stead and Pope, 2010). Interactions between leeches and amphibians seem to be opportunistic, as these ectoparasites do not show particular adaptations to exploit amphibians as hosts (Elliot & Dobson, 2015). Generally, leeches use amphibians as food source, without leading the hosts to death (Getz, 2011; Rocha et al., 2012); however, some studies documented that leeches represented a serious threat for local amphibian populations. In the lake area of Dungeness (Kent, UK), where the Marsh frog (*Pelophylax ridibundus*) represents the most important host of the leech *Hirudo medicinalis*, several Smooth newts (*Lissotriton vulgaris*) were found dead with leeches bites (Wilkin and Scofield, 1990; Elliot and Dobson, 2015). In a pond in Luxemburg, adults of the Common toad (*Bufo bufo*) were heavily parasitized by *H. medicinalis,* being unable to reproduce (Hoffmann, 1960); *H. medicinalis* represented a threat also for the Moor frog (*Rana arvalis*) from Gotland (Sweden) (Merilä and Sterner, 2002), and authors pointed out that strong negative effects of leeches may be enhanced by environmental changes (Berven and Boltz, 2001; Ayres and Comesaña Iglesias, 2008).

Interactions between amphibians and leeches were mostly observed in freshwater environments, while data from terrestrial environments remain scarce (Rocha et al., 2012). During several herpetological activities performed in Madagascar, Rocha et al. (2012) documented the parasitism of *Malagabdella* leeches on at least four species of Mantellid tree frogs. In their report, authors highlighted the scarcity of observations carried out on leeches-amphibians interaction in terrestrial environments. Very few data exist on the interactions between the *Hydromantes* terrestrial salamanders (for furhter discussion on nomenclature see Wake, 2013) and the leech *Batracobdella algira*. *Hydromantes* salamanders are amphibians of high conservation value, including species endemic to very small areas (Lanza et al., 2006). While environmental and human factors affecting their distribution have been investigated, few studies have been devoted to the interaction with pathogens and parasites (Pasmans et al., 2013). Until now, reports of interactions between leeches and *Hydromantes* salamanders is limited to a list of records on the occurrence of this ectoparasite, with little information on interspecific interactions, on ecological factors that can promote parasitism, and on their potential impacts (Lanza et al., 2006; Manenti et al., 2016).

In this paper, we provide a brief overview of both *Hydromantes* and *B. algira,* followed by new data on the ecology of *B. algira*, reporting for the first time information related to its effects on *Hydromantes*. In particular, we tested whether leeches may be detrimental to salamanders' body condition.

2. Materials and methods

2.1. The ectoparasite: Batracobdella algira.

After the first description of *Batracobdella algira* by Moquin-Tandon (1846), few studies dealt with this species, mostly describing development and distribution. *B. algira* is a greenish dorsoventrally flattened leech belonging to the group of jawless leeches (Ben Ahmed et al., 2015). It is mostly distributed in the Mediterranean basin (Minelli, 1979; Nesemann, 1991; Ben Ahmed et al., 2015), where it parasitizes several species of amphibians (Ben Ahmed et al., 2015; Manenti et al., 2016); it is the only known ectoparasite of the Sardinian *Hydromantes* salamanders (Lanza et al., 2006). This leech is also identified as a vector of some pathogenic micro-parasites, such as the protists *Lankesterella* and the protozoan *Trypanosoma* (Jiménez Sánchez, 1997). *Batracobdella algira* has been reported for different freshwater habitats (both lentic and lotic) and was even found in subterranean ones, generally sticking themselves to hosts like *Discoglossus* and *Pelophylax* frogs (Minelli, 1979). In both surface and subterranean terrestrial habitats it was found parasitizing *Hydromantes* in different localities of Sardinia (Mertens, 1929; Manenti et al., 2016). However there are very few ecological observations on *B. algira*, and almost no investigations have been carried out on the factors affecting its distribution and phenology (Nesemann, 1991). Breeding occurs only once in its life: adult individuals ($\geq$20 mm) mostly breed during spring, producing several eggs hatching after nearly 3 weeks (Ben Ahmed et al., 2009; Romdhane et al., 2015). Parents provide pre- and post-hatch parental cares and later, 2-3 weeks after eggs hatched, they die (Romdhane et al., 2015).

2.2. The hosts: Sardinian Hydromantes *salamanders.*

Sardinian *Hydromantes* (*H. flavus, H. supramontis, H. imperialis, H. sarrabusensis, H. genei*) are lungless salamanders endemic to Sardinia (Sillero et al., 2014), all showing allopatric distribution (Chiari et al., 2012). *Hydromantes* are mostly epigean species which exploit a variety of different terrestrial habitats (Lanza et al., 2006; Manenti, 2014; Costa et al., 2016). However, these salamanders have a narrow thermal niche and require high humidity level (Spotila, 1972). They are strongly influenced by environmental conditions (Lunghi et al., 2016a), and thus, when external climate becomes unsuitable (too hot and too harsh), they refuge underground (Ficetola et al., 2012; Lunghi et al., 2014a), where suitable microclimate is present throughout the year (Lunghi et al., 2015a). These salamanders have direct development and mostly reproduce in underground habitats (Lanza et al., 2006). Females lay about 6-14 eggs in caves characterized by stable microclimate and low predation pressure (Lunghi et al., 2014b; Lunghi et al., 2015b; Salvidio et al., 2017); the mother attends and protects eggs and hatchlings for more than nine months (Oneto et al., 2010, 2014). The Italian IUCN Red List classifies the majority of the Sardinian *Hydromantes* (*Speleomantes*) species as vulnerable to extinction risks (Rondinini et al., 2013); all these species are therefore deserving protection.

2.3. Surveys

From September 2015 to May 2017, we performed surveys throughout the whole distribution range of the Sardinian *Hydromantes* species, exploring underground environments where individuals are easily detectable (Ficetola et al., 2012; Lunghi et al., 2016b). To prevent the spread of pathogens (Stegen et al., 2017), salamanders were handled using disposable latex gloves, and the used instruments were cleaned with bleach before every survey. *Hydromantes* were searched by VES (Visual Encounter Surveys; Crump and Scott, 1994). Salamanders were hand-inspected in order to detect *Batracobdella algira*. When leeches were observed, we first recorded the salamander body part at which the ectoparasites were attached and then leeches were removed using tweezers. For each leech we estimated the body size (during the relaxation phase) defining four categories (in mm; Small = up to 5; Medium = from 5.1 to 10; Large = from 10.1 to 20; X-Large > 20). When possible, for each salamander we recorded: the linear distance from the cave entrance (measured by a laser meter, accuracy ~2 mm; hereafter depth), snout-vent length (SVL) and total length (TL) (using a transparent plastic rule) and weight (using a digital scale, accuracy 0.01 g); weighted salamanders were all free of leeches. Salamander age class was assessed on the basis of SVL, considering as juveniles all individuals with SVL < 45 mm (< 40 for *H. genei*) (Lanza et al., 2006). Adults were sexed on the basis of the presence/absence of male secondary sexual characters (mental gland and premaxillary teeth) (Lanza et al., 2006). Body Condition Index (BCI) was estimated using the Residual Index (Labocha et al., 2014), an appropriated method to estimate amphibians' physical condition (Băncilă et al., 2010). To calculate the BCI we performed a regression analysis using log-transformed weight and the total length of salamanders (logBM and logBL), as this improves the linearity of the relationship (Labocha et al., 2014). We then extracted the residual of each individual, representing the difference between the observed and the predicted body mass of salamanders for a given size. We considered the total length because tail is usually used as energy storage (Scott et al., 2007). For *H. flavus* sites (see Table 1), if present, we seasonally recorded the physical parameters of the dripping water (pH, permanent hardness and NO_2) using multi-parameter stripes. Considering that the presence of dripping water was not constant across caves and time (in each cave it was observed during 2.37 ± 0.26 seasons), for each parameter we calculated the average value.

2.4. Statistical analyses

For each site where leeches were observed, we estimated the average frequency of parasitized salamanders. We run a binomial generalized linear mixed model (GLMM) to test if the presence of leeches on salamanders was related to the depth of salamanders and to the age and sex of the host. In these analyses we only considered the three species for which we collected data on salamanders' depth (*Hydromantes flavus*, *H. imperialis* and *H. sarrabusensis*). In a first GLMM we used as dependent variable the presence/absence of the ectoparasite, with both salamander age class and depth as independent variables; we used the year, month of survey, cave identity and host-species as random factors. We did not consider the size of salamanders (total length) as it was strongly correlated with age class ($r = 0.85$, $P < 0.001$). The same analysis was repeated replacing age class with sex, in order to test if adult males and females are parasitized with different frequencies. We then tested whether leech presence is related to salamander BCI. To avoid pseudoreplications, in following analyses we only considered measures from marked individuals and from just one survey per cave (the one in which collected the highest number of data) (Lunghi and Veith, 2017). We run a binomial GLMM to evaluate the difference between the BCI of parasitized salamanders with the non-parasitized ones; the presence/absence of leeches was used as dependent variable, salamanders BCI as independent variable and cave identity, year, month of survey and host-species as random variables. Given the difference in BCI between parasitized and non-parasitized salamanders, and that the number of leeches parasitizing a single salamander was variable (see results), we run a GLMM considering only parasitized salamanders to assess how salamanders BCI varies in relation to the number of leeches stucking simultaneously. We used leech abundance (log-transformed) as dependent variable, while salamanders BCI and leech size were independent variables; year, month of survey, cave identity and host-species were used as random factors. Furthermore, for the caves where *Hydromantes flavus* was present, we used a binomial generalized linear model to assess whether the presence of leeches was related to the physical water parameters; in this analysis we merged data from all surveys. We used the presence of leeches inside caves as dependent variable (if the leeches were observed at least once, they were considered present), while the average water parameters were used as independent variable.

3. Results

We performed 162 surveys in 26 different caves (average 6.38 ± 5.1 surveys per cave), overall exploring 1,366 m of underground environments, and examining 2,629 salamanders (Table 1); we measured and weighed 513 salamanders (*Hydromantes flavus*: *N* =287; *H. supramontis*: *N* = 66; *H. imperialis*: *N* = 76; *H. sarrabusensis*: *N* =84). and, for them, we calculated BCI. The BCI of salamanders did not show any correlation with salamanders' length, species and interaction between them (all $P > 0.05$).

We observed 131 individuals of *Batracobdella algira* (Small = 35, Medium = 16, Large = 31, X-Large = 43) in nine caves inhabited by four *Hydromantes* species (*H. flavus* =5; *H. supramontis* = 1; *H. imperialis* = 2; *H. sarrabusensis* =1) (Table 1); 107 salamanders were found with parasites (8 juveniles, 37 females and 62 males). About one-third of the observed leeches (32.82%) were adults, as our largest category corresponds to the size of sexual mature leeches (Romdhane et al., 2015). Almost all the observed leeches (130) were parasitizing *Hydromantes* (Fig. 1a), while one was found attached to a stone located 6.1 m from the cave entrance (Fig. 1b). Leeches were found attached to the hosts' tail (*N* = 12), limbs (*N* = 24), back (*N* = 14), belly (*N* = 7), flanks (*N* = 36) and head (*N* = 11). Populations of *Hydromantes* showed a different frequency of parasitized salamanders, (chi-squared test; $\chi^2_3 = 19.475$, $P < 0.001$), which ranged from < 1% to > 30% (Table 1).

The presence of leeches was significantly higher in adult salamanders ($\chi^2 = 17.44$, df= 1, $P < 0.001$) and in individuals close to the cave entrance ($\chi^2 = 715.37$, df= 1, $P < 0.001$). Analysis carried out on adults only showed significant correlation with both sex ($\chi^2 = 98619.26$, df= 1, $P < 0.001$) and depth ($\chi^2 = 310.28$, df= 1, $P < 0.001$); leeches were more frequent on females and in adults close to the cave entrance.

Overall, the BCI of parasitized salamanders did not differ from that of not parasitized ones (χ^2 = 0.33, df= 1, P = 0.568) (Fig. 2). Parasitized salamanders carried in average 1.57 ± 0.23 leeches (max: 11). Within parasitized salamanders, those having higher parasite load showed lower BCI ($F_{1,80.41}$ = 7.21, P = 0.009), while smaller leeches were found to be more aggregated than bigger ones ($F_{3,82.95}$ = 46.94, $P < 0.001$).

For seven caves inhabited by *H. flavus*, we recorded data describing chemical features of underground water (Table 1). The presence of leeches was significantly higher in sites with high water hardness (χ^2 = 7.59, df = 1, $P < 0.004$), while no significant correlation with other water parameters was found (for both pH and NO_2, $P > 0,05$).

4. Discussion

Our study adds new information on the ecology of *Batracobdella algira* and on its relationships with *Hydromantes* salamanders, describing for the first time the parasitism on *Hydromantes sarrabusensis* (Lanza et al., 2006; Manenti et al., 2016). Considering that all Sardinian *Hydromantes* species show an allopatric distribution (Chiari et al., 2012), it is unlikely that this ectoparasite moves from one *Hydromantes* species to another. Two hypotheses may help in explaining the presence of this ectoparasite on all Sardinian *Hydromantes*. On the one hand, other amphibian species could drive the spread of *B. algira* among all *Hydromantes* species. For example, *Hydromantes* could get in touch with *Discoglossus sardus*, a species which is found in both epigean and subterranean environments, and which is paratized by *B. algira* (Minelli, 1979). On the other hand, no information exists on the gene flow between leech populations, and it is possible that leeches paratizing the different *Hydromantes* species actually are isolated. If this was the case, current diversity of leeches in Sardinia would be higher than assumed. However, none of these hypotheses has been tested yet.

Manenti et al. (2016) reported a strong spatial autocorrelation of caves in which *B. algira* was present, and also identified *H. flavus* as the species showing the highest frequency of parasitized salamanders. Considering only the distribution of *H. flavus* (limited to a single massif; see Lanza et al., 2006), we observed a particular distribution of leeches. Caves in which we observed *B. algira* were located along the major longitudinal length of the massif, mostly avoiding lower altitudes (min and max recorded altitude, 267 and 1029 m a.s.l.) (Fig. 3). The presence of *B. algira* decreased with altitude, where evergreen forests are replaced by sporadic xeric shrubs (AA. VV., 2006). Forests may be preferred by leeches because evergreen trees buffer seasonal variation of local climate, and because it represents a suitable habitat to search for hosts.

In this study we identified one cave parameter related to the presence of leeches: we found that *B. algira* was associated to caves where the hardness of the dripping water is high. To our knowledge this is the first report concerning the relationship between *B. algira* and physical/chemical environmental features. Hard water can be an indicator of water flowing through in active karst systems, where water determine the alteration of rocks (Culver and Pipan, 2009). Water flowing through the karst might play a role in the underground diffusion of leeches, and *B. algira* might more easily spread in caves of active karst systems.

Salamanders' flanks and limbs seem to be the body parts where leeches more frequently are found sticked on (chi-squared test; $\chi^2_5 = 38.03$, $P < 0.001$), while they are rarely observed on the belly. Where *Hydromantes* get in touch with its ectoparasites is still unknown. Moreover, it is likely that leeches tend to avoid to stick on the ventral body parts of the salamanders, which generally adhere to the substrate (Bicca-Marques and Calegaro-Marques, 2016). In our study, we identified a higher frequency of parasitized salamanders in cave areas close to the surface, which are the cave zones where prey availability for salamanders is higher (Ficetola et al., 2013; Manenti et al., 2015; Lunghi et al., 2017).

However, it is still not clear if salamanders encounter leeches more frequently in areas close to the cave entrance, or if the necessity of getting more food due to the presence of the ectoparasite induce salamanders to frequent more often foraging sites (Ficetola et al., 2013). We also identified a tendency of female salamanders to be more parasitized. This is probably well explained by body-size of salamanders: in these species, females are bigger than males (Lanza et al., 2006) and indeed, previously was assessed that bigger salamanders were the most parasitized by leeches (Manenti et al., 2016).

The BCI of parasitized salamanders was not significantly lower than that of non-parasitized ones (Fig. 2); however, a negative effect on salamanders' BCI was observed when they were highly parasitized. The host-parasite relationship is generally negative for the host, which is going to suffer the cost of the parasite nourishment, activity mostly determined by both host tolerance and parasite invasiveness (DuRant et al., 2015; Budischak et al., 2017; Fleskes et al., 2017; Miller and Cotter, 2017). However, our observations were limited to the salamanders' body condition, and future investigations are needed to explore other aspects of this host-parasite relationship such as the possible transfer of pathogenic micro-parasites, changes in the host immunologic response and in behavior.

Adult salamanders were more frequently parasitized than young individuals and, within adults, females showed higher frequency of parasitism. Our findings agree with a previous study where bigger salamanders were observed to be the most parasitized (Manenti et al., 2016). The number of leeches simultaneously recorded on a single salamander was variable, and was mostly related to the size of parasites. Given that these leeches carry their eggs in a specific area of their ventral part (Ben Ahmed et al., 2009), they likely release the newborns on a 'high suitable' host before dying (Romdhane et al., 2015). In this scenario, selected salamanders will have to provide nourishment to several hatchlings, event that surely affects salamanders' body condition.

5. Conclusion

The present study provides one of the firsts contributions on the ecology of *Batracobdella algira* (Jiménez Sánchez, 1997; Ben Ahmed et al., 2015; Romdhane et al., 2015; Manenti et al., 2016). Given the wide distribution of this species (Ben Ahmed et al., 2015), further research considering the whole range of the species will be needed to better understand its ecology, in order to assess whether local adaptations are occurring. Our first assessment on *B. algira-Hydromantes* relationship did not highlight any particular negative effect on salamanders body condition (Stead and Pope, 2010). However, further studies are needed to better understand the dynamics occurring between these leeches and cave salamanders, as well as to evaluate the impact of *B. algira* on the long-term performance of *Hydromantes* and on the whole amphibian communities.

Acknowledgments

We would like to thank all speleologists for their assistance during field work. E. Lunghi was supported by National Speleological Society, The Mohamed bin Zayed Species Conservation Fund and Instrumentl. The study was authorized by the Italian Ministry of Environment (9384/PNM of 12/05/2015).

References

AA. VV., 2006. Gestione del Sito di Importanza Comunitaria "Monte Albo ITB021107". POR Sardegna 2000-2006 ASSE I Misura 1.5 Rete Ecologica Regionale.

Al-Khleif, A., Roth, M., Menge, C., Heuser, J., Baljer, G., Herbst, W., 2011. Tenacity of mammalian viruses in the gut of leeches fed with porcine blood. Journal of Medical Microbiology 60, 787-792.

Ayres, C., Comesaña Iglesias, J., 2008. Leech presence on Iberian Brown frog, *Rana iberica*, (Amphibia: Anura: Ranidae) from north-western Spain. Acta Herpetol. 3, 155-159.

Băncilă, R.I., Hartel, T., R., P., Smets, J., Cogălniceanu, D., 2010. Comparing three body condition indices in amphibians: a case study of yellow-bellied toad *Bombina variegata*. Amphibia-Reptilia 31, 558-562.

Ben Ahmed, R., Romdhane, Y., Tekaya, S., 2015. Checklist and distribution of marine and freshwater leeches (Annelida, Clitellata, Hirudinea) in Tunisia with identification key. Ecologica Montenegrina 2, 3-19.

Ben Ahmed, R., Ropelewska, E., Bielecki, A., Cichocka, J., 2009. *Batracobdella algira* Moquin-Tandon, 1846 (Hirudinida: Glossiphoniidae) – morphometric analysis and internal morphology. Wiadomooeci Parazytologiczne 55, 353-358.

Berven, K.A., Boltz, R.S., 2001. Interactive effects of leech (*Desserobdella picta*) infection on Wood frog (*Rana sylvatica*) tadpole fitness traits. Copeia 2001, 907-915.

Beukema, W., de Pous, P., 2010. Exceptional leech predation on *Amietophrynus mauritanicus* (Anura, Bufonidae) in Tunisia. Herpetol. Notes 3, 289-290.

Bicca-Marques, J.C., Calegaro-Marques, C., 2016. Ranging behavior drives parasite richness: a more parsimonious hypothesis. American Journal of Primatology 78, 923-927.

Brisola Marcondes, C., Coutinho- Abreu, I.V., Valenzuela, J., Hurd, H., 2017. Blood sucking, vector-parasite relationship, and transmission of diseases, in: Brisola marcondes, C. (Ed.), Arthropod Borne Diseases. Springer International Publishing, Cham, pp. 47-57.

Budischak, S.A., O'Neal, D., Jolles, A.E., Ezenwa, V.O., 2017. Differential host responses to parasitism shape divergent fitness costs of infection. Functional Ecology.

Chiari, Y., van der Meijden, A., Mucedda, M., Lourenço, J.M., Hochkirch, A., Veith, M., 2012. Phylogeography of Sardinian cave salamanders (Genus *Hydromantes*) is mainly determined by geomorphology. PLoS ONE 7, e32332.

Costa, A., Crovetto, F., Salvidio, S., 2016. European plethodontid salamanders on the forest floor: local abundance is related to fine-scale environmental factors. Herpetol. Conserv. Biol. 11, 344-349.

Crump, M.L., Scott, N.J., 1994. Visual Encounter Surveys, in: Heyer, W.R., Donnelly, M.A., McDiarmid, R.W., Hayek, L.C., Foster, M.S. (Eds.), Measuring and monitoring biological diversity: standard methods for Amphibians. Smithsonian Institution Press, Washington, pp. 84-92.

Culver, D.C., Pipan, T., 2009. The biology of caves and other subterranean habitats. Oxford Unuiversity Press, New York.

Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E., Speare, R., 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5, 735-748.

DuRant, S.E., Hopkins, W.A., Davis, A.K., Romero, L.M., 2015. Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*). The Journal of Experimental Biology 218, 2297-2304.

Elliot, J.M., Dobson, M., 2015. Freshwater Leeches of Britain and Ireland: Keys to the Hirudinea and a Review of their Ecology. The Freshwater Biological Association, Ambleside.

Elliot, J.M., Kutschera, U., 2011. Medicinal leeches: historical use, ecology, genetics and conservation. Freshwater Reviews 4, 21-41.

Ficetola, G.F., Pennati, R., Manenti, R., 2012. Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. Amphibia-Reptilia 33, 251-259.

Ficetola, G.F., Pennati, R., Manenti, R., 2013. Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? Popul. Ecol. 55, 217-226.

Fleskes, J.P., Ramey, A.M., Reeves, A.B., Yee, J.L., 2017. Body mass, wing length, and condition of wintering ducks relative to hematozoa infection. Journal of Fish and Wildlife Management 8, 89-100.

Getz, W.M., 2011. Biomass transformation webs provide a unified approach to consumer–resource modelling. Ecology Letters 14, 113-124.

Hoffmann, J., 1960. Notules Hirudinologiques. Archives de la section de Sciences Naturelles, Physiques et Mathematiques de l'Institut Grand-ducal de Luxembourg 27, 285-291.

Howard, R.D., 1978. The influence of male-defended oviposition sites on early embryo mortality in Bullfrogs. Ecology 59, 789-798.

Jiménez Sánchez, M.S., 1997. Contribución al conocimiento de la parasitofauna de la *Rana perezi* Seoane, 1885 (Amphibia: Ranidae) de la provincia de Ávila, Parasitología. Universidad Complutense de Madrid - Facultad de Farmacia, Madrid.

Labocha, M.K., Schutz, H., Hayes, J.P., 2014. Which body condition index is best? Oikos 123, 111-119.

Lanza, B., Pastorelli, C., Laghi, P., Cimmaruta, R., 2006. A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). Atti Mus. Civ. Stor. Nat. Trieste 52, 5-135.

Lunghi, E., Manenti, R., Canciani, G., Scarì, G., Pennati, R., Ficetola, G.F., 2016a. Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. J. Therm. Biol. 60, 79-85.

Lunghi, E., Manenti, R., Ficetola, G.F., 2014a. Do cave features affect underground habitat exploitation by non-troglobite species? Acta Oecol. 55, 29-35.

Lunghi, E., Manenti, R., Ficetola, G.F., 2015a. Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? PeerJ 3, e1122.

Lunghi, E., Manenti, R., Ficetola, G.F., 2016b. *Speleomantes flavus* (Stefani, 1969) (Geotritone del Monte Albo); *S. genei* (Temminck & Schlegel, 1838) (Geotritone di Gené); *S. imperialis* (Stefani, 1969) (Geotritone imperiale); *S. sarrabusensis* (Lanza et al., 2001) (Geotritone dei Sette Fratelli); *S. supramontis* (Lanza, nascetti & Bullini, 1986) (Geotritone del Supramonte), in: Stoch, F., Genovesi, P. (Eds.), Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali, Roma, pp. 212-215.

Lunghi, E., Manenti, R., Ficetola, G.F., 2017. Cave features, seasonality and subterranean distribution of non-obligate cave dwellers PeerJ 5, e3169.

Lunghi, E., Manenti, R., Manca, S., Mulargia, M., Pennati, R., Ficetola, G.F., 2014b. Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments. Salamandra 50, 105-109.

Lunghi, E., Murgia, R., De Falco, G., Buschettu, S., Mulas, C., Mulargia, M., Canedoli, C., Manenti, R., Ficetola, G.F., 2015b. First data on nesting ecology and behaviour in the Imperial cave salamander *Hydromantes imperialis*. North-West. J. Zool. 11, 324-330.

Lunghi, E., Veith, M., 2017. Are Visual Implant Alpha tags adequate for individually marking European cave salamanders (genus *Hydromantes*)? Salamandra 53, 541-544.

Manenti, R., 2014. Dry stone walls favour biodiversity: a case-study from the Appennines. Biodivers. Conserv. 23, 1879-1893.

Manenti, R., Lunghi, E., Canedoli, C., Bonaccorsi, M., Ficetola, G.F., 2016. Parasitism of the leech, *Batracobdella algira* (Moquin-Tandon, 1846), on Sardinian cave salamanders (Genus *Hydromantes*) (Caudata: plethodontidae). Herpetozoa 29, 27-35.

Manenti, R., Lunghi, E., Ficetola, G.F., 2015. Distribution of spiders in cave twilight zone depends on microclimatic features and trophic supply. Invertebr. Biol. 134, 242-251.

Merilä, J., Sterner, M., 2002. Medicinal leeches (*Hirudo medicinalis*) attacking and killing adult amphibians. Ann. Zool. Fenn. 39, 343-346.

Mertens, R., 1929. *Glossiphonia algira* als parasit von *Hydromantes genei genei*. Bl. Aquar.- Terrar.-Kde, Stuttgart 40, 206-207.

Miller, C.V.L., Cotter, S.C., 2017. Resistance and tolerance: the role of nutrients on pathogen dynamics and infection outcomes in an insect host. Journal of Animal Ecology.

Minelli, A., 1979. Fauna d'ltalia. Hirudinea. Calderini, Bologna.

Mock, B.A., Gill, D.E., 1984. The infrapopulation dynamics of trypanosomes in red-spotted newts. Parasitology 88, 267-282.

Moquin-Tandon, A., 1846. Monographie de la famillie des Hirudinees, Paris.

Moser, W.E., Govedich, F.R., J., K.D., 2009. Annelida, Hirudinida (Leeches), in: Likens, G.E. (Ed.), Encyclopedia of Inland Waters (3 volumes set). Elsevier Academic Press, New York.

Nehili, M.I., C.Mehlhorn, H., Ruhnau, K., Dick, W.N., M., 1994. Experiments on the possible role of leeches as vectors of animal and human pathogens: a light and electron microscopy study. Parasitology Research 8, 277-290.

Nesemann, H., 1991. Zoogeography and composition of leech fauna of Danubian lowland rivers in the Kisalföld compared with some molluscs (Hirudinea, Gastropoda) Miscellanea Zoologica Hungarica 6, 35-51.

Oneto, F., Ottonello, D., Pastorino, M.V., Salvidio, S., 2010. Posthatching parental care in salamanders revealed by infrared video surveillance. J. Herpetol. 44, 649-653.

Oneto, F., Ottonello, D., Pastorino, M.V., Salvidio, S., 2014. Maternal care and defence of young by the plethodontid salamander *Speleomantes strinatii* (Aellen, 1951). Scripta Herpetologica. Studies on Amphibians and Reptiles in honour of Benedetto Lanza, 129-138.

Pasmans, F., Van Rooij, P., Blooi, M., Tessa, G., Bogaerts, S., Sotgiu, G., Garner, T.W.J., Fisher, M.C., Schmidt, B.R., Woeltjes, T., Beukema, W., Bovero, S., Adriaensen, C., Oneto, F., Ottonello, D., Martel, A., Salvidio, S., 2013.

Resistance to chytridiomycosis in european plethodontid salamanders of the genus *Speleomantes*. PLoS ONE 8, e63639.

Rigbi, M., Levy, H., Eldor, A., Iraqi, F., Teitelbaum, M., Orevi, M., Horovitz, A., Galun, R., 1987. The saliva of the medicinal leech *Hirudo medicinalis*-II. Inhibition of platelet aggregation and of leukocyte activity and examination of reputed anaesthetic effects. Comparative Biochemistry and Physiology 88C, 95-98.

Rocha, R., Borda, E., Andreone, F., Rosa, G.M., 2012. First reports of leech parasitism in Malagasy anurans. Comparative Parasitology 79, 352-356.

Romano, A., Di Cerbo, A.R., 2007. Leech predation on amphibian eggs. Acta Zoologica Sinica 53, 750-754.

Romdhane, Y., Ben Ahmed, R., Tekaya, S., 2015. Insemination and embryonic development in the leech: *Batracobdella algira* (Hirudinea, Annelida). Invertebrate Reproduction & Development 59, 17-25.

Rondinini, C., Battistoni, A., Peronace, V., Teo li, C., 2013. Lista Rossa IUCN dei Vertebrati Italiani. . Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Roma.

Salvidio, S., Palumbi, G., Romano, A., Costa, A., 2017. Safe caves and dangerous forests? Predation risk may contribute to salamander colonization of subterranean habitats. Sci. Nat. 104, 20.

Salzet, M., Capron, A., Stefano, G.B., 2000. Molecular crosstalk in host–parasite relationships: schistosome– and leech–host interactions. Parasitology Today 16, 536-540.

Sawyer, R.T., 1981. Leech biology and behavior, in: Muller, K.J., Nichols, J.G., Stent, G.S. (Eds.), Neurobiology of the Leech. Cold Spring Harbor Laboratory Publications, New York, pp. 7-26.

Scott, D.E., Casey, E.D., Donovan, M.F., Lynch, T.K., 2007. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. Oecologia 153, 521-532.

Sillero, N., Campos, J., Bonardi, A., Corti, C., Creemers, R., Crochet, P.-A., Isailović, J.C., Denoël, M., Ficetola, G.F., Gonçalves, J., Kuzmin, S., Lymberakis, P., de Pous, P., Rodríguez, A., Sindaco, R., Speybroeck, J., Toxopeus, B., Vieites, D.R., Vences, M., 2014. Updated distribution and biogeography of amphibians and reptiles of Europe. Amphibia-Reptilia 35, 1-31.

Sket, B., Trontelj, P., 2008. Global diversity of leeches (Hirudinea) in freshwater. Hydrobiologia 595, 129-137.

Spotila, J.R., 1972. Role of temperature and water in the ecology of lungless salamanders. Ecol. Monogr. 42, 95-125.

Stead, J.E., Pope, K.L., 2010. Predatory leeches (Hirudinida) may contribute to amphibian declines in the Lassen Region, California. Northwestern Naturalist 91, 30-39.

Stegen, G., Pasmans, F., Schmidt, B.R., Rouffaer, L.O., Van Praet, S., Schaub, M., Canessa, S., Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., Martel, A., 2017. Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. Nature 544, 353-356.

Tiberti, R., Gentilli, A., 2010. First report of freshwater leech *Helobdella stagnalis* (Rhyncobdellida: Glossiphoniidae) as a parasite of an anuran amphibian. Acta Herpetol. 5, 255-258.

Trauth, S.E., Neal, R.G., 2004. Geographic range expansion and feeding response by the leech *Macrobdella diplotertia* (Annelida: Hirudinea) to wood frog and spotted salamander egg masses. Journal of the Arkansas Academy of Science 58, 139-141.

Veith, M., Viertel, B., 1993. Veränderungen an den Extremitäten von Larven und Jungtieren der Erdkröte (*Bufo bufo* L.): Analyse möglicher Ursachen. Salamandra 29, 184-199.

Wake, D.B., 2013. The enigmatic history of the European, Asian and American plethodontid salamanders. Amphibia-Reptilia 34, 323-336.

Wells, K.D., 2007. The ecology and behavior of amphibians. The University of Chicago Press, Chicago and London.

Wilkin, P.J., Scofield, A.M., 1990. The use of a serological technique to examine host selection in a natural population of the medicinal leech, *Hirudo medicinalis*. Freshwater Biology 23, 165-169.

Table 1: Data of each single monitored cave inhabited by *Hydromantes* species. In this table are shown: Coordinates; Elevation (meters a.s.l.); Length of the explored cave area (meters); average values (±ES) of the water parameters (pH, permanent Hardness and NO_2); number of the examined salamanders; total number of leeches observed; average frequencies (±ES) of the parasitized salamanders. * indicate one single data collection.

Cave	***Hydromantes* species**	**Coor_X**	**Coor_Y**	**Elevation**	**Explored area**	**pH**	**Hardness (°dH)**	**NO_2 (mg/L)**	**Examined salamanders**	**Total leeches**	**Parasitized salamanders (%)**
Cave_fla1	*H. flavus*	9.59	40.49	267	147	7.8 ± 0.6	20	30 ± 2	112	67	31.06 ± 8.02
Cave_fla2	*H. flavus*	9.52	40.46	1029	105	7.87 ± 0.53	18.33 ± 1.67	20.00 ± 1.53	675	33	5.94 ± 2.01
Cave_fla3	*H. flavus*	9.61	40.51	116	99	7.73 ± 0.35	16.67 ± 3.33	101.67 ± 7.64	17	0	
Cave_fla4	*H. flavus*	9.61	40.51	116	15	8.00 ± 0.4	20.00	10.00 ± 1.33	12	0	
Cave_fla5	*H. flavus*	9.64	40.56	777	12				23	12	28.33 ± 13.84
Cave_fla6	*H. flavus*	9.61	40.51	107	18	7.60 ± 0.4	17.50 ± 2.5	10.00	2	0	
Cave_fla7	*H. flavus*	9.65	40.54	265	54				5	0	
Cave_fla8	*H. flavus*	9.68	40.58	94	48	8.00 ± 0.4	18.33 ± 1.67	33.33 ± 1.67	22	0	
Cave_fla9	*H. flavus*	9.62	40.55	902	9				32	3	9.37*
Cave_fla10	*H. flavus*	9.64	40.57	954	18				39	0	
Cave_fla11	*H. flavus*	9.53	40.47	679	45	8.4*	20*	0*	58	2	1.14 ± 1.14
Cave_fla12	*H. flavus*	9.68	40.56	116	24				7	0	
Cave_sup1	*H. supramontis*	9.34	40.08	937	90				40	5	10*
Cave_sup2	*H. supramontis*	9.33	40.17	142	30				170	0	
Cave_sup3	*H. supramontis*	9.29	40.15	206	36				191	0	
Cave_sup4	*H. supramontis*	9.36	40.19	200	150				81	0	
Cave_imp1	*H. imperialis*	8.57	39.53	180	33				304	0	
Cave_imp2	*H. imperialis*	9.27	39.51	833	39				170	3	2.14 ± 1.50
Cave_imp3	*H. imperialis*	9.27	39.51	820	72				25	1	3.33 ± 3.33
Cave_sar1	*H. sarrabusensis*	9.26	39.17	765	6				261	5	0.38 ± 0.26
Cave_sar2	*H. sarrabusensis*	9.26	39.17	780	6				26	0	
Cave_sar3	*H. sarrabusensis*	9.26	39.17	718	6				3	0	
Cave_gen1	*H. genei*	8.25	39.21	200	36				23	0	
Cave_gen2	*H. genei*	8.31	39.15	412	139				266	0	
Cave_gen3	*H. genei*	8.44	39.07	234	117				47	0	
Cave_gen4	*H. genei*	8.31	39.15	440	12				18	0	

Figure 1. Two adults of *Batracobdella algira*. (A) parasitizing *Hydromantes flavus*, (B) attached to a stone.

Figure 2. Boxplots showing BCI of *Hydromantes*. On left side (0) BCI of salamanders free from leeches, while on the right side (1) BCI of the parasitized salamanders.

Figure 3. Distribution of studied sites for *Hydromantes flavus*. The map shows the altitude levels of the studied area; the polygon represents the whole distribution area of *H. flavus*. Yellow circles indicate sites where *B. algira* was not observed, while green squares indicate sites in which the leech was present.

Figure 1

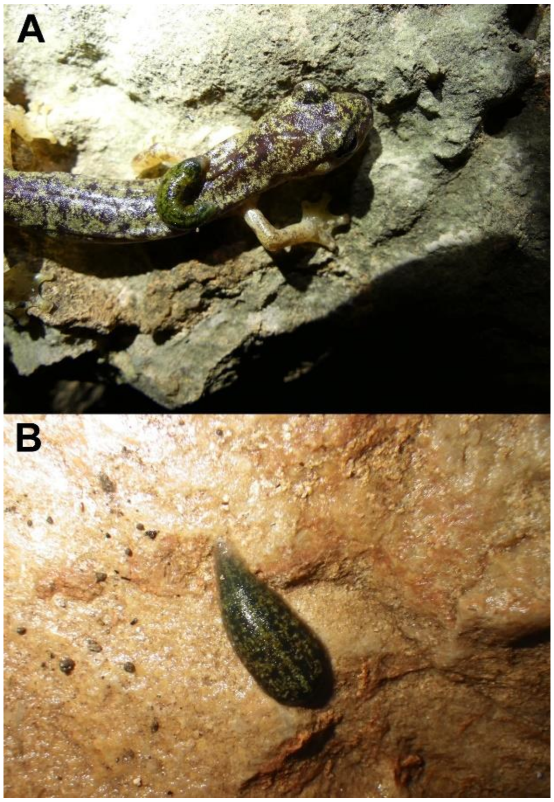


Figure 2

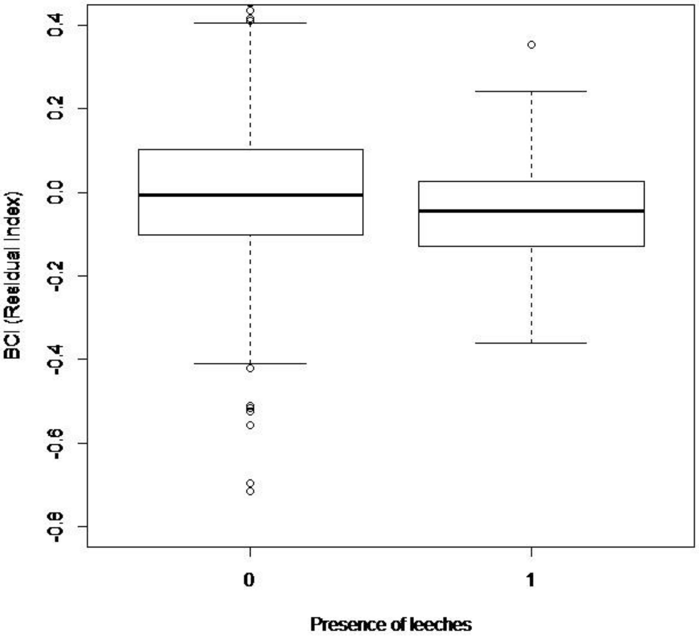
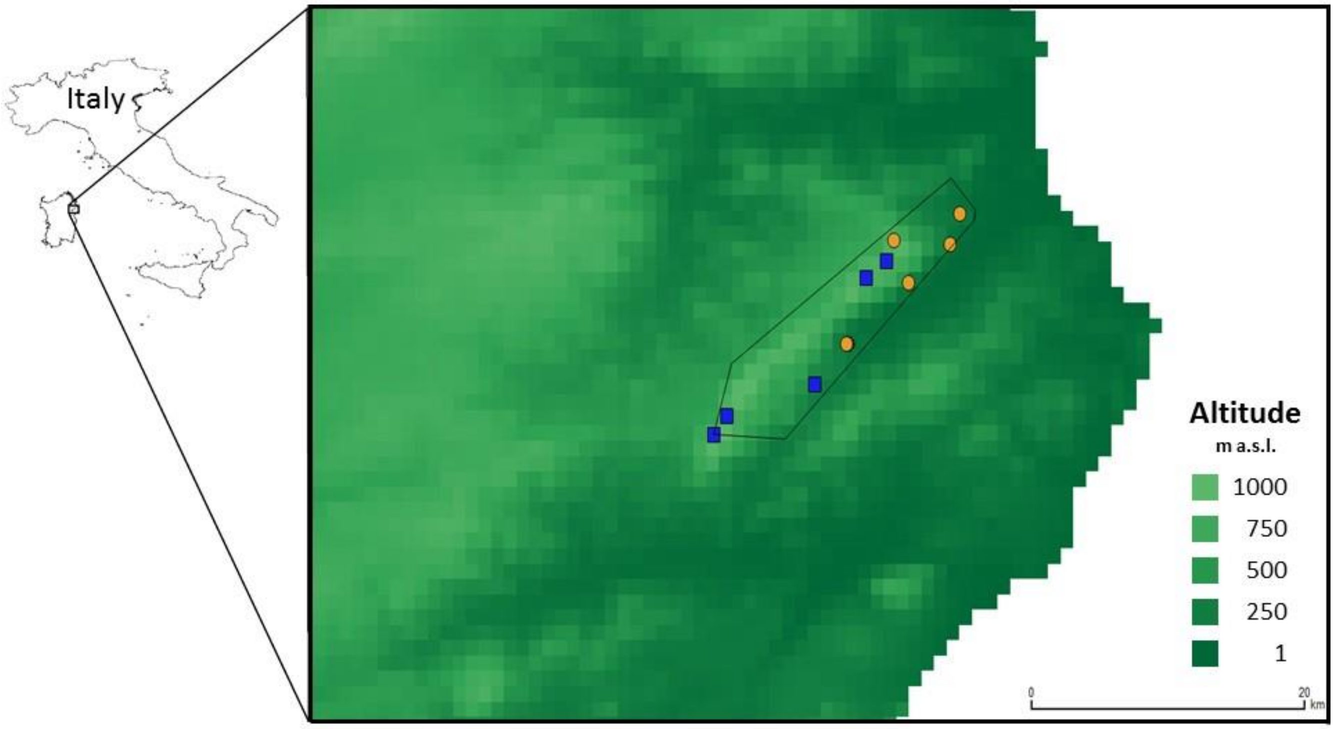


Figure 3



Chapter VIII

NORTH-WESTERN JOURNAL OF ZOOLOGY 11 (2): 324-330
Article No.: 151505

http://biozoojournals.ro/nwjz/index.html

First data on nesting ecology and behaviour in the Imperial cave salamander *Hydromantes imperialis*

Enrico LUNGHI[1,2,3]*, Rosalba MURGIA[4,5], Gianni DE FALCO[4],
Salvatore BUSCHETTU[4], Cinzia MULAS[6], Manuela MULARGIA[7],
Claudia CANEDOLI[8], Raoul MANENTI[9] and Gentile Francesco FICETOLA[8,10,11]

1. Natural Oasis, Via di Galceti 141, 59100 Prato, Italy.
2. Universität Trier Fachbereich VI Raum- und Umweltwissenschaften Biogeographie,
Campus I, Gebäude N Universitätsring 15, 54286 Trier, Germany.
3. Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy.
4. Speleo Club Oristanese, Via Canepa 8, 09170 Oristano, Italy.
5. IMC-International Marine Centre, Torregrande, 09170 Oristano, Italy.
6. Gruppo Grotte Ogliastra, Via Pascoli snc, 08046 Perdasdefogu, Italy.
7. Speleo Club Nuoro, Via Tunisi 3, 08100 Nuoro, Italy.
8. Dipartimento di Scienze dell'Ambiente e del Territorio e di Scienze della Terra,
Università degli Studi di Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy.
9. Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy.
10. Laboratoire d'Ecologie Alpine (LECA), Université Grenoble-Alpes, F-38000 Grenoble, France.
11. CNRS, LECA, F-38000 Grenoble, France.
*Corresponding author, E. Lunghi, E-mail: enrico.arti@gmail.com

Received: 24. November 2014 / Accepted: 03. May 2015 / Available online: 11. November 2015 / Printed: December 2015

Abstract. During the dry season, the European Plethodontid salamanders (genus *Hydromantes*) usually occupy underground environments (i.e. caves), where they can find cold temperatures and high moisture. *Hydromantes* breed in hypogean environments, where they usually lay eggs in hidden shelters. Mothers perform a long-lasting parental care of the eggs, which also continues after hatching. Due to the cryptic habitat and behaviour, their breeding biology is poorly known. Most of the available data refer to observations in captivity, while data from wild populations are scarce and deal with the findings of single nests. Here we report the first study on the Imperial cave salamander *H. imperialis* nesting ecology and behaviour, by performing quantitative observations on multiple nests. We found four nests in a cave located in Central Sardinia. We monitored them through five months, recording environmental features. Nests were associated with cold, humid and dark sectors of the cave, but sectors with nests did not show greater climatic stability than the superficial ones. Nests were continuously attended by females; temporary desertion became more frequent when temperatures were high and it was later in the season. Newborns were attended by their mothers for up to 52 days after hatching. The comparison of breeding biology across multiple *Hydromantes* species suggests earlier hatch in population/species living in warmer areas, with similar post-hatch brood attendance among species.

Keywords: Microclimate, temperature, humidity, parental cares, brood attendance, cave fauna, amphibians.

Introduction

Cave salamanders of the genus *Hydromantes* Gistel, 1848 are fully terrestrial and lay eggs in subaerial environments (Lanza et al. 2006). Such features force these salamanders to live and reproduce in environments with specific microclimate, such as cold temperature and high moisture (Ficetola et al. 2012, Lunghi et al. 2014a). In Europe there are eight species of *Hydromantes*, five of which are endemic to Sardinia (Sillero et al. 2014). Seven of these species belong to the sub-genus *Speleomantes*, while one species belongs to the sub-genus *Atylodes* (Wake 2013). In Mediterranean ar-eas, cave salamanders can exploit both epigean and hypogean environments; however outdoor environments are avoided during the hottest and driest seasons (Cimmaruta et al. 1999, Lanza et al. 2006). On the other hand, hypogean environments represent a shelter in which *Hydromantes* can find suitable microclimatic conditions even during unfavourable periods (e.g. hot and dry summers) (Salvidio et al. 1994, Vignoli et al. 2008, Ficetola et al. 2012, Lunghi et al. 2014a). In these habitats, *Hydromantes* select the most appropriate zone to breed and to perform their long-lasting egg care (Stefani & Serra 1966, Papinuto 2005, Lunghi et al. 2014b). Until few years ago the knowledge on the reproduction of these salamanders was quite scarce (Lanza et al. 2006). Recent studies per-formed both in natural and semi-natural condi-tions improved our understanding of their reproductive biology, and showed the complexity of parental cares in these salamanders (Stefani & Serra 1966, Papinuto 2005, Lanza et al. 2006, Oneto et al. 2010, Oneto et al. 2013, Lunghi et al. 2014b, Oneto et al. 2014). Observations in both natural and controlled environments can be useful to understand the breeding behaviour of these salamanders. Oneto et al. (2010, 2014) recently studied the parental cares of *H. strinatii*, by performing observations on individuals under semi-natural conditions. *H. strinatii* females constantly attended the nests, keeping their body in touch with the eggs (Oneto et al. 2010). During egg care, the mother rarely moved from the nest; however, temporary desertion of the nest became more frequent after all eggs had hatched (Oneto et al. 2010). Post-hatching parental care lasted six weeks, during which newborns tried to keep their body in touch with the mother as much as they could. During this time, the mothers moved away from the nest only occasionally, and they actively defended the broods against conspecific intruders (Oneto et al. 2014). However, observations performed in wild conditions, and on a wider range of species, are needed to assess the generality of results obtained under controlled conditions. Until now, information on the breeding behaviour of *Hydromantes* in nature was extremely limited and available for three species only. Papinuto (2005) observed a clutch of *H. genei* in an abandoned mine, and noticed that the mother stayed constantly close to the eggs, rarely moving away from the nest and only for a short time. Newborns left the nest after two weeks. Lunghi et al. (2014b) reported two observations: a clutch of *H. flavus* in a deep zone of a cave system and a clutch of *H. italicus* in an abandoned mine. In these species, the mothers were never observed far from the nest, and post-hatch parental cares lasted from one week to one month, in *H. flavus* and *H. italicus*, respectively. However, due to the difficulty of accessibility and finding cave salamander nests, all the studies from natural environments reported observations on single nests, without possibilities to compare the nesting behaviour of different females of the same population, and only recorded few quantitative data.

Here we report the first study on the Imperial cave salamander *H. imperialis* nesting ecology and behaviour, by performing quantitative observations on multiple nests. First, we assessed whether the selection of nesting areas was related to cave environmental features. Specifically, we tested two non-exclusive hypotheses: *i*) cave sectors with eggs are characterized by high humidity, low light and temperature (as observed for the adults of other *Hydromantes* species (Ficetola et al. 2013)); *ii*) sectors with eggs have particularly stable microclimatic conditions (Lunghi et al. 2014b). Second, we quantitatively recorded the brooding behaviour of females, and evaluated whether it was related to specific microhabitat features (Oneto et al. 2010). In particular we assessed whether the periods in which females leave the nest unattended (Oneto et al. 2010) were related to specific environmental conditions.

Material and methods

Surveys were conducted during a herpetological field activity performed in a cave located in Oristano District in Central Sardinia (39° 53'N, 8° 58' E). The presence of *H. imperialis* was assessed using visual encounter surveys in June 2014, following the same procedure described by Ficetola et al. (2013). After the first observation of clutches, the site was repeatedly surveyed until the com-plete abandonment of all nests by mothers and newborns: from 15 June to 25 October 2014 the cave was surveyed 20 times. Eggs or brooding females were never touched or directly disturbed. Using a metric wheel we measured the maximum depth of the cave (from the entrance to the deepest point) and determined the position of each nest (distance from the entrance and height from the ground). We also measured the height and width of each nest's entrance. Starting from the entrance, we divided the cave environment in 3-meter sectors. This size approximately corresponds to the size of *Hydromantes* home-ranges during their underground activity (Salvidio et al. 1994). In each sector we recorded microclimatic parameters [air temperature, relative humidity and max/min incident light (illuminance)] using an EM882 multi-function Environmental Meter (PCE Instruments; minimum illuminance 0.01 lux). Data on temperature and relative humidity was recorded 17 times through the whole period. As previous studies suggest that *Hydromantes* select cold, humid and dark sectors of caves (Ficetola et al. 2012, Lunghi et al. 2014a), we used one-tailed t-test to evaluate whether nest sites were associated with these microhabitat conditions. We used Levene's test to assess whether eggs were associated with areas with particularly stable microclimate (as hypothesized by Lunghi et al. 2014b), by testing whether sectors in which nest were present varied significantly in respect to temperature and humidity, compared to the first sector of the cave (parameters recorded within the cave, 3 m from the cave entrance).

Moreover, we used Generalized linear mixed models (GLMM) assuming binomial error distribution to test if mothers move away from their nest under specific microclimatic features or in particular periods. To analyse mother's choice to move away from nest, we considered as dependent variable female attendance to the nest dur-ing each survey. As independent variables, we considered microclimatic conditions (temperature, relative humid-ity), day of survey, and presence of hatched eggs. Nest identity was considered as random factor. We used the Aikake's Information Criterion corrected for small sample size (AICc) to identify the combination of variables that explains attendance/nonattendance of females to the nest (Stephens et al. 2007). If a model showed AICc higher than a simpler nested model, it was not included among the candidate models (Richards et al. 2011). We tested significance of each variable included into the best model (i.e. the model with lowest AICc) using a likelihood ratio test. Prior to performing analyses, humidity was transformed using square-root arcsine, while illuminance was log-transformed to better meet the assumptions of parametric analyses. All statistical analysis were performed in R 3.1.1 (R Development Core Team 2014).

Figure 1. Seven eggs provided with peduncle that draws them close (third nest; 15 June).

Results

We found four clutches of *Hydromantes imperialis* during the first survey performed on 15 June 2014. The maximum cave depth was 30 m. Nests were located at different points through the development of the cave, into small holes present on cave walls (Table 1). Sectors with nests showed significantly higher humidity ($t_{7.03}$ = -2.26, *P* = 0.029) and lower illuminance (t_{7} = 2.05, *P* = 0.04) compared to sectors without nests. Furthermore, sectors with nests tended to have lower tempera-ture, although this test was marginally non-significant ($t_{8.68}$ = 1.66; *P* = 0.067). Considering tem-perature, not a single nest was located in cave sec-tors with lower variability than the first sector of the cave (Levene's test: all *P* ≥ 0.29). Similarly, sec-tors with nests did not show lower variability than the first sector in respect to humidity (all *P* ≥ 0.10).

Clutches were composed of 7-11 eggs each, all provided with a peduncle (Fig. 1). During the first surveys (June) eggs were transparent and the embryos inside were perfectly observable (Fig. 2 and Fig. 3a). In early July almost half of the yolk was absorbed and embryos started to have a defined shape, allowing us to recognize various morphology features of the animals. In late July-early August embryos had filled up all the available space inside the eggs, making their aspect more dark and opaque (Fig. 3b-c). The first newborns were observed on 16 August in the nest furthest from the entrance, while in the other nests the first newborns appeared on 25 and 28 August (Table 1; Fig. 3d). Eggs from the same nest did not hatch simultaneously, but required up to 10 days for hatch completion (Table 1).

Figure 2. First nest during a survey on 15 June: eggs show high transparency and embryos and yolk are clearly recognisable.

Table 2. Candidate AIC models created using the following dependent factors: Day (day of survey); Eggs hatch (presence of hatched eggs); Temp (air temperature); Hum (relative humidity); Day*T (interaction between day of survey and air temperature).

Day	Eggs hatch	Temp	Hum	Day*T	df	AICc	delta	weight
-1.612		-4.615		0.08958	5	31.0	0.00	0.71
-0.04962		1.0960			4	33.8	2.77	0.18
					2	34.7	3.70	0.11

During surveys mothers constantly took care of their broods, rarely moving away from them (Table 1). The best-AIC model suggested that nests were more frequently unattended by mothers when temperature was higher (nearly significant effect; χ_{21} = 3.404, *P* = 0.06) and in the last part of breeding season (χ_{21} = 4.52, *P* = 0.03) (Table 2). A significant positive interaction between

Table 1. Position and morphology of nests and data on their occupancy: Distance (distance from entrance); Height (height from the ground); Size of the nest-hole (height and width of the entrance of nests); First born (date of first eggs hatched); All born (date when all eggs were hatched); Empty (date when the nest was completely uninhabited); Nest unat-tended (date when mother left unattended the nest). * The fourth nest was not checked between 14 and 26 September due to the high risk of stepping on salamanders that were covering the ground floor.

	Distance from entrance	Height	Size of the nest-hole	First born	All Born	Empty	Nest unattended
1st nest	14.8 m	100 cm	2 x 2 cm	28/08	3/09	25/10	17/08
2nd nest	17.6 m	50 cm	5 x 2 cm	25/08	3/09	20/09	7/09
3rd nest	20 m	20 cm	3 x 2 cm	28/08	7/09	13/09	7/09
4th nest	21.7 m	150 cm	4 x 3 cm	16/08	25/08	27/09*	9/08

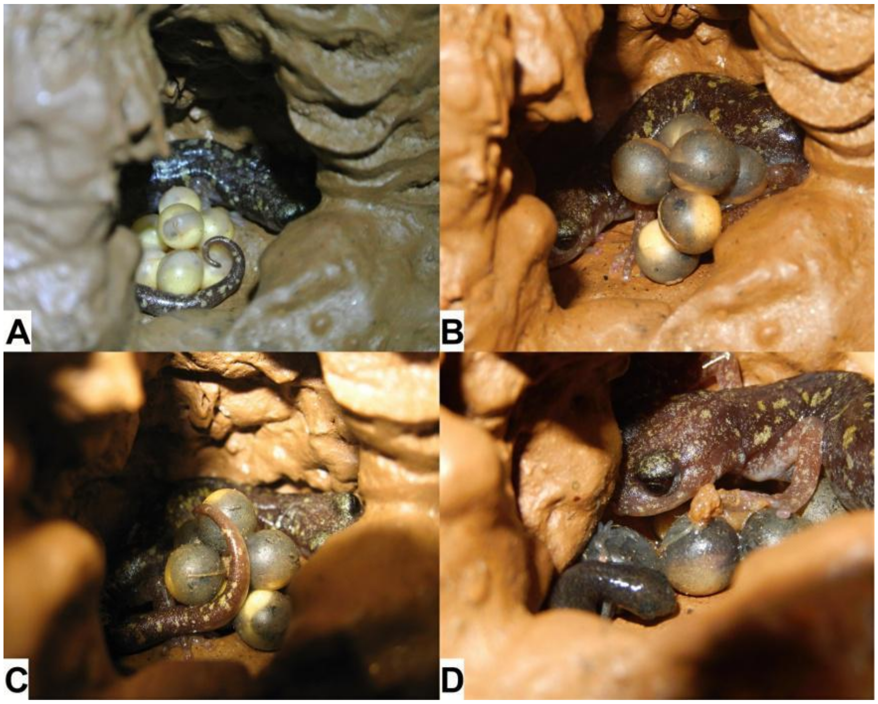


Figure 3. Third nest observed at different times. A) On 23 June eggs were transparent and embryos in early developmental stage are detectable; B) On 9 August embryos were grown up and showed an advanced body-shape; C) On 17 August yolks were continu-ously less visible due to embryos development; D) First salamander hatched on 28 Au-gust.

Table 3. Mean hatch date and length of post-hatch parental cares, in different studies on *Hydroman-tes* nests, from natural and semi-natural environments. Mean annual temperature is the mean an-nual temperature of the outdoor environment, obtained from the world climate (Hijmans et al. 2005).

Species	Study	Mean hatch date	Number of clutches	Mean outdoor temperature	Post-hatch parental cares (days)
H. genei	(Papinuto 2005)	31 August	1	13.7	14
H. imperialis	present study	24 August	4	15.0	6-52
H. italicus	(Lunghi et al. 2014b)	11 September	1	12.0	30
H. flavus	(Lunghi et al. 2014b)	26 August	1	15.9	7
H. strinatii	(Oneto et al. 2010, 2014)	6 October	3	10.2	42

temperture and day of survey indicated that the effect of temperature was particularly important later in the season ($\chi_{21} = 5.10$, $P = 0.024$).

Before leaving the nests unattended, females changed eggs position, by moving them to the deepest and most hidden part of the nest-holes. After hatching, newborns followed their mothers into and outside the nests for 6-52 days (mean: 27). Sometimes some newborns explored areas just outside the nest, while the mother remained inside with the rest of her brood. This behaviour was observed 6 times, most frequently for the deepest nest: we found newborn salamanders outside of the fourth nest 4 times and only one time outside of the first and the second. By the 25 October all nests were abandoned.

Discussion

This study describes the first finding of multiple nests for the genus *Hydromantes* (*Speleomantes*) under natural conditions, by analysing the first collection of quantitative parameters on environmental features potentially related to nest site choice and parental care behaviour for the genus. Before this study, *Hydromantes* clutches in natural environments have been observed only few times (Stefani & Serra 1966, Papinuto 2005, Lunghi et al. 2014b), while clutches of *H. imperialis* were only observed in captivity (Lanza et al. 2006). Clutches of *H. imperialis* were inside small holes located on cave walls, as observed by studies on the other species (Stefani & Serra 1966, Papinuto 2005, Lunghi et al. 2014b). Females look for hidden places in order to protect their eggs as much as possible (Lanza et al. 2006, Pastorelli & Laghi 2006, Oneto et al. 2010). Normally, observed females laid their eggs not too close to the entrance, in ar-eas that present suitable microclimatic features (cool temperature, high moisture and darkness), with a pattern of microhabitat selection similar to the one observed by individuals of other *Hydro-mantes* species (Ficetola et al. 2013, Lunghi et al. 2014a). During previous observations, females with eggs were always relatively far from the en-trance, and previous work suggested that these areas might be characterized by high microcli-matic stability (Lunghi et al. 2014b). Females of *H. imperialis* also selected nest-holes relatively far from the entrance, and the recording of microcli-matic parameters in multiple occasions allowed to test this hypotheses. Actually, the cave sectors selected for nesting did not show particularly high microclimatic stability, compared to the areas of the cave closest to the surface, i.e. where the high-est microclimatic variability is expected (Romero 2009). This suggests that the microclimatic stability, that is characteristic of the whole cave environment (Romero 2009), is sufficient to maintain suitable microhabitat conditions during the several months required for egg development (Lanza et al. 2006). To lay their eggs, females probably select the first suitable area of the cave (cold and humid enough) which is at the same time not too far from food resources (Ficetola et al. 2013). This choice might help females to obtain food without distancing too much from their clutches. Female choice might therefore result from the trade-off between environmental features, clutch security and food availability.

Clutch size was similar to the one previously reported for other species (Lanza et al. 2006). For all the nests, hatching was relatively synchronous, toward the end of the summer, similar to what was observed in all the other *Hydromantes* species (Papinuto 2005, Lanza et al. 2006, Lunghi et al. 2014b) (Table 3). The end of the summer may be an extremely suitable period for juveniles, as it generally corresponds to the end of the dry sea-son, and thus juveniles may move outside of the caves, where food availability is higher. Neverthe-less, across the different *Hydromantes* species, av-erage hatch date was significantly related to the mean annual temperature: eggs hatched signifi-cantly earlier in populations living in the warmest areas (Table 3; linear regression, weighted for the number of clutches, between mean hatch date and mean annual temperature: $F_{1,3} = 52.2$, $P = 0.005$, $R_2 = 0.93$). Cave temperature is strongly related to the mean annual outdoor temperature (Romero 2009), and in amphibians high temperature increases metabolism, accelerating embryo development (Morrison & Hero 2003). Overall, the good synchrony of hatches, with earlier hatch in the warmer locali-ties, suggests that the whole breeding cycle may follow a similar seasonal pattern across the whole *Hydromantes* genus.

Within a nest, eggs hatched over a relatively long period (Table 1). As was observed for *H. strinatii* (Oneto et al. 2010), eggs from all 4 nests of *H. imperialis* never hatched in the same day: a single clutch required up to 10 days for all newborns to come out of their eggs (Fig. 3d).

Mothers look carefully after their broods, attending and protecting them against predators and infections (Lanza et al. 2006, Oneto et al. 2014). However, it is already known that some-times mothers leave their nest unattended (Oneto et al. 2010). From our observations we could see that the mothers' choice to distance for a while from their nest was related to some environmental features. Mothers left nests mostly when air tem-perature was relatively high and during later pe-riods. However, before moving away, mothers moved the eggs in a concealed part of the nest. During our observations we always saw mothers curled on their eggs inside holes; however, in two instances, before leaving the nest, mothers hid the eggs in the backside of nest walls, making their detection extremely difficult (or impossible). For all species, after hatching, mothers continued to occupy the nest with newborns in average for one month, with great differences among females (min 6 days, max 52 days). The post-hatch nest attendance overlaps with what has been observed in other species (Table 3), suggesting a lack of inter-specific differences in post-hatching behaviour, but rather some variation among females, which could depend on both environmental features and physical status. As observed by Oneto et al. (2010) newborns never feed before leaving definitively the nest. Time of permanence inside the nest after hatching might thus also depend on how long would last the yolk absorption by newborns (Lunghi et al. 2014b). However, in contrast to the observations performed on *H. strinatii* (Oneto et al. 2010), in our study newborns were more inclined to explore areas around the nest, even without their mother, as also reported by Papinuto (2005). This difference in behaviour might be due to inter-specific behavioural differences or to the different environmental conditions (natural vs. semi-natural) which may influence the behaviour of both mother and newborns.

Our study adds new insights on the brooding behaviour of European cave salamanders; however information remains limited to a handful of species (Table 3), and for each species no more than one population has been investigated. Fur-ther researches, comparing the results of multiple nests and multiple populations will be necessary to better understand the complex breeding behaviour of these cryptic salamanders.

Acknowledgements. We are grateful to many friends that took part in this long survey: Valentina Mirimin, Sandro Corona, Francesca Fais, Sandro Murru, Silvestro Papinuto, Francesco Papinuto, Denise Atzei, Luca Buschettu, Giancarlo Cadeddu, Rita Cadeddu, Alfredo Camedda, Franca Contu, Gabriele Espis, Salvatore Manca, Carla Meli, Gianfranco Muzzetto, Simona Putzolu, Cristina Santona, Loredana Tegas. This study was allowed by the Italian Ministry of the Environment, out withstanding of the DPR 357/97 , Prot. 0040002.

References

Cimmaruta, R., Forti, G., Nascetti, G. Bullini, L. (1999): Spatial distribution and competition in two parapatric sibling species of European plethodontid salamanders. Ethology Ecology & Evolution 11: 383-398.

Ficetola, G.F., Pennati, R. Manenti, R. (2012): Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. Amphibia-Reptilia 33: 251-259.

Ficetola, G.F., Pennati, R. Manenti, R. (2013): Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? Population Ecology 55: 217-226.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G. Jarvis, A. (2005): Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology 25: 1965-1978.

Lanza, B., Pastorelli, C., Laghi, P. Cimmaruta, R. (2006): A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). Atti del Museo Civico di Storia Naturale di Trieste 52.

Lunghi, E., Manenti, R. Ficetola, G.F. (2014a): Do cave features affect underground habitat exploitation by non-troglobite species? Acta Oecologica 55: 29-35.

Lunghi, E., Manenti, R., Manca, S., Mulargia, M., Pennati, R. Ficetola, G.F. (2014b): Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments. Salamandra 50: 105-109.

Morrison, C. Hero, J.M. (2003): Geographic variation in life-history characteristics of amphibians. Journal of Animal Ecology 72: 270-279.

Oneto, F., Ottonello, D., Pastorino, M.V. Salvidio, S. (2010): Posthatching Parental Care in Salamanders Revealed by Infrared Video Surveillance. Journal of Herpetology 44: 649-653.

Oneto, F., Ottonello, D., Pastorino, M.V. Salvidio, S. (2013): Nuovi dati sul comportamento di cura parentale nei caudati: *Hydromantes* (*Speleomantes*) *strinatii* (Amphibia, Plethodontidae). Atti IX Congresso Nazionale della Societas Herpetologica Italica, Bari-Conversano 26-30 settembre 2012. pp. 204-207. In: Scillitani, G., Liuzzi, C., Lorusso, L., Mastropasqua, F. Ventrella, P. (eds), Atti IX Congresso Nazionale della Societas Herpetologica Italica, Bari-Conversano 26-30 settembre 2012. Eds. Pineta, Conversano (Bari).

Oneto, F., Ottonello, D., Pastorino, M.V. Salvidio, S. (2014): Maternal care and defense of young by the plethodontid salamander *Speleomantes strinatii* (Aellen, 1951). Scripta Herpetologica: Studies on Amphibians and reptiles in honour of Benedetto Lanza. pp. 129-136. In Capula, M. Corti, C. (eds), Scripta Herpetologica: Studies on Amphibians and reptiles in honour of Benedetto Lanza. Edizioni Belvedere, Latina.

Papinuto, S. (2005): Sul ritrovamento ed il monitoraggio di una nidiata di *Speleomantes genei* (Temminck & Schlegel, 1838) (Amphibia Urodela Plethodontidae) in una galleria mineraria dell'Inglesiente (Sardegna sud-occidentale). Sardegna Speleologica 22: 3-6.

Pastorelli, C. Laghi, P. (2006): Predazione su *Speleomantes italicus* (Amphibia, Caudata, Plethodontidae) da parte di *Meta menardi* (Arachnida, Araneane, Metidae). Riassunti 6° Congresso Nazionale della Societas Herpetologica Italica. In Riassunti 6°

Congresso Nazionale della Societas Herpetologica Italica. Stilgrafica, Roma.

R Development Core Team. (2014): R: A language and environment for statistical computing. Foundation for Statistical Computing, Vienna.

Richards, S.A., Whittingham, M.J. Stephens, P.A. (2011): Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. Behavioral Ecology and Sociobiology 65: 77-89.

Romero, A. (2009): Cave biology. Cambridge University Press, New York.

Salvidio, S., Lattes, A., Tavano, M., Melodia, F. Pastorino, M.V. (1994): Ecology of a *Speleomantes ambrosii* population inhabiting an artificial tunnel. Amphibia-Reptilia 15: 35-45.

Sillero, N., Campos, J., Bonardi, A., Corti, C., Creemers, R., Crochet, P.-A., Isailović, J.C., Denoël, M., Ficetola, G.F., Gonçalves, J., Kuzmin, S., Lymberakis, P., de Pous, P., Rodríguez, A., Sindaco, R., Speybroeck, J., Toxopeus, B., Vieites, D.R. Vences, M. (2014): Updated distribution and biogeography of amphibians and reptiles of Europe. Amphibia-Reptilia 35: 1-31.

Stefani, R. Serra, G. (1966): L'oviparità in *Hydromantes genei* (Temm. et Schl.). Bollettino di Zoologia 33: 283-291.

Stephens, P.A., Buskirk, S.W., Hayward, G.D. Del Rio, C.M. (2007): A call for statistical pluralism answered. Journal of Applied Ecology 44: 461-463.

Vignoli, L., Caldera, F. Bologna, M.A. (2008): Spatial niche of the Italian cave salamander, *Speleomantes italicus* (Dunn, 1923) (Plethodontidae, Amphibia), in a subterranean system of Central Italy. Italian Journal of Zoology 75: 59-65.

Wake, D.B. (2013): The enigmatic history of the European, Asian and American plethodontid salamanders. Amphibia-Reptilia 34: 323-336.

Chapter IX

Accepted in Salamandra, November 2017

Comparative reproductive biology of European cave salamanders (genus *Hydromantes*): nesting selection and multiple annual breeding

LUNGHI ENRICO[1,2,3,*], CLAUDIA CORTI[2], RAOUL MANENTI[4], BENEDETTA BARZAGHI[4], SALVATORE BUSCHETTU[5], CLAUDIA CANEDOLI[6], ROBERTO COGONI[7], GIOVANNI DE FALCO[5], FRANCESCA FAIS[5], ALESSANDRA MANCA[5], VALENTINA MIRIMIN[5], MANUELA MULARGIA[8], CINZIA MULAS[8], MARTINA MURARO[4], ROSALBA MURGIA[5,10], MICHAEL VEITH[1], GENTILE FRANCESCO FICETOLA[4,11]

1) *Department of Biogeography, University of Trier, Trier, Germany*
2) *Natural History Museum of University of Florence, Section Zoology "La Specola", Firenze,* Italy
3) *Natural Oasis, Prato, Italy*
4) *Department of Environmental Science and Policy, University of Milan, Milano, Italy*
5) *Speleo Club Oristanese,* ***Via Canepa 8,*** *09170* ***Oristano, Italy***
6) Department of Environment and Territor Science, University of Milan-Bicocca, Milano, Italy
7) *Unione Speleologica Cagliaritana, Quartu Sant'Elena (CA), Italy*
8) *Speleo Club Nuoro, Nuoro, Italy*
9) *Gruppo Grotte Ogliastra, Perdasdefogu (OG), Italy*
10) *Fondazione IMC - International Marine Centre – Onlus, 09170 Oristano*
11) Univ. Grenoble Alpes, CNRS, Laboratoire d'Écologie Alpine (LECA), *Grenoble, France*

Corresponding author: ENRICO LUNGHI, e-mail: enrico.arti@gmail.com

Correspondence: Biogeography Department, Trier University, Universitätsring 15, 54286 Trier, Germany; Tel.:+39 3391604627; +49 15905329542

Running title: Reproduction in European cave salamanders

Abstract

Information on the life history of European cave salamanders remains limited. Despite a handful of studies carried out both under natural and controlled conditions, one of the least known aspects concerns the reproductive and breeding behaviour. Here we present information on the breeding behaviour of eight European *Hydromantes* species collected during four years of intensive monitoring (2014-2017). We provide information on the breeding phenology, suggesting that the breeding seasons are likely linked to environmental variables. Nesting sites were used repeatedly in different years by different females. Our data indicated a seasonality of mating activity and the possibility of sperm storage. Furthermore, we report on the presence of a single large-sized oviductal egg in *H. imperialis*, possibly indicating invariable clutch size. Finally, we documented oviparity in *Hydromantes sarrabusensis*, the only species that was suggested to be viviparous.

Keywords

Speleomantes, Plethodontidae, Cave, Amphibia, Urodela, Mating behaviour, Mediterranean, Biospeleology, Seasonality

Introduction

European cave salamanders (genus *Hydromantes*; see WAKE 2013 for discussion on nomencalture) include eight species distributed in Italy and in a small part of South-Eastern France (LANZA et al. 2006a, SILLERO et al. 2014). The two main characteristics of these amphibians are the lack of lungs and direct development (LANZA et al. 2006b). Lack of lungs force these animals to fully rely on cutaneous and bucco-pharyngeal respiration only (LANZA et al. 2006b), which occurs efficiently only in specific environmental conditions, i.e., high moisture and cool temperature (SPOTILA 1972). Underground habitats (e.g., caves, mines, cracks and other epikarst-environments) are therefore optimal refuges from adverse outside environmental conditions (FICETOLA et al. 2012, CULVER & PIPAN 2014, LUNGHI et al. 2014a). Even though underground detection of salamanders is higher in summer, these habitats are exploited throughout the year, suggesting that they are extremely suitable for these species (LUNGHI et al. 2015a); moreover, besides the suitable microclimate, they mostly lack predators (PASTORELLI & LAGHI 2006, MANENTI et al. 2016, SALVIDIO et al. 2017a), and, therefore, represent an optimal choice to carry out a delicate life-phase such as reproduction.

Giving the elusive habits of *Hydromantes*, many of their life-history traits are still poorly known, and reproduction is among the less known traits. During the last years, several studies on the biology and physiology of these salamanders have been performed to better understand their reproduction (all related papers are gathered in the latest review written by LANZA et al. 2006). Sexual maturity in *Hydromantes* is generally reached at the third or fourth year, while in the big-sized Sardinian species is likely reached a bit later (SALVIDIO 1993, SALVIDIO et al. 2017b). Courtship has been observed throughout the year, while the deposition of spermatophores was observed only in autumn (for further details on courthship see also BRUCE et al. 2000, LANZA et al. 2006b). However, given that in these species the pachytene spermatocytes do not degenerate during the cold season, it is likely that European cave salamanders can mate throughout the year (MERTENS 1923, LANZA 1959).

Besides some physiological analyses, only a few studies performed in controlled conditions were focused on *Hydromantes'* breeding behaviour (DURAND 1967, LANZA et al. 2006b, ONETO et al. 2010, ONETO et al. 2014). Recently some authors monitored clutches in nature, improving the knowledge on this topic and allowing comparison with findings obtained in controlled conditions (PAPINUTO 2005, LUNGHI et al. 2014b, LUNGHI et al. 2015b, MURGIA et al. 2016). The first field report on the breeding behaviour was provided by PAPINUTO (2005), who monitored one clutch of *H. genei* in an abandoned mine. The female was observed curled up on her eggs for nearly five months until hatching occurred. This behaviour not only allows the transfer of her skin secretion to the eggs providing protection against bacteria and fungi, but also protects the nest from intruders (LANZA et al. 2006b, ONETO et al. 2014). Monitoring activities on clutches was also carried out on *H. italicus, H. flavus* and *H. imperialis* (LUNGHI et al. 2014b, LUNGHI et al. 2015b, MURGIA et al. 2016). Findings of these studies were generally consistent with those observed for *H. strinatii* in controlled conditions (ONETO et al. 2010, ONETO et al. 2014). Embryos require a long time to develop before being ready to hatch, and during this span of time the mother rarely leaves the clutch unattended (LANZA et al. 2006b, LUNGHI et al. 2014b, ONETO et al. 2014). After hatching, the mother provides parental care to the hatchlings for some weeks before they leave the nest (ONETO et al. 2010, LUNGHI et al. 2015b). MURGIA et al. (2016) also observed that the highest density of nest sites was found in a section of a cave showing the most stable microclimate, characterized by high humidity levels (close to saturation) and a yearly fluctuation of temperature of only 2°C.

Nevertheless, many aspects of the breeding biology of cave salamanders remain poorly known. *a)* Does *Hydromantes* breed more than once a year? All studies on European cave salamanders carried out in nature report that only one breeding season occurs during the warmer periods of the year (PAPINUTO 2005, LUNGHI et al. 2014b, LUNGHI et al. 2015b, MURGIA et al. 2016). *b)* Do cave salamanders show nest site selection? Observations performed in nature described the location chosen by females to nest, but it is not known whether and how females select the breeding site, nor if nest-site fidelity occurs. The present long-term study attempts to answer the above mentioned questions, adding unpublished insights to the behaviour of these salamanders. Furthermore, for the first time oviparity in *Hydromantes sarrabusensis* is reported, a species that was thought to be viviparous (LANZA et al. 2006b).

Materials and Methods

Data collection

During a period of four years (2014 - 2017) we performed extensive field activities with the aim to cover as much as possible the distribution range of the eight *Hydromantes* species (LANZA et al. 2006a). Overall, we surveyed >150 underground sites (caves and mines) where the salamanders were present. Twenty-four of them (seven for *H. flavus,* three for *H. supramontis,* three for *H. imperialis,* three for *H. genei*, three for *H. sarrabusensis* and five for *H. italicus*) were repeatedly surveyed during the entire study period. Surveys on *H. flavus* were performed during all seasons, while for the other species they were mainly concentrated in spring/early summer and autumn. During each survey, we first checked if salamanders were performing courtship behaviour. These salamanders adopt the so called "vaccination": after mounting on the female, males use their pre-maxillary teeth to produce scratches on the females skin in order to transfer the edonic secretions produced by their mental gland directly in the female circulatory system; all these actions are performed with the purpose to increase the female interest and make her willing to collect the spermatophora (LANZA et al. 2006b). We then measured snout–vent length (SVL, using a plastic transparent rule) to the nearest mm in females salamanders, considering gravid those showing fully developed eggs (~3-5 mm in diameter) visible in the abdominal cavity; when possible, we also recorded salamanders' distance from the cave entrance. Using Linear Mixed Models, we tested whether gravid females occupy different cave areas when compared to the non-gravid ones. For this analysis we used the square-root transformed distance from the cave entrance as dependent variable, female condition (gravid/non-gravid) and species identity as dependent variables, and cave identity as random factor. We then used Linear Models to test whether gravid females showed larger size. To avoid pseudo-replication, for this analysis we only used the females collected during the survey where the highest number of individuals was measured, plus individually marked females (LUNGHI & VEITH 2017). In this analysis, SVL was used as dependent variable, while condition (gravid/non-gravid), species identity and the interaction between these two, as independent variables.

Extended monitoring

Hydromantes imperialis. One cave located in the Oristano Province was monitored for 3 years (2014 to 2016). During 2014, the monitoring of clutches started in early summer and lasted until late summer, when nests became empty (LUNGHI et al. 2015b). In 2015 and 2016, surveys always started in January and ended when nests were empty: we performed 19 surveys in 2015 and 9 in 2016. We used an endoscope to check for mothers and eggs inside rock cracks, when inspection by bare eyes was not possible. On September 14th, 2016, four gravid females were marked using Visual Implant Elastomers (SALVIDIO 2013).

Hydromantes sarrabusensis. On May 17th, 2016, we found in two sites some females with externally visible developed eggs. One female per site, apparently in advanced pregnancy, was placed in a terrarium on site. Soil and stones were placed in the terrarium to imitate the natural substratum and holes were drilled to allow air circulation. Females were regularly fed with invertebrates captured on site (Diptera, Coleoptera and Orthoptera).

Results

Gravid females

Out of 787 observed females, 112 from 29 different underground environments were observed carrying eggs in their abdomen (Table 1). The number of externally visible eggs ranged from six to ten (Fig. 1a); in two cases (in *Hydromantes imperialis*) females showed just one single large egg (~10 mm, Fig. 1b). Gravid females were found at an average depth (± SD) of 19.99 m ± 2.90 (N = 57, min = 3 m, max = 102 m); no significant differences between the position of gravid and non-gravid females were found (condition-gravid, $F_{1,523}$ = 0.592 and P = 0.441; no significant differences between species: $F_{6,26}$ = 1.30, P = 0.29). The smallest gravid females observed for the mainland species *Hydromantes strinatii* and *H. ambrosii* was 52 mm (SVL), while for the Sardinian species was 56 mm long (*H. flavus* and *H. imperialis*). The average SVL of gravid females was significantly larger than for those in which we did not detect eggs (N of females analysed= 454; ANOVA: $F_{1,438}$ = 59.16, P < 0.001) (Fig. 2). SVL was significantly different among species ($F_{7,438}$ = 8.7, P < 0.001), but the interaction between species and egg presence was not significant ($F_{7,438}$ = 0.71, P = 0.655), indicating that the pattern is the same across all the species.

Courtship, breeding behaviour and clutches

From May to September, couples of different species were observed performing the so called "vaccination" (Fig. 3a-b). In two occasions, we observed the exchange of a spermatophore. On September 17th, 2014, a female and a male of *H. italicus* were found sharing the same crack in a cave located in the Prato Province. The female showed visible developed eggs in her abdomen and a spermatophora attached to the cloaca. On May 28th, 2017, in a cave located in the Oliena area, a female without any visible egg was observed with a spermatophora attached to the cloaca.

One female of *H. genei*, one of *H. supramontis* and two of *H. ambrosii* were observed close to oviposition on September 11th 2016, in a cave located in the Carbonia-Iglesias Province, on May 18th, 2017, in a cave located in the Oliena area, and on June 22th, 2017, in a cave located in La Spezia Province, respectively. During manipulation an egg was almost extruded from the cloaca (Fig. 4a-b). On September 7th, 2016, a large number of *H. supramontis* hatchlings were found in a cave located in the Dorgali area; more than 70 small salamanders (total length 25-30 mm) were found on the walls and on the cave floor. Finally, on April 24th, 2017, a female *H. supramontis* was found guarding a litter of young in a cave in the Baunei area (Fig. 5).

Prolonged monitoring

Hydromantes imperialis. Seven clutches were found in 2015 (Table 2; the first one on March 22nd). The first hatchlings were observed on August 21st and all nests were empty on September 30th. In 2016, nine clutches were found (Table 2): the first on February 21st, the first hatchlings appeared on July 19th, all nests were empty on August 14th. In 2017, the first oviposition was recorded on March 8th. The cave was occupied by a few males ($N = 10$), by gravid females (carrying externally visible eggs, $N = 17$) and by females who already oviposited ($N = 3$) (Table 2) on this date. Ten nests were counted on April 29th (Table 2). Overall, 13 different nesting sites were detected (Table 2) and the majority were used for more than one season: ten were used at least twice, while three of them were used consecutively for three or even four times (Table 2). All the nesting sites were located between 15 m and 30 m from the cave entrance and showed variable shapes (holes, cracks, cervices) and positions (height from the ground).

Hydromantes sarrabusensis. On April 22nd, 2017, one female produced a clutch of 14 eggs in the terrarium (Fig. 6), while the second female was still retaining the eggs in her abdomen.

Discussion

In *Hydromates*, body size of gravid females is highly variable; the body size of the smallest gravid females recorded in this study for both mainland and Sardinian species, was smaller than observed by SALVIDIO (1993) during individual dissections performed on one of the smallest *Hydromantes* species, *H. strinatii*. The case of *H. imperialis* females carrying a single large egg is noteworthy (Fig. 1b) since the clutch size for *Hydromantes* reported by LANZA et al. (2006b) varies from 6 to 14 eggs; however, there are no reports on the correlation between clutch and egg size, nor for unusual large eggs. A dissimilarity in the number of fertilised and unfertilised eggs has been never reported; therefore, additional studies are urgently required to understand the significance of single large eggs.

Our observations on the mating behaviour mostly correspond to the literature, describing aseasonal courtship (LANZA et al. 2006b). We observed mating throughout the year, and both gravid and non-gravid females collecting spermatophores. If mating occurs throughout the year, but egg laying (at least for Sardinian species) is mostly restricted to spring (Table 2), it likely indicates that *Hydromantes* females may be able to store sperm, as known for other plethodontids (ADAMS et al. 2005). ONETO et al. (2010) rearing wild salamanders for breeding experiments temporarily reported that they captured "apparently gravid females" in June 2007 with visible fully developed eggs. The females were placed in a terrarium without any contact with males. Egg laying occurred after more than four months, and 45 weeks later authors observed the first hatchlings. Two females of the present study were observed picking up a spermatophora each: one had visible fully developed eggs, while the other did not show any egg presence in her abdomen. In plethodontids male competition for reproduction is well documented (HOUCK et al. 1985, VERRELL 1991, HOUCK & VERRELL 1993), and females have the opportunity to mate with several males by storing their sperm (HOUCK & ARNOLD 2003). Therefore, according to our observations sperm storage may also occur in European *Hydromantes*.

Another interesting information concerns the time of egg retention. The recapture of the marked gravid female (*H. imperialis*) and the observation of the two *H. sarrabusensis* females kept in the terrarium show that fully developed eggs are carried over a very long period (up to 12 months), confirming the observations reported for other *Hydromantes* species (ONETO et al. 2010). Females need to allocate a large amount of resources for reproduction, and the long period lasting from fecundation to egg deposition is followed by more than six months of clutch attendance and parental care (LUNGHI et al. 2014b, LUNGHI et al. 2015b; Table 2). Therefore, the reproductive cycle is extremely long and likely lasts at least two years. Considering the limited lifespan of these salamanders (max. 11 years in captivity) (SNIDER & BOWLER 1992) and that sexual maturity is reached at around the third-fourth year (SALVIDIO 1993), it implies that females can only complete a limited number of breeding cycles during lifetime. This limits the potential growth rate of populations and is an additional concern for the conservation of these strictly endemic species.

Hydromantes salamanders are usually reported to lay eggs in springs while hatching mostly occurs in late summer – early autumn (LANZA et al. 2006b, LUNGHI et al. 2015b, MULARGIA et al. 2016, SERRA & ARGIOLAS 2016). Most of our observations match the above schedule (see Table 2), although in some cases a different seasonal pattern occurred. A female of *H. genei* was observed in September close to lay eggs (Fig. 4a), while previous studies on this species reported clutches in spring only (STEFANI & SERRA 1966, PAPINUTO 2005). For *H. supramontis* we observed hatchlings in a nest on late April (Fig. 5) and a female close to lay eggs in late May (Fig. 4b); the only available information on this species reports an egg clutch observed in February (MULARGIA et al. 2016). These data suggest that breeding activity may be not constant, and thus might occurs in different periods. Given that these observations of non-standard breeding activity were performed in different caves, such differences might be related to local environmental condition (e.g., temperature, humidity). However, additional data are needed to confirm this hypothesis.

Given the length of the *Hydromantes* breeding cycle (LANZA et al. 2006b, ONETO et al. 2010, LUNGHI et al. 2015b, MURGIA et al. 2016), the choice of the breeding period has a strong impact on the breeding success. It is generally assumed that caves are characterised by relatively stable environments with limited seasonal variation. However, recent analyses suggest that the impact of seasonal variation on underground communities may be strong (LUNGHI et al. 2017). The earliest observations of *H. imperialis* clutches ranged between late February (in 2016) to late March (in 2015; Table 2), suggesting some variation in the phenology of *Hydromantes* among years. Meteorological data showed that February 2015 was much colder than February 2016 [average air temperature: 2015: 9.4 ± 0.1°C; 2016: 12.8 ± 0.1 °C; data from the nearest weather station (Cagliari; www.eurometeo.com)], suggesting that meteorological conditions could affect breeding phenology. Underground microclimate is partially affected by the variation of outdoor conditions (LUNGHI et al. 2015a), and this might influence underground activity because of the peculiar physiology of *Hydromantes* (SPOTILA 1972, LANZA et al. 2006b, LUNGHI et al. 2016). However, in the above mentioned cave the nesting area has a rather stable microclimate (LUNGHI et al. 2015b, MURGIA et al. 2016).

In the visited cave, several nesting sites were used in different years, suggesting nesting site selection (Table 2). This is the first time that such behaviour was observed in *Hydromantes*. The repeated use of nesting sites cannot only represent site fidelity, because it is unlikely that a female breeds for two consecutive years. Our data suggest that different females use the same nesting site, as the majority of nests were used multiple times (Table 2). The reason of such behaviour is still unknown, and might be related to favourable environmental conditions that drive all females to breed together in such a reduced space (LUNGHI et al. 2015). Breeding site defence might be an alternative hypothesis. Females actively defend their offspring against intruders (ONETO et al. 2014), and laying eggs in deep crevices might improve egg protection. In the study cave, the number of such sites may be somehow limited, thus increasing the probability that the most suitable ones are repeatedly used.

Until the present study, the breeding biology of *H. sarrabusensis* was uncertain. Only a single report on a captive female suggested ovoviviparity, mostly based on the record of the birth of one salamander (LANZA & LEO 2001). Such information is strongly in contrast with the available knowledge related to the other *Hydromantes* species, which are known to be oviparous. Conversely, our data indicate that *H. sarrabusensis* is oviparous and thus shares the reproductive mode with its congeners (Fig. 6). The case of a record of “viviparity” might have been the result of stress caused by the prolonged cooled condition at which females were kept.

The study of underground habitats is extremely challenging, especially because animals exploit small environments that humans cannot access and where observations are extremely difficult to obtain. Therefore, only the future collection of a large amount of data will allow a comprehensive understanding of the breeding biology of subterranean cave salamanders. As we have presented novel interesting information on the breeding behaviour of European cave salamanders we hope to stimulate future investigations.

Acknowledgments

We thank S. Salvidio and another anonymous reviewer to helpful comments which improved our manuscript. The study was conducted with the authorization of the Italian Ministry of the Environment, 9384/PNM of 12/05/2015. E. Lunghi is founded by The Mohamed bin Zayed Species Conservation Fund, Instrumentl and National Speleological Society.

References

ADAMS, E. M., A. G. JONES & S. J. ARNOLD (2005): Multiple paternity in a natural population of a salamander with long-term sperm storage. ‒ Molecular Ecology, **14:** 1803-1810.

BRUCE, R. C., R. G. JAEGER & L. D. HOUCK (2000): The biology of Plethodontid salamanders. - Springer Science+Business Media, LLC, New York, U.S.A.

CULVER, D. C. & T. PIPAN (2014): Shallow subterranean habitats: Ecology, evolution, and conservation. ‒ Oxford University Press, New York, U.S.A.

DURAND, J. P. (1967): Sur la reproduction ovipare d'*Hydromantes italicus strinatii* Aellen (Urodèle, Plethodontidae). ‒ Comptes rendus hebdomadaires des séances de l'Académie des sciences, Paris, **264:** 854-856.

FICETOLA, G. F., R. PENNATI & R. MANENTI (2012): Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. ‒ Amphibia-Reptilia, **33:** 251-259.

HOUCK, L. D. & S. J. ARNOLD (2003): Courtship and mating behavior. ‒ pp. 383-424 in: SEVER, D. M. (ed.): Phylogeny and reproductive biology of Urodela (Amphibia) - Science Publishers, Enfield, New Hampshire.

HOUCK, L. D., S. J. ARNOLD & R. A. THISTED (1985): A statistical study of mating choice: Sexual selection in a plethodontid salamander (*Desmognathus ochrophaeus*). ‒ Evolution, **39:** 370-386.

HOUCK, L. D. & P. A. VERRELL (1993): Studies of courtship behavior in Plethodontid salamanders: A review‒ Herpetologica, **49:** 175-184.

LANZA, B. (1959): Il corpo ghiandolare mentoniero dei «Plethodontidae» («Amphibia, Caudata»). ‒ Monitore zoologico italiano, **67:** 15-53.

LANZA, B., F. ANDREONE, M. A. BOLOGNA, C. CORTI & E. RAZZETTI (2006a): Fauna d'italia. Amphibia. ‒ Calderini, Bologna.

LANZA, B. & P. LEO (2001): Prima osservazione sicura di riproduzione vivipara nel genere *Speleomantes* (Amphibia: Caudata: Plethodontidae). ‒ in: BARBIERI, F., F. BERNINI & M. FASOLA (eds.): Atti 3° Congresso Nazionale Societas Herpetologica Italica (Pavia, 14-16 settembre 2000), Pianura, Cremona.

LANZA, B., C. PASTORELLI, P. LAGHI & R. CIMMARUTA (2006b): A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). ‒ Atti del Museo Civico di Storia Naturale di Trieste, **52:** 5-135.

LUNGHI, E., R. MANENTI, G. CANCIANI, G. SCARÌ, R. PENNATI & G. F. FICETOLA (2016): Thermal equilibrium and temperature differences among body regions in european plethodontid salamanders. ‒ Journal of Thermal Biology, **60:** 79-85.

LUNGHI, E., R. MANENTI & G. F. FICETOLA (2014a): Do cave features affect underground habitat exploitation by non-troglobite species? ‒ Acta Oecologica, **55:** 29-35.

LUNGHI, E., R. MANENTI & G. F. FICETOLA (2015a): Seasonal variation in microhabitat of salamanders: Environmental variation or shift of habitat selection? ‒ PeerJ, **3:** e1122.

LUNGHI, E., R. MANENTI & G. F. FICETOLA (2017): Cave features, seasonality and subterranean distribution of non-obligate cave dwellers‒ PeerJ, **5:** e3169.

LUNGHI, E., R. MANENTI, S. MANCA, M. MULARGIA, R. PENNATI & G. F. FICETOLA (2014b): Nesting of cave salamanders (*Hydromantes flavus* and *H. Italicus*) in natural environments. ‒ Salamandra, **50:** 105-109.

LUNGHI, E., R. MURGIA, G. DE FALCO, S. BUSCHETTU, C. MULAS, M. MULARGIA, C. CANEDOLI, R. MANENTI & G. F. FICETOLA (2015b): First data on nesting ecology and behaviour in the Imperial cave salamander *Hydromantes imperialis*. ‒ North-Western Journal of Zoology, **11:** 324-330.

LUNGHI, E. & M. VEITH (2017): Are visual implant alpha tags adequate for individually marking European cave salamanders (genus *Hydromantes*)? ‒ Salamandra, **53:** 541-544.

MANENTI, R., E. LUNGHI, C. CANEDOLI, M. BONACCORSI & G. F. FICETOLA (2016): Parasitism of the leech, *Batracobdella algira* (Moquin-Tandon, 1846), on Sardinian cave salamanders (genus *Hydromantes*) (Caudata: Plethodontidae). ‒ Herpetozoa, **29:** 27-35.

MERTENS, R. (1923): Zur biologie des höhlenmolches, *spelerpes fuscus* bonaparte. ‒ Blätter für Aquarien- und Terrarien-Kunde, **34:** 171-174.

MULARGIA, M., G. DE FALCO, S. BUSCHETTU, R. MURGIA & C. MULAS (2016): Primo ritrovamento di una nidiata di *Hydromantes supramontis* (Lanza, Nascetti et Bullini, 1986). ‒ Sardegna Speleologica, **28:** 79-81.

MURGIA, R., G. DE FALCO, S. BUSCHETTU, F. FAIS, V. MIRIMIN & C. MULAS (2016): Microclima di grotta e deposizioni del geotritone *Hydromantes imperialis*. ‒ Sardegna Speleologica, **28:** 76-78.

ONETO, F., D. OTTONELLO, M. V. PASTORINO & S. SALVIDIO (2010): Posthatching parental care in salamanders revealed by infrared video surveillance. ‒ Journal of Herpetology, **44:** 649-653.

ONETO, F., D. OTTONELLO, M. V. PASTORINO & S. SALVIDIO (2014): Maternal care and defence of young by the Plethodontid salamander *Speleomantes strinatii* (Aellen, 1951). ‒ Scripta Herpetologica. Studies on Amphibians and Reptiles in honour of Benedetto Lanza**:** 129-138.

PAPINUTO, S. (2005): Sul ritrovamento ed il monitoraggio di una nidiata di *Speleomantes genei* (Temminck & Schlegel, 1838) (Amphibia Urodela Plethodontidae) in una galleria mineraria dell'inglesiente (Sardegna sud-occidentale). – Sardegna Speleologica, **22:** 3-6.

PASTORELLI, C. & P. LAGHI (2006): Predation of *Speleomantes italicus* (Amphibia: Caudata: Plethodontidae) by *Meta menardi* (Arachnida: Araneae: Metidae). – pp. 45-48: Atti del 6° Congresso Nazionale della Societas Herpetologica Italica (Roma, 27.IX-1.X.2006), Roma.

SALVIDIO, S. (1993): Life history of the European plethodontid salamander *Speleomantes ambrosii* (Amphibia, Caudata). – Herpetological Journal, **3:** 55-59.

SALVIDIO, S. (2013): Homing behaviour in *Speleomantes strinatii* (Amphibia Plethodontidae): A preliminary displacement experiment. – North-Western Journal of Zoology, **9:** 429-433.

SALVIDIO, S., G. PALUMBI, A. ROMANO & A. COSTA (2017a): Safe caves and dangerous forests? Predation risk may contribute to salamander colonization of subterranean habitats. – The Science of Nature, **104:** 20.

SALVIDIO, S., F. PASMANS, S. BOGAERTS, A. MARTEL, M. VAN DE LOO & A. ROMANO (2017b): Consistency in trophic strategies between populations of the sardinian endemic salamander *Speleomantes imperialis*. – Animal Biology, **67:** 1-16.

SERRA, B. & S. ARGIOLAS (2016): Monitoraggio di una popolazione di *Hydromantes imperialis* (Stefani, 1969) nel Gerrei. Segnalazione del ritrovamento di una nidiata. – Sardegna Speleologica, **28:** 82-85.

SILLERO, N., J. CAMPOS, A. BONARDI, C. CORTI, R. CREEMERS, P.-A. CROCHET, J. C. ISAILOVIĆ, M. DENOËL, G. F. FICETOLA, J. GONÇALVES, S. KUZMIN, P. LYMBERAKIS, P. DE POUS, A. RODRÍGUEZ, R. SINDACO, J. SPEYBROECK, B. TOXOPEUS, D. R. VIEITES & M. VENCES (2014): Updated distribution and biogeography of amphibians and reptiles of europe. – Amphibia-Reptilia, **35:** 1-31.

SNIDER, A. T. & J. K. BOWLER (1992): Longevity of reptiles and amphibians in North-American collections (2nd ed.) S.S.A.R. – Herpetological Circulars, **21:** 1-44.

SPOTILA, J. R. (1972): Role of temperature and water in the ecology of lungless salamanders. – Ecological Monographs, **42:** 95-125.

STEFANI, R. & G. SERRA (1966): L'oviparità in *Hydromantes genei* (Temm. et Schl.). – Bollettino di Zoologia, **33:** 283-291.

VERRELL, P. A. (1991): Male mating success in the mountain Dusky salamander, *Desrnognathus ochrophaeus*: Are small, young, inexperienced males at a disadvantage? – Ethology, **88:** 277-286.

WAKE, D. B. (2013): The enigmatic history of the European, Asian and American Plethodontid salamanders. – Amphibia-Reptilia, **34:** 323-336.

Table 1. Number of females with visible abdominal eggs of eight species of *Hydromantes* observed from different caves per month. Last columns show the number of surveyed caves, the total number of surveys performed and the total number of females observed per each species. Each cave was surveyed max once per month; x = no surveys performed.

Species	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	***N* caves**	***N* surveys**	***N* females**
H. strinatii	x	x	x	x	x	7	x	x	x	x	x	x	1	1	40
H. ambrosii	x	x	x	x	x	10	x	x	x	x	x	x	4	10	113
H. italicus	x	x	x	x	x	3	x	x	1	x	x	x	5	5	27
H. flavus	1	0	0	12	2	1	0	0	4	3	0	0	10	47	282
H. supramontis	x	x	x	0	1	x	x	x	2	x	x	x	3	10	82
H. imperialis	x	x	9	1	7	1	6	x	25	x	x	x	7	14	157
H. sarrabusensis	x	x	x	x	7	x	x	x	1	x	x	x	2	8	46
H. genei	x	x	x	x	3	x	x	x	3	x	x	x	3	10	40

Table 2: Data on monitored clutches of *Hydromantes imperialis*. First column = numbered nests; per each year columns = date in which eggs were observed for the first time, date in which new-borns were observed for the first time, date in which the nest was empty; wide dash = nest used during the monitoring year; “n.o.” = hatchlings not observed; “Failed” = breeding was interrupted. Data for 2014 were taken from Lunghi et al. (2015).

	2014			2015			2016			2017
Nest site	First eggs observation	Hatchlings	Empty	First eggs observation	Newborns	Empty	First eggs observation	Newborns	Empty	First eggs observation
1	15.VI	28.VIII	25.X	18.IV	n.o.	27.VII	21.II	n.o.	11.V	12.III
2	15.VI	25.VIII	20.IX	4.IV	21.VIII	30.IX	5.III	n.o.	19.VII	8.III
3	15.VI	28.VIII	13.IX	–	–	–	–	–	–	
4	15.VI	16.VIII	27.IX	–	–	–	–	–	–	29.IV
5				22.III	Failed		1.VI	n.o.	24.VI	
6				28.III	Failed		6.IV	n.o.	24.VI	
7				18.IV	26.VIII	30.IX	5.III	19/07	14.VIII	8.III
8				18.IV	21.VIII	26.VIII	1.VI	n.o.	19.VII	
9				26.IV	n.o.	27.VII	–	–	–	
10				–	–	–	5.III	n.o.	11.V	2.IV
11	–	–	–	–	–	–	6.IV	n.o.	11.V	25.IV
12	–	–	–	–	–	–	11.V	n.o.	24.VI	8.III
13										29.IV
14										1.V
15										25.V

Figure legends

Figure 1. Gravid females of various cave salamanders species. (A) *Hydromantes flavus* with eight visible developed eggs; (B) *H. imperialis* showing a single large-sized egg. Females of (C) *H. genei* and (B) *H. supramontis* almost dropping an egg from their cloaca.

Figure 2. Boxplots showing average SVL (mm) of non-gravid (N, white) and gravid (Y, pink) females for each *Hydromantes* species (*H. strinatii*, *N* = 33 (N) and *N* = 7 (Y); *H. ambrosii*, *N* = 103 (N) and *N* = 9 (Y); *H. italicus*, *N* = 21 (N) and *N* = 4 (Y); *H. flavus*, *N* = 97 (N) and *N* = 11 (Y); *H. genei*, *N* = 17 (N) and *N* = 4 (Y); *H. imperiali*s, *N* = 48 (N) and *N* = 24 (Y); *H. sarrabusensis*, *N* = 24 (N) and *N* = 6 (Y); *H. supramontis*, *N* = 43 (N) and *N* = 3 (Y)).

Figure 3. *Hydromantes imperialis* (A) and *H. ambrosii* (B) during courtship.

Figure 4. (A) Female of *H. supramontis* attending the nest with her brood and (B) Female of *H. sarrabusensis* with her clutch.

Figure 1

Figure 2

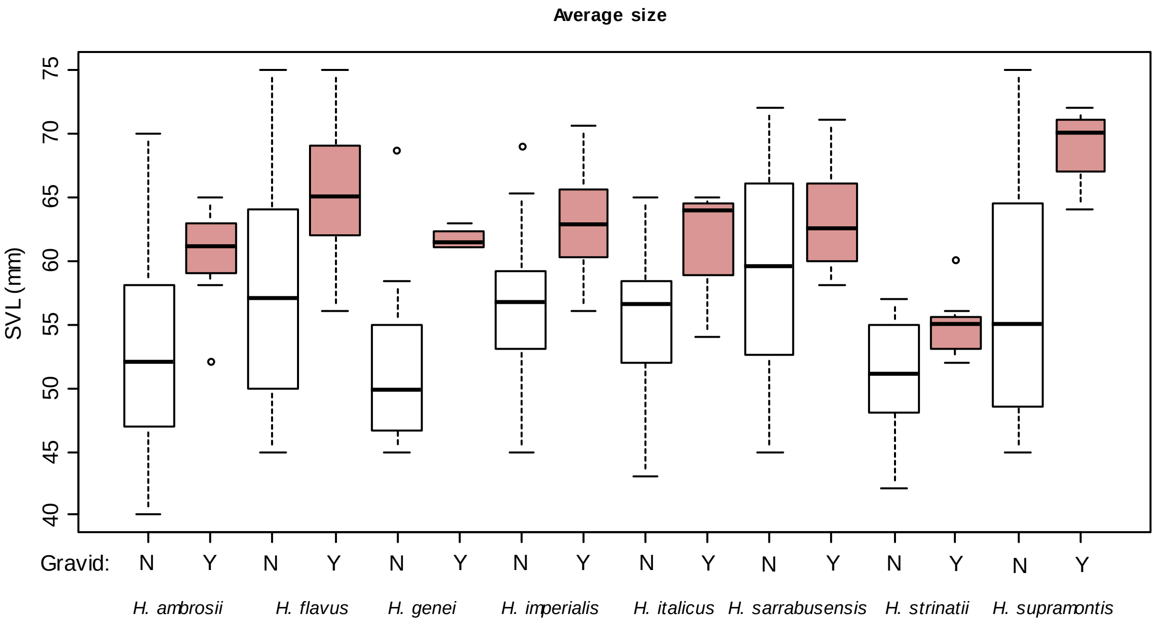


Figure 3

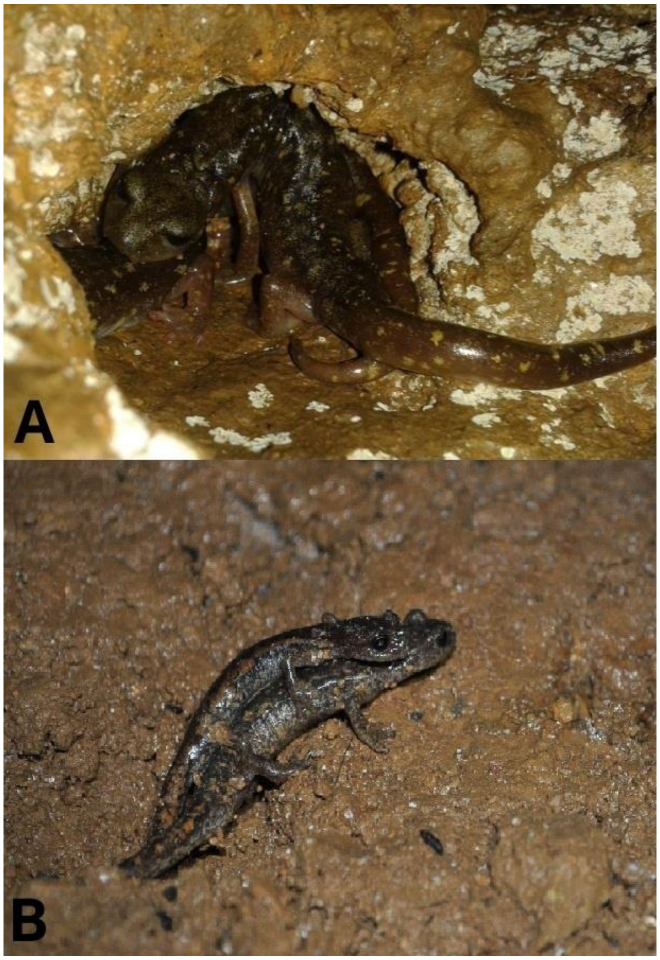


Figure 4

Chapter X

SALAMANDRA **53**(4) | 541–544 | 30 October 2017CorrespondenceISSN0036–3375

Correspondence

Are Visual Implant Alpha tags adequate for individually marking European cave salamanders (genus *Hydromantes*)?

Enrico Lunghi[1,2,3] & Michael Veith[1]

1) Universität Trier Fachbereich VI Raum- und Umweltwissenschaften Biogeographie, Universitätsring 15, 54286 Trier, Germany
2) Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", Via Romana 17, 50125 Firenze, Italy
3) Natural Oasis, Via di Galceti 141, 59100 Prato, Italy

Corresponding author: Enrico Lunghi, e-mail: enrico.arti@gmail.com

Manuscript received: 13 February 2017
Accepted: 9 May 2017 by Alexander Kupfer

During the last decades, studies on species ecology has improved considerably, becoming continuously more focused on individual needs (Emata & Marte 1992, Link & He-sed 2015, Mazerolle et al. 2007). To this end, researchers increasingly use Capture-Mark-Recapture (CMR) techniques, which allow to obtain ecological and behavioural data on individuals (Emata & Marte 1992, Pierce et al. 2014, Sanchez-Camara & Booth 2004). One of the cru-cial aspects is the selection of appropriate marking meth-ods, a decision that may strongly affect the outcome of a study (Brannelly et al. 2014, Davis & Ovaska 2001, Ro-bison-Cox 1998, Swanson et al. 2013). Therefore, many studies aim at evaluating the efficiency of marking meth-ods (Buckmeier & Irwin 2000, Jerry et al. 2001, Lemarié et al. 2000, Potts 2012, Woods 2005) and related activi-ties (Anholt et al. 1998, Kinkead et al. 2006) in various species.

Visual Implant Alpha tags (VIA; Northwest Marine Technology, Inc., Shaw Island, Washington) are one of the more widely used marking methods (Buckley et al. 1994, Heard et al. 2008, Measey et al. 2001, Osbourn et al. 2011). VIA are biocompatible tags (standard size 1.2 × 2.7 mm or large size 2 × 5 mm) that have black text on a fluorescent and coloured background on one side (see Fig. 1) meant for being implanted subcutaneously; their combination of an alphanumeric code (one letter and two numbers) with four colours facilitates up to 10,000 differ-ent markings. Tags are inserted under the skin of animals by means of a specialized injector. However, despite the number of successful studies (Arce et al. 2003, Crook & White 1995, Emata & Marte 1992, Turek et al. 2014), this method seems to have some limitations that will prevent its use in various circumstances. Firstly, there may be handling problems related to tag implantation, e.g., the insertion of tags seems to be not that easy in some instances, requiring a team of experts and maybe controlled conditions, render-ing this method not always practical in the field (Heard et al. 2008). In some species, anaesthesia and/or skin cuts are required to properly implant the tags (Buchan et al. 2005, Gower et al. 2006). Furthermore, tags may be accidentally flipped over during insertion or slip beneath muscle tis-sue, or come to rest under heavily pigmented skin, all of which will affect their readability (Heard et al. 2008, Isely et al. 2004, Wagner et al. 2013). Secondly, there are some concerns about tag retention, which seems to be not high enough in several circumstances (Isely & Grabowsky 2004, Pillai et al. 2009, Replinger & Wood 2007) and probably positively correlated with the sizes of individu-als, making this method not the best choice to mark early life-stages of small-sized amphibian species (Davis et al. 2014, Isely et al. 2004). Finally, some authors reported that wounds produced by the injector may require a long time to heal, i.e., several days, consequently paving ways for tag expulsion and pathogen infections (Buckmeier & Irwin 2000, Heard et al. 2008, Replinger & Wood 2007, Robison-Cox 1998). As far as amphibians are concerned, VIA tags were tested only in few species (Buchan et al. 2005, Gower et al. 2006, Heard et al. 2008, Kaiser et al. 2009, Kinkead et al. 2006, Osbourn et al. 2011, Pittman et al. 2008). Considering the potential problems it is extreme-ly important to test VIA tags in other study species prior to their application since the method may produce biased data.

Two types of marking techniques were successfully applied in previous studies on European cave salamanders (genus *Hydromantes*, see also Wake 2013), i.e., Visual Implant Elastomers (Salvidio 2013) and photographic recognition (Salvidio et al. 1994); however, to the best of our knowledge, VIA was never tested before for them. Here we report on our tests and evaluation of VIA tagging in two species of European cave salamanders, *Hydromantes italicus* and *H. flavus*.

VIAs are provided as plastic sheets (100 tags each) in which the single tags are attached by at their short sides. To load the injector, a label must be placed inside the nee-dle, and it is released from the sheet by twisting the in-jector. However, this sometimes produces an imperfect tag separation, creating sharp edges that represent a serious danger to individuals. In such cases we cut away the sharp parts using small scissors. Given the limited plasticity of tags (although being soft their shape will not change) and considering possible dangers due to sub-optimal tag load-ing, we preferred to apply tags only on limbs, as these body parts do not hold vital organs and can be regenerated if necessary (Lanza et al. 2006, Scaravelli et al. 2002). We also avoided tagging tails, as these salamanders are able to autotomise them (Lanza et al. 2006). We chose to ap-ply standard-size VIAs only in individuals large enough to withstand the size of the needle (for standard tag ~2 mm). We first applied alpha tags on three individuals of *H. itali- cus* from a cave in the northern Tuscan Apennines (lat. 43.90°, long. 11.11°); however, the dark colouration of the ventral side obscured the tags even right after applica-tion, so that we aborted the test with this species. We then applied them to individuals of *H. flavus* from six caves in Monte Albo, northwestern Sardinia (lat. 40.53°, long. 9.63°). This species has a pale/white ventral colouration, so that tag readability would probably be better. Perforating the salamander skin with the injector was not always easy, and the large wound left behind by the injector nee-dle allowed tags to easily slip out from their subcutaneous position. In these cases, we manually reinserted the tags using tweezers. In a few instances tags slid between mus-cles and became partially illegible, while in one case the tag flipped and became unreadable thus. Well-positioned tags were easily readable with bare eyes (Fig. 1). After tag application, individuals were observed for 15 minutes to monitor their physical well-being. Injections into *H. itali- cus* legs were performed by only one operator, while the application of VIA tag in *H. flavus* were carried out by at least two operators in most of the surveys. All operations were performed while wearing sterile latex gloves, and all equipment was disinfected with bleach and then washed with water after each survey.

We marked 114 individuals of *Hydromantes flavus* (61 females, 50 males, and 3 juveniles) with VIA tags. No salamander incurred any problematic injury. Time required for individual marking differed substantially depending on the number of operators, ranging from an average of 90 seconds needed by one operator to an average of 15 sec-onds with two operators. Within twelve surveys, we recap-tured 14 different individuals (~12% of total marked), and only one of them was recaptured twice. None of these sala-manders showed injuries. First recaptures were made af-ter six days and last recaptures were made after 272 days. During our first revisits (within 14 days), four out of eight recaptured salamanders showed incompletely healed tag-ging wounds, making us aware of an apparent risk of tag loss. In fact, in two recaptured individuals, tags were par-tially hanging from the wound, and in one of them we had

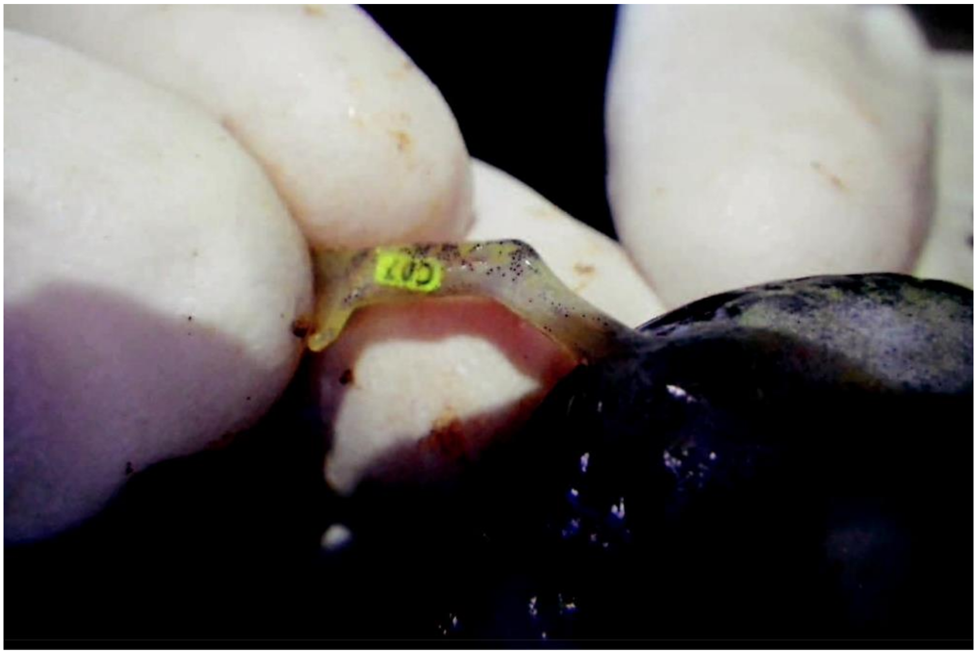


Figure 1. Example of a successfully implanted VIA tag in the forelimb of a *Hydromantes flavus*.

to implant a new one, as the previous tag was lost during handling. Proper tag retention (not counting flipped and slipped tags) in recaptured individuals occurred in 60% within the first 30 days and 50% thereafter.

Given the low number of recaptures, we were not able to provide any relevant information on the sizes of the studied populations. Thus, we only discuss the reliability of VIA marking technique here. VIA tags represent one of the newest commonly used marking methods, which seems to be fit for use in a wide range of species (Emata & Marte 1992, Gower et al. 2006, Turek et al. 2014, Woods 2005). Some of its advantages are the relatively low costs of tags (around 0.80–0.90 $ each) and a fixed code for each tag. However, the use of this marking method encounters several obstacles in some species, which may significant-ly influence the entire study outcome (Heard et al. 2008, Replinger & Wood 2007, Wagner et al. 2013). During our test of VIA tags on *Hydromantes*, we basically experienced all problems previously noted by other authors (Buckmei-er & Irwin 2000, Davis et al. 2014, Heard et al. 2008, Ise-ly & Grabowsky 2004). Our major concerns were related to the survival of individuals. The most risky phase of the marking procedure occurred during the insertion of tags, i.e., performing the injection without assistance was more or less impossible. Also, the needle size for standard tags basically equals the average width of limbs in adult *Hydro- mantes* and, considering both needle rigidity and length, it is easy to imagine that an imperfect application can be harmful to the salamanders. We did not use any anaesthesia, so the presence of a second operator was essential for keeping individuals immobile and succeeding in tag application. The size of the needle strongly limits the range of salamander species this method can be used on; thus, considering the body size of *Hydromantes* species (Lanza et al. 2006), only few adults and almost no juveniles were suitable for being marked with VIA tags. This renders this method unsuited for long-term studies aiming at record-ing data on, e.g., individual growth. Tag retention was low and probably influenced by the length of time wounds re-quired to heal. Open wounds provided a chance for easily losing tags. This was observed in 50% of the individuals re-captured during the first 14 days, so that it is reasonable to assume that it may frequently occur in tagged salamanders. Seemingly low recapture rates may therefore plausibly ex-plained simply by tag loss. Even if VIAs were retained by the marked salamanders, the readability of their tags was not always adequate since improper tag implantation may impede their detection.

Acknowledgements

We thank M. Mulargia for help in the field, S. Lötters for the review of an early draft, and A. Kupfer for commenting on the last manuscript versions. E. Lunghi was supported by The Mohamed bin Zayed Species Conservation Fund, Instrumentl and The National Speleological Society. Our study was conducted un-der Italian Ministerial Authorization 9384/PNM of 12/05/2015 is-sued by the Ministry of the Environment.

References

Anholt, B. R., S. Negovetic & C. Som (1998): Methods for anaesthetizing and marking larval anurans. – Herpetological Review, **29**: 153–154.

Arce, S. M., B. J. Argue, D. A. Thompson & S. M. Moss (2003): Evaluation of a fluorescent, alphanumeric tagging system for penaeid shrimp and its application in selective breeding programs. – Aquaculture, **228**: 267–278.

Brannelly, L. A., L. Berger & L. F. Skerratt (2014): Compari-son of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*. – Herpetological Conservation and Biology, **9**: 428–435.

Buchan, A., L. Sun & R. S. Wagner (2005): Using alpha numeric fluorescent tags for individual identification of amphibians. – Herpetological Review, **36**: 43–44.

Buckley, R. M., J. E. West & D. C. Doty (1994): Internal microtag systems for marking juvenile reef fishes. – Bulletin of Marine Science, **55**: 848–857.

Buckmeier, D. L. & E. R. Irwin (2000): An evaluation of soft visual implant tag retention compared with anchor tag retention in channel catfish. – North American Journal of Fisheries Management, **20**: 296–298.

Crook, D. A. & R. W. G. White (1995): Evaluation of subcutaneously implanted visual implant tags and coded wire tags for marking and benign recovery in a small scaleless fish, *Galaxias truttaceus* (Pisces : Galaxiidae). – Marine and Freshwater Research, **46**: 943–946.

Davis, J. L., M. E. Barnes, J. L. Kientz & A. G. Galinat (2014): Effects of fish length and anatomical placement on retention of visible implant alpha tags in hatchery-reared rainbow trout. – North American Journal of Fisheries Management, **34**: 932– 937.

Davis, T. M. & K. Ovaska (2001): Individual recognition of amphibians: Effects of toe clipping and fluorescent tagging on the salamander *Plethodon vehiculum*. – Journal of Herpetology, **35**: 217–225.

Emata, A. C. & C. L. Marte (1992): The use of a visual implant tag to monitor the reproductive performance of individual milkfish *Chanos chanos* Forsskal. – Journal of Applied Ichthy-ology, **8**: 314–317.

Gower, D. J., O. V. Oommen & M. Wilkinson (2006): Marking amphibians with alpha numeric fluorescent tags: Caecilians lead the way. – Herpetological Review, **37**: 302.

Heard, G. W., M. P. Scroggie & B. Malone (2008): Visible implant alphanumeric tags as an alternative to toe-clipping for marking amphibians – a case study. – Wildlife Research, **35**: 747–759.

Isely, J. J. & T. B. Grabowsky (2004): Occurrence, size, and tag retention of sneaker male hatchery rainbow trout. – North American Journal of Aquaculture, **66**: 234–236.

Isely, J. J., D. G. Trested & T. B. Grabowsky (2004): Tag retention and survivorship of hatchery rainbow trout marked with large-format visible implant alphanumeric tags. – North American Journal of Aquaculture, **66**: 73–74.

Jerry, D. R., T. Stewart, I. W. Purvis & L. R. Piper (2001): Evaluation of visual implant elastomer and alphanumeric internal tags as a method to identify juveniles of the freshwater cray-fish, *Cherax destructor*. – Aquaculture, **193**: 149–154.

Kaiser, K., M. Alloush, R. M. Jones, S. Marczak, K. Mar-tineau & M. Oliva (2009): Use of visual implant alpha (vialpha) fluorescent tags in a small hylid frog with a new technique for application. – Herpetological Review, **40**: 421–422.

Kinkead, K. E., J. D. Lanham & R. R. Montanucci (2006): Comparison of anesthesia and marking techniques on stress and behavioral responses in two *Desmognathus* salamanders. – Journal of Herpetology, **40**: 323–328.

Lanza, B., C. Pastorelli, P. Laghi & R. Cimmaruta (2006): A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). – Atti del Museo Civico di Storia Naturale di Trieste, **52**: 5–135.

Lemarié, D. P., D. R. Smith, R. F. Villella & D. A. Weller (2000): Evaluation of tag types and adhesives for marking freshwater mussels (Mollusca: Unionidae). – Journal of Shell-fish Research, **19**: 247–250.

Link, W. A. & K. M. Hesed (2015): Individual heterogeneity in growth and age at sexual maturity: A gamma process analy-sis of capture–mark–recapture data. – Journal of Agricultural, Biological, and Environmental Statistics, **20**: 343.

Mazerolle, M. J., L. L. Bailey, W. L. Kendall, J. A. Royle, S. J. Converse & J. D. Nichols (2007): Making great leaps forward: Accounting for detectability in herpetological field stud-ies. – Journal of Herpetology, **41**: 672–689.

Measey, G. J., D. J. Gower, O. V. Oommen & M. Wilkinson (2001): Permanent marking of a fossorial caecilian, *Gegeneo- phis ramaswamii* (Amphibia: Gymnophiona: Caeciliidae). – Journal of South Asian Natural History, **5**: 141–147.

Osbourn, M. S., D. J. Hocking, C. A. Conner, W. E. Peterman & R. D. Semlitsch (2011): Use of fluorescent visible implant alphanumeric tags to individually mark juvenile ambystomatid salamanders. – Herpetological Review, **42**: 43–47.

Pierce, B. A., K. D. Mcentire & A. A. E. Wall (2014): Popu-lation size, movement, and reproduction of the Georgetown salamander,- *Eurycea naufragia*. – Herpetological Conservation and Biology, **9**: 137–145.

Pillai, B. R., L. Sahoo, K. D. Mahapatra, R. Ponzoni, S. Sahu, S. Mohanty, Vijaykumar & S. Sahu (2009): Evaluation of the new fluorescent internal tag (soft visible implant alphanu-meric tag) in the freshwater prawn, *Macrobrachium rosenber- gii*. – The Israeli Journal of Aquaculture, **61**: 345–350.

Pittman, S. E., A. L. Jendrek, S. J. Price & M. E. Dorcas (2008): Habitat selection and site fidelity of cope's gray treefrog (*Hyla chrysoscelis*) at the aquatic-terrestrial ecotone. – Journal of Herpetology, **42**: 378–385.

Potts, D. D. (2012): Investigating new methods to improve ageing and study movement patterns of larval great lakes sea lamprey (*Petromyzon marinus*) populations. – Department of Biology – University of Michigan, Flint.

Replinger, W. E. & J. B. Wood (2007): A preliminary investigation of the use of subcutaneous tagging in Caribbean reef squid *Sepioteuthis sepioidea* (Cephalopoda: Loliginidae). – Fisheries Research, **84**: 308–313.

Robison-Cox, J. F. (1998): A record linkage approach to imputation of missing data: Analyzing tag retention in a tag-recapture experiment. – Journal of Agricultural, Biological, and Environmental Statistics, **3**: 48–61.

Salvidio, S. (2013): Homing behaviour in *Speleomantes strinatii* (Amphibia Plethodontidae): A preliminary displacement experiment. – North-Western Journal of Zoology, **9**: 429–433.

Salvidio, S., A. Latters, M. Tavano, F. Melodia & M. V. Pas-torino (1994): Ecology of a *Speleomantes ambrosii* population inhabiting an artificial tunnel. – Amphibia-Reptilia, **15**: 35–45.

Sanchez-Camara, J. & D. J. Booth (2004): Movement, home range and site fidelity of the weedy seadragon *Phyllopteryx tae- niolatus* (Teleostei: Syngnathidae). – Environmental Biology of Fishes, **70**: 31–41.

Scaravelli, D., P. Laghi & C. Pastorelli (2002): Rinvenimento in natura di *Speleomantes italicus* (Dunn, 1923) con un arto rigenerato. – in: Salvidio, S. & M. V. Pastorino (eds): Pri-mo convegno nazionale biologia dei geotritoni europei genere *Speleomantes*, Genova e Busalla (GE), 26 e 27 ottobre 2002, Programma e Riassunti, Università di Genova, Genova.

Swanson, J. E., L. L. Bailey, E. Muths & W. C. Funk (2013): Factors influencing survival and mark retention in postmetamor-phic boreal chorus frogs. – Copeia, 2013(4): 670–675.

Turek, K. C., M. A. Pegg & K. L. Pope (2014): Short-term evaluation of visible implant alpha tags in juveniles of three fish species under laboratory conditions. – Journal of Fish Biology, **84**: 971–981.

Wagner, J. P., R. B. Blaylock & M. S. Peterson (2013): Evaluation of internal tag performance in hatchery-reared juvenile spotted seatrout. – North American Journal of Fisheries Management, **33**: 783–789.

Woods, C. M. C. (2005): Evaluation of vi-alpha and pit-tagging of the seahorse *Hippocampus abdominalis*. – Aquaculture International, **13**: 175–186.

Chapter XI

Submitted to Biodiversity & Conservation, November 2017

N-mixture models reliably estimate the abundance of terrestrial salamanders

Gentile Francesco Ficetola[1,2], Benedetta Barzaghi[1], Andrea Melotto[1], Martina Muraro[1], Enrico Lunghi[3,4,5], Elia Lo Parrino[1], Arianna Urso[1], Claudia Canedoli[6], Giorgio Scarì[1], Roberta Pennati[1], Franco Andreone[7], Raoul Manenti[1]

1) Department of Environmental Science and Policy, University of Milan, Milano, Italy

2) Laboratoire d'Ecologie Alpine (LECA), CNRS, Université Grenoble Alpes, Grenoble, France7) Via Isalle 4, 08029 Siniscola, Italy

3) Universität Trier Fachbereich VI Raum-und Umweltwissenschaften Biogeographie, Universitätsring 15, 54286 Trier, Germany

4) Museo di Storia Naturale dell'Università di Firenze, Sezione di Zoologia "La Specola", ViaRomana 17, 50125 Firenze, Italy

5) Natural Oasis, Via di Galceti 141, 59100 Prato, Italy

6) DISAT, Università degli Studi di Milano-Bicocca. Piazza della Scienza 1, 20126 Milano, Italy
7) Museo Regionale di Scienze Naturali, Torino, Italy

Abstract

Accurate measures of species abundance are essential to identify conservation strategies. *N*-mixture models are increasingly used to estimate abundance on the basis of species counts. In this study we tested whether abundance estimates obtained using *N*-mixture models provide consistent results with more traditional approaches requiring capture (capture-mark recapture and removal sampling). We focused on two endemic, threatened species of salamanders, for which accurate abundance data are needed for periodical assessment of conservation status: the the Lanza's Alpine salamander *Salamandra lanzai* and the Ambrosi's cave salamander *Hydromantes ambrosii*. For Alpine salamanders, detection probability was low (0.14). Nevertheless, even if a few surveys were performed, abundance estimates obtained using *N*-mixture models were mostly consistent with the ones obtained through capture-mark-recapture. For cave salamanders, detection probability was higher (~0.6). In this case, we obtained an excellent match between the estimates of *N*-mixture models and the ones of removal sampling. Overall, *N*-mixture models provide reliable abundance estimates that are well comparable with the ones of more traditional approaches, and offer several additional advantages such as a smaller sampling effort and no need of manipulating individuals, which in turn reduces the risk of harming animals and spreading infectious diseases.

Keywords: Amphibian conservation: capture-mark-recapture: detectability: *Hydromantes ambrosii*: monitoring: removal sampling: *Salamandra lanzai*.

Introduction

Estimating species abundance is a pivotal task of species monitoring. Unfortunately, in most of cases detecting individuals of the target species can be challenging. Very often we are not able to detect all individuals present in a given place, and this may happen for several reasons, such as their elusive behaviour, cryptic habits or simply because of the limited ability of surveyors (Mazerolle et al. 2007). Therefore, the number of observed individuals rarely represents a reliable estimation of the number of individuals actually present in a given area.

Multiple approaches have been developed to estimate the true number of present individuals. Among them, approaches requiring multiple sessions of capture have a considerable success. For instance, in capture-mark-recapture (CMR) approaches animals of a population are captured, individually marked or photographed to allow identification, and released at the capture site. The frequency of marked individuals observed in subsequent capture sessions is then used to estimate abundance (Chao 2001; Mazerolle et al. 2007). Removal sampling (sometimes named catch-effort model) is an alternative approach, which requires the systematic capture and removal of individuals. Population size is then estimated on the basis of the decline in catch size during sequential capture sessions (Chao and Chang 1999; Chao 2001; Petranka and Murray 2001). These studies have been particularly useful to obtain reliable estimates of population size, and have revealed long term temporal trends, allowing to identify the factors determining the survival of individuals and the decline of populations (Schmidt 2004; Schmidt et al. 2005; Lindstrom et al. 2010; O'Donnell and Semlitsch 2015; Romano et al. 2016).

However, approaches requiring the capture and identification are generally labour intensive, as many individuals need to be captured and identified to obtain reliable population estimates. Therefore, the broad scale monitoring of the abundance of wildlife has been a challenge for decades (Pollock et al. 2002). In the last years formal approaches have been proposed to estimate animal abundance from repeated counts at fixed sites, without marking individuals to identify them (Royle and Nichols 2003; Royle 2004). The number of individuals detected in a given site is counted using standard monitoring techniques (e.g. point counts, observation in small plots, fixed area transects), and each site is generally surveyed in multiple occasions. The repeated counts in a given site are then used to jointly estimate the detectability of individuals and population size on the basis of *N*-mixture (or hierarchical) models (Royle and Nichols 2003; Royle 2004; Kéry et al. 2009; Dail and Madsen 2011). As they do not require capture or manipulation of individuals, such models might allow to collect abundance information over larger areas compared to traditional approaches, can be also appropriate for protected species, and have been proposed for broad-scale assessment of populations (Kéry et al. 2009; Griffiths et al. 2015; Ficetola et al. 2017). The usefulness of *N*-mixture models to estimate abundance of amphibians and reptiles is advocated since several years (Mazerolle et al. 2007; Kéry et al. 2009; Romano et al. 2017) and, given their high cost-effectiveness, these approaches have also been suggested to obtain broad scale estimates of the population trends of amphibians and reptiles (Ficetola et al. 2017). For instance, repeated counts analysed with *N*-mixture models have been proposed for the periodic monitoring of several species of amphibians and reptiles listed in the EU Habitat Directive at the national scale (Stoch and Genovesi 2016). However, these approaches are not yet widely used to estimate population abundance, perhaps because practitioners remain unsure about their efficiency, compared to more traditional techniques requiring capture. Studies comparing the performance of mixture models with more traditional approaches (e.g. CMR) found similar abundance and density estimates (e.g. Priol et al. 2014; Courtois et al. 2016) but, until now, such comparative analyses have focused on a limited range of species.

In this study, we compared population estimates obtained using *N*-mixture models with estimates obtained applying more traditional approaches, i.e. removal sampling and capture-mark-recapture. We focused on two threatened species of salamanders endemic of Italy and adjacent areas: the Lanza's alpine salamander *Salamandra lanzai*, and the Ambrosi's cave salamander *Hydromantes ambrosii*. Both species are fully terrestrial and do not require water for reproduction (Lanza et al. 2007), thus other traditional approaches to estimate the abundance of amphibians (e.g. egg counts, monitoring of breeding sites) cannot be used. Therefore, the reliability of monitoring approaches based on the observation of unmarked active individuals is a key aspect to provide effective information for management plans.

Methods

Study area and sampling

Lanza's Alpine Salamander *Salamandra lanzai*

Salamandra lanzai is endemic of a small area of the Alps, between NW Italy and W France, and lives at altitudes of 1200-2650 m. This salamander is viviparous, and individuals are active at the surface from late spring until early autumn, mostly at night and during humid periods (Andreone 2007). The study was performed at the Pian del Re area (approx. 44.70°N, 7.10°E; altitude: 2000-2150m; Fig 1), which is an alpine meadow with multiple small streams, and is the type locality of *S. lanzai*.

Repeated counts. We defined 28 rectangular (20 × 10 m) plots, across the whole Pian del Re. Each plot was surveyed by a 6-8 people team, actively searching and counting all the salamanders at the surface for 10-15 min. Plots were surveyed 2-3 times (average: 2.3 surveys per plot) in the period 16-19 August 2015 after dusk, between 9.00 pm and 1.00 am.

Capture-mark-recapture data were obtained from previously published studies performed in the same area in 1992-2003, which sampled a surface of approx. 41,000 m^2 (Fig. 1; Andreone et al. 1999; Andreone et al. 2007).We positioned the 28 plots performed in 2015 as follows: eight were inside the study area were Andreone et al.(1999; 2007) performed they CMR study, 17 were nearby the Andreone et al. (1999; 2007) study area (<250 m from the area), and three were 500-750 apart (Fig. 1).

Cave salamander *Hydromantes ambrosii*

The Ambrosi's cave salamander *H. Ambrosii* (see Wake 2013 for discussion on nomenclature) is endemic of a small area of peninsular Italy. This terrestrial salamander does not live exclusively in caves, as from autumn to spring it is often active at the surface. However, during the dry and hot Mediterranean summer it often moves to underground refugia, where its detectability is highest (Lanza et al. 2006; Lunghi et al. 2015). In June 2017, we monitored ten cavities in Central Italy using both repeated counts and removal sampling. We considered the Spelerpes cave (44.13°N, 9.78°E), six cavities within the Pignone karst Area (44.18°N, 9.72°E) and the Alta di Castè cave (44.12°N, 9.77°E). Explored depth of caves ranged between 9 and 48m. For repeated counts, each cave was monitored by 6-7 observers during daytime in sunny, dry days, by actively searching and counting all the salamanders visible on both cave walls and floor, as described by Lunghi et al. (2015). Each cave was surveyed two times within 3-10 days, to ensure meeting assumption of population closure. Survey effort was approx. 0.5 man/hour for each m of explored cave. Subsequently, we performed a three-sample removal experiment (Chao and Chang 1999). Immediately after the end of the last count survey, 6-7 people collected and stored salamanders in specific fauna boxes (40x25x15 cm) which were left inside caves. Removal session ended after 10 minutes without captures. At the end of the third session of capture, animals were released in the same cave areas in which they were found. Individuals were manipulated with disposable nitrile gloves, and all the equipment was disinfected following guidelines for preventing the spread of infectious diseases (Speare et al. 2004).

Data analysis

For both species, repeated counts were analysed using *N*-mixture models for closed populations (Royle 2004). We used Akaike's Information Criterion (Burnham and Anderson 2002) to select the most appropriate error distribution (Poisson or zero-inflated Poisson); we did not consider negative binomial errors as can produce infinite abundance estimates, particularly when detection probability is limited (Dennis et al. 2015). In models, we used 100 + the maximum observed species abundance as upper bound to approximate an infinite summation of the likelihood, since preliminary analyses suggested that this value provides robust estimates (Ficetola et al. 2017). For *S. lanzai*, activity is often higher early after dusk (Andreone et al. 2007), thus we considered hour of survey as a covariate potentially affecting detection probability; all surveys were conducted within four days, with constant meteorological conditions (similar temperature; no rain). For both species we then used empirical Bayes methods to estimate the posterior distribution of the abundance (mean and 95% Bayesian credible intervals) (Royle and Dorazio 2008). *N*-mixture models were run using the unmarked package in R 3.3.3 (Fiske and Chandler 2011).

To estimate population size from removal sampling of *H. ambrosii*, we used the sampling coverage estimator for heterogeneous model of Chao and Chang (1999), which is able to estimate population size with low bias, assuming that capture rate can be different among individuals. In preliminary analyses, we also tried using methods assuming homogeneous detection probabilities (Chao and Chang 1999), and obtained very similar estimates.

Results

Lanza's Alpine salamander

During repeated surveys, we obtained 63 salamander detections (range: 0-9 individuals per plot in each survey). In *N*-mixture models, we used a zero-inflated Poisson model as it showed AIC values lower than the Poisson model (model without covariates: AIC: 180.9 vs. 207.2). *N*-mixture models estimated an average detection probability of 0.14 (95% CI: 0.02-0.62). The model including hour of survey as covariate showed a higher AIC value than the model without hour (AIC = 182.5), and hour did not have a significant effect on salamander detection (z = -0.20, P = 0.608), therefore we kept the model without covariates. The estimated number of individuals ranged between 0.4 and 14.7 individuals per plot (Fig. 1).

Andreone et al. (1999; 2007), using capture-mark-recapture, estimated a density of ~300 individuals / ha. If we only consider the six plots within the CMR study area, the average density of salamander was 141 individuals / ha (95% CI: 38 - 450 individuals / ha). However, if we also include the plots nearby the CMR study area, the average density of salamanders was much higher, and closer to the estimates obtained using CMR. For instance, if we also consider plots within 250 m, the average density was 254.3 individuals / ha (95% CI: 130 – 544).

Ambrosi's cave salamander

During repeated counts, we obtained 599 salamander detections overall (range: 0-123 detections per cave in each survey). In *N*-mixture models, we used a Poisson error distribution as it showed AIC values lower than zero-inflated Poisson models (AICc: 510.4 vs. 512.4). *N*-mixture models estimated an average detection probability of 0.62 (95% CI: 0.59-0.76); empirical Bayes estimates indicated population abundances between 13 and 135 individuals / site (Table 1).

During the removal experiment, we captured 507 individuals (range: 0-99 individuals per cave in each session). In removal models, estimates of population size ranged between 13 and 244 individuals per cave (Table 1). The depletion method was unable to estimate population size for the two cavities with less captured individuals (False Snake and Ambrosi's sinkhole), in which zero individuals were captured during the second and third capture sessions.

Overall, *N*-mixture models and removal provided highly correlated and consistent estimates of population densities, with overlapping confidence intervals in most of populations (Table 1). In the populations with more individuals, *N*-mixture models tended to estimate smaller population sizes (Table 1). Nevertheless, a linear regression model, relating log-transformed abundance estimated with the two approaches revealed a strongly significant relationship ($R^2 = 0.91$; $F_{1,6} = 57.2$, $P < 0.001$), with a slope not significantly different from one ($B = 1.32$, 95% CI = 0.89 - 1.74) and an intercept not significantly different from zero ($k = -1.00$, 95% CI = -2.63 – 0.61).

Discussion

Accurate estimates of population size provide baseline data for many studies on population ecology, are essential to assess the conservation status of populations, and allow to identify management priorities. For instance, in the European Union the Habitat Directive protects several hundreds of animal species, and requires regular reports on the conservation status of all species protected by the directive. Such reports should include measures of population size and trends for all these species across the continent. Obtaining quantitative measures of population size of hundreds species over broad areas requires considerable monitoring efforts, and volunteers are a key resource for such a broad scale monitoring (Kéry et al. 2009; Sewell et al. 2010; Bonardi et al. 2011; Sewell et al. 2012; Griffiths et al. 2015; Maes et al. 2015). Approaches based on the capture of individuals such as capture-mark-recapture or removal sampling can provide reliable estimates of population size, but also have drawbacks. First, the capture of many individuals often requires more time than just observing their presence, therefore it can be less effective if we need monitoring many populations. For instance, for cave salamanders the removal sampling required approx. 80 man-days of work, while only 28 man-days were required for the visual surveys of *N*-mixutre models. Second, some techniques used to mark amphibians are expensive, or can harm individuals and pose ethical issues (e.g. toe clipping) (McCarthy and Parris 2004). Finally, the manipulation of specimens can pose threats to the study populations, such as the risk of transmission of infectious diseases (Lunghi and Veith 2017). Actually, European salamanders currently face the risk of infection by the chytrid fungus, *Batrachochytrium salamandrivorans,* which is lethal to most salamanders and is causing dramatic declines to several populations (Spitzen-van der Sluijs et al. 2013; Martel et al. 2014; Stokstad 2017). Under these circumstances, protocols requiring the capture of individuals by a large number of volunteers cannot be advocated. *N*-mixture models just require the observation of individuals, and thus are a promising alternative that do not require the manipulation of individuals. Our study shows that this approach provides reliable estimates of population size, which are highly comparable with the ones obtained by more traditional approaches.

Simulations suggest that population sizes estimated through *N*-mixture models generally have a limited bias (Ficetola et al. 2017), but the accuracy of these analyses still requires assessment. If detection probability is ~0.15 (as we recorded for the Lanza's alpine salamander) and sites are surveyed only a few times, simulations suggested that the relative bias of mixture models is 45-50%, i.e. they can over- or underestimate population size by approx. 50%. The accuracy of mixture models increases in easily detectable species and, if detection probability is 0.5, the expected bias is ~12-20%, and the correlation between true and estimated population size is expected to be ~0.9 (Fig. S2 in Ficetola et al. 2017). Testing the validity of these predictions is difficult, as in real populations we hardly know the true population size. Nevertheless, if we compare mixture models with CMR and removal sampling, we obtain measures of bias that are in agreement with these predictions. For alpine salamanders, the differences between

CMR and mixture models was 17-52% (depending if we consider all transects nearby the CMR study area, or only transects within the CMR study area; see below). For cave salamanders, the average relative bias was 26%, and the correlation between the two population size estimates was 0.95. The match between empirical data and simulations confirms the robustness of conclusions obtained through the virtual ecologist approach (Zurell et al. 2010), and supports mixture models as a reliable tool for the analysis of monitoring data.

In the Lanza's alpine salamander example, the two population estimates (*N*-mixture vs. CMR) were not performed in the same year. Salamander populations can undergo strong temporal variation, for instance in response to habitat modifications, climatic variation and variation of biotic factors (e.g. Schmidt et al. 2005; Whiteman and Wissinger 2005; Salvidio et al. 2016), and population fluctuations can occur even in absence of evident habitat changes (Pechmann et al. 1991). Despite we do not have quantitative data on this, the available information suggests that the study populations did not undergo strong variation of abundance through time. For instance, for *S. lanzai*, CMR estimates of abundance obtained in 1992 and in 2003 were very similar (Andreone et al. 1999; Andreone et al. 2007). Furthermore, the study area is a protected site, for which no major habitat modifications occurred in the last 20 years. Differences between CMR and *N*-mixture models were stronger if we only consider the eight plots falling within the CMR area. However, only eight plots were inside the target area, and sampled just 1,600 m^2, which represent 3.9% of the surface sampled by CMR. Therefore, the imperfect match between the estimates probably occurred because the sampling effort inside the target area was too low. Conversely, if all the plots nearby the Andreone et al. (1999; 2007) study area are considered, *N*-mixture models sampled a much larger surface (5,400 m^2). Salamander distribution is not homogeneously distributed across the landscape (Fig. 1), and the more intense effort probably allows a better representation of the overall landscape. The quality of population estimates generally increases at high sampling efforts (Boitani and Fuller 1999) which, in this case, is related to both the number of surveys per plot, and the total area covered by plots. When planning surveys, both these parameters must be defined a priori. Increasing the surface of each plot, and increasing the number of plots, are alternative approaches to increase sampling efforts. Deciding the best strategy (a few large or several small plots) strongly depends on parameters such as population density, detection probability, spatial heterogeneity and logistic constraints, and should be decided a priori, on the basis of study aims and resources availability. For instance, the number of individuals that are detected at each survey is generally higher in larger plots. Therefore, large plots and / or a large number of surveys per plot are a more effective strategy for species with limited detectability, while surveying several small transects can be preferable if populations have high detection probability (MacKenzie et al. 2006; Guillera-Arroita 2012; Ficetola et al. 2017; Guillera-Arroita 2017).

With cave salamanders, detection probability estimates were very high (≥ 0.4) using both approaches. High detection probability has already been demonstrated in other species of cave salamanders, particularly during their underground activity phase (Lindstrom et al. 2010; Ficetola et al. 2012; Lunghi et al. 2015), and this favors studies on the ecology and dynamics of cave salamander populations (e.g. Lunghi et al. 2015; Salvidio et al. 2016). The match between mixture models and removal sampling was excellent for the caves with fewer salamanders. Mixture models tended to underestimate population size in the two caves where removal estimated more individuals (Table 1, Fig. 2). It should be remarked that capture rate, estimated by removal sampling, is unrelated to both cave depth and salamander abundance ($|r| \leq 0.4$ and $P > 0.25$ for both correlation), suggesting that this does not occur because sampling quality was weaker in larger caves and / or in caves with more salamanders. Overall, the slope of the regression between population sizes estimated with the two approaches was not significantly different from one, and in most of cases abundance estimates were extremely similar, confirming that *N*-mixture models are an excellent approach to estimate the abundance of these animals.

We showed that *N*-mixture models can provide effective measures of the abundance of populations, but just measuring abundance provides limited information for conservation. An additional advantage of *N*-mixture models is the possibility of including covariates as potential predictors of species abundance also at very fine spatial scale (Romano et al. 2017). Assessing the factors that can determine differences in abundances among sites, or differences in abundance in a site surveyed during different years would provide more complete information and, for instance, can allow the identification of threatening factors that should be targeted by conservation strategies (Nichols and Williams 2006; Purse and Golding 2015). During surveys, experienced observers can also record parameters representing habitat quality or threats (Purse and Golding 2015) that can be successfully integrated within *N*-mixture models to provide quantitative management indications (e.g. Romano et al. 2017). The elaboration of comprehensive monitoring schemes, that allow the integration of species abundance data with information on habitat features and on drivers of abundance is not easy (Sutherland et al. 2015), but will provide essential information for more effective management.

Compliance with Ethical Standards

The authors declare no conflict of interest. All research involving animals was conducted under the authorization of National Authorities (Ministero dell'Ambiente della Natura e del Mare; *H. ambrosii*: *9384/PNM/2015*, 20624/PNM/2016*; S. lanzai: 14382/PNM/2015, 12273/PNM/2015)*.

Aknowledgments

We thank M. Bertolotto and the Pignone municipality for logistic support, and all the Scienze Naturali students helping during fieldwork.

References

Andreone F 2007. *Salamandra lanzai* Nascetti, Andreone, Capula, Bullini, 1998. Pages 215-221 *in* Lanza B, Andreone F, Bologna MA, Corti C, Razzetti E eds.) Fauna d'Italia, Vol XLII: Amphibia. Calderini, Bologna.

Andreone F, Bergò PE, Mercurio V 2007. La salamandra di Lanza *Salamandra lanzai*. Fusta Editore, Saluzzo (CN), Italy.

Andreone F, Clima V, De Michelis S 1999. On the ecology of *Salamandra lanzai* Nascetti, Andreone, Capula & Bullini, 1988. Number and movement of individuals, and influence of climate on activity in a population of the upper Po Valley. Herpetozoa 12: 3-10.

Boitani L, Fuller TK editors. 1999. Research techniques in animal ecology. Columbia University Press, New York.

Bonardi A, Manenti R, Corbetta A, Ferri V, Fiacchini D, Giovine G, Macchi S, Romanazzi E, Soccini C, Bottoni L, Padoa Schioppa E, Ficetola GF 2011. Usefulness of volunteer data to measure the large scale decline of "common" toad populations. Biol Conserv 144: 2328-2334.

Burnham KP, Anderson DR 2002. Model selection and multimodel inference: a practical information-theoretic approach. Springer Verlag, New York.

Chao A 2001. An overview of closed capture-recapture models. J Agric Biol Environ Stat 6: 158-175.

Chao A, Chang SS 1999. An estimating function approach to the inference of catch-effort models. Environmental and Ecological Statistics 6: 313-334.

Courtois E, Michel E, Martinez Q, Pineau K, Dewynter M, Ficetola GF, Fouquet A 2016. Taking the lead on climate change: Modeling and monitoring the fate of an Amazonian frog. Oryx 50: 450-459.

Dail D, Madsen L 2011. Models for estimating abundance from repeated counts of an open metapopulation. Biometrics 67: 577-587.

Dennis EB, Morgan BJT, Ridout MS 2015. Computational Aspects of N-Mixture Models. Biometrics 71: 237-246.

Ficetola GF, Pennati R, Manenti R 2012. Do cave salamanders occur randomly in cavities? An analysis with *Hydromantes strinatii*. Amphibia-Reptilia 33: 251-259.

Ficetola GF, Romano A, Salvidio S, Sindaco R 2017. Optimizing monitoring schemes to detect trends in abundance over broad scales. Anim Conserv in press.

Fiske I, Chandler R 2011. unmarked: An R package for fitting hierarchical models of wildlife occurrence and abundance. J Stat Softw 43: 1-23.

Griffiths RA, Foster J, Wilkinson JW, Sewell D 2015. Science, statistics and surveys: a herpetological perspective. J Appl Ecol 52: 1413-1417.

Guillera-Arroita G 2012. Designing studies to detect differences in species occupancy: power analysis under imperfect detection. Methods Ecol Evol 3: 860-869.

Guillera-Arroita G 2017. Modelling of species distributions, range dynamics and communities under imperfect detection: advances, challenges and opportunities. Ecography 40: 281-295.

Kéry M, Dorazio RM, Soldaat L, Van Strien A, Zuiderwijk A, Royle JA 2009. Trend estimation in populations with imperfect detection. J Appl Ecol 46: 1163-1172.

Lanza B, Andreone F, Bologna MA, Corti C, Razzetti E editors. 2007. Fauna d'Italia, vol. XLII, Amphibia. Calderini, Bologna.

Lanza B, Pastorelli C, Laghi P, Cimmaruta R 2006. A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). Atti Mus Civ St Nat Trieste 52 (Suppl.): 5-135.

Lindstrom J, Reeve R, Salvidio S 2010. Bayesian salamanders: analysing the demography of an underground population of the European plethodontid *Speleomantes strinatii* with state-space modelling. BMC ecology 10: 4.

Lunghi E, Manenti R, Ficetola GF 2015. Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? PeerJ 3: e1122.

Lunghi E, Veith M 2017. Are Visual Implant Alpha tags adequate for individually marking European cave salamanders (genus *Hydromantes*)? Salamandra in press.

MacKenzie DI, Nichols JD, Royle JA, Pollock KH, Bailey LA, Hines JE 2006. Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence. Academic Press, Burlington, MA.

Maes D, Isaac NJB, Harrower CA, Collen B, van Strien AJ, Roy DB 2015. The use of opportunistic data for IUCN Red List assessments. Biol J Linn Soc 115: 690-706.

Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt BR, Tobler U, Goka K, Lips KR, Muletz C, Zamudio KR, Bosch J, Lötters S, Wombwell E, Garner TWJ, Cunningham AA, Spitzen-van der Sluijs A, Salvidio S, Ducatelle R, Nishikawa K, Nguyen TT, Kolby JE, Van Bocxlaer I, Bossuyt F, Pasmans F 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346: 630-631.

Mazerolle MJ, Bailey LL, Kendall WL, Royle JA, Converse SJ, Nichols JD 2007. Making great leaps forward: Accounting for detectability in herpetological field studies. J Herpetol 41: 672-689.

McCarthy MA, Parris KM 2004. Clarifying the effect of toe clipping on frogs with Bayesian statistics. J Appl Ecol 41: 780-786.

Nichols JD, Williams BK 2006. Monitoring for conservation. Trends Ecol Evol 21: 668-673.

O'Donnell KM, Semlitsch RD 2015. Advancing Terrestrial Salamander Population Ecology: The Central Role of Imperfect Detection. J Herpetol 49: 533-540.

Pechmann JHK, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons JW 1991. Declining amphibian populations: the problem of separating human impacts from natural fluctuations. Science 253: 892-895.

Petranka JW, Murray SS 2001. Effectiveness of removal sampling for determining salamander density and biomass: A case study in an Appalachian streamside community. J Herpetol 35: 36-44.

Pollock KH, Nichols JD, Simons TR, Farnsworth GL, Bailey LL, Sauer JR 2002. Large scale wildlife monitoring studies: statistical methods for design and analysis. Environmetrics 13: 105-119.

Priol P, Mazerolle MJ, Imbeau L, Drapeau P, Trudeau C, Ramiere J 2014. Using dynamic N-mixture models to test cavity limitation on northern flying squirrel demographic parameters using experimental nest box supplementation. Ecool Evol 4: 2165-2177.

Purse BV, Golding N 2015. Tracking the distribution and impacts of diseases with biological records and distribution modelling. Biol J Linn Soc 115: 664-677.

Romano A, Costa A, Basile M, Raimondi R, Posillico M, Scinti Roger D, Crisci A, Piraccini R, Raia P, Matteucci G, De Cinti B 2017. Conservation of salamanders in managed forests: Methods and costs of monitoring abundance and habitat selection. For Ecol Manage 400: 12-18.

Romano A, Novaga R, Costa A 2016. *Olim palus*, where once upon a time the marsh: distribution, demography, ecology and threats of amphibians in the Circeo National Park (Central Italy). Acta Herpetol 11: 197-212.

Royle JA 2004. *N*-mixture models for estimating population size from spatially replicated counts. Biometrics 60: 108-115.

Royle JA, Dorazio RM 2008. Hierarchical Modeling and Inference in Ecology: The analysis of data from populations, metapopulations and communities. Academic Press, Lonon.

Royle JA, Nichols JD 2003. Estimating abundance from repeated presence-absence data or point counts. Ecology 84: 777-790.

Salvidio S, Oneto F, Ottonello D, Pastorino MV 2016. Lagged influence of North Atlantic Oscillation on population dynamics of a Mediterranean terrestrial salamander. International Journal of Biometeorology 60: 475–480.

Schmidt BR 2004. Declining amphibian populations: The pitfalls of count data in the study of diversity, distributions, dynamics, and demography. Herpetol J 14: 167-174.

Schmidt BR, Feldmann R, Schaub M 2005. Demographic processes underlying population growth and decline in *Salamandra salamandra*. Conserv Biol 19: 1149-1156.

Sewell D, Beebee TJC, Griffiths RA 2010. Optimising biodiversity assessments by volunteers: The application of occupancy modelling to large-scale amphibian surveys. Biol Conserv 143: 2102-2110.

Sewell D, Guillera-Arroita G, Griffiths RA, Beebee TJC 2012. When Is a Species Declining? Optimizing Survey Effort to Detect Population Changes in Reptiles. Plos One 7: e43387.

Speare R, Berger L, Skerratt LF, Alford RA, Mendez D, Cashins SD, Kenyon N, Hauselberger K, Rowley JJL 2004. Hygiene protocol for handling amphibians in field studies. James Cook University, Amphibian Diseases Group. URL : http://www.jcu.edu.au/school/phtm/PHTM/frogs/field-hygiene.doc, Townsville, Australia.

Spitzen-van der Sluijs A, Spikmans F, Bosman W, de Zeeuw M, van der Meij T, Goverse E, Kik M, Pasmans F, Martel A 2013. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. Amphibia-Reptilia 34: 233-239.

Stoch F, Genovesi P editors. 2016. Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. ISPRA, Roma.

Stokstad E 2017. Saving Europe's salamanders. Science 357: 242-245.

Sutherland WJ, Roy DB, Amano T 2015. An agenda for the future of biological recording for ecological monitoring and citizen science. Biol J Linn Soc 115: 779-784.

Wake DB 2013. The enigmatic history of the European, Asian and American plethodontid salamanders. Amphibia-Reptilia 34: 323-336.

Whiteman HH, Wissinger SA 2005. Amphibian population cycles and long-term data sets. Pages 177-184 *in* Lannoo ML (ed Amphibian Declines: Conservation Status of United States Species. California University Press, Berkley.

Zurell D, Berger U, Cabral JS, Jeltsch F, Meynard CN, Munkemuller T, Nehrbass N, Pagel J, Reineking B, Schroder B, Grimm V 2010. The virtual ecologist approach: simulating data and observers. Oikos 119: 622-635.

Table 1. Population abundances in ten populations of *Hydromantes ambrosii*, estimated with different approaches. *N* max: max number of individuals detected in one single survey session.

Cave	*N* max	*N*-mixture models		Removal sampling		
		Abundance	95% CI	Capture rate	Abundance	95% CI
Pignone left entrance	27	33.8	29 / 39	0.5	24	*
Pignone right entrance	38	50.1	45 / 56	0.702	53	51 / 63
Pignone main cave	38	53.3	48 / 59	0.636	59	57 / 70
Pignone – False snake’s hole	5	11.0	7 / 16	†	†	
Pignone – Ambrosi’s sinkhole	3	8.7	5 / 13	†	†	
Fornace	30	43.3	38 / 49	0.38	76	*
Fornace left entrance	15	23.8	19 / 29	0.6	20	19 / 33
Pignone abandoned mine	52	57.8	54 / 63	0.386	114	92 / 240
Spelerpes	6	13.1	9 / 18	0.426	13	*
Alta di Castè	123	144.5	138 / 152	0.382	244	219 / 300

† The method was unable to estimate population size

* Estimation of 95% CI was not available

Figure legends

Figure 1. Plots used to assess the abundance of *Salamandra lanzai*, and spatial variation of abundance estimates. The violet line is the approximate limit of the area sampled with capture-mark-recapture (Andreone et al. 2007).

Figure 2. Abundance of *H. ambrosii*: comparison between removal sampling and *N*-mixture models. Error bars are 95% confidence intervals of each estimate, the black dashed line has intercept = zero and slope = 1.

Figure 1

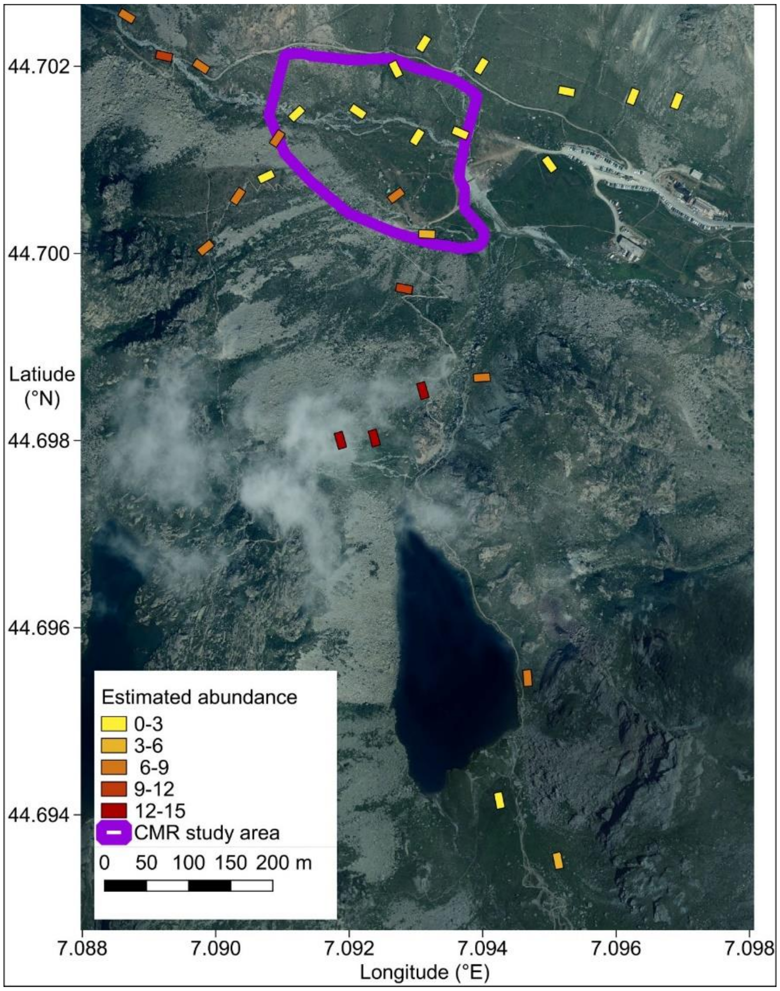
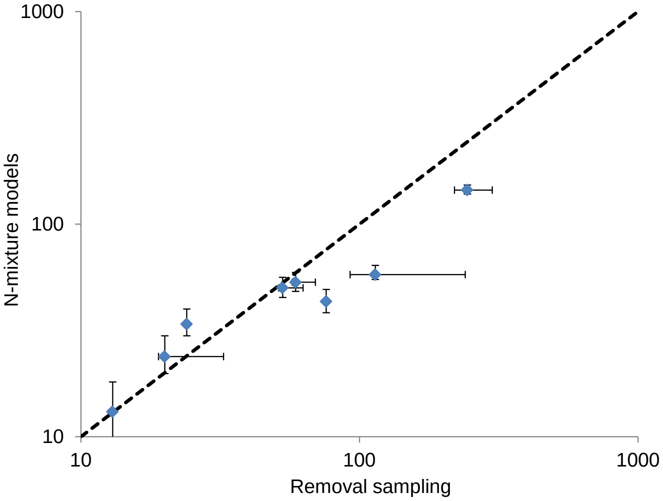


Figure 2



Supplementary materials
Chapter III

SUPPORTING INFORMATION

Microhabitat analyses challenge broad-scale patterns of niche evolution in terrestrial salamanders

Gentile Francesco Ficetola, Enrico Lunghi, Claudia Canedoli, Emilio Padoa-Schioppa, Roberta Pennati, Raoul Manenti

Appendix A. Supplementary results: niche analyses repeated measuring niches using different approaches, and supplementary figures.

SUPPLEMENTARY RESULTS

Niche analyses repeated measuring niches using different approaches

Microhabitat niche measured using the tolerance limit approach

Species distribution is often defined by tolerance limits for the fundamental niche. For instance, the northern limit of species may be defined by the tolerance limit to low temperatures (e.g. Hutchinson 1957, Whittaker et al. 2001). The approach to microhabitat niche in the main text compared the conditions at all the presence points where individuals were observed. We repeated the analyses by using the limits instead. For all study species, within cave distribution was determined by temperature, humidity and light, being positively related to humidity and negatively related to light and temperature within the observed range of these variables (Fig. 2, Table 1). We therefore calculated the tolerance limit of each species as the value of each variable below which there are 95% of salamander records (for temperature and light), or the value above which there are 95% of records (humidity). For instance, for *H. flavus* the tolerance limit for temperature and humidity were 18.1°C and 88.4%, respectively, because 95% of sectors with salamanders showed temperature ≤ 18.1°C and humidity ≥ 84.4%. The between-species differences in tolerance limits were then calculated as the pairwise Euclidean distances. Spiders were not considered in this analysis, as we never found clear relationships between spiders and salamanders (Table 1).

Microhabitat distance measured using the tolerance limit approach (Table A5) was positively correlated to microhabitat distances on the basis of Shoener's *D* (Mantel's test: $r = 0.45$, $P = 0.01$), while it was unrelated to phylogenetic distance ($r = 0.001$, P 0.997) and was not positively related to bioclimatic distance ($r = -0.30$, $P = 0.26$), confirming the results presented in the main text.

Bioclimatic niche measured using annual climatic features

We repeated the bioclimatic analysis using mean temperature and summed annual precipitation, instead than temperature and precipitation measured in the activity period (autumn, winter and spring). Niche overlap using annual features (Table A5) was nearly identical to the overlap obtained considering climate during the activity period (Mantel's test: $r = 0.98$, $P = 0.0001$; compare Fig. 2 with Fig. A4). Bioclimatic distance measured using annual climate was unrelated to microhabitat distance ($r = -0.2$, $P = 0.28$), while it remained positively related to both genetic ($r = 0.49$, $P = 0.004$) and geographical distance ($r = 0.56$, $P = 0.004$), confirming the results in the main text.

Bioclimatic niche measured incorporating spatial autocorrelation

Spatial autocorrelation might affect the results of PCA-env, therefore we repeated the bioclimatic analysis also including the prediction of a spatial generalized additive model as an additional variable. Niche overlap measured using this additional variable (Table A5) was strongly correlated to the overlap measured using standard PCA-env (Mantel's test: $r = 0.75$, $P = 0.0006$). Also in this analysis, bioclimatic distance was unrelated to microhabitat distance ($r = 0.003$, $P = 0.99$), while it remained positively related to both genetic ($r = 0.60$, $P = 0.002$) and geographical distance ($r = 0.75$, $P = 0.0002$), confirming the results in the main text.

Table A1. Caves and cave sectors sampled for the microhabitat analyses, and presence localities used for the bioclimatic analyses.

Species	Microhabitat analyses			Bioclimatic analyses
	N caves surveyed	*N* sectors	*N* sectors with presence	*N* presence localities
Hydromantes ambrosii	40	151	93	56
H. flavus	33	69	22	43
H. genei	29	183	66	54
H. imperialis	27	223	98	60
H. italicus	57	245	84	122
H. sarrabusensis	8	12	5	5
H. strinatii	61	178	124	179
H. supramontis	23	119	32	37

Table A2. Relationships between the occurrence of nine species of salamanders in underground sectors and microhabitat features: best-AIC generalized linear mixed models taking into account imperfect detection. R^2_C: conditional R^2 (Nakagawa and Schielzeth 2013); B: unstandardized regression coefficients.

	Best AIC models			
	R^2_C	B	χ^2_1	P
H. ambrosii	0.315			
Max light		-0.44	15.1	<0.001
Temperature		-0.13	9.8	0.002
H. flavus	0.99			
Max light		-29.93	5.3	0.020
Humidity		169.73	5.0	0.025
Humidity (Q)		-61.65	4.4	0.035
H. genei	0.73			
Min. light		-0.63	2.5	0.111
Humidity		17.51	10.1	0.002
H. imperialis	0.39			
Min. light		-1.32	8.6	0.003
Temperature		-0.31	14.6	<0.001
H. italicus	0.57			
Max. light		-0.62	29.1	<0.001
Humidity		8.92	17.8	<0.001
H. sarrabusensis	0.70			
Max. light		-1.45	6.0	0.014
H. strinatii	0.44			
Max. light		-0.39	9.8	0.002
Temperature		1.24	10.6	0.001
Temperature (Q)		-0.04	13.5	<0.001
H. supramontis	0.80			
Min. light		-1.53	3.7	0.055
Temperature		-0.50	12.3	<0.001

Table A3. Niche overlap between terrestrial salamanders, measured at both the microhabitat and the broad scale (bioclimatic) levels. In bold, significance values that remained significant after sequential Bonferroni's correction.

a) Niche overlap at the microhabitat-level

	H.ambrosii	*H.flavus*	*H.genei*	*H.imperialis*	*H.italicus*	*H.sarrabusensis*	*H.strinatii*
			Schoener's *D*				
H.flavus	0.201						
H.genei	0.24	0.443					
H.imperialis	0.451	0.408	0.633				
H.italicus	0.334	0.518	0.608	0.607			
H.sarrabusensis	0.467	0.681	0.322	0.262	0.444		
H.strinatii	0.719	0.221	0.534	0.429	0.429	0.759	
H.supramontis	0.335	0.613	0.835	0.482	0.785	0.359	0.57
			Significance of niche equivalency tests				
H.flavus	**<0.001**						
H.genei	**<0.001**	**<0.001**					
H.imperialis	**<0.001**	**<0.001**	**<0.001**				
H.italicus	**<0.001**	**<0.001**	**<0.001**	**<0.001**			
H.sarrabusensis	0.465	**<0.001**	**<0.001**	0.017	**<0.001**		
H.strinatii	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.907	
H.supramontis	0.023	0.554	0.099	**<0.001**	**<0.001**	0.081	**<0.001**

b) Niche overlap at the broad-scale

	H.ambrosii	*H.flavus*	*H.genei*	*H.imperialis*	*H.italicus*	*H.sarrabusensis*	*H.strinatii*
			Shoener's *D*				
H.flavus	0.220						
H.genei	0.028	0.081					
H.imperialis	0.408	0.366	0.065				
H.italicus	0.264	0.052	0.001	0.236			
H.sarrabusensis	0.072	0.188	0.018	0.488	0.004		
H.strinatii	0.504	0.287	0.012	0.219	0.331	0.053	
H.supramontis	0.201	0.465	0.042	0.457	0.055	0.378	0.299
			Significance of niche equivalency tests				
H.flavus	**<0.001**						
H.genei	**<0.001**	**<0.001**					
H.imperialis	**<0.001**	**<0.001**	**<0.001**				
H.italicus	**<0.001**	**<0.001**	**<0.001**	**<0.001**			
H.sarrabusensis	**<0.001**	**<0.001**	**<0.001**	**0.015**	**<0.001**		
H.strinatii	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
H.supramontis	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**	0.086	**<0.001**

Table A4. Results of commonality analysis evaluating the unique, common and total contribution of genetic and geographical distance on the bioclimatic differentiation of niches.

Independent variables	Contribution		
	Unique	Common	Total
Genetic distance	0.116	0.166	0.282
Geographical distance	0.105	0.166	0.272

Table A5. Alternative approaches to the measure of niche overlap between salamander species. a): niche distances at the microhabitat-level: Euclidean distances calculated using species tolerance limits; b) niche overlap at the broad scale (bioclimatic), calculated using annual values of temperature of precipitation; c) niche overlap at the bioclimatic scale, calculated also including a term representing spatial autocorrelation. In bold, significant values after sequential Bonferroni's correction. In a), no significance values can be calculated.

a) Niche distances at the microhabitat-level: Euclidean distances calculated using species tolerance limits

	H.ambrosii	*H.flavus*	*H.genei*	*H.imperialis*	*H.italicus*	*H.sarrabusensis*	*H.strinatii*
H.flavus	29.16						
H.genei	26.07	4.30					
H.imperialis	16.69	15.46	11.72				
H.italicus	26.12	4.01	2.88	11.72			
H.sarrabusensis	29.53	3.89	3.52	14.71	4.90		
H.strinatii	12.21	17.55	14.57	8.93	14.92	18.02	
H.supramontis	23.85	7.65	5.41	8.81	3.67	7.84	13.51

b) Niche overlap at the broad-scale, calculated using annual values of temperature of precipitation

Shoener's *D*

	H.ambrosii	*H.flavus*	*H.genei*	*H.imperialis*	*H.italicus*	*H.sarrabusensis*	*H.strinatii*
H.flavus	0.276						
H.genei	0.013	0.157					
H.imperialis	0.469	0.369	0.072				
H.italicus	0.257	0.032	0.001	0.206			
H.sarrabusensis	0.044	0.184	0.025	0.498	0.002		
H.strinatii	0.541	0.276	0.008	0.231	0.271	0.032	
H.supramontis	0.246	0.451	0.065	0.359	0.028	0.415	0.307

Significance of niche equivalency tests

H.flavus	**<0.001**						
H.genei	**<0.001**	**<0.001**					
H.imperialis	**<0.001**	**<0.001**	**<0.001**				
H.italicus	**<0.001**	**<0.001**	**<0.001**	**<0.001**			
H.sarrabusensis	**<0.001**	**<0.001**	**<0.001**	**0.017**	**<0.001**		
H.strinatii	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
H.supramontis	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.113	**<0.001**

c) Niche overlap at the broad-scale, integrating an additional term representing spatial autocorrelation

Shoener's *D*

	H.ambrosii	*H.flavus*	*H.genei*	*H.imperialis*	*H.italicus*	*H.sarrabusensis*	*H.strinatii*
H.flavus	0.238						
H.genei	0.069	0.506					
H.imperialis	0.249	0.426	0.235				
H.italicus	0.387	0.217	0.000	0.205			
H.sarrabusensis	0.008	0.247	0.070	0.452	0.009		
H.strinatii	0.349	0.240	0.170	0.162	0.243	0.027	
H.supramontis	0.189	0.468	0.443	0.492	0.174	0.302	0.249

H.flavus	**<0.001**						
H.genei	**<0.001**	**<0.001**					
H.imperialis	**<0.001**	**<0.001**	**<0.001**				
H.italicus	**<0.001**	**<0.001**	**<0.001**	**<0.001**			
H.sarrabusensis	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**		
H.strinatii	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
H.supramontis	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.020**	**<0.001**

Supplementary figures

Figure A1. Distribution of a) caves sampled for the microhabitat analyses; b) presence localities used for the broad scale, macroecological analyses.

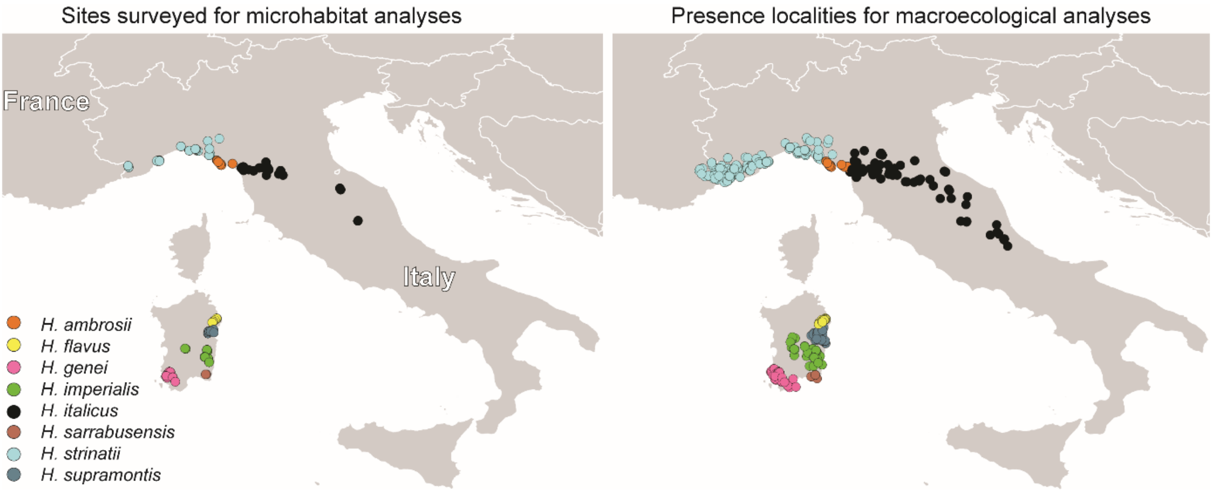


Figure A2. Relationships between microhabitat features and presence of salamanders. The plots show the three relationships that are significantly non-linear (See Table 1).

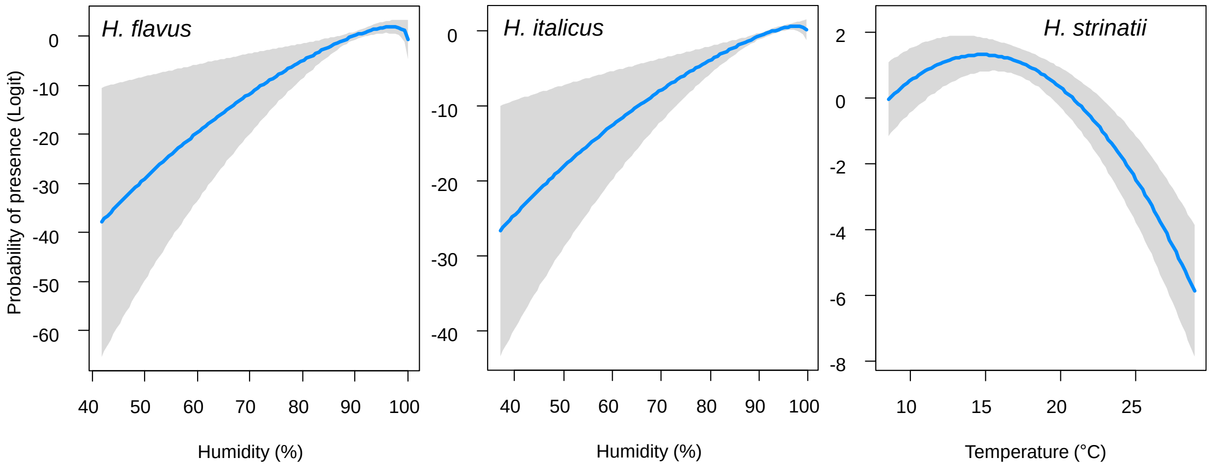


Figure A4. Niche differences among species according to a) microhabitat and b) bioclimatic analyses (multidimensional scaling plots), using annual temperature and precipitation instead than conditions during the activity seasons. Blue arrows are environmental variables added to plots using vector fitting.

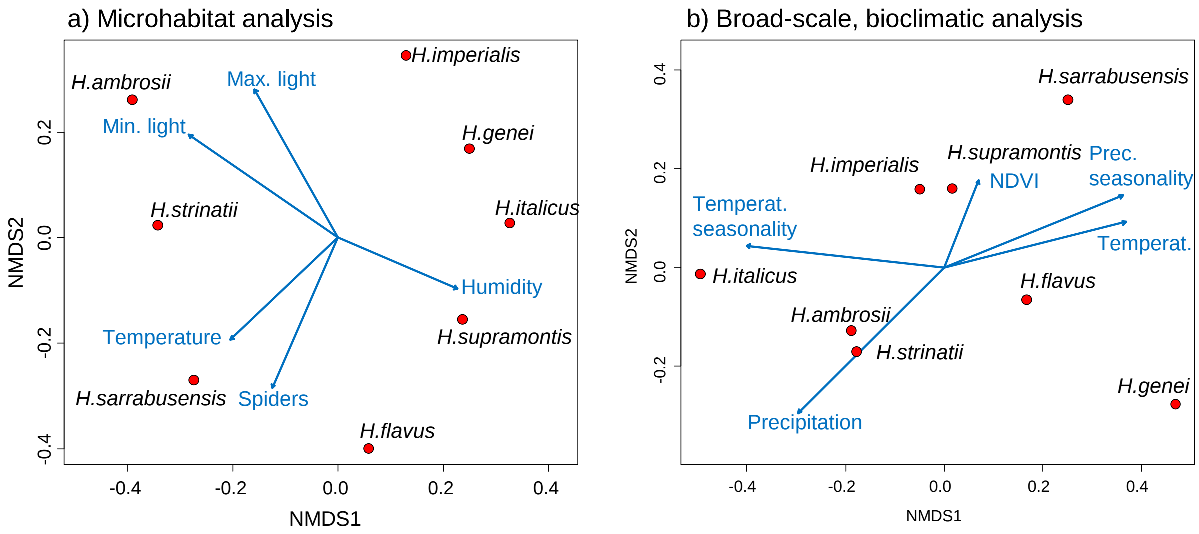
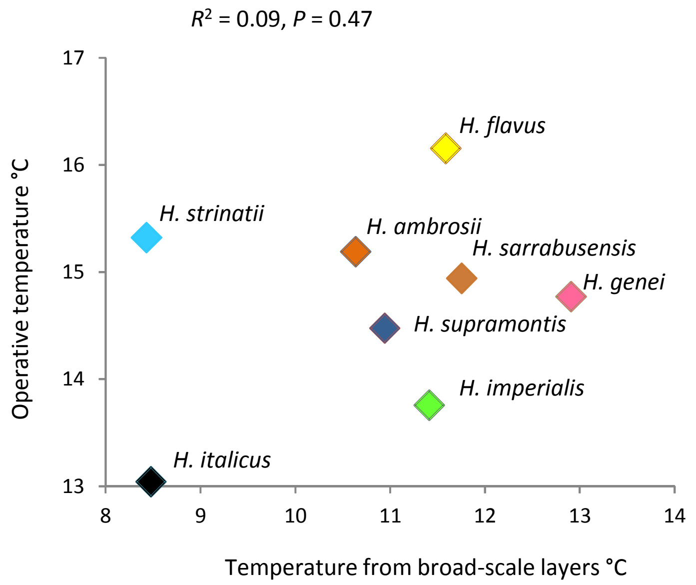


Figure A5. Relationship between operative temperature (mean temperature of sectors with active salamanders), and mean annual temperature measured on the basis of broad-scale variables.



Supplementary references

Hutchinson, G. E. 1957. Population studies: animal ecology and demography. Concluding remarks. — Cold Spring Harbor Symposia on Quantitative Biology 22: 415-427.

Nakagawa, S. and Schielzeth, H. 2013. A general and simple method for obtaining R^2 from generalized linear mixed-effects models. — Methods in Ecology and Evolution 4: 133-142.

Whittaker, R. J. et al. 2001. Scale and species richness: towards a general, hierarchical theory of species diversity. — J. Biogeogr. 28: 453-470.

CURRICULUM VITAE

Enrico Lunghi

PERSONAL INFORMATION

Name	**Enrico Lunghi**
Address	**Via Raffaello Luti 17, 59100 Prato (PO), Italy**
Telephone	**+39 3391604627 / +49 15905329542**
E-mail	**enrico.arti@gmail.com**
Nationality	**Italian**
Date and place of birth	**11/02/1985 Prato**

Memberships:

Societas Herpetologica Italica
Societas Europaea Herpetologica
Deutsche Gesellschaft für Herpetologie und Terrarienkunde
Natural Oasis
Unione Speleologica Calenzano
National Speleological Society

Education and training

Current position (from March 2015): Ph.D. student at Trier University, department of Biogeography

Date	October 2012 – July 2014
Name of organization	University of Florence, Faculty of Natural and Human Science
Principal subjects/occupational skills covered	Deep study on biodiversity and wildlife conservation
• Title of qualification awarded	**Master in Natural and Human Science – curriculum Management and Conservation of -Wildlife**

Date	September 2013 – February 2014
Name of organization	Erasmus at Tuebingen University
Principal subjects/occupational skills covered	Chemical Ecology, Landscape Genetic, Experiments on plant ecology
• Title of qualification awarded	

Date	24 August – 6 September 2013
Name of organization	University of Trento and MuSe
Principal subjects/occupational skills covered	Field activities and lectures
• Title of qualification awarded	**Tropical Rainforest Biodiversity: Field and GIS Tools for Assessing, Monitoring and Mapping - Tanzania**
Date	September 2010 – October 2012
Name of organization	University of Milan, Faculty of Natural Science
Principal subjects/occupational skills covered	I focused my studies mostly on Ecology, Zoology and Herpetology
• Title of qualification awarded	**Bachelor degree in Natural Science**

Work experiences and field activities

Dates	March - May 2017
Activity	Project: Study on biology and ecology of natural populations of genus *Testudo* in Italy, aimed at finding useful information for the proper relocation in nature of confiscated individuals
Position	**Researcher**
Name and address of employer	Natural History Museum of Florence University, sez. zoology “La Specola”

Dates	April 2016 – August 2016
Activity	Collaboration
Position	**Teaching assistantship**
Name and address of employer	University of Trier

Dates	August 2016
Activity	Project: Ethological experiments in underground environments
Position	**Researcher**
Name and address of employer	CNRS-Moulis

Dates	July 2015 – December 2015
Activity	Collaboration
Position	**Research assistantship**
Name and address of employer	University of Trier

Dates	January 2015 - Current days
Activity	Collaboration
Position	**Researcher**
Name and address of employer	Natural History Museum of Florence University, sez. zoology “La Specola

Dates	July 2014 – current days
Activity	Association
Position	**Field Guide and Researcher**
Name and address of employer	Natural Oasis

Dates	July 2013 – current days
Activity	Project: Herpetological field activities
Position	**Researcher**
Name and address of employer	University of Milan

Dates	October 2012 – current days
Activity	Photography of Nature
Position	**Field guide and Researcher**
Name and address of employer	Naturalfoto

Personal skills and competences

First language: **Italian**

Other languages:

- **English** IELTS (March 2014) Average score 6

  August 2009: English course at St. Giles School, Brighton

  September – December 2007: English course at Callan School, London

- **Spanish** Average good knowledge
  From August 2014 to January 2015 I spent 3 months in Spain to learn my third language

Other activities

Organizing Commettee (President): Biospeleology Congress, Cagliari (Italy) 2017
Scientific Commettee: Biospeleology Congress, Cagliari (Italy) 2017
Scientific Commettee: Premio di Laurea Rodolfo Giannotti II edizione (2016), Federazione Speleologica Toscana

Collaboration with Scientific Journals

Review Editor (November 2017 – current days): Frontiers in Ecology and Evolution, Editorial Board of Conservation

Reviewer: Amphibia-Reptilia, Invertebrate Biology, North-Western Journal of Zoology, Journal of Animal Ecology, Oryx.

DNA sequence deposited

Uncultured bacterium clone Rho1 16S ribosomal RNA gene, partial sequence GenBank: MF445653.1

Awards

2016 Grant obtained from The Mohamed bin Zayed Species Conservation Fund for the project **“**Conservation assessment of the subterranean aquatic planarians of Italy”. [Group leader: Raoul Manenti]

2016 Grant obtained from The Mohamed bin Zayed Species Conservation Fund for the project “Improving the knowledge of Sardinian grass snake (*Natrix natrix cetti*)”. [Group leader: Enrico Lunghi]

2016 National Speleological Society's Ralph W. Stone Graduate Fellowship.

2015 Grant obtained from The Mohamed bin Zayed Species Conservation Fund for the project “Assessing the status of European cave salamanders (genus *Speleomantes* and *Atylodes*)”. [Group leader: Enrico Lunghi]

2015 Genomic pop-up challenge organized by crowdfunding website (https://www.instrumentl.com/campaigns/population-dynamics-speleomantes-flavus/) with the project “Population dynamics of *Speleomantes flavus*”.

Publications

Scientific papers (peer reviewed)

Lunghi E., Corti C., Manenti R., Barzaghi B., Buschettu S., Canedoli C., Cogoni R., De Falco G., Fais F., Manca A., Mirimin V., Mulargia M., Mulas C., Muraro M., Murgia R., Veith M., Ficetola G. F. (in press). Comparative reproductive biology of European cave salamanders (genus *Hydromantes*): nesting selection and multiple annual breeding. Salamandra.

Lunghi E., Guillaume O., Blaimont P., Manenti R. (in press). The first ecological study on the oldest allochthonous population of European cave salamanders (*Hydromantes* sp.). Amphibia-Reptilia.

Manenti R., Lunghi E., Ficetola G. F. (2017). Cave exploitation by an usual epigean species: a review on the current knowledge on fire salamander breeding in caves. Biogeographia 32: 31-46.

Lunghi E., Veith M. (2017). Are Visual Implant Alpha tags appropriate to individually mark European cave salamanders (genus *Hydromantes*)? Salamandra 53(4): 541-544.

Lunghi E., Ficetola G. F., Barzaghi B., Vitillo C., Mulargia M., Manenti R. (2017). Melanism in European plethodontid salamanders. Spixiana 40(1): 157-160.

Lunghi E., Manenti R., Ficetola G. F. (2017). Cave features, seasonality and subterranean distribution of non-obligate cave dwellers. PeerJ 5: e3169.

Lunghi E., Monti A., Binda A., Piazzi I., Salvadori M., Cogoni R., Riefolo L. A., Biancardi C., Mezzadri S., Avitabile D., Ficetola G. F., Mulargia M., Manca S., Blaimont P., Di Cerbo A. R., Manenti R. (2017). Cases of albinism and leucism in amphibians in Italy: new reports. Natural History Sciences Atti della Società Italiana di Scienze Naturali e del Museo Civico di Storia Naturale di Milano 4(1): 73-80.

Lunghi E., Deschandol F., Cornago L., Cogoni R. (2016). Dark colorations in Sardinian Grass Snake (*Natrix natrix cetti*). Herpetological Bulletin 137: 28-29.

Lunghi E., Manenti R., Canciani G., Scarì G., Pennati R., Ficetola G. F. (2016). Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. Journal of Thermal Biology 60: 79-85.

Manenti R., Lunghi E., Canedoli C., Bonaccorsi M., Ficetola G. F. (2016). Parasitism of the leech, *Batracobdella algira* (Moquin-Tandon, 1846), on sardinian cave salamanders (Genus *Hydromantes*) (Caudata: plethodontidae). Herpetozoa 29 (1/2): 27-35.

Lunghi E., Mulargia M., Mulargia M. (2016). Evidence of malformation in The European cave salamanders, *Hydromantes flavus*. Herpetological Bulletin 135: 34-35.

Lunghi E., Corti C., Cencetti T. (2015). Oophagy in the Smooth snake (*Coronella austriaca*). Herpetological Bulletin 134: 35-36.

Lunghi E., Murgia R., De Falco G., Buschettu S., Mulas C., Mulargia M., Canedoli C., Manenti R., Ficetola G. F. (2015). First data on nesting ecology and behaviour in the Imperial cave salamander *Hydromantes imperialis*. North-Western Journal of Zoology 11(2): 324-330.

Lunghi E., Manenti R., Ficetola G. F. (2015). Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? PeerJ 3: e1122.

Manenti R., Lunghi E., Ficetola G. F. (2015). Distribution of spiders in cave twilight zone depends on microclimatic features and trophic supply. Invertebrate Biology 134(3): 242-251.

Lunghi E. Manenti R., Ficetola G. F. (2014). Do cave features affect underground habitat exploitation by non-troglobite species? Acta Oecologica 55: 29-35.

Lunghi E., Manenti R., Manca S., Mulargia M., Pennati R., Ficetola G. F. (2014). Nesting of cave salamanders (*Hydromantes flavus* and *H. italicus*) in natural environments. Salamandra, 50(2): 105-109.

Conference papers

Manenti R., Lunghi E., Canedoli C., Pennati R., Scarì G., Cimmaruta R., Ficetola G. G. (2017). Integrating micro-scale analyses in the study of niche similarity: Preliminary results in the hybrid zone between *Hydromantes italicus* and *H. ambrosii*. 19th European Congress of Herpetology (SEH), Salzburg.

Lunghi E., Ceccolini F., Cianferoni F., Cornago L., Mulargia M., Cogoni R., Manenti R., Corti C., Ficetola G. F. (2016). Dati preliminari sulla dieta autunnale dei geotritoni della Sardegna (genere *Speleomantes*). XI Congresso della Societas Herpetologica Italica, Trento.

Muraro M., Manenti R., Pennati R., Lunghi E. (2016). Primi risultati di uno studio di popolazione del geotritone *Speleomantes italicus* in ambiente ipogeo: valutazione dell'affidabilità del metodo del removal sampling. XI Congresso della Societas Herpetologica Italica, Trento.

Lunghi E. (2014). Progetto (geo)salamandra 2014. VIII Congresso della Federazione Speleologica Toscana, Campiglia Marittima.

Lunghi E., Bruni G., Manenti R., Ficetola G. F. (2014). Use of Visible implant elastomer on two amphibians orders (Anura and Caudata): data on efficiency and reliability. X Congresso della Societas Herpetologica Italica, Genova.

Ficetola G. F., Lunghi E., Pennati R., Manenti R. (2014). Variation of ecological niche in cave salamanders (genus *Hydromantes*). X Congresso Nazionale della Societas Herpetologica Italica, Genova.

Lunghi, E., Manenti, R., Ficetola, G. F. (2014). Distribuzione e fenologia del geotritone italiano (*Hydromantes italicus*) sull'Appennino centro-settentrionale: dati preliminari sulla presenza e sull'osservabilità in ambiente ipogeo. X Congresso Nazionale della Societas Herpetologica Italica, Genova.

Conference abstracts

Manenti R., Lunghi E., Canedoli C., Pennati R., Scarì G., Cimmaruta R., Ficetola, G. F. (2017). Integrating micro-scale analyses in the study of niche similarity: preliminary results in the hybrid zone between *Hydromantes italicus* and *H. ambrosii.* 19th European Congress of Herpetology (SEH), Salzburg.

Modica C., Lunghi E., Veith M. (2017). Test of photographic identification on *Speleomantes supramontis* (Lanza, Nascetti, Bullini, 1986). Biospeleology Congress. Abstracts and Photographs exhibition.

Lunghi E., Bruni G., Manenti R., Ficetola G. F. (2017). What frogs do in caves? Preliminary data of a multiseason study. Biospeleology Congress. Abstracts and Photographs exhibition.

Muraro M., Manenti R., Ficetola G. F., Lunghi E. (2017). Analisi sulla variabilità fenotipica degli arti in *Spelomantes italicus.* Biospeleology Congress. Abstracts and Photographs exhibition.

Barzaghi B., Lunghi E. (2017). Studio preliminare su una delle più grandi popolazioni di *Speleomantes imperialis*: Spelerpes, la grotta delle meraviglie. Biospeleology Congress. Abstracts and Photographs exhibition.

Di Gregorio S., Lunghi E. (2017). Analisi sull'abbondanza di una comunità presente in ambiente ipogeo in relazione al ciclo giorno/notte. Biospeleology Congress. Abstracts and Photographs exhibition.

Demontis C., Lunghi E., Cogoni R., Tuveri E., Orrù G. (2017). Antibacterial Activity Of The Skin Secretions Of *Hydromantes sarrabusensis.* Biospeleology Congress. Abstracts and Photographs exhibition.

Manenti R., Ficetola G. F., Lunghi E., Canedoli C. (2017). Informazioni preliminari sulla fauna delle grotte delle province di Pavia e di Alessandria, con cenni sul limite settentrionale del geotritone *Speleomantes strinatii* (Amphibia, Urodela, Plethodontidae). Biospeleology Congress. Abstracts and Photographs exhibition.

Book sections

Lunghi, E., Manenti, R., Ficetola, G.F., 2016. *Speleomantes flavus* (Stefani, 1969) (Geotritone del Monte Albo); *S. genei* (Temminck & Schlegel, 1838) (Geotritone di Gené); *S. imperialis* (Stefani, 1969) (Geotritone imperiale); *S. sarrabusensis* (Lanza et al., 2001) (Geotritone dei Sette Fratelli); *S. supramontis* (Lanza, nascetti & Bullini, 1986) (Geotritone del Supramonte). In Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. eds F. Stoch, P. Genovesi, pp. 212-215. ISPRA, Roma.

Ficetola, G. F., Lunghi, E., Fiacchini,D., Salvidio, S., 2016. *Speleomantes ambrosii* (Lanza, 1955) (Geotritone di Ambrosi), *S. strinatii* (Aellen, 1958) (Geotritone di Strinati). In Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. eds F. Stoch, P. Genovesi, pp. 208-211. ISPRA, Roma.

Books

Tommaso Cencetti and Enrico Lunghi. Conoscere gli anfibi e i rettili della Toscana: I Serpenti. Edizioni Medicea Firenze. Prato.

Roberto Cogoni, Enrico Lunghi, Renata Manconi, Raoul Manenti and Manuela Mulargia (Eds). Biospeleology Congress. Abstracts and Photographs exhibition.

Popular publications

Ficetola G. F., Lunghi E., Canedoli C., Manenti R. (2015). I geotritoni: cinque ricchezze nascoste della Sardegna. Sardegna Speleologica 27: 80-87.

Lunghi E. (2014). Grotta dolce Grotta. L’utilizzo delle grotte come sito riproduttivo. TALP, 48: 48-55.

Supervised thesis

Master Thesis, Domenico Avitabile (2017): Studio sulla dieta primaverile e autunnale in sei specie di geotritone (genere *Hydromantes*). University of Milan.

Bachelor Thesis, Martina Muraro (2015): Analisi quantitative e qualitative della struttura delle popolazioni del geotritone *Hydromantes italicus* in ambiente ipogeo. University of Milan.

Bachelor Thesis, Lorenzo Cornago (2015): La dieta autunnale in due specie sarde del genere *Hydromantes*. University of Milan.

Bachelor Thesis, Samuele Di Gregorio (2015): Studio della distribuzione spaziale di una comunità in ambiente ipogeo in relazione al ciclo giorno/notte e ai fattori biotici. University of Milan.