

ROLE OF CAVE FEATURES FOR AQUATIC TROGLOBIONT FAUNA
OCCURRENCE: EFFECTS ON “ACCIDENTALS”
AND TROGLOMORPHIC ORGANISMS DISTRIBUTION

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Subterranean aquatic habitats may be of particular interest from the zoological point of view. Most of the studies dealing with aquatic troglobiont fauna focus on troglobionts, such as organisms spending their entire life cycle in caves. Few studies are dedicated to non obligate cave dwellers whose occurrence is usually considered as accidental or random. The focus of this paper is on identifying the role played by cave features in the occurrence of aquatic troglobiont fauna and assessing the relationship between species with and without troglomorphic features and their cave habitat requirements. Organisms' occurrence and environmental features were assessed during a repeated survey of all the caves occurring in a karstic valley of north-western Italy. We surveyed 39 natural caves from March to December 2013 sampling the waterbodies inside them for fire salamander larvae and invertebrates occurrence. 11 aquatic taxa were recorded during this study including 2 aquatic troglobiont species, *Niphargus ambulator* Karaman, 1975 and *Dendrocoelum* sp. n. All the taxa did not occur randomly. Aquatic troglobionts preferred deep and dark caves while other non obligate cave dwellers occurred in shallower and brighter caves. The most important variables assessing distribution within caves were distance from the entrance and the temperature variation range of the pools. The occurrence of troglomorphic organisms was positively affected by pool distance from the cave entrance and pool area.

Key words: freshwater spring, *Dendrocoelum*, *Salamandra*, underground.

INTRODUCTION

Groundwater plays a key role for humans as it represents a major source of potable water supply in many countries and, globally, is the largest source of available freshwater. From the human perspective, since the prehistory, the interest of groundwater has focused on the operational management for potable or agricultural supply (BALLAND 1992). However, underground freshwater environments such as aquifers, natural springs, draining galleries and the hyporheic zone of rivers can be of particular interest also from a zoological perspective (STOCH *et al.* 2009). Many subterranean aquatic habitats contain exceptional communities of organisms (CULVER & PIPAN 2009, ROMERO 2009) which are receiving an increasing interest by zoologists. They are studied both from a biological point of view focusing on the evolutionary processes involved in cave colonization and on the description of new species (BOTELLO *et al.* 2013, JEAN *et al.* 2012, LEIJS *et al.* 2011, TRONTELJ *et al.* 2012) and, from a

conservation point of view involving the understanding of the features affecting survival and the management of the surrounding landscape (EBERHARD *et al.* 2009, GIBERT & CULVER 2009, GUZIK *et al.* 2010, HUMPHREYS 2006). Organisms inhabiting groundwater are named stygobionts (the term “stygo” is applied to aquatic organisms instead of “troglo” that refers to terrestrial ones) and following the recent classification proposed by (SKET 2008) they are distinct in different categories.

Generally obligate aquatic cave dwellers organisms are defined as troglobiont, such as organisms that evolved specific adaptations to underground freshwater habitats, in which they spend their entire life cycle. Aquatic troglobionts often show morphological features associated with their underground habitat. These characters, such as blindness and depigmentation, are commonly referred to as troglomorphisms (PIPAN & CULVER 2012, ROMERO 2009). The biological relevance of troglomorphism has been recently challenged by (ROMERO 2011) who provided several cases of troglomorphic species being not associated to cave environment. Moreover considerable morphological differences are frequently encountered among troglobiont animals. Some cave-dwellers even lack troglomorphic characters while some appear to be less, others more troglomorphic. Recently CULVER *et al.* (2010) tried to understand the diversity of troglomorphic features among 56 amphipods of the genus *Troglobromus* through ecological gradients among different underground environments. As trophic availability is supposed to decrease when the subterranean environment becomes more deep, antennal length was expected to elongate in response to a stronger selection pressure for food location, but they found no support for this prediction (CULVER *et al.* 2010).

It is also possible to recognize three categories of aquatic non-obligate cave dwellers (SKET 2008) that are often associated with caves although generally without showing troglomorphic features: subtroglophiles, eutroglophiles and troglonexes. Subtroglophiles are considered as “species inclined to perpetually inhabit a subterranean freshwater habitat” although remaining associated to the epigeous environment for some biological function; eutroglophiles are “essentially epigeous species” able to form permanent subterranean populations and troglonexes are considered as accidentals or stray aquatic organisms for cave environments. Recently, LUNGI *et al.* (2014), considering terrestrial communities of non strict cave dwelling organisms, found that also usually epigeous organisms did not occur randomly in caves, but selected them on the basis of their features suggesting that terms such as troglonexes or accidentals may not being reliable to the true occurrence patterns of these species.

As in the case of terrestrial species, most of the studies dealing with aquatic troglobiont fauna focus on aquatic troglobiont or subtroglophiles (DEHARVENG

et al. 2009), while few researches are devoted to troglaphiles and troglonexes (KORBEL *et al.* 2013, LARSON *et al.* 2013, ROMERO 2009, SCHMIDT & HAHN 2012).

In a survey of the oligochaetes inhabiting groundwater in southern Europe (GIANI *et al.* 2001) found more than 80% of non-troglobiont species and observed a species richness highly similar to that found in the surface waters of the same area. In another interesting study (WILHELM *et al.* 2006), described how aquatic eutroglophile and troglonexene species can make aquatic troglobiont vulnerable to replacement if the availability of energy (frequency and quantity of food available) in caves increases.

However, studies analyzing mechanisms determining the occurrence in caves of troglaphiles and troglonexes remain scarce (GRAENING *et al.* 2006). The focus of this paper is assess the category of species occurring in caves, describing their spatial distribution and analyzing if it can be explained by some environmental variables, considering all the species, thus not only aquatic troglobionts, but also species that are usually found also outside caves. Moreover, this study investigates the relationship between species with and without troglomorphic features and their cave habitat requirements.

The study design considers the data resulting from an extensive survey of all the caves occurring in a karstic valley of north-western Italy. The specific questions that the study wants to answer to are: 1) do troglonexes and troglaphiles occur randomly in caves? 2) how within caves do their requirements differ from aquatic troglobionts? and 3) which cave features are associated with the occurrence of troglomorphic organisms? As the proper categorisation of the aquatic taxa found was not feasible and, despite the correlation of troglomorphy and ecology is not perfect, we considered the broadly accepted ecological the occurrence of troglomorphic features to a priori assign taxa.

MATERIAL AND METHODS

Study area and surveys

We surveyed 39 natural caves occurring in the upper Cosia Valley (Como district) in Lombardy (NW Italy) (Fig. 1). We used the data of the CAI Speleo Club of Erba and the annotations of the Regional Speleological Register (RSR) to localize the caves and to record preliminary information of them. Preliminary surveys to verify the hydrological features of the caves (caves without water inside, caves with water only from water steam, temporary or permanent emitting or absorbing caves with waterbodies inside) were performed from 2009 to 2011. All the caves occurring in the RSR were located and explored except for three, annotated without water inside that could not been located. The caves were mainly emitting caves or had internal streams not far from the entrance; the maximum horizontal and vertical depth were 86 and 18 m respectively.

In all the caves with waterbodies inside ($n = 15$) from March to December 2013, we performed repeated surveys in all the seasons (for each cave minimum 4, maximum 6 and

average 4.5 surveys) in order to assess aquatic organisms occurrence and to record cave environmental features. All the caves hosted one or more pools generally of percolated water, sometimes linked by small connections with low water flow reaching also the outside environment as in the case of emitting caves.

Aquatic organisms occurrence was assessed by performing in each caves' pool 3 minutes of visual encounter survey followed by 7 minutes sampling by moving the substrate using a thin-mesh dip net to collect invertebrates. In the samplings were included all the invertebrates larger than 3 mm, thus excluding small aquatic troglobiont gastropods that require specific sampling technique with pool substrate analysis in laboratory (PEZZOLI 2008, 2010). Aquatic troglobionts were identified at the species level; molluscs, hirudineans, dipterans and plecopterans were identified at the genus level, but chironomids were included in the analyses at the family level, while trichopterans and coleopterans were identified at the family level and the two families recorded were considered together in the analyses. Moreover were sampled and considered also the larvae of the fire salamander that are known to represent the top predators of several underground waterbodies (LANC *et al.* 2012, MANENTI *et al.* 2011, 2013b). On the whole 31 pools were sampled.

We characterized cave general features by recording a) their depth measured from the entrance, b) if the waterbodies were temporary (i.e. if at least in one of the surveys the waterbodies were empty), c) if the waterbodies were in at least one survey directly connected through cave entrance to epigeous streams or pools, d) the distance from the entrance of the first waterbody encountered, e) average water depth, f) average water temperature, g) average water pH.

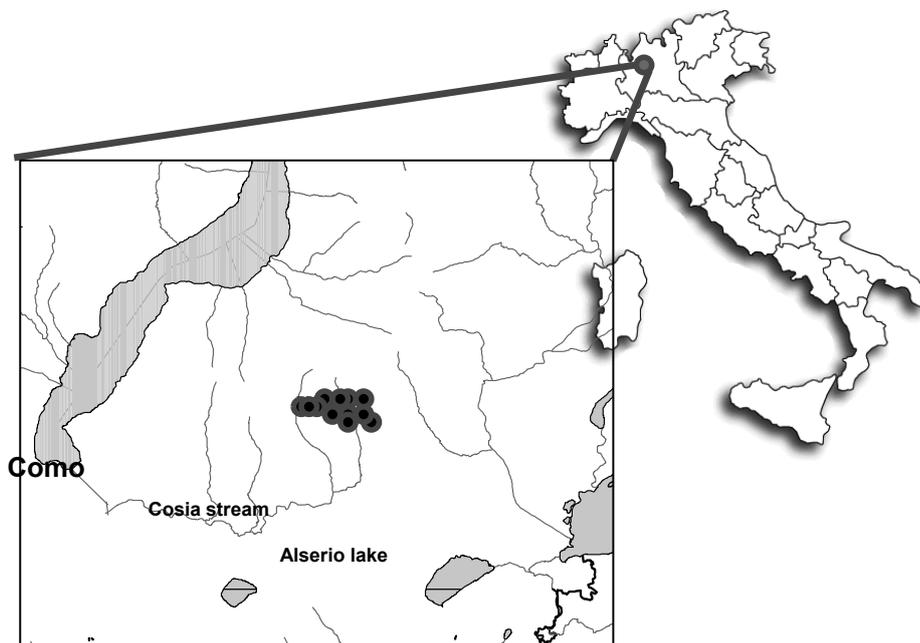


Fig. 1. Location of the study area Lombardy (Italy). Black circles identify the cave locations.

We characterized also pool features within caves by recording: a) pool distance from the entrance b), maximum illuminance received by the pool measured with a EM882 multi-function light-meter (PCE Instruments), c) pool width, d) pool maximum depth, e) maximum depth range reached by the pool during the surveys, measured as the difference between the maximum and the minimum depth recorded, f) pool average temperature and g) maximum temperature range reached by the pool during the surveys, measured as the difference between the maximum and the minimum temperature recorded.

Statistical analyses

Detectability analysis. A site is surely “occupied” if a species is found at that site, but the lack of detection of a species during all sampling occasions does not necessarily indicates that the species is absent (MACKENZIE 2006). This can lead to an underestimation of occupancy and might influence the results of analyses increasing the risk of data over-interpretation. Presence 5.5 (HINES 2006) was used to assess the probability of detection per visit as well as the probability of occupancy (ψ) of the species.

Site occupancy rate is estimated using a simple probabilistic argument to allow for species detection probabilities. If ψ is the probability that a site is occupied and $p[j]$ the probability of detecting the species in the j^{th} survey, given it is present at the site, MACKENZIE *et al.* (2002) use a probabilistic argument to describe the observed detection history for a site over a series of surveys.

For the pools data set, it was assumed that the probability of detection of species at a given survey might be affected by 4 survey-specific covariates: cave identity, water temperature, date, water depth. For each species, models were made assuming that detection probability depends on all the possible combinations of these covariates; the model with the lowest Akaike’s information criterion (AIC) and the highest weight was considered as the minimum adequate model describing species detectability (BURNHAM & ANDERSON 2002). It was then calculated the misdetection rate as the percentage difference between the observed occupancy and the occupancy estimated from the PRESENCE best model for each species occupancy. *Salamandra salamandra* (L., 1758) larvae are known to show high detectability levels (MANENTI *et al.* 2009, MANENTI *et al.* 2013b), but no data are available on the detectability of the invertebrates constituting the aquatic troglobiont fauna. To avoid data over-interpretation the taxa that occurred in less than a third of the caves were excluded from the analyses that follow.

Relationships between cave and pools features and aquatic troglobiont fauna occurrence. Two constrained redundancy analyses (RDA) were performed to evaluate the relative roles of caves, of aquatic habitats within caves and features on the multivariate structure (i.e., taxa composition) of aquatic troglobiont fauna communities, considering the species (5) that were observed in at least a third of the caves. RDA is a canonical analysis, combining the properties of regression and ordination techniques that allows evaluating how much of the variation of the structure of one dataset (e.g., community composition in a woodland; endogenous data-set) is explained by independent variables (e.g., habitat features; exogenous datasets) (BORCARD *et al.* 2011).

The first RDA was performed assuming aquatic taxa as endogenous dataset and the matrix of cave features as exogenous; the second RDA was performed assuming aquatic taxa as endogenous dataset and the matrix of pool features as exogenous. Accordingly to the current zoological knowledge (MANENTI *et al.* 2009, PEZZOLI 2008, PRZHIBORO 2009,

ROSSARO & BETTINETTI 2001, ROSSARO *et al.* 2006), the 5 considered taxa can be described as follow: 1) aquatic troglobiont and troglomorphic species such as *Niphargus ambulator* Karaman, 1975, an amphipod endemic of the study area and its surroundings, and *Dendrocoelum* n. sp., a new species of troglomorphic planarian apparently endemic of the study area that is going to be described; 2) larvae usual inhabitants of cold springs and small creeks or streams as the Chironomids of the genus *Micropsectra* and *Procladius* and the trichopterans of the families Limnephilidae and Brachycentridae and 3) vertebrate predators larvae of *Salamandra salamandra* with huge impact on macrobenthos communities often occurring in both springs and caves.

Factors determining the occurrence of troglomorphic organisms within caves. Generalized linear mixed models with binomial error distribution were used within an information-theoretic approach to assess whether the occurrence of troglomorphic organisms within caves was most likely explained by i) pool morphology, ii) pool microclimatic features or iii) pool stability. The presence/absence of at least a depigmented or blind species was considered as dependent variable. To take into account the heterogeneity (variation linked to landscape position or other factors) between the caves, cave identity was included as random factor in models.

First it was built a model representing pool morphology (including the variables pool distance from the entrance, pool area and the average of pool maximum depth recorded during surveys), second it was built a model representing pool climatic features (including the average of maximum illuminance and of water temperature recorded during surveys) and third it was built a model representing pool stability (including the variables maximum depth range and maximum temperature range reached by the pool during the surveys). Subsequently it was used the small-sample-size corrected version of Akaike's information criterion (AICc) to assess the relative support of the three models. AICc trades-off explanatory power vs number of predictors; parsimonious models explaining more variation have the lowest AICc values. It was then calculated the evidence ratio of the models which provides a measure of the relative likelihood of one hypothesis versus another. Finally it was also used likelihood ratio to assess the significance of the independent variables in the model in order to facilitate the interpretation of their role.

All the analyses were performed in the R 3.01 environment using the *vegan*, *car*, *AICmodavg*, *MuMIn* and *Himsc* packages (OKSANEN *et al.* 2005, R Development Core Team 2012).

RESULTS

Among the 15 caves 11 aquatic taxa were recorded (Table 1). Five of them occurred in at least a third of the caves. Their detection probability was generally high with a misdetection rate < 3% (Table 1). All the caves hosted internal pools and streams not receiving water from upstream epigeous water bodies that are quite scarce in the surveyed karstic area. At most some of the surveyed caves were temporary springs for downstream pools or little streams. Thus the accidental drift from upstream epigeous waterbodies should be excluded.

The relationship between the 5 taxa and cave features was significant ($P < 0.01$) and explained 76% of variation (Fig. 2). The first RDA axis was represented by shallow caves reaching high illuminance and explained 55% of the variance described by the RDA, while the second was represented by

Table 1. List of the recorded taxa, percentage of caves in which they have been observed and their occupancy estimation (psi = detection probability as estimated by PRESENCE). Psi has been calculated only for species occurring in at least a third of the caves with waterbodies.

Species	Number of caves	% pools observed	% pools occupied (psi estimate)	PRESENCE best model
Crustacea Amphipoda				
<i>Niphargus ambulator</i>	5	28	28.01	<i>P</i> constant
Triclada				
<i>Dendrocoelum</i> sp. n.	5	28	30.22	Water depth
Insects				
Elmidae, larvae	1	4		
<i>Tipula</i> sp.	1	4		
Chironomidae <i>Procladius</i> sp. and <i>Micropsectra</i> sp.	6	16	16.23	<i>P</i> constant
<i>Nemoura</i> sp.	1	8	–	
Trichoptera (Limnephilidae and Brachycentridae)	6	28	28.01	<i>P</i> constant
Hirudinea				
<i>Erpobdella</i> sp.	1	4		
Molluscs				
<i>Pisidium</i> sp.	1	4		
Nematomorpha				
Gordiidae	3	12		
Amphibians				
<i>Salamandra salamandra</i> larvae	5	32	32.01	<i>P</i> constant

caves with temporary waterbodies and explained 20.5 % of the variance described by the RDA. *Dendrocoelum* sp. n. and *Niphargus ambulator* were negatively associated to both axes; thus they preferred deep and dark caves with permanent waterbodies. Chironomids, trichopterans and, at a lower degree, salamander larvae were positively associated to first axis and negatively to the second; thus they occurred in shallower and brighter caves with permanent waterbodies.

The relationship between the 5 taxa and pool features was significant ($P < 0.0001$) and explained 57% of variation (Fig. 3). The first RDA axis was represented by pools near the cave entrance reaching higher illuminance and explained 43.6% of the variance described by the RDA, while the second was

represented by pools characterized by high ranges of temperature variation and explained 7% of the variance described by the RDA.

Niphargus ambulator was linked to pools far from cave entrance, while chironomids and trichopteran larvae to pools near cave entrance. *Dendrocoelum* sp. n. and fire salamander larvae distribution was not affected the first axis, with salamander larvae associated to pools with higher temperature ranges. In most of the caves and in the 50% of the pools where *Dendrocoelum* sp. n. occurred we found also *N. ambulator* specimens; also trichopteran and fire salamander larvae coexisted frequently.

The best model explaining the occurrence of troglomorphic organisms was the pool morphology model (Table 2) even if the evidence ratio toward the second best model, such as the pool microclimatic model was just 1.1. The morphological features affecting troglomorphic organisms occurrence were both the distance from the entrance and the pool surface.

DISCUSSION

Our results showed that both troglomorphic aquatic troglobionts and other *taxa* generally considered as crenobiont (i. e. living in springs) or stream dwelling occurred in the caves surveyed.

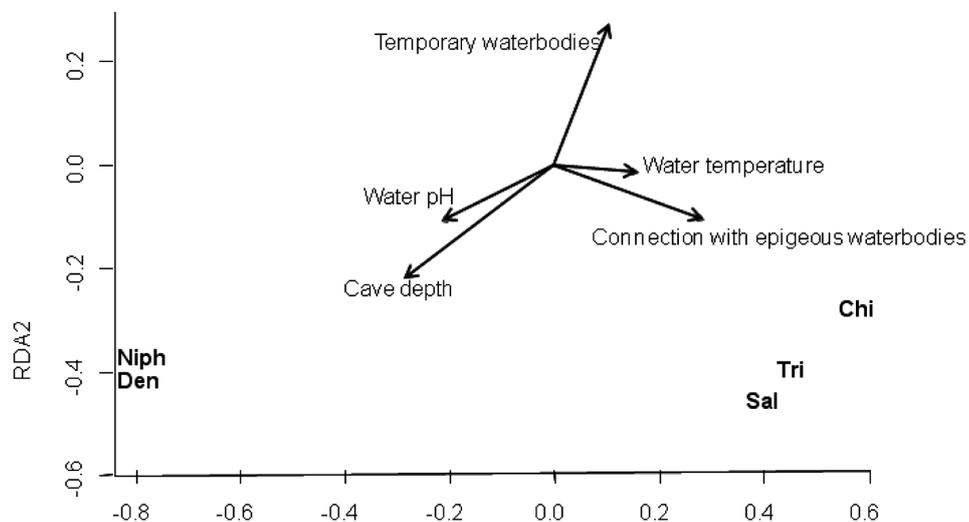


Fig. 2. Constrained redundancy analyses showing the relationship between cave features and taxa distribution. Niph = *Niphargus ambulator*, Den = *Dendrocoelum* sp. n., Chi = Chironomidae, genera *Procladius* and *Micropsectra*, Tri = trichopteran families Limnephilidae and Brachycentridae, Sal = *Salamandra salamandra*. Constraining variables are represented by arrows.

Table 2. Results of generalized linear mixed models describing the occurrence of troglobiontic organisms on the basis of the morphological, microclimatic and stability hypotheses.

Model	Independent variable	B	χ^2	P	AIC
Pool morphology					30.77
	Distance from the entrance	1.10	8.21	<0.01	
	Pool average depth	-0.29	0.01	0.89	
	Pool area	3.41	4.53	0.03	
Pool climate					32.57
	Illuminance	-0.36	1.55	0.21	
	Pool average temperature	-1.8	4.42	0.03	
Pool stability					37.02
	Pool maximum depth range	-1.22	0.39	0.53	
	Pool maximum temperature range	-1.12	0.25	1.01	

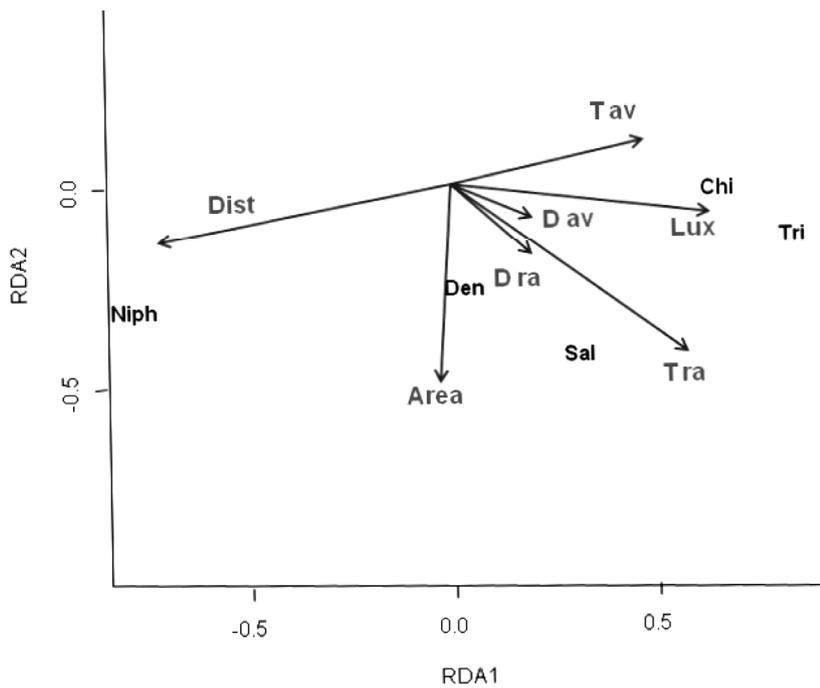


Fig. 3. Constrained redundancy analyses showing the relationship between underground pools features and taxa distribution. Taxa acronyms as in Fig. 1. Dist = distance from cave entrance, T av = average temperature, Lux = maximum illuminance, D av = average depth, T ra = temperature maximum range, D ra = depth maximum range. Constraining variables are represented by arrows.

The number of different taxa sampled was lower than that of the epigeous waterbodies of the study area (MANENTI 2010). Apart from the 2 aquatic troglobionts, only Gordidae, which larvae are parasitic of invertebrates, have both juvenile and adult aquatic stages. The other organisms found are larvae of terrestrial and flying organisms which have to enter the caves to breed. The only aquatic vertebrates found were the fire salamander larvae confirming the inclination toward the hypogeous waterbodies of this species (IANC *et al.* 2012, MANENTI 2008, MANENTI *et al.* 2009). If the fire salamanders can be considered close to subtroglaphiles in some karstic areas (BRESSI & DOLCE 1999, MANENTI *et al.* 2011), chironomids and trichopterans could be supposed as accidentals, thus considered troglonexes. However, our results underline that their occurrence was not random. *N. ambulator* and *Dendrocoelum* sp. n. are considered as aquatic troglobionts and are linked to deep caves with stable waterbodies. Chironomids, trichopterans and, at a lower level, salamanders to shorter ones. From a side in shorter caves it is possible that waterbodies are more easily reached by adults during breeding. For example in the fire salamander, cave accessibility is one of the fundamental requisites for cave exploitation (MANENTI *et al.* 2011). Moreover shorter caves can host climatic features more closed to those found by trichopterans and chironomids in epigeous springs.

The requirements of the different taxa, were underlined considering their distribution inside caves. The most important variables assessing distribution were distance from the entrance and the temperature variation range of the pools. The troglobiont *N. ambulator* occurred in distant pools, while chironomids and trichopterans near the cave entrance. *N. ambulator* is a species with a narrow distribution range, mostly represented by the study area (KARAMAN 1975) that likely chooses stable areas with less contact to the external area and without daylight and other predators.

Several *Niphargus* species can be found in epigeous habitats and also aquatic troglobionts have been observed during nighttimes outside caves (DANIELOPOL *et al.* 1999, FLOT *et al.* 2010, VERVIER & GIBERT 1991). The species *N. stygius*, is more widespread (MEZEK *et al.* 2010) and inhabits several groundwater systems of Lombardy around the study area (PEZZOLI 2010) being easily detectable also in springs and streams near the spring. It will be interesting to perform night survey to assess if *N. ambulator* distribution changes and to compare the habits of *N. stygius* that could explain why one species is strictly endemic and the other more widespread.

For chironomids and trichopterans larvae occurrence, pools that are not far from the cave entrances do not differ extremely to spring like conditions, except from illuminance, being considerably more dark; as recently pointed out for some Odonata (MANENTI *et al.* 2013c), flying insects can reach these environments and breed inside. The endemic troglobiont *Dendrocoelum* sp. n.

was not and the fire salamander larvae were only limitedly affected by the distance from the cave entrance. Both are predator species able to catch both *Niphargus* and insect larvae; in caves and in conditions of total darkness, fire salamander larvae adopt a wide foraging strategy that allow them to enhance the probability to catch a prey (MANENTI *et al.* 2013). It is possible that also this planarian uses a wide foraging strategy and both species likely exploit both inner and outer pools of the caves.

The variation range of temperature affected mostly fire salamander larvae. They occurred in pools with the higher variation range. This factor is likely related to their development requirements. The species grows slowly at temperatures of 0–5 degrees while much more quickly at higher temperatures (GIOVINE 1996, ZAKRZEWSKI 1987). Most studies focusing on aquatic subterranean environments ecology and biology focus mainly on aquatic troglobiont species (ROMERO 2009), while the factors determining trogloniles and troglonenes occurrence and distribution in caves is less studied. Caves communities primarily depend on the initial input of energy and nutrients from the surface that mostly occurs through two main mechanisms: passive transport (mostly by water and air), and the entering of organisms. For this reason both troglonenes and eu- or sub-trogloniles entering caves play important roles as source of energy becoming for example often prey for cave dwellers organisms (SMITHERS 2005).

All trogloniles and troglonenes species encountered during surveys were species inhabiting springs and headwaters; our results underline that they are able to actively colonize also the caves first sectors that can offer more stable waterbodies and, with the exclusion of the fire salamander larvae, with less predation pressure (MANENTI *et al.* 2013c, RINGIA & LIPS 2007). Finally it is interesting to note that the best model explaining the occurrence of troglomorphic organisms inside caves involved pools morphology, troglomorphic species being linked to larger pools far from the cave entrance. Further studies should assess if the distribution inside caves changes during nighttimes and which relationships could occur between the aquatic troglobiont and eutrogloniles, subtrogloniles and eutroglonenes found. Overall this study concentrated on a narrow karstic area, sampling all the existing caves inside it. The data shown on this paper may constitute a basis for further studies characterising different karstic areas or covering more extended areas.

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