



Behavioural drivers of ecotone exploitation: activity of groundwater animals in spring

Raoul Manenti^{1,2} · Matteo Galbiati^{1,2} · Stefano Lapadula¹ · Martina Forlani^{1,2} · Benedetta Barzaghi^{1,2} · Andrea Melotto³ · Gentile Francesco Ficetola¹

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Abstract

Border habitats such as interfaces and ecotones are promising research targets being likely areas of high species richness and genetic and phenotypic diversity. Springs are intriguing habitats exploited by both subterranean and surface species. For subterranean species, springs can provide higher trophic resources but can be risky in terms of predation and UV radiation, while for surface species, springs can be safer but less productive environments. We coupled field surveys and laboratory experiments to understand how predation risk and physical constraints, like light occurrence, affect spring exploitation by both a subterranean (*Niphargus thuringius*) and a surface crustacean amphipod species (*Echinogammarus stammeri*). From March to May 2021, we surveyed multiple springs and evaluated the activity (both during day and night) of the amphipods and of their predators. Furthermore, in a subterranean laboratory, we reared 80 *N. thuringius* and 80 *E. stammeri* under safe and risky conditions with both constant darkness and diel light variation assessing their activity and survival. Risky conditions were represented by the occurrence of meso-predators alone or coupled with the presence of a top predator. In the field, *N. thuringius* activity was negatively related to the density of predators, while laboratory experiments revealed a main role played by light treatments and night period. *E. stammeri* activity in the field was higher close to surface while in laboratory conditions decreased during time. In laboratory conditions, predation risk negatively affected survival of both amphipods. Our findings reveal that physical constraints play a key role in affecting the exploitation of ecotones and can mediate antipredator responses, thus providing selective pressures for the exploitation of border environments.

Significance statement

Understanding environmental pressures acting on ecotones is a key point to verify if new adaptations may occur at the border between two distinct habitats. Using both field and laboratory approaches, we show that, in springs, the behaviour of subterranean invertebrates is affected by surface physical constraints which can mediate the effects of predation risk. Behavioural strategies to avoid predation, such as nocturnal activity, may promote spring ecotone exploitation by groundwater animals, such as amphipod crustaceans.

Keywords Boundary · Transition · Interface · Source · Landscape of fear · Border · Salamander · *Niphargus* · Interaction · Ecotone

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✉ Raoul Manenti
raoul.manenti@unimi.it

¹ Department of Environmental Science and Policy, Università degli studi di Milano, Via Celoria, 26, 20133 Milan, Italy

² Laboratorio di Biologia Sotterranea, Parco Regionale del Monte Barro, Località Eremo, 23851 Galbiate, Italy

³ Centre of Excellence for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch 7600, South Africa

Introduction

The interest in boundary environments and edges of ecological systems dates back to the nineteenth century, when the concept of ecotones was developed (Odum 1953). The concept of ecotone itself implies the existence of an active interface between two or more habitats, involving the interactions of different continuums harbouring specific biotic communities, which exploit different energy flows and are subjected to differing environmental pressures (Howarth 1993;

Parsons 1991). These interactions can determine unique features that do not exist in either of the adjacent environments (Kark 2017; Kark and van Rensburg 2006).

Several studies on ecotones have suggested that these areas of transition can be characterised by high genetic diversity, phenotypic divergence and species abundance (Connell 1978; Ficetola et al. 2019; Kark 2017; Schneider et al. 1999). Ecotones can sustain unique populations/ecotypes or species that are less represented or do not occur in another environment (Cantonati et al. 2006; Kark 2017), especially if stress conditions are moderate and metabolic costs affordable (Howarth 1993). In addition, some ecotones can be systems where populations are diverging to new lines/phenotypes as a consequence of the interplay between strong selective gradients and gene flow across the interface (Schilthuizen 2000; Smith et al. 2000). Thus, understanding the importance of ecological pressures of ecotones in shaping species adaptive shifts and promoting biodiversity remains a key research field that can provide insights about evolutionary processes modulating niche exploitations. The interest is particularly high given that human activities often determine the creation of new boundaries, or the modification of existing ones (Lindenmayer and Fischer 2006; Murcia 1995).

Springs are typical ecotones that separate two strongly distinct environments: surface freshwater habitats and groundwaters. While the surface and underground water environments are well studied (Bertoli et al. 2021; Cozzoli et al. 2020; Malard et al. 1997; Niemiller et al. 2018), the boundaries between them (i.e. spring habitats) are less considered, especially for their role as potential drivers of unique evolutionary processes. This can be partly linked to the human difficulty in perceive transitions (Pirni 2016; Sturz and Bodily 2016) and to the complexity of ecotones: boundaries can shift over time and space and according to the processes considered in the analysis (Gibert et al. 1997; Manenti and Pezzoli 2019). Processes acting in spring habitats are complex, being the result of the interplay of both the subterranean and the epigeal habitat features and organisms (Alfaro and Wallace 1994; Cantonati et al. 2006), but their study could address some of the evolutionary riddles posed by subterranean-dwelling animals (Vandel 1920).

Recent analyses suggest that the occurrence of stygobionts (i.e. obligate groundwater dwelling animals) in spring habitats could be more common than usually thought, and is driven by their active exploitation of these environments (Barzaghi et al. 2021; Manenti and Barzaghi 2021; Manenti and Piazza 2021). For stygobionts, springs can be attractive environments because they often have much higher availability of trophic resources compared to underground environments (Culver and Pipan 2014). For surface species, springs (and their groundwater side) can be safer habitats due to the scarcity of predators (Clements et al. 2016; Manenti

and Barzaghi 2020). The features of ecotones, especially light occurrence and predation risk, can act differently in stygobiont and surface species. Exposure to light and UV radiations can be detrimental for stygobionts (Manenti and Barzaghi 2021) and can play selective effects on their spring populations. For instance, *Niphargus* amphipods are eyeless and depigmented stygobionts, and still, they can detect light (Borowsky 2011; Fišer et al. 2016). This capability has been associated to the necessity to recognise and avoid surface risky habitats (Fišer et al. 2016), but could be also interpreted as an adaptation to exploit springs during night, when predation risk is lower and UV radiation is absent (Manenti and Barzaghi 2021). Unlike stygobionts, surface species usually are pigmented and protected from UV radiation; they are thus often active during daytime, but they can be easily detectable by predators with daylight in the surface side of springs. Predation risk is associated to the concept of landscape of fear (LOF) (Gaynor et al. 2019), an often overlooked aspect in freshwater studies (Manenti and Barzaghi 2020). Habitats with high number of predators shape prey activities in different ways than safer habitats (Melotto et al. 2019). The density and variety of predators can differ between downstream and spring sites; springs can be safer habitats for surface animals like epigeal amphipods that have more efficient antipredator mechanisms, but riskier for stygobiont ones inhabiting and adapted to groundwaters where predators are scarce or absent (Barzaghi et al. 2017; Zhai et al. 2020). Springs are inhabited by salamander larvae and other predators (e.g. dragonfly larvae) that can prey on both stygobiont and surface amphipods, or affect their activity due to non-consumptive effects and the consequent LOF (Manenti and Pezzoli 2019; Melotto et al. 2019). Finally, patterns of occurrence of stygobiont and surface species in springs strongly differ among geographical regions and localities (Cantonati et al. 2020; Culver et al. 2012; Manenti and Piazza 2021), suggesting that local conditions can play major roles and single or few seasons of surveys only provide an incomplete picture of the role of biotic and abiotic pressures interacting in spring systems, while hiding broad and main processes. Experimental tests are required to clarify how key factors, such as light availability and predation risk, influence the exploitation of springs by stygobiont and surface species.

With this paper, we combine field surveys with laboratory experiments in order to disentangle the relative role of light exposure and predation risk in ecotone exploitation by both stygobiont and surface-dwelling species. Both in the field and in laboratory conditions, we assessed the activity variation in two amphipod species, the stygobiont *Niphargus thuringius* and the surface-dwelling *Echinogammarus stammeri*, under different regimes of light and predation risk exposure. For both field and experimental data, we tested

three hypotheses related to the constraints likely experienced by amphipods trying to exploit springs.

- (1) Predation risk hypothesis: predator abundance limits amphipod activity in the field and affects activity and survival in experimental exposure, particularly for stygobionts.
- (2) Ecotone condition hypothesis: the exposure to light occurring at the interface between groundwater and surface water limits stygobiont exploitation of spring habitats, and interacts with predation risk in affecting surface amphipod activity.
- (3) Diel activity hypothesis: spring exploitation affects diel activity of stygobionts that can exploit them when conditions are favourable (such as during night when darkness occurs), and interacts with predation risk in affecting surface amphipod activity.

Material and methods

Study species and area

Niphargus thuringius is a widespread crustacean (Amphipoda) in the Prealpine area of Northern Italy. The species shows strong specialisation for living underground (is eyeless and depigmented, i.e. is troglomorphic), is common in subterranean systems and is also recorded in numerous spring habitats (Pezzoli 2010; Stoch 2000). *Echinogammarus stammeri* is a common species in surface areas both in the Po plain and in Prealpine headwaters where it often occurs in springs (Casellato et al. 2006; Dezfuli et al. 1991; Stoch 2000). Here, we focussed on 15 springs between the districts of Lecco and Como in Lombardy (Northern Italy) where the occurrence of both species has been previously ascertained.

Field samplings

From March to May 2021, we monitored 15 springs in the range of *N. thuringius* and *E. stammeri*. We included natural springs, for which only the surface side was accessible, and artificial catching buildings connecting streams to draining galleries, which are subterranean tunnels built to collect groundwater that provide access to the subterranean side of the emergences (Manenti 2014; Manenti et al. 2009). In the study area, both amphipod species can be preyed by fire salamander larvae (*Salamanca salamandra*) and dragonfly larvae, especially of the genus *Cordulegaster*, which can occur also in groundwaters (Manenti and Ficetola 2013; Manenti et al. 2013). Each spring was divided in 1–4 non-adjacent plots; plots were randomly placed along the boundary between groundwater and surface; average plot length

was 126 cm and average plot width 90 cm. In total, we had 25 plots that we surveyed twice at night (h: 21–24) and twice during daytime (h: 13–16:30); the same observer (SL) performed all the surveys. On all the surveys, we recorded the number of *N. thuringius* and *E. stammeri* individuals and of fire salamander larvae, i.e. the number of individuals/larvae detected in 10-min visual surveys. To this purpose, we approached the plot without lightening it and, once at the border of the pool, we lightened it with a torch (Petzl Ultra Vario) and counted the individuals. At the end of each night survey and at least 7 days before the successive survey, we also performed in each plot a dip-netting sampling by moving the substrate for 5 min and using a thin-mesh dip net (20 cm large with a 50-cm-long stick) to collect all the invertebrates and salamander larvae occurring at the point, with the purpose to record the number of predator taxa and individuals for *N. thuringius* and *E. stammeri*. At each survey, we also recorded maximum illuminance (incident light; using a PCE-170 A Lux Meter), maximum width, length and depth of every sampled point, water temperature at the bottom and the % cover of submerged leaves.

Experimental system

Rearing and experiments were performed within the study area in the laboratory of subterranean biology “Enrico Pezzoli” located in the Monte Barro Regional Park. The laboratory is a 60-m-long artificial ancient draining gallery which is naturally inhabited by *N. thuringius*. The laboratory is completely lightless, equipped for aquatic animals rearing and, during the period of the experiment, had an average (\pm SE) temperature of 10.620 (\pm 0.002) °C.

To assess how predation risk and dark/light conditions affect the behaviour of amphipods depending on their degree of adaptation to subterranean environment, we designed microcosms with two distinct light treatments and three different conditions representing risk of predation (Fig. 1). Half of the microcosms were set in constant darkness, while half was set with a photoperiod of 12 h (from 7 a.m. to 7 p.m.) using a NICREW 3W 28cm white LED light for aquariums (illuminance provided to the microcosms: 1800 lux). The conditions representing risk of predation included controls (no predator); meso-predators (four fire salamander larvae, allowed to wander across the microcosm); and meso-predators with top predators (four salamander larvae wandering across the microcosm, plus one large *Cordulegaster boltonii* dragonfly larva in a small plastic transparent cage inside the microcosm; see below). Salamander larvae are considered to be meso-predators as in nature they are often preyed by dragonfly larvae (Manenti et al. 2013).

In April 2021, we collected 80 individuals of *N. thuringius* and 80 adult individuals of *E. stammeri* from two different spring sites each in a hilly area (Mount of Brianza, Lecco

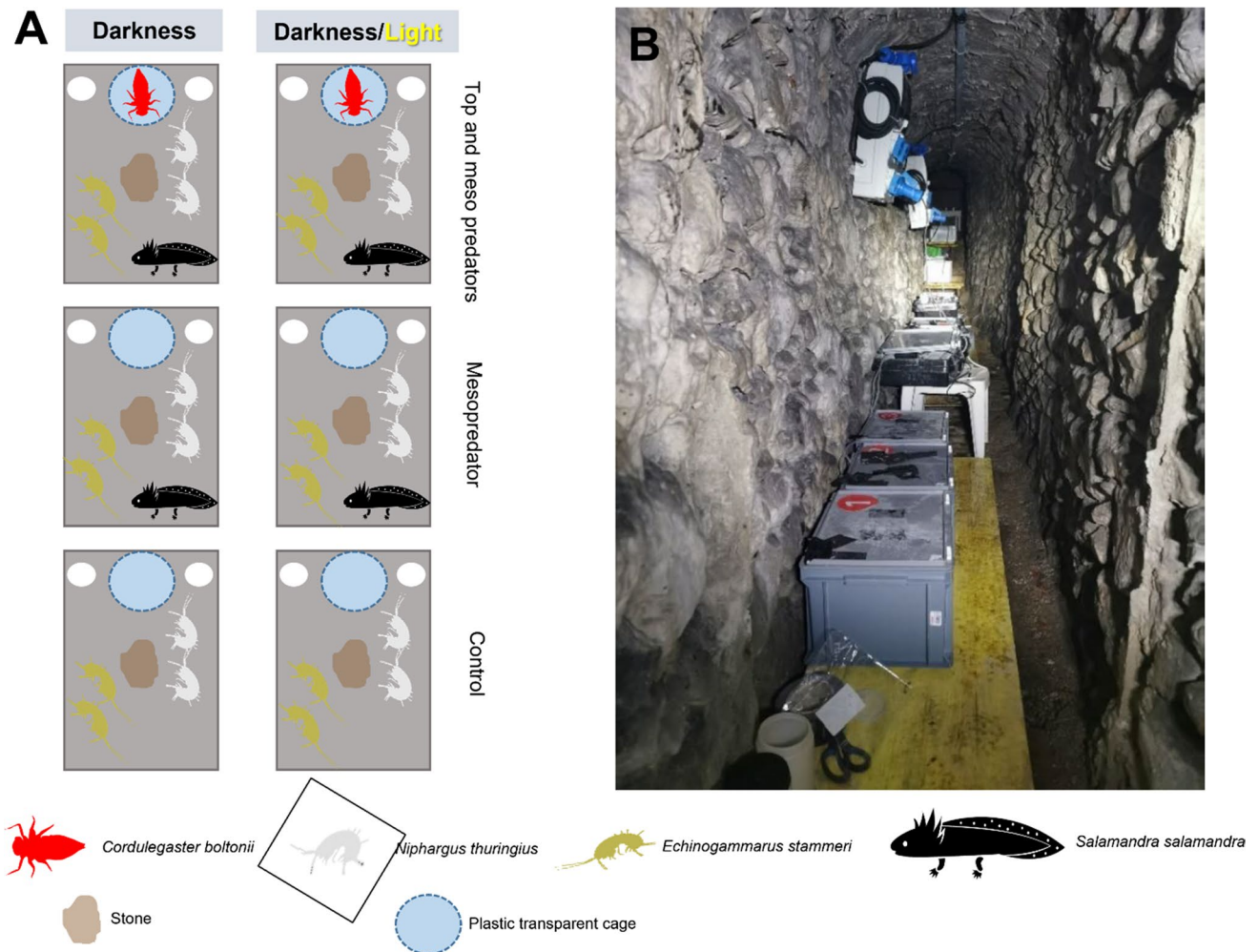


Fig. 1 **A** Schema of the experiment; organisms and elements are not scaled and have been enlarged to increase visibility. White circles represent plastic bowls used for feeding amphipods and salamanders. **B** Setting in the subterranean laboratory “Enrico Pezzoli”

district). Animals were transferred into the subterranean biology laboratory and mixed in a tank; then, we randomly assigned 5 *N. thuringius* and 5 *E. stammeri* individuals of similar sizes (range 8–12 mm) to each of 16 microcosms. Microcosms were 40 × 30 × 22 cm tanks, with 8 cm of water and sharing the same features, i.e. a 5-cm stone as shelter in the right side, two Cabilock white plastic bowls (6 cm diameter) and a pierced transparent bottle (10 cm of diameter). All microcosms had the same water temperature. Animals were let acclimatise for 15 days to avoid behavioural alterations caused by stress (Ginet 1960). After acclimatisation, we added top and meso-predators to tanks according to the different risk treatments (Fig. 1). Dragonfly larvae were collected in a different spring of Mount of Brianza; their sizes ranged between 3.1 and 3.7 cm. Salamander larvae were collected from two streams of the karst locality “Alpe del Viceré” in the Como district; the average total length at collection was 32.4 mm. The two Cabilock white plastic bowls

were used to place food for amphipods and salamanders; amphipods were fed ad libitum with Tetra Cory Wafers, tablets composed mainly by vegetal protein extracts with added cereals, molluscs and crustaceans. Preliminary tests showed that both *Niphargus* and *Echinogammarus* voraciously fed on them. Tablets were gently changed/added twice a week in one of the plastic bowls. Fire salamander larvae were fed with 20 live chironomid larvae placed twice a week in the other plastic bowl. Each dragonfly larva was fed with 10–12 live chironomid larvae per week which were placed into the cage. Feeding was performed using a red-light lamp during night and also during day for microcosms that were in total darkness to minimize disturbance for amphipods.

After starting the experiment, we recorded the number of active individuals, i.e. individuals that were outside shelters (represented by the stone and the lower side of the bowls), every 3 days both during daytime (4:30 p.m.) and during night-time (9:30 p.m.). The same operator (MG) performed

all the recordings; at each survey, he gently removed the microcosm lid and recorded the number of active individuals; again during night and for microcosms of the total darkness treatment, a red-light lamp was used during surveys. After each night survey, he also assessed the total number of surviving individuals of each amphipod species (thus recording also sheltered individuals) by gently moving the stone and the feeding bowls. The rearing and monitoring lasted 30 days.

Ethics

The rearing experiment was planned following ABS/ASAB guidelines for ethical treatment of animals. The collection and maintenance of fire salamander larvae and their prey and predator invertebrate species was in accordance with the Regional Law 10-31/03/2008 (Lombardy Region); the study design has been approved by the regional ethical committee (permit n. 18303_13.12.2019). After the end of the experiment, each individual of the different species was released in the site of collection.

Statistical analyses

Field sampling data We used data from field samplings to assess the relative role of LOF with respect to ecotone level and day/night period in affecting activity of stygobiont and surface amphipods. We used generalised linear mixed effect models (GLMMs) to assess the factors determining the abundance of the two species (number of individuals detected during both diurnal and night visual surveys). Spring sites where dip-netting did not reveal target species occurrence in at least one of the plots were not included in the analysis. Simulation analyses showed that this approach is appropriate to assess the factors determining variation of activity for species with low detection probability (Barker et al. 2017). The number of recorded individuals of the two focal taxa was the dependent variable of each model. As fixed factors, we considered density of active fire salamander larvae (N larvae/area of the plot), distance from the edge and the period of the day (night or day). As covariates that could affect salamander and amphipod detection, we considered also the maximum depth and the % cover of submerged leaves. We used a negative binomial error distribution to take into account overdispersion. Random factors included plot and spring identity to take into account non-independence of observations (Pinheiro and Bates 2000).

Experimental data We used generalised linear mixed models (GLMMs) with binomial error distribution to assess the factors determining individuals' activity in laboratory. We built a separate GLMM for each species; the two-column matrix composed by the number of active individuals and by the inactive ones

was the dependent variable. As fixed factors, we considered the typology of predation risk (predation risk hypothesis; no predators, one predator, two predators), the light treatment (ecotone hypothesis) and the period of the day (diel activity hypothesis). As covariate, we considered the time (in days) since the beginning of the experiment to account for individuals' experience increase and other possible variations. We included the rearing tank identity as random factor. We also tested the occurrence of two-way interactions between all the factors.

To assess factors affecting mortality, we built another binomial GLMM using the same factors except for the period of the day, as mortality was assessed only once at the end of the experiment. As dependent variable, we considered a two-column matrix composed by the number of dead individuals vs. the number of surviving ones. For all models, we assessed the significance of the fixed factors using likelihood ratio tests (Bolker et al. 2008) and we checked model assumptions by verifying the absence of multicollinearity issues through VIF calculation and plotting residuals versus fitted values, versus each covariate (Zuur et al. 2010). For the typology of predation risk, in case of significance, we assessed differences between the three levels by performing a post hoc Tukey test using the function `glht` of the R package `multcomp`. We also tested overdispersion issues by preliminarily building the same GLM models using quasibinomial distribution. All statistical analyses were performed in R 4.1.1.

Results

Field surveys

We detected *Niphargus thuringius* in 10 of the 25 plots belonging to seven different spring sites, while *Echinogammarus stammeri* occurred in 5 sampling plots belonging to five different springs. The two species co-occurred in 3 plots. Besides *C. boltonii* and *S. salamandra*, through dip-netting, we also recorded the occurrence of additional potential predators of *N. thuringius* and *E. stammeri*, i.e. stygobiont planarians of the genus *Dendrocoelum*; dragonfly larvae of the genera *Calopteryx* and *Aeshna*; and Dytiscidae coleopterans. *Cordulegaster boltonii* was the most widespread dragonfly; its larvae occurred in four springs; in one of them, they reached a density of 12 larvae in a plot of 0.15 m² (density 80 larvae/m²). The richness of predatory taxa for both species detected in the same point was of 4; the maximum number of individuals of a predator taxon, represented by fire salamander larvae, was 70 for *N. thuringius* and 43 for *E. stammeri*. Syntopy between fire salamander larvae and *N. thuringius* occurred in half of the plots. The maximum number of *N. thuringius* individuals recorded was 30 in a subterranean point during day, while in surface plots, we detected

a maximum of 10 individuals during day. For *E. stammeri*, a maximum of 3 individuals was detected during daytime in surface plots and no individuals have been observed in subterranean plots. Visual surveys never detected individuals of the two amphipod species in sites where dip-netting did not reveal their occurrence.

In plots belonging to sites where *N. thuringius* occurrence was detected through dip-netting, the number of individuals observed visually was on average (\pm SE) 2.7 ± 1.2 if salamander larvae were present and of 6.5 ± 2.1 if salamander larvae were absent. The number of observed *N. thuringius* individuals was negatively related to the density of active salamander larvae, while it did not vary significantly between night and day and with the distance from the boundary (Table 1).

E. stammeri individuals were significantly more abundant in surface plots ($\chi^2 = 7.78$, $P < 0.001$), while we did not record effects of predator density and day/night period (Table 2).

Activity variation in laboratory conditions

For the stygobiont *N. thuringius*, in constant darkness conditions, the average (\pm SE) proportion of active individuals was $0.43 (\pm 0.03)$ during day and $0.45 (\pm 0.04)$ during night. In tanks with diel variation of darkness/light conditions, the average proportion of active individuals was $0.11 (\pm 0.03)$ during daytime and of $0.27 (\pm 0.03)$ during night-time.

The number of active *N. thuringius* individuals was significantly higher in tanks with constant darkness treatment than in those with varying darkness/light and during night (Table 3). Moreover, a significant interaction between predation risk and period of the day suggests that, during night, they were more active if predation risk was low (Table 3). Finally, a significant interaction between light treatment and period of the day suggests that, in tanks with total darkness treatment, *N. thuringius* was more active during daytime than in tanks with varying darkness/light (that were lightened during day; Table 3; Fig. 2).

For the epigeal *E. stammeri*, in constant darkness conditions, the average (\pm SE) proportion of active individuals

Table 2 Results of the generalised linear model assessing factors determining the abundance of *Echinogammarus stammeri* at spring plots. In bold are reported significant values

Factor	Estimate	DF	AIC	χ^2	<i>P</i>
Distance from the edge	0.12	1	65.96	7.78	< 0.001
Density of fire salamander larvae	0.03	1	58.28	0.11	0.73
Period of the day	1.29	1	56.76	1.37	0.24
Maximum depth	-0.04	1	58.66	0.15	0.69
Decaying leaves abundance	-0.58	1	58.32	0.48	0.48

was $0.44 (\pm 0.04)$ during day and $0.43 (\pm 0.05)$ during night. In tanks with diel variation of darkness/light conditions, the average proportion of active individuals was $0.46 (\pm 0.05)$ during day and of $0.44 (\pm 0.05)$ during night.

The number of active *E. stammeri* individuals significantly decreased through time since the onset of the experiment (Table 4) while no differences occurred among predation risk, light treatments and day/night period for this surface amphipod (Table 4; Fig. 3).

Survival in laboratory conditions

Overall, 34 individuals (42.4%) of *N. thuringius* survived after 30 days of experiment. In constant darkness, the rate of survival was of 55%, while in the treatment with diel light variation, the survival was 30%. With both predators, the average survival rate was 13.3%, while it increased at 50% with only fire salamander larvae and was 75% without predators. The mortality of *N. thuringius* was positively affected by both predation risk ($\chi^2 = 40.84$, $P < 0.01$) and light variation during rearing ($\chi^2 = 4.33$, $P = 0.03$). Tukey's post hoc tests showed that mortality significantly increased with different predator species (Table 5).

Overall, only 8 individuals of *E. stammeri* survived after 30 days of experimental rearing. In constant darkness, the survival was 20%, while under light variation, survival was 15%. With both predators, survival rate was 3.3%, while it

Table 1 Results of the generalised linear model assessing factors determining the abundance of *Niphargus thuringius* at spring plots. In bold are reported significant values

Factor	Estimate	DF	AIC	χ^2	<i>P</i>
Distance from the edge	0.002	1	187.54	1.03	0.30
Density of fire salamander larvae	-0.28	1	193.97	7.46	< 0.01
Period of the day	-0.204	1	186.83	0.32	0.57
Maximum depth	0.04	1	188.17	1.66	0.19
Decaying leaves abundance	-0.978	1	189.19	2.68	0.10

Table 3 Results of the generalised linear model testing the factors affecting activity of *Niphargus thuringius* during the rearing experiment. Significant relationships are in bold

Factor	Estimate	DF	χ^2	<i>P</i>
Predation risk	-0.10	2	0.36	0.83
Light/darkness treatment	-2.05	1	29.91	< 0.01
Period of the day (night)	0.27	1	6.50	< 0.01
Predation risk: period of the day	-1.16	2	7.07	0.02
Light/darkness treatment: period of the day	1.21	1	11.28	< 0.01
Time since the beginning of the experiment	-0.01	1	-1.29	0.25

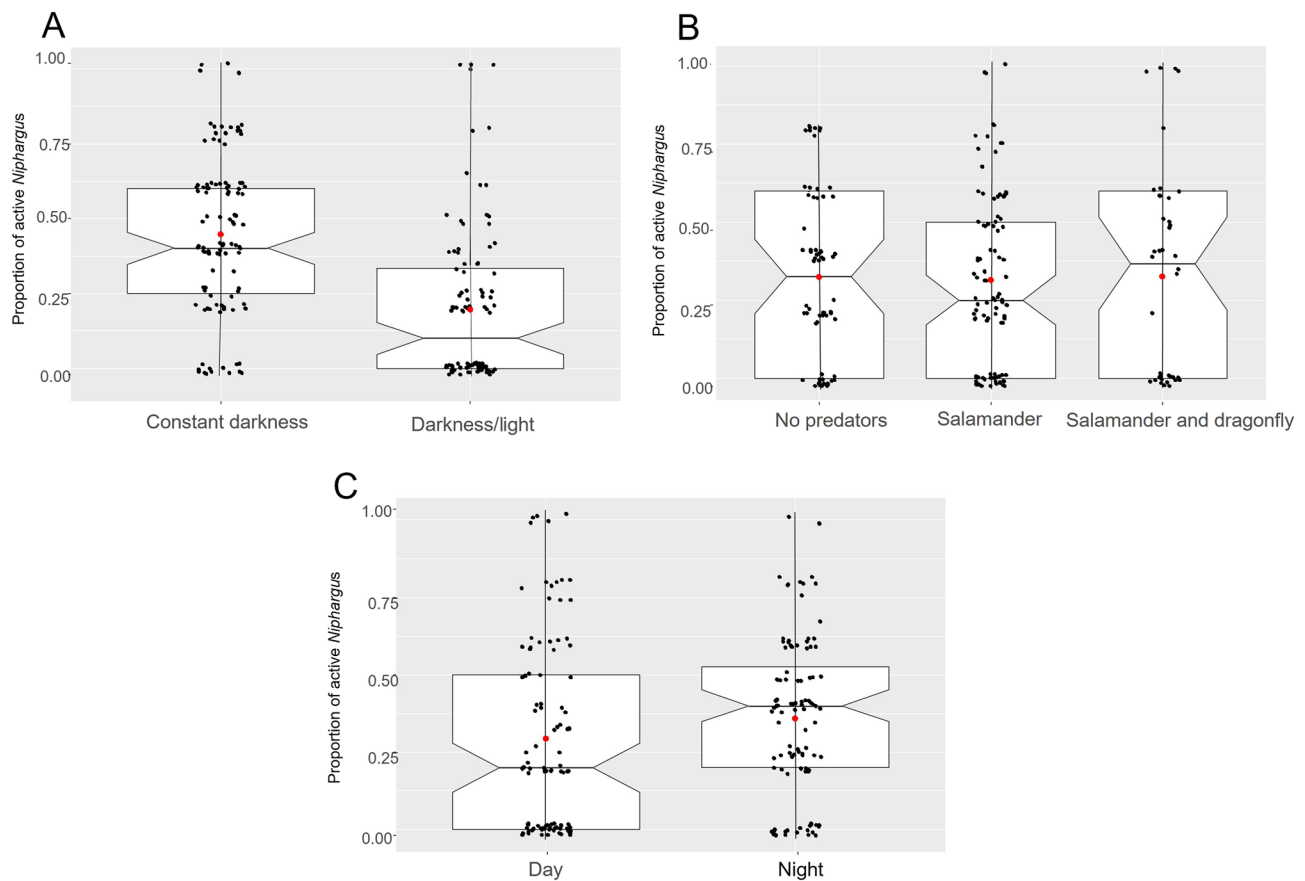


Fig. 2 Activity of *Niphargus thuringius* in experimental conditions. Notched boxplots with mean points of the relationship between the proportion of active *N. thuringius* and light treatment (A), predation risk (B) and period of the day (C). Red dot identifies the mean; the

boxes identify the interquartile range (50% of the data) and are separated by the line representing the median; the “notch” represents the 95% confidence interval of the median; the vertical line identifies the range between maximum and minimum

Table 4 Results of the generalised linear model testing the factors affecting activity of *Echinogammarus stammeri* during the rearing experiment. Significant relationships are in bold

Factor	Estimate	DF	χ^2	<i>P</i>
Predation risk	−0.39	2	4.12	0.11
Light/darkness treatment	0.09	1	0.07	0.79
Period of the day (night)	−0.07	1	0.15	0.69
Time since the beginning of the experiment	−0.05	1	12.22	< 0.01

was 27% when only salamander larvae were present, and reached the 25% without predators. The mortality of *E. stammeri* was significantly affected by predation risk ($\chi^2 = 8.17$, $P = 0.01$); in particular with two predators, mortality was significantly higher than with one ($z = 3.02$, $P = 0.04$), but no differences occurred between two predators and control condition (Table 6).

Discussion

Our results suggest that both light conditions and predation risk play a major role in affecting the activity of stygobiont animals at the edge with surface. The combination of field surveys with laboratory tests can increase our ability to understand complex behavioural, ecological and evolutionary processes, and the consistency of results obtained with the two approaches allows to evaluate the realism of conclusions (Ficetola and De Bernardi 2006; Miner et al. 2005; Skelly and Kiesecker 2001). Combining field and laboratory approaches allowed a more complete understanding of the complexity of interactions between biotic and abiotic constraints interplaying in springs, even though the low occurrence in the field and the limited survival in laboratory conditions limited the power of inference for the surface species. In the field, we detected support for the importance of predation risk in affecting spring habitat exploitation by stygobiont, because the number of active *Niphargus thuringius* clearly decreased with the number of fire salamander

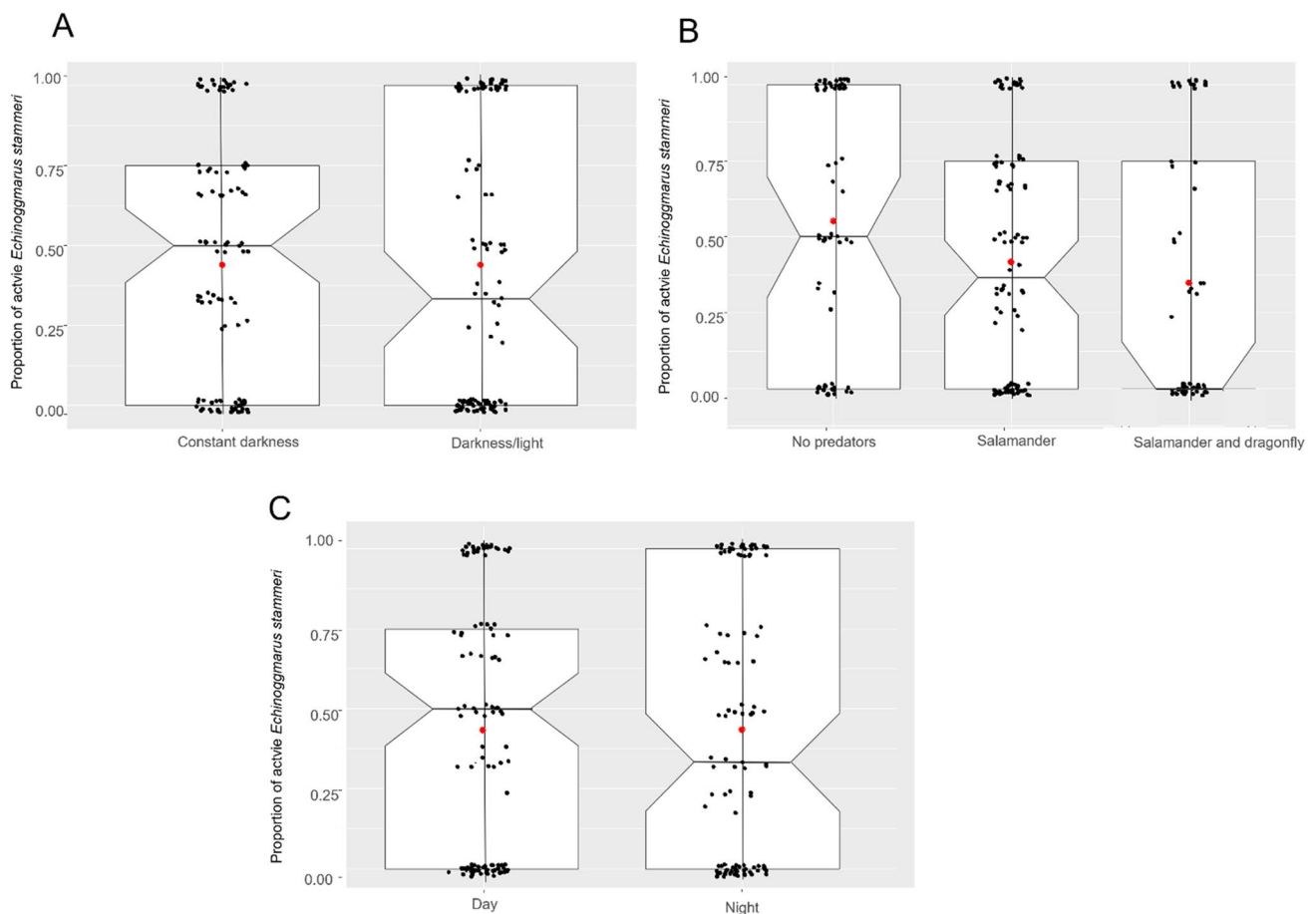


Fig. 3 Activity of *Echinogammarus stammeri* in experimental conditions. Notched boxplots with mean points of the relationship between the proportion of active *E. stammeri* and light treatment (A), predation risk (B) and period of the day (C). Red dot identifies the mean;

the boxes identify the interquartile range (50% of the data) and are separated by the line representing the median; the “notch” represents the 95% confidence interval of the median; the vertical line identifies the range between maximum and minimum

Table 5 Results of a Tukey post hoc test of the differences among predation risk treatments performed on the GLMM built to assess factors that influenced *Niphargus thuringius* mortality at the end of the rearing experiment. In bold are reported significant values

Factor	Estimate	SE	z	P
Salamander alone vs no predators	2.67	1.11	2.41	0.04
Salamander and dragonfly vs no predators	5.13	1.21	4.24	< 0.01
Salamander and dragonfly vs salamander alone	2.46	0.69	3.52	< 0.01

Table 6 Results of a Tukey post hoc test of the differences among predation risk treatments performed on the GLMM built to assess factors that influenced *Echinogammarus stammeri* mortality at the end of the rearing experiment. In bold are reported significant values

Factor	Estimate	SE	z	P
Salamander alone vs no predators	-0.80	0.66	-0.13	0.99
Salamander and dragonfly vs no predators	2.27	1.14	1.99	0.18
Salamander and dragonfly vs salamander alone	2.42	1.09	3.02	0.04

larvae. This is consistent with a recent study performed on the whole community of spring habitats that suggested that fire salamander larvae play a negative role on the occurrence of multiple invertebrates, including *N. thuringius* (Manenti and Pezzoli 2019). In the field, we did not observe differences among the number of individuals detected during day and night sampling, conversely to previous studies performed on other *Niphargus* species (Kureck 1967; Manenti and Barzaghi 2021; Müller et al. 1963). However, these studies only focused on the surface side of springs, recording higher activity at night, when light, UV radiation and other factors acting during daytime do not pose constraints to gather trophic resources in surface (Kureck 1967; Manenti and Barzaghi 2021). Nevertheless, field surveys can only provide a snapshot of patterns and processes that are complex and dynamic, and would require continuous, long-term data for a full understanding (Cantonati et al. 2006; Parsons 1991). For instance, also meso-predators such as salamander larvae can follow a diel activity, being influenced by the presence of other predator species in spring habitats (Manenti et al. 2016; Oswald et al. 2020) and, together with shelter availability, this can enhance or limit the extent of their ecotonal zone exploitation, and in turn shape the occurrence and activity of stygobiont prey.

Laboratory conditions highlighted that light exposure is a main constraint for the activity of stygobionts. Conversely, the effect of light exposure was mostly absent for the epigeal amphipods, for which we did not detect clear effects of photoperiod or time of the day. In crustaceans, photophobic reactions are widespread among depigmented stygobionts while tend to lack in their pigmented surface relatives (Banta 1910; Fišer et al. 2016; Ginet 1960; Park et al., 1941; Vandel, 1964). Photophobic behaviours reduce the risk of damages caused by UV radiation (Block et al. 2009; Ciroso-Perez et al. 2015; Jacobs et al. 2005; Rudh and Qvarnstrom 2013), and may reduce the exposure to risky conditions found in surface environments. Such behaviour has been observed in multiple *Niphargus* amphipods (Borowski 2011; Fišer et al. 2016; Ginet 1960; Vandel 1964). The significant interaction that we recorded between light treatment and period of the day with *N. thuringius* individuals being more active during night in tanks that were dark than in those that had light, suggests that photophobic behaviours can be particularly useful for populations inhabiting spring ecotones to avoid exposure to UV radiation when daylight comes.

After the sunset, light constraints (UV radiations and detectability by visual predators) are absent in springs, and conditions can become favourable for the exploitation of surface freshwaters (Manenti and Barzaghi, 2021). Likewise, in our experiment, *N. thuringius* individuals were more active during night, when all microcosms were completely dark. Differences in diel activity are expected to occur in

organisms with activity patterns regulated by the photoperiod (Bancila et al. 2021; Falcón et al. 2020) and it is worth noting that in our experiment responses to day period and light were detected in the stygobiont amphipod, but not in the epigeal one. Furthermore, the interaction between day period and predation risk highlighted that during night-time, stygobionts were more active in tanks without predation risk than in those with predators. Similarly, Bancila et al. (2021) detected a complex interplay between light conditions and environmental features, including not only predation risk, but also food availability, in shaping activity of salamander larvae. Interactive effects of night/light conditions with environmental features suggest that the differences observed between field and laboratory conditions could be linked to the complex and difficult to be controlled interplay of multiple factors acting on spring ecotones in the wild.

The strong decrease of *N. thuringius* survival highlights that, if antipredator strategies are not developed, the abundance of predators that is typical of surface habitats can cause high mortality of stygobionts. Therefore, epigeal predators can severely limit the abundance of stygobiont fauna at the boundary between underground and surface environments. Consistently, the low densities of subterranean-dwelling invertebrates often recorded in cave sectors and subterranean streams close to surface (Lunghi et al. 2017; Manenti et al. 2020) can be a result of the higher predation risk that they can experience with respect to inner groundwater areas. For instance, higher mortality of stygobionts has been recorded in sites with light variation between day and night, implying that light can increase their detectability by visual-oriented predators, especially considering that generally stygobiont species are blind or with limited visual ability (Parimuchová et al. 2021).

With the progress of the experiment, the number of active *E. stammeri* decreased, suggesting that likely less cautious ones have been preyed upon first. *E. stammeri* showed a very high mortality, even in control conditions. Similar differences in mortality between stygobiont and surface amphipods have already been described, but in conditions of starvation (Hervant et al. 1999), while in our experimental system, food was available ad libitum. The low survival rates of the surface species might be linked to both competition and predation by the stygobiont one, and suggests the need for a further control condition for the epigeal species in future studies. *Niphargus* amphipods are often considered generalist species feeding on detritus (Luštrik et al. 2011; Tachet 2010), but opportunistic predatory behaviours can be more frequent than usually reported (Ercoli et al. 2019; Fišer et al. 2010), and predator species have been recently recognised (Premate et al. 2021). However, we never observed direct predation by *Niphargus* on *E. stammeri*. The very high mortality of *E. stammeri* may also explain the low coexistence of the two species detected in field surveys. Further

studies are required for a more complete understanding of interactions between subterranean and surface amphipods in springs.

In ecotonal areas, the activity of stygobionts is limited by light conditions and predator occurrence, compared to what happens in deeper underground environments. However, these issues can be compensated by the higher availability of trophic resource, compared to deeper groundwater habitats (Culver et al. 2012; Moldovan et al. 2018), possibly making these boundary environments attractive contexts during night or favourable conditions. In springs, stygobiont species experience environmental conditions completely different from the ones occurring in caves, with more trophic availability but also high UV stress and predation risk. The exploitation of spring habitats by subterranean invertebrates is affected by surface physical constraints which can mediate the effects of predation risk and provide multiple and interplaying selective pressures with the potential to promote adaptive shifts at morphological and/or behavioural level (Melotto et al. 2019). The outcomes of our study suggest that specific experimental studies are needed to assess whether there is intraspecific variation between stygobiont populations inhabiting groundwater and spring environments.

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Declarations

Competing interests The authors declare no competing interests.

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