diversity

MDPI

Article

Both Light Stimuli and Predation Risk Affect the Adult Behavior of a Stygobiont Crustacean

Matteo Galbiati [1,2], Stefano Lapadula [1,2,*], Martina Forlani [1,2], Benedetta Barzaghi [1,2] and Raoul Manenti [1,2]

[1] Dipartimento di Scienze e Politiche Ambientali, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

[2] Laboratorio di Biologia Sotterranea "Enrico Pezzoli", Parco Regionale del Monte Barro, Località Eremo, 1, 23851 Galbiate, Italy

* Correspondence: stefano.lapadula.1999@gmail.com

Abstract: Stygobiont species show common, typical traits derived from their adaptation to subterranean life. Due to the general absence of light in cave environments, the majority of them are eyeless. Although the absence of eyes generally does not allow them to perceive luminous stimuli, some stygobionts still present phototaxis. Previous studies determined that different species of the eyeless amphipod crustaceans of the genus *Niphargus* are able to react to light; this has been interpreted as an adaptation to avoid dangerous surface habitats, even if recent studies suggest that this could also be an adaptation to exploit them when a situation is less dangerous (i.e., during the night). *Niphargus thuringius* is a stygobiont amphipod that can also be observed in spring environments despite possessing all the main morphological features of subterranean organisms, such as depigmentation and a lack of eyes. In the present study, we test how the species respond to light stimuli according to the light cycle and predation risk experienced during a conditioning period. We assessed the reactions to light stimuli of adult individuals of *N. thuringius* after 30 days of rearing in microcosms with different conditions of light occurrence (total darkness or a light/darkness daily cycle) and predation risk (without predators, with one predator, and with two predators). Both light stimuli during the test and rearing conditions affected the behavior of *Niphargus thuringius*. With light stimuli, individuals presented a strong photophobic response. Moreover, individuals reared in conditions of high predation risk preferred a more sheltered environment during behavioral tests than individuals reared in safe conditions. Our results add a new species to those of stygobiont amphipods known to display negative phototaxis, confirming that this pattern is widespread and conserved in the field. *N. thuringius* could be a good candidate model to perform further studies aiming to assess if differences occur between spring populations and populations present in deeper groundwater.

Keywords: ecotone; spring; cave

check for updates

Citation: Galbiati, M.; Lapadula, S.; Forlani, M.; Barzaghi, B.; Manenti, R. Both Light Stimuli and Predation Risk Affect the Adult Behavior of a Stygobiont Crustacean. *Diversity* **2023**, *15*, 290. https://doi.org/10.3390/d15020290

Academic Editor: Piero G. Giulianini

Received: 3 January 2023
Revised: 12 February 2023
Accepted: 14 February 2023
Published: 16 February 2023

1. Introduction

Animals living in ecotones are worth investigating in the scientific field for a variety of reasons. Populations living at the edge of their species range generally afford ecotones with more or less steep gradients of biotic and microenvironmental pressure changes depending on the features of the adjacent habitats [1,2]. Various studies have shown that biodiversity, population densities, and species variation tend to peak in ecotonal habitats, even if exceptions to these outlines occur [3,4]. The evidence suggests that ecotones may also be differentiation hotspots where new species/ecotypes may evolve [5,6]. As such, species inhabiting ecotones deserve increased research investment.

However, studying species exploiting ecotones can be challenging because ecotonal habitats generally display both features that are typical of their bordering habitats as well as their own peculiar characteristics. This is the case for spring habitats that separate groundwater and surface water [7,8]. While in groundwater, the absence of light causes trophic resources to be an important limiting factor, both the occurrence of primary producers and a high supply from the surrounding terrestrial environment favor the occurrence of well-developed trophic webs in surface freshwaters [9,10]. At the same time, groundwater is a much more stable environment in terms of environmental conditions and hydroperiods, which can also be safer for consumers and mesopredator species in terms of predation risks with respect to the surface side of springs [11–14]. The transition between the two environments can be more or less steep, but borders can be particularly obvious in springs during the daytime when sunlight strictly demarks the limits [15].

This light-mediated limit is usually considered as particularly strong for groundwater inhabitants called stygobionts [16,17]; they often present specific morphological, physiological, and behavioral adaptations to living in subterranean, light-deprived environments. These features are commonly referred to as troglomorphisms [18,19] and strongly link stygobionts to groundwater for their entire life cycle, including reproduction, and likely limit their possibility to exploit surface environments [19–22].

The main adaptations of stygobionts involve: (1) morphological changes, such as the partial or total disappearance of visual organs and pigmentation; (2) physiological changes, with effects on metabolism and life cycle; and (3) behavioral changes, mainly related to the presence or absence of circadian rhythms.

Morphological adaptations involve a more or less pronounced lack of pigmentation [23,24]. At the same time, the reduction in or absence of visual organs (eyes or ocelli, in general) is particularly important in stygobionts. As a cave environment is completely dark, possessing functioning eyes would not only be useless, but a disabling form of energy expenditure [25]. Some crustaceans have visual structures in the larval or embryonic stages that gradually degrade as they grow [23,26,27].

Some stygobiont species, which should be blind due to their pronounced microphthalmia or complete anophthalmia, are able to respond effectively to light stimuli [28,29]. This aspect has been highlighted in the literature, especially in the genus of underground amphipods *Niphargus*, which show pronounced photophobic behavior [17,28]. Such a behavioral response to light has been suggested as a mechanism to prevent the dispersal of stygobiont *Niphargus* crustaceans into surface environments [16,17] where UV rays may be dangerous for a depigmented animal. Light avoidance can also work as a useful behavioral mechanism, allowing a relatively safe exploitation of the trophic resources occurring in springs and in those surface environments that border groundwater [15,16,30]. A stygobiont that would maximize access to resources and reduce the negative impacts of light could use the advantage of crossing such boundary at night, when UV radiation is absent [15]. To exploit springs during the day, it should avoid photic areas and instead inhabit dark microhabitats under stones and other available shelters [16].

In addition, predators, such as fire salamander and dragonfly larvae that usually occur in European springs, may be a strong selection agent in spring habitats [31,32], driving subterranean amphipods, such as those of the genus *Niphargus*, to reduce their activity in surface areas or sectors close to the surface [33,34]. The impact on stygobiont species and their exploitation of surface habitats by competition and predation posed by surface-dwelling species has rarely been quantified in the literature. Being eyeless, stygobiont species are likely vulnerable to be visually oriented predators in springs during the day; simultaneously, they can have more of an advantage during the night and in dark microhabitats. However, stygobiont species that are adapted to food-deprived groundwater areas might be poor competitors in richer surface environments where greater and more rapid metabolic performances are advantageous [35]. Nonetheless, it is unknown which environmental constraints, ranging from predation and interspecific interactions to light impact and physical pressures, play a major role in affecting subterranean animals' exploitation of springs. The aim of this study is to add experimental evidence to the photophobic responses present in *Niphargus* amphipods, by testing the effects of light stimuli on the species *N. thuringius*, and to assess if the conditions of predation risk experienced by adults may affect/interact with such responses. We hypothesize that if predation risk plays a major role in affecting the exploitation of surface habitats by organisms considered strictly stygobiont, adults of *N. thuringius* reared in risky conditions display responses similar to the reactions driven by light stimuli.

2. Materials and Methods

2.1. Study Species

Niphargus thuringius is a stygobiont amphipod crustacean found in the Prealpine area of Northern Italy. The species has a strong specialization for living underground (it is blind and depigmented), is common in subterranean environments, and is also recorded in numerous spring habitats [34,36,37].

In Lombardy, 18 species of amphipods have been observed to date, 14 of which belong to the genus *Niphargus*, and of these, as many as 13 are stygobites [37]. *Niphargus thuringius* is the species with the greatest ecological value [37], capable of populating both groundwater and springs.

2.2. Experimental System

Rearing and experiments were performed inside the study area in the laboratory of subterranean biology "Enrico Pezzoli", located in the Monte Barro Regional Park (NW Italy). The laboratory was a 60 m long, ancient, artificial draining gallery, which was naturally inhabited by *N. thuringius*. The laboratory was completely absent of light, equipped for rearing aquatic animals, and, during the period of the experiment, had an average (±SE) temperature of 10.628 (±0.002) °C.

To assess how light stimuli, predation risk, and dark/light conditions experienced by the object of study affected the behavior of amphipods, depending on their degree of adaptation to the subterranean environment, we designed microcosms with two distinct light treatments and three different conditions representing risk of predation (Figure 1). Half of the microcosms were exposed to constant darkness, while the other half were exposed to a varying photoperiod of 12 h (from 7 a.m. to 7 p.m.) using a NICREW 3W 28 cm white LED light for aquariums. The illuminance rate in the tanks was 1800 lux. All the tanks had the same water temperature level. The conditions representing risk of predation included the following: controls (no predator); meso-predators (four fire salamander larvae (*Salamandra salamandra*), permitted to wander across the microcosm); and meso-predators plus one top-predator (four salamander larvae wandering across the microcosm, plus one large dragonfly larva (*Cordulegaster boltonii*) placed in a small cage inside the microcosm; see below). Salamander larvae are considered to be meso-predators as, in nature, they are often preyed on by dragonfly larvae [32]. Fire salamanders were widespread in the study area [38] and often bred in springs and subterranean environments along with *Niphargus* amphipods [13,33].

In April 2021, we collected 80 individuals of *N. thuringius* from two different spring sites, each in a hilly area (Mount of Brianza, Lecco district). Both springs have been artificially managed to collect water; they are short draining galleries—"bottini di presa"—characterized by a very low water depth at the first site (Figure 1A) and by a pool with an abundant mud substrate (Figure 1B) at the second site. Both are perennial and the occurrence of *N. thuringius* has been assessed by multiple surveys [39].

The animals were transferred to the subterranean biology laboratory and mixed in a tank; then, we randomly assigned 5 *N. thuringius* individuals to 16 microcosms. Initially, we planned to obtain 18 microcosms, such as three replicates for each condition presented in Figure 2. However, we were able to collect fewer animals than expected, and we only had two replicates for each control condition, for which we expected a higher survival rate (and, thus, a final number of animals comparable to that of the predator treatments). The partially imbalanced final dataset was accounted for in the statistical analyses. Microcosms were present in 40 × 30 × 22 cm tanks, with an 8 cm water depth, sharing the same features, i.e., a 5 cm stone as shelter on the right-hand side; two Cabilock, white, plastic bowls (6 cm in diameter); and a pierced, transparent bottle (10 cm in diameter). The white plastic bowls were refilled each week, one with chironomids for the fire salamander larvae and two fresh Tetra Cory ShrimpWafers tablets. The animals were left to acclimatize for 15 days to avoid behavioral alterations caused by stress (Ginet, 1960). Following the acclimatization stage, we added top and meso-predators to the tanks, according to the different risk treatments (Figure 2). In the tanks containing two predators, the fire salamander larvae were free to wander around and the dragonfly larva was placed in the pierced bottle. Dragonfly larvae were collected from a different spring on the Mount of Brianza. Fire salamander larvae were collected from two of the initial straits of two streams in the karst locality of "Alpe del Viceré" in the Como district. The rearing lasted for 30 days.

Figure 1. Sites of collection of *Niphargus thuringius* amphipods used during the experiment. (**A**) Short draining gallery in the locality of Valle Tolsera, Airuno municipality (Lecco, Northern Italy). (**B**) "Bottino di presa" in the municipality of Castello di Brianza (Lecco, Northern Italy).

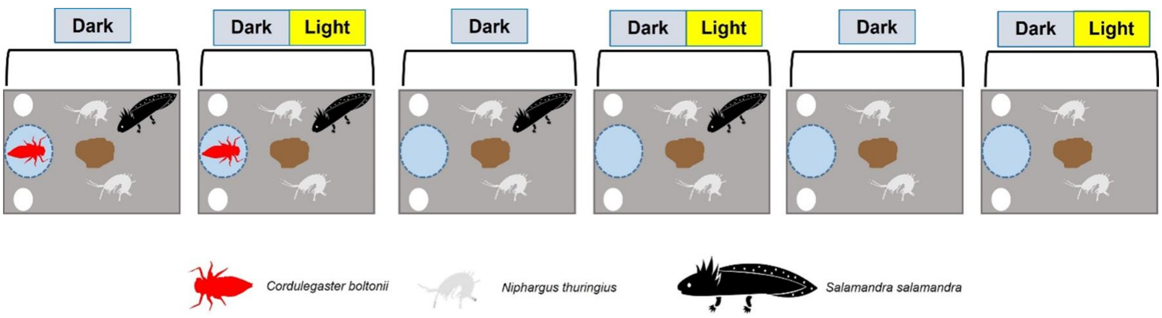

Figure 2. Disposition and composition of the microcosms in the laboratory of Subterranean Biology "Enrico Pezzoli". In brown, the stone provided as a form of shelter; in white, two Cabilock, white, plastic bowls (6 cm in diameter); in blue, a pierced, transparent bottle (10 cm in diameter).

2.3. Behavioural Tests

At the end of the 30-day experiment, a test to assess the response to light stimuli was conducted on the surviving *N. thuringius*. This behavioral test was performed for all the surviving animals during the 31st day, from the beginning of the experiment.

For this behavioral test, black, plastic arenas measuring 14 cm × 7 cm and containing 2 cm of water were constructed. The procedure consisted of placing *N. thuringius* individuals at the center of the arena, which was half-covered; then two tests were conducted for each individual: the first one consisted of illuminating the arena with a flashlight perpendicular to it to keep one side illuminated and the side in shadow; the second test consisted of keeping the arena completely in the dark. During the test, the light was placed 25 cm over the experimental arena, providing ~250 lumen of illuminance. In total-darkness conditions, we used an IR night visor to observe the amphipods. Each test was repeated twice for each individual; each trial lasted 3 min, with a minute of acclimatation between one trial and another. Four arenas were managed simultaneously by the same observers.

The entire test arena was covered with a box to avoid interference from the external environment. Both tests had a duration time of three minutes and were conducted in a random order to avoid any form of bias. At the end of the three minutes, after uncovering the box, the positions of the *Niphargus thuringius* were recorded using a ruler placed at the base of the arena.

Movement was recorded starting from a value of zero placed at the midpoint of the arena; the scale ranged from −7 cm to +7 cm; and negative values referred to the covered half and positive values to the uncovered half of the arena, which was illuminated during the tests.

At the end of the test, the amphipods were released into the collection site.

2.4. Statistical Analyses

To understand the patterns of the individuals' activity in laboratory conditions, we built a linear mixed model (LMM), which can also deal with partially imbalanced datasets [40,41]. The amphipods' preference for the side location (i.e., distance from the center) was the response variable, and light-exposure treatment (dark and light) was included as the fixed factor. We used orthogonal contrasts to perform the comparison between the rearing conditions. Orthogonal contrasts allow for pairwise comparisons without increasing type I and II errors, as they would occur when using post hoc tests [42]. To test the hypothesis that the predation risk of the rearing habitat affects the adult behavior of groundwater-dwelling amphipods, we used contrasts to compare the risk-rearing (two and one predators) and no-risk (without predators) conditions. Subsequently, for the no-risk conditions, we compared the tanks exposed to a daylight photoperiod to those in constant darkness. We then contrasted the tanks with two predators with those with only fire salamander larva, and then we contrasted, in both risky conditions, the tanks exposed to a daylight photoperiod against those exposed to constant darkness. Each contrast was included as a fixed factor in the model.

The microcosms' identity was considered as a random factor for each treatment, to account for the different number of replicates and individuals belonging to the same replicate.

We assessed the significance of the fixed factors using a Wald F test [43], and we verified the models' assumptions by verifying the absence of multicollinearity issues through a VIF calculation [44]. All statistical analyses were performed in R (version 3.6.0) using lmerTest, lme4, and glmmTMB packages.

3. Results

Overall, 34 individuals (42.4%) of *N. thuringius* survived and were used for the behavioral test. For both predators, the survival rate was 13.3%, while it increased to approximately 50% only for fire salamander larvae and was 75% without predators. In particular, at the end of the 30 days of the rearing period, in the microcosms that contained both *Cordulegaster boltonii* and *Salamandra salamandra* larvae, only 4 *Niphargus thuringius* out of the original 30 survived. In the tanks containing only *Salamandra salamandra* larvae, the surviving *Niphargus thuringius* increased to 16 out of 30 individuals. The tanks without predators showed a very high survival outcome of *Niphargus thuringius* individuals, with 16 surviving individuals out of the original 20. In the constant-darkness condition, the rate of survival was 55%, while in intermittent-light/darkness conditions, the survival rate was 30%.

Behavioral tests conducted on surviving *Niphargus thuringius* revealed a strong negative phototaxis presented by this species: indeed, a significantly higher number of animals preferred the covered side of the arena (Table 1; Figure 3). Adults responded to light stimuli, irrespective of the light conditions for rearing experiments (Table 1). Moreover, the hypogean amphipods that survived the rearing test with predators presented a significant preference for the borders of the covered (and safer) part of the test arena in comparison to those obtained from the microcosms without predators (Table 1; Figure 4).

Table 1. Results of the LMM considering the relationship between the behavioral response of the surviving *Niphargus thuringius* with light stimuli during the test and rearing conditions experienced (statistically significant effects ($p < 0.05$)) are highlighted in bold). NumDF = degree of freedom at the numerator; DenDF = degree of freedom at the denominator.

.	Estimate	NumDF	DenDF	F	*p*
Light stimuli	−4.76	1	145	49.04	**<0.001**
Reared with predators vs. without predators	−0.68	1	145	9.10	**<0.01**
Tanks without predators, intermittent-light conditions vs. constant darkness	0.54	1	145	1.01	0.31
Tanks with two predators vs. tanks with only one predator	−0.98	1	145	2.51	0.11
Tanks with two predators, intermittent-light conditions vs. constant darkness	−4.85	1	145	3.63	0.06
Tanks with one predator, intermittent-light conditions vs. constant darkness	−0.83	1	145	2.18	0.14

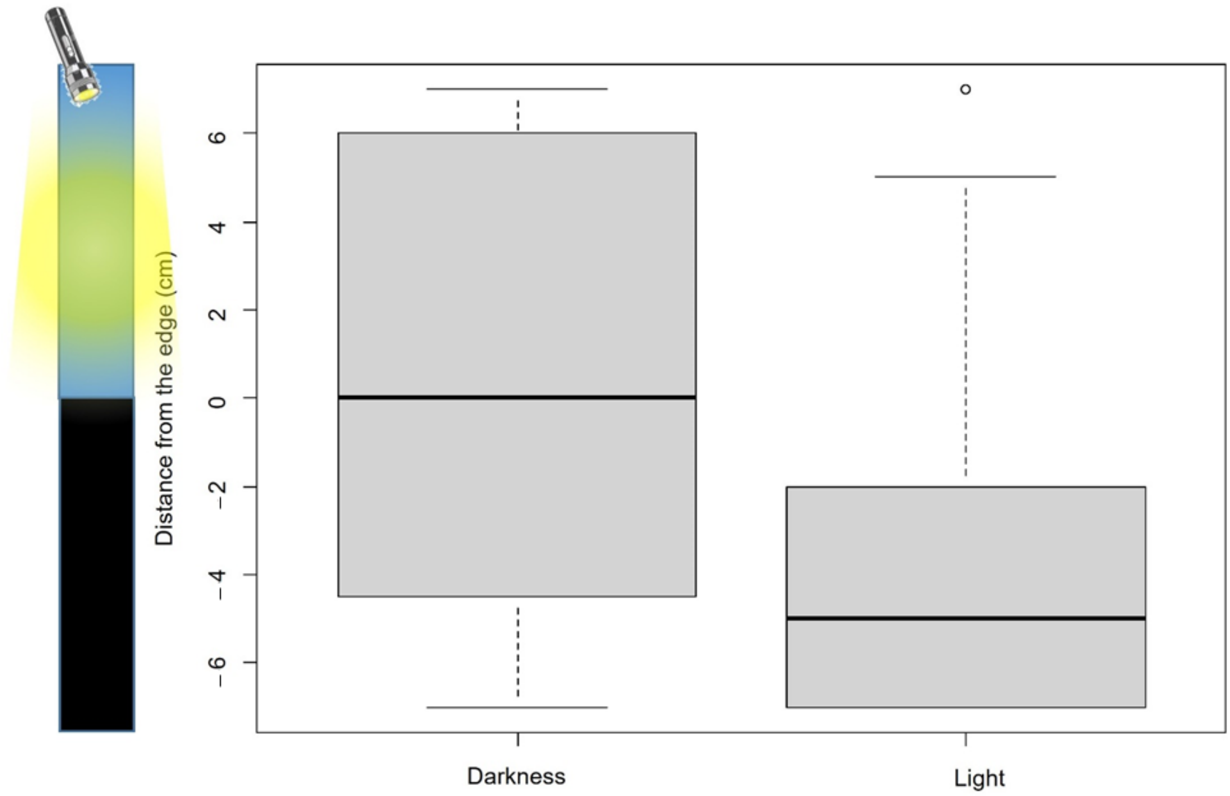

Figure 3. Box–whisker plot of the relationship between the distribution of *N. thuringius* in the presence or absence of a light source during the behavioral tests. Values of the disposition of individuals are assessed in relation to the center of the arena corresponding to 0. Negative values indicate an arrangement towards the covered half of the arena, whereas positive values indicate an arrangement towards the uncovered half of the arena.

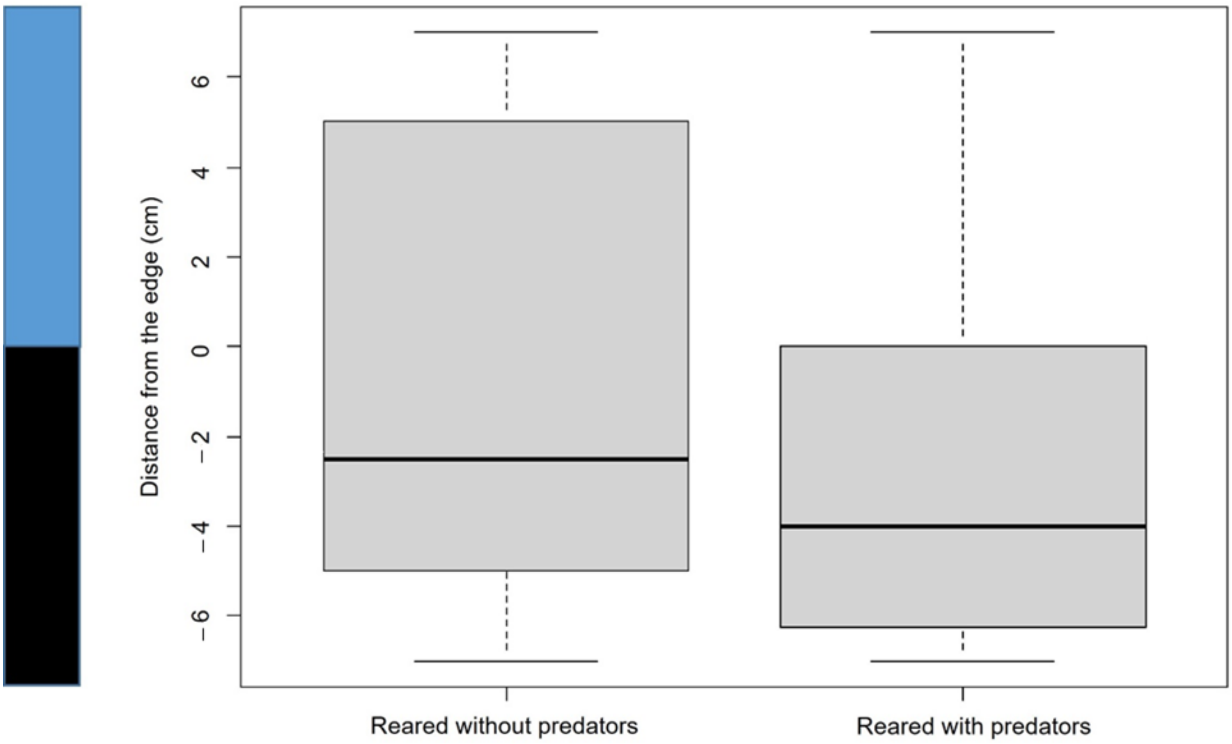

Figure 4. Box–whisker plot of the relationship between the distribution of *N. thuringius* during the behavioral tests and their condition of rearing (with or without predators). Values of the disposition of individuals are assessed in relation to the center of the arena corresponding to 0. Negative values indicate an arrangement towards the covered half of the arena, whereas positive values indicate an arrangement towards the uncovered half of the arena.

4. Discussion

Our results show that the stygobiont amphipods of the species *Niphargus thuringius*, which are as eyeless as all the representatives of the genus *Niphargus*, respond to light stimuli and avoid lighted areas, preferring darker ones.

The photophobic behavior of *Niphargus* amphipods is already known in the literature for other species [45,46], and has been tested multiple times in recent years [17,28]. While some authors have observed in the literature that subterranean amphipods seem to display stronger photophobic responses than those that inhabit the surface [17], others observed no particular differences or surface species displaying stronger photophobia [47,48], suggesting that negative phototaxis is a widespread characteristic in freshwater amphipods, even if not strictly linked to subterranean environments. Our results confirm that this pattern is widespread and conserved among niphargids. *N. thuringius* is one of the species in the North of Italy with the highest ecological plasticity; it can be observed both in deep groundwater and surface habitats close to springs [34,37]. The adult individuals collected for this experiment belonged to two different springs characterized by artificial buildings, named "bottini di presa", used to collect water in the past. Both sites are obsolete and contain unused buildings; past surveys revealed a very limited occurrence of other aquatic invertebrates in the area, suggesting a very poor occurrence of trophic resources [49]. It is, thus, possible that *N. thuringius* individuals living in these sites are attracted by the higher number of trophic resources that occur in the small creeks that originate from the two springs sampled in our experiment. Being able to perceive light and exploit surficial sites when UV light is absent—during the night or day, sheltering under leaves and materials that provide shadow—could limit the risk of light exposure to these unpigmented amphipods. On the other hand, if we consider that the individuals collected for this experiment were obtained from spring areas bordering an epigean environment and, thus, somehow likely used to perceive light during the day, it can be assumed that individuals obtained from deeper-lying habitats, not familiar with variations in light-intensity levels, can present even more marked responses. In this case, a stronger response displayed by individuals obtained from deeper-lying environments would not only suggest that avoiding light is a highly conserved behavioral pattern, but that it is also linked to the necessity to always avoid surface, sub-optimal, risky habitats and remain in the optimal subterranean environment, as already suggested for different species of *Niphargus*. In our case study, no differences occurred between individuals that experienced light/darkness and total darkness; thus, phototaxis seems to not be affected by short conditioning periods of rearing. Recent studies performed on the stygobiont amphipod *Stygobromus tenuis* determined that this species is more sensitive than surface amphipods to variations in light wavelengths, although they are not strongly photonegative [47,48]. *S. tenuis* shows increased photonegativity in response to long wavelengths of light and an augmented positive phototaxis in response to shorter wavelengths [47]; this pattern could reflect adaptations to light stimuli occurring in spring ecotones. Our results suggest that *N. thuringius* could be a good candidate model to perform further studies aiming to assess if differences exist between spring populations and populations occurring in deeper groundwater.

The fact that *N. thuringius* can respond to light stimuli, despite being eyeless, also suggests that this species should possess non-visual, extra-ocular photoreceptors. Light detection can also occur directly at the level of the diencephalon, as demonstrated in different fish species [50]; this fact was previously hypothesized for some stygobiont crustaceans, including *Niphargus* amphipods [51], but was never demonstrated in the field. Moreover, photoreceptors can also occur at the skin level, a fact previously well-reported in the literature for planarians [52], even if it seems less likely in animals characterized by a strong exoskeleton, such as amphipods. Further studies, using molecular tools, such as in situ hybridization for neural genes involved in light-detection activity, could be performed, and good candidates for this practice are invertebrates, such as ascidians, whose larvae can display negative phototaxis [53].

The most intriguing result of our experiment is that adult individuals of *N. thuringius* displayed different choices when considering the test arena's sectors, according to the conditions in which they were reared during the 30 days prior to the test. Amphipods reared with predators stayed closer to the border of the covered half of the arena than individuals reared without predators. This is one of the first attempts to assess the behavioral effects of predation risk on *Niphargus* amphipods. While recent phylogenetic studies revealed that predator–prey interactions are important in shaping antipredator morphological structures in *Niphargus* species [54], behavioral studies do not acknowledge this concept, which is similar for studies conducted on stygobiont crustaceans. An enhanced sensitivity to different predator cues exists in surface-dwelling freshwater amphipods [55–58], and this suggests that similar investigations can also be performed on subterranean-dwelling species. In our experiment, we used adult individuals, for which it was not possible to know their age or previous experience. The individuals were more cautious if reared with epigean predators (such as the fire salamander and dragonfly larvae) than individuals reared without predators. Therefore, only 30 days were sufficient enough to affect the adult behavior of subterranean-dwelling amphipods. This study provides new insights into the potential role played by predation risk in affecting the exploitation of surface habitats by stygobiont organisms. Predation by surface species can be a factor limiting the activity of stygobionts. However, a limitation of our study that requires further investigations is linked to the relatively low number of both collected (which reduced the number of replicates performed) and surviving individuals. Surviving amphipods were those that were more cautious in general, or that already experienced predation risks during their lifetime. In any case, they were able to survive the attacks from predators; it is also likely that they developed cautious behavior as a response to the predation risk they experienced. Further studies could assess if *N. thuringius* can distinguish predators' chemical cues from other cues and assess if differences between populations present in springs and deeper groundwater exist.

The limitation of working with adults, without a knowledge of their experiences and age, is a problem that can affect different studies focusing on stygobiont species. Stygobionts are in fact characterized by long life cycles and reduced fecundity, which makes it difficult to work with newborn larvae/individuals that could allow us to disentangle the effect of the environment/experience on their behavioral patterns.

The behavioral outcomes presented in this study deserve further research and could represent, together with investigations focusing on the study of the circadian cycles of subterranean amphipods, interesting points for comparative studies between surface and subterranean species as well as between populations of the same species of stygobionts. In conclusion, it can be inferred from the results obtained during this experimental work that low light intensity and a reduced presence of predators could represent decisive factors for stygobiont species, such as *Niphargus thuringius*, in the successful exploitation of ecotonal environments.

Author Contributions: Conceptualization, R.M., M.G. and S.L.; methodology, M.G., M.F., B.B. and R.M.; writing—original draft preparation, M.G. and S.L.; writing—review and editing, R.M., S.L. and M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The animal study protocol involving fire salamander larvae was approved by the Institutional Review Board (or Ethics Committee) of Lombardy Region (protocol code n. 18303 13.12.2019).

Data Availability Statement: The data are available upon reasonable request.

Acknowledgments: We are grateful to Andrea Melotto for his suggestions on the behavioral tests.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Carroll, T.M.; Thorp, J.H. Ecotonal shifts in diversity and functional traits in zoobenthic communities of karst springs. *Hydrobiologia* **2014**, *738*, 1–20. [CrossRef]
2. Kark, S. Effects of Ecotones on Biodiversity. In *Encyclopedia of Biodiversity*, 2nd ed.; Levin, S.A., Ed.; Academic Press: Waltham, MA, USA, 2013; pp. 142–148.
3. Konec, M.; Prevorčnik, S.; Sarbu, S.M.; Verovnik, R.; Trontelj, P. Parallels between two geographically and ecologically disparate cave invasions by the same species, Asellus aquaticus (Isopoda, Crustacea). *J. Evol. Biol.* **2015**, *28*, 864–875. [CrossRef] [PubMed]
4. Thorp, J.H. Chapter 4—Functional Relationships of Freshwater Invertebrates. In *Thorp and Covich's Freshwater Invertebrates*, 4th ed.; Thorp, J.H., Rogers, D.C., Eds.; Academic Press: Boston, MA, USA, 2015; pp. 65–82.
5. Kark, S.; van Rensburg, B.J. Ecotones: Marginal or central areas of transition? *Isr. J. Ecol. Evol.* **2006**, *52*, 29–53. [CrossRef]
6. Schilthuizen, M. Ecotone: Speciation-prone. *Trends Ecol. Evol.* **2000**, *15*, 130–131. [CrossRef] [PubMed]
7. Di Lorenzo, T.; Cipriani, D.; Bono, P.; Rossini, L.; De Laurentiis, P.; Fiasca, B.; Pantani, C.; Galassi, D.M.P. Dynamics of groundwater copepod assemblages from the Mazzoccolo karstic spring (central Italy). *Meiofauna Mar.* **2005**, *14*, 97–103.
8. Galassi, D.M.P.; Stoch, F.; Fiasca, B.; Di Lorenzo, T.; Gattone, E. Groundwater biodiversity patterns in the Lessinian Massif of northern Italy. *Freshw. Biol.* **2009**, *54*, 830–847. [CrossRef]
9. Pacioglu, O.; Robertson, A. The invertebrate community of the chalk stream hyporheic zone: Spatio-temporal distribution patterns. *Knowl. Manag. Aquat. Ecosyst.* **2017**, *418*, 10. [CrossRef]
10. Premate, E.; Borko, Š.; Delić, T.; Malard, F.; Simon, L.; Fišer, C. Cave amphipods reveal co-variation between morphology and trophic niche in a low-productivity environment. *Freshw. Biol.* **2021**, *66*, 1876–1888. [CrossRef]
11. Barzaghi, B.; Ficetola, G.F.; Pennati, R.; Manenti, R. Biphasic predators provide biomass subsidies in small freshwater habitats: A case study of spring and cave pools. *Freshw. Biol.* **2017**, *62*, 1637–1644. [CrossRef]
12. MacAvoy, S.E.; Braciszewski, A.; Tengi, E.; Fong, D.W. Trophic plasticity among spring vs. cave populations of Gammarus minus: Examining functional niches using stable isotopes and C/N ratios. *Ecol. Res.* **2016**, *31*, 589–595. [CrossRef]
13. Manenti, R.; Ficetola, G.F. Salamanders breeding in subterranean habitats: Local adaptations or behavioural plasticity? *J. Zool.* **2013**, *289*, 182–188. [CrossRef]
14. von Fumetti, S.; Nagel, P.; Baltes, B. Where a springhead becomes a springbrook—A regional zonation of springs. *Fundam. Appl. Limnol.* **2007**, *169*, 37–48. [CrossRef]
15. Manenti, R.; Barzaghi, B. Diel activity of *Niphargus* amphipods in spring habitats. *Crustaceana* **2021**, *94*, 705–721.

16. Fišer, C.; Pipan, T.; Culver, D.C. The vertical extent of groundwater metazoans: An ecological and evolutionary perspective. *Bioscience* **2014**, *64*, 971–979. [CrossRef]
17. Fišer, Z.; Novak, L.; Lustrik, R.; Fiser, C. Light triggers habitat choice of eyeless subterranean but not of eyed surface amphipods. *Sci. Nat.* **2016**, *103*, 7. [CrossRef] [PubMed]
18. Culver, D.C.; Pipan, T. *Biology of Caves and Other Subterranean Habitats*, 2nd ed.; Oxford University Press: New York, NY, USA, 2019; pp. 1–301.
19. Romero, A. *Cave Biology: Life in Darkness*; Cambridge University Press: New York, NY, USA, 2009.
20. Mammola, S. Finding answers in the dark: Caves as models in ecology fifty years after Poulson and White. *Ecography* **2019**, *42*, 1331–1351. [CrossRef]
21. Poulson, T.L.; White, W.B. The cave environment. *Science* **1969**, *165*, 971–981. [CrossRef]
22. Romero, A. The Evolution of Cave Life. *Am. Sci.* **2011**, *99*, 144–151. [CrossRef]
23. Durand, J.P. Ocular Development and Involution in European Cave Salamander, *Proteus anguinus* Laurenti. *Biol. Bull.* **1976**, *151*, 450–466. [CrossRef]
24. Moldovan, O.T.; Kováč, L.; Halse, S. *Cave Ecology*; Springer: Cham, Switzerland, 2018.
25. Moran, D.; Softley, R.; Warrant, E.J. The energetic cost of vision and the evolution of eyeless Mexican cavefish. *Sci. Adv.* **2015**, *1*, e1500363. [CrossRef]
26. Friedrich, M. Biological Clocks and Visual Systems in Cave-Adapted Animals at the Dawn of Speleogenomics. *Integr. Comp. Biol.* **2013**, *53*, 50–67. [CrossRef] [PubMed]
27. Jeffery, W.R. Cavefish as a model system in evolutionary developmental biology. *Dev. Biol.* **2001**, *231*, 1–12. [CrossRef] [PubMed]
28. Borowsky, B. Responses to light in two eyeless cave dwelling amphipods (*Niphargus ictus* and *Niphargus frasassianus*). *J. Crustac. Biol.* **2011**, *31*, 613–616. [CrossRef]
29. Soares, D.; Niemiller, M.L. Sensory Adaptations of Fishes to Subterranean Environments. *Bioscience* **2013**, *63*, 274–283. [CrossRef]
30. Manenti, R.; Piazza, B. Between darkness and light: Spring habitats provide new perspectives for modern researchers on groundwater biology. *PeerJ* **2021**, *9*, e11711. [CrossRef]
31. Barzaghi, B.; Blaimont, P.; Manenti, R. Detection of non-consumptive effects of predation and intraspecific aggression in fire salamander larvae: Environmental issues. *North-West J. Zool.* **2020**, *16*, 74–77.
32. Manenti, R.; Siesa, M.E.; Ficetola, G.F. Odonata occurence in caves: Active or accidentals? A new case study. *J. Cave Karst Stud.* **2013**, *75*, 205–209. [CrossRef]
33. Manenti, R.; Lunghi, E.; Barzaghi, B.; Melotto, A.; Falaschi, M.; Ficetola, G.F. Do salamanders limit the abundance of groundwater invertebrates in subterranean habitats? *Diversity* **2020**, *12*, 161. [CrossRef]
34. Manenti, R.; Pezzoli, E. Think of what lies below, not only of what is visible above, or: A comprehensive zoological study of invertebrate communities of spring habitats. *Eur. Zool. J.* **2019**, *86*, 272–279. [CrossRef]
35. Wilhelm, F.M.; Taylor, S.J.; Adams, G.L. Comparison of routine metabolic rates of the stygobite, Gammarus acherondytes (Amphipoda: Gammaridae) and the stygophile, Gammarus troglophilus. *Freshw. Biol.* **2006**, *51*, 1162–1174. [CrossRef]
36. Pezzoli, E.; Lemme, M. I Molluschi delle "Acque sotterranee". X° Contributo per la Provincia di Brescia (VIII° Regione Lombardia). Revisione delle stazioni edite e proseguimento della mappatura sul territorio. Particolare ricerca sulla tanatocenosi che si accumula nelle vasche di decantazione dei manufatti di captazione di sorgenti. *Monogr. Nat. Brescia* **2003**, *26*, 1–240.
37. Stoch, F. Isopodi ed anfipodi (Crustacea, Malacostraca) della Provincia di Bergamo: Note sulle specie rinvenute nelle grotte e nelle sorgenti. In *I Molluschi Delle Sorgenti e Delle 'Acque Sotterranee', IX Aggiornamento al Censimento*; Pezzoli, E., Spelta, F., Eds.; Monografie di Natura Bresciana: Brescia, Italy, 2000; Volume 24, pp. 231–241.
38. Ficetola, G.F.; Manenti, R.; De Bernardi, F.; Padoa-Schioppa, E. Can patterns of spatial autocorrelation reveal population processes? An analysis with the fire salamander. *Ecography* **2012**, *35*, 693–703. [CrossRef]
39. Pezzoli, E. I Molluschi crenobionti e stigobionti presenti in Italia. Censimento delle stazioni: VII aggiornamento. *Quad. Civ. Staz. Idrobiol. Milano.* **1996**, *21*, 111–118.
40. Hesselmann, G. Applying Linear Mixed Effects Models (LMMs) in Within-Participant Designs With Subjective Trial-Based Assessments of Awareness—A Caveat. *Front. Psychol.* **2018**, *9*, 788. [CrossRef]
41. Piepho, H.-P. Multiple Treatment Comparisons in Linear Models When the Standard Error of a Difference is not Constant. *Biom. J.* **2000**, *42*, 823–835. [CrossRef]
42. Field, A.; Miles, J.; Field, Z. *Discovering Statistics Using R*; SAGE Publications: London, UK, 2015.
43. Bolker, B.M.; Brooks, M.E.; Clark, C.J.; Geange, S.W.; Poulsen, J.R.; Stevens, M.H.H.; White, J.S. Generalized linear mixed models: A practical guide for ecology and evolution. *Trends Ecol. Evol.* **2008**, *24*, 127–135. [CrossRef]
44. Zuur, A.F.; Ieno, E.N.; Walker, N.J.; Saveliev, A.A.; Smith, G.M. *Mixed Effects Models and Extensions in Ecology with R*; Springer: Berlin/Heidelberg, Germany, 2009; p. 680.
45. Ginet, R. Ecologie, éthologie et biologie de" Niphargus"(Amphipodes Gammaridés hypogés). *Ann. Speleol.* **1960**, *15*, 127–237.
46. Vandel, A. *Biospeleologie: La Biologie des Animaux Cavernicoles*; Gauthiers-Villars: Paris, France, 1964; Volume XVIII, p. 619.
47. Kennedy, M.D.; Barberio, A.; Connaughton, V.P. Novel experimental apparatus for laboratory measurements of phototaxis: A comparison between amphipod species. *J. Crustac. Biol.* **2022**, *42*, ruab085. [CrossRef]
48. Kennedy, M.D.; Connaughton, V.P. Differential effects of fluoxetine on the phototactic behavior of 3 amphipod species (Crustacea; Amphipoda). *Environ. Toxicol. Pharmacol.* **2022**, *93*, 103889. [CrossRef]

49. Manenti, R.; Ficetola, G.F.; Bianchi, B.; De Bernardi, F. Habitat features and distribution of *Salamandra salamandra* in underground springs. *Acta Herpetol.* **2009**, *4*, 143–151.
50. Breder, C.M.J.; Rasquin, P. Comparative Studies in the Light Sensitivity of Blind Characins from a Series of Mexican Caves. *Bull. Am. Mus. Nat. Hist.* **1947**, *89*, 5.
51. Merker, E. Lichtsinn und allgemeine LIchtempfindlichkeit. *Zool Anz.* **1929**, *4*, 29–32.
52. Viaud, G. Recherches experimentales sur le Phototropisme des Planaires. *Behaviour* **1950**, *2*, 163–216. [CrossRef]
53. Pasini, A.; Manenti, R.; Rothbacher, U.; Lemaire, P. Antagonizing Retinoic Acid and FGF/MAPK Pathways Control Posterior Body Patterning in the Invertebrate Chordate Ciona intestinalis. *PLoS ONE* **2012**, *7*, e46193. [CrossRef]
54. Premate, E.; Zagmajster, M.; Fišer, C. Inferring predator–prey interaction in the subterranean environment: A case study from Dinaric caves. *Sci. Rep.* **2021**, *11*, 21682. [CrossRef]
55. James, W.R.; McClintock, J.B. Anti-predator responses of amphipods are more effective in the presence of conspecific chemical cues. *Hydrobiologia* **2017**, *797*, 277–288. [CrossRef]
56. Jermacz; Kletkiewicz, H.; Krzyżyńska, K.; Klimiuk, M.; Kobak, J. Does global warming intensify cost of antipredator reaction? A case study of freshwater amphipods. *Sci. Total Environ.* **2020**, *742*, 140474. [CrossRef]
57. Jermacz, Ł.; Andrzejczak, J.; Arczyńska, E.; Zielska, J.; Kobak, J. An enemy of your enemy is your friend: Impact of predators on aggregation behavior of gammarids. *Ethology* **2017**, *123*, 627–639. [CrossRef]
58. Jermacz, Ł.; Dzierżyńska-Białończyk, A.; Kobak, J. Predator diet, origin or both? Factors determining responses of omnivorous amphipods to predation cues. *Hydrobiologia* **2017**, *785*, 173–184. [CrossRef]