

Population divergence in aggregation and sheltering behaviour in surface- versus cave-adapted *Asellus aquaticus* (Crustacea: Isopoda)

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Aggregation (gathering together) and sheltering (hiding in cover) are basic behaviours that can reduce the risk of predation. However, both behaviours have costs, such as increased competition over resources and high prevalence of contact-spread parasites (aggregation) or lost opportunities for foraging and mating (sheltering). Therefore, variation in these behaviours is expected between populations with varying levels of predation risk. We compared aggregation and sheltering in surface- (various predators) and cave-adapted (no predator) populations of the isopod *Asellus aquaticus* in a common garden experiment. Given that the cave environment is constantly dark, we also tested for population variation in light-induced behavioural plasticity. Variation in sheltering was explained by habitat type: cave individuals sheltered less than surface individuals. We found high between-population variation in aggregation with or without shelters and their light-induced plasticity, which was not explained by habitat type. Cave individuals decreased (habituation) whereas surface individuals increased sheltering with time (sensitization). We suggest that population variation in sheltering is driven by predation, whereas variation in aggregation must be driven by other, unaccounted environmental factors, in a similar manner to light-induced behavioural plasticity. Based on habituation/sensitization patterns, we suggest that predation-adapted populations are more sensitive to disturbance related to routine laboratory procedures.

ADDITIONAL KEYWORDS: aggregation – *Asellus aquaticus* – cave adaptation – shelter use.

INTRODUCTION

Behaviour is perhaps the most plastic quantitative phenotypic trait (West-Eberhard, 2003). However, despite high plasticity and the potential for a moment-by-moment optimization to the prevailing environment, geographical between-population variation within species in behaviour is evident, suggesting local (genetic) adaptation in behaviour (e.g. Foster, 1999; Foster & Endler, 1999). There are several examples of between-population behavioural divergence, such as the effect of varying predation pressure on behaviour shown in common frog (*Rana temporaria*) tadpoles (Van Buskirk & Arioli, 2005), nine-spined sticklebacks (*Pungitius pungitius*) (Herczeg *et al.*, 2009a) or guppies (*Poecilia reticulata*) (Magurran & Seghers, 1991, 1994). Such

environmentally driven population divergence in behaviour has been found to be based genetically in several species (see Breden *et al.*, 1987; Brown *et al.*, 2007; Herczeg *et al.*, 2013; Laine *et al.*, 2014).

Aggregation (gathering together) is a basic behaviour exhibited by many animals (Parrish & Edelman, 1999; Hassall & Tuck, 2007; Kullmann *et al.*, 2008; Broly *et al.*, 2012); nevertheless, it does not necessarily imply any social organization (e.g. antelopes gather for water; Allaby, 1994). Aggregation can be seen as a form of evolutionary adaptation with multiple advantages (Pitcher, 1986): it might help in avoidance of desiccation in some terrestrial animals (Brockett & Hassall, 2005), it can improve feeding efficiency (Heupel & Simpfendorfer, 2005), and it can provide a mechanism of defence from predators (Broly *et al.*, 2013). However, aggregation behaviour can also have costs, such as higher vulnerability to contact-spread parasites, increased competition for resources and conspicuousness of the group

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to predators (Pitcher, 1986; Romey, 1995). Hence, decreased aggregation is expected in populations where the benefits of the behaviour are low or where the disadvantages outweigh the benefits. For instance, the negative covariance between predation pressure and the social costs of aggregation was supported experimentally (Herczeg *et al.*, 2009b).

Sheltering (hiding under cover) is one of the most straightforward antipredator behaviours, when it is not applied against some other environmental effect, such as harsh sunlight [i.e. small reptiles hide and cool during the hottest hours of the day (Castilla *et al.*, 1999; Arribas, 2013)]. Sheltering behaviour has obvious benefits, but it also incurs costs owing to lost opportunities connected to alternative beneficial behaviours, such as mate-searching or foraging (e.g. Sih, 1992; Cooper & Frederick, 2007). We know from previous studies that sheltering behaviour successfully reduces the risk of predation (Cooper & Frederick, 2007; Kullmann *et al.*, 2008). Furthermore, a previous study showed that a marine isopod, *Idothea balthica*, traded sheltering for food (Vesakoski *et al.*, 2008). The antipredatory role of aggregation and sheltering has been supported in many invertebrate and vertebrate taxa, with populations showing considerable behavioural variation explained by predation pressure (e.g. mammals: Fryxell, 1991; amphibians: Watt *et al.*, 1997; birds: Forsman *et al.*, 1998; fish: Griffiths *et al.*, 2003; *Gammarus pulex*: Kullmann *et al.*, 2008; Thünken *et al.*, 2010; *Forficula auricularia*: Raveh *et al.*, 2014; *Pyrrhocoris apterus*: Svádová *et al.*, 2014; reptiles: Gardner *et al.*, 2016; *Cirolana harfordi*: Salma & Thomson, 2018).

Environmental conditions in subterranean environments differ drastically from those of the surface. Caves and related habitats are characterized by the absence of light, food scarcity, simplified communities, and seasonal and yearly environmental variation being low to non-existent. Furthermore, being separated physically from each other, caves represent natural replicates of environmental conditions with no gene flow between them (Culver & Sket, 2000; Gibert & Deharveng, 2002; Tobler *et al.*, 2008; Culver & Pipan, 2009). Importantly, most caves are free from both vertebrate and invertebrate predators (Culver & Pipan, 2019; White *et al.*, 2019; but see Culver, 1975). In fact, predator avoidance was suggested previously to be a main factor behind cave colonization in several insect species (Rivera *et al.*, 2002; Juan & Emerson, 2010) and in the Mexican tetra, *Astyanax mexicanus* (Romero, 1985). Furthermore, recent results indicate the importance of low predation pressure behind occurrence of the salamanders *Eurycea lucifuga* and *Speleomantes strinatii* in caves (Salvidio *et al.*, 2017; Bradley & Eason, 2018, respectively). Intuitively, in populations with a longer history of cave adaptation

(troglobionts and/or eutroglophiles; *sensu* Sket, 2008), the loss or reduction of antipredatory responses is expected in comparison to subtroglophile (Sket, 2008) or surface populations. In line with this, empirical results show that predator recognition is maintained in cave-dwelling populations of the Pyrenean newt (*Calotriton asper*), a recent colonist, whereas such behaviour is lost in the highly adapted olm (*Proteus anguinus*) (Manenti *et al.*, 2020). Despite the consensus on the importance of differences in predation pressure between surface and subterranean habitats, differences in antipredator behaviours between cave and surface populations are rarely tested, at best.

Here, we studied population variation in aggregation and sheltering behaviour of the common water louse, *Asellus aquaticus* (Linnaeus, 1758). In a common garden experiment, we tested aggregation behaviour with and without shelter, in addition to sheltering in general, in a highly specialized cave population (no predation and permanent darkness) and three surface populations (various predators present, with diurnal light cycle). We also applied light treatments (presence/absence) in common garden settings. Aggregation would hardly increase the foraging efficiency of a detritivorous species feeding on a wide variety of stationary food sources (e.g. living and dead plant material, together with bacteria and fungi growing on them, for surface populations, see Moore, 1975; Graça *et al.*, 1993; Bloor, 2011; and endogenous bacterial mats in the studied cave, see Herczeg *et al.*, 2020). Hence, we expected that the main environmental driver of the variation in aggregation behaviour in our study system would be the presence or absence of predation. The same was expected for sheltering behaviour, because sheltering in the natural populations is unlikely to provide defence against various forms of environmental harshness, apart from predation. Therefore, we predicted that predation-adapted surface *A. aquaticus* populations would be more risk averse (showing higher levels of aggregation and sheltering) than the cave population adapted to the lack of predation. Expectations regarding light-induced plasticity were less straightforward. Although the vision of cave-adapted *A. aquaticus* is highly reduced, they still sense light (see Protas *et al.*, 2011; Pérez-Moreno *et al.*, 2018; Re *et al.*, 2018) and show negative phototaxis (Fišer *et al.*, 2016). Assuming that surface *A. aquaticus* populations experience higher predation risk during the day than at night, we predicted that all populations would show higher aggregation and shelter use when tested in light, with the response being the strongest in cave-adapted individuals owing to light avoidance. We included only one cave population in our study; hence, we could not make generalizations

about *A. aquaticus* behaviour in caves and, practically speaking, we compared four populations and not two habitats. However, we compared a cave population that has been isolated for > 60 000 years from the closest surface populations (Pérez-Moreno *et al.*, 2017) with one surface population inhabiting a water body directly connected to the cave and two surface populations that are isolated from the cave but still in the geographical vicinity. Hence, we still expected that any patterns where the cave population was clearly divergent from the three surface populations would result from adaptation to the cave environment.

MATERIAL AND METHODS

STUDY SYSTEM

Asellus aquaticus is widespread in a wide variety of surface freshwater habitats across the Western Palaearctic (Verovnik *et al.*, 2005) and successfully colonized caves in Central Europe on several independent occasions (Verovnik *et al.*, 2005, 2009). Like other cave-dwelling species, cave-adapted *A. aquaticus* exhibit typical troglomorphic adaptations, such as reduced eyes and loss of pigmentation (Pérez-Moreno *et al.*, 2018; Re *et al.*, 2018).

Three surface populations and one cave population of *A. aquaticus* were used in the experiments. All populations live within or in the vicinity of Budapest, Hungary. The Molnár János Cave (47.518°N, 19.03608°E) is a water-filled cave of hydrothermal origin with a water temperature of 23–24 °C all year round. Despite the absence of physical barriers, the population inhabiting the Molnár János Cave has been isolated genetically from surface populations (including the Malom Lake population described below) for ≥ 60 000 years, and it shows the aforementioned troglomorphic adaptations (Pérez-Moreno *et al.*, 2017). The only available food source for this cave population of *A. aquaticus* is endogenous bacterial mats. Visible organic material from the surface does not enter the cave (G.B., personal observation), which is supported by Erőss *et al.* (2006) and Bodor *et al.* (2015), whose results indicate no direct hydrological connection between the Rózsadomb recharge area and the discharge area of the Boltív Spring (connected to the Molnár János Cave–Malom Lake system). The outflow of the cave to the surface forms a small lake (Malom Lake) right at the cave entrance (47.518°N, 19.03608°E), harbouring the first sampled surface population. Malom Lake receives natural sunlight, but the water temperature is similar that of the cave all year round. Guppies (*P. reticulata*) were introduced to Malom Lake during the 20th century. In the absence of other native fish

species, guppies, forming a high-density population, are the main predators of *A. aquaticus* in the Malom Lake (Berczik, 1956; G.B., personal observation). The remaining two surface populations, Gótés Lake (47.59556°N, 19.04142°E) and Dunakeszi Peat-moor (47.615613°N, 19.126392°E) are subject to a natural surface light regimen and temperature fluctuations typical for the region. These surface populations are members of diverse natural communities consisting of competitor and predator species. *Asellus aquaticus* is known to be a food source for fish and larval dragonflies in surface habitats (Harris *et al.*, 2013). The cave-dwelling population experiences low biotic complexity, with a small number of competitors and absence of predators in a stable and predictable environment.

COLLECTION AND HOUSING OF THE EXPERIMENTAL ANIMALS

Adult male individuals were collected on 30 October 2019 ($N = 30$ per population). Samples were collected by hand sorting with a mesh net, except for the subterranean animals from the Molnár János Cave, where a modified Sket bottle was used (Chevaldonné *et al.*, 2008) and cave diving was necessary. The autumn of 2019 was exceptionally warm in the region; hence, the stable temperature of thermal water at Malom Lake and Molnár János Cave (23–24 °C) was available in the surface habitats during sampling. After collection, animals were transported immediately to the facilities of the Biological Institute of Eötvös Loránd University (Budapest, Hungary).

We used only males to rule out the potential importance of aggregation for mating. Animals were sexed visually under a stereo microscope, based on the shape of the second pleopod. We collected only adults > 4 mm in length (Hasu *et al.*, 2007). All populations were divided randomly into two equal subgroups, and individuals from the subgroups were housed together in transparent plastic containers (volume, 5 L; dimensions: 32 cm × 21 cm × 11 cm, length × width × height, respectively). Individuals were kept in these containers throughout the experiment (except during behavioural tests, see experimental set-up below). Water collected at the source habitats was used to fill the containers and was refilled regularly as the water level dropped. We also provided small stones as shelters. Containers were placed in custom-made, light-controlled chambers (see experimental set-up below). Surface populations were acclimated to a daily light cycle (10 h light–14 h dark; controlled by a timer), whereas the cave-dwelling population was acclimated to complete darkness, and all handling processes were done under red light. The temperature in the laboratory varied between 23 and 24 °C. Animals did not receive food during the acclimation period and experiments.

EXPERIMENTAL SET-UP

To video record the behaviour of the animals in different light conditions, custom-made recording chambers were built (100 cm × 55 cm × 105 cm, length × width × height). All chambers were equipped with two light sources: light-emitting diodes (LEDs) imitating daylight (Colour Temperature = 4500K, Colour Rendering Index > 90) at the top and infrared LEDs (920 nm) at the bottom. This infrared wavelength is outside the visible range of *A. aquaticus* (Dember & Richman, 2012). The chambers were closed at the sides with non-transparent, black plastic boards meaning that light did not scatter inside/outside of the chambers. Inside each chamber, we mounted a webcam (Logitech C920 FullHD; Logitech, Lausanne, Switzerland) that was modified to improve the quality of videos recorded in infrared light. OBS STUDIO software (OBS Studio Contributors) was used to capture videos at 5 frames/s at HD Ready resolution (1280 × 720 pixels).

We tested sheltering behaviour and aggregation in the presence of shelters on 1 November 2019. The experimental set-up consisted of circular arenas (Petri dishes, diameter 140 mm). The bottom of each Petri dish was coarsened with emery paper to enable normal movement by the animals (Fišer *et al.*, 2019). These Petri dishes were used to house the tested groups (see next paragraph). Five shelters, made of red glass were placed into each Petri dish (red glass reduces light intensities and filters the spectrum of light; Devigne *et al.*, 2011). The shelters were square shaped (3 cm wide), and each one was placed at equal distances from the others inside the arenas. Based on their preference for narrow spaces that provide thigmotactic (tactile) stimulation, surface *A. aquaticus* is considered highly thigmotactic (Fišer *et al.*, 2019). Therefore, one side of the shelters was raised slightly with a tiny piece of glass so that animals of different size could crawl underneath and receive thigmotactic stimuli.

Animals within a population were divided randomly into six groups (five individuals in each group, sampled from the two holding tanks per population randomly). These groups of five were tested in separate Petri dishes. The groups were placed into small removable cylinders (diameter = 28 mm) located in the centres of the Petri dishes. The total of 24 Petri dishes were divided randomly between two recording chambers. After ~3 min of acclimation, the cylinders were removed and the video recording started. Each group was tested in both the presence and the absence of light, with treatments having a different order in the recording chambers. After 150 min of video recording, light regimens were changed in the recording chambers, and a second video recording of 150 min started. Infrared light was on for all tests, because it was needed for the video recording. Aggregation in the

absence of shelters was tested on 5 November 2019. The experimental set-up was similar to that in the previous experiment, except that the behaviour of individuals was assessed in the absence of shelters.

For the video analyses, the first and last 30 min (hereafter, recording periods) of every 150 min video recording were analysed manually to see how behaviour changed throughout the assays. Each behavioural variable was recorded in every third minute within these recording periods, which resulted in a total of 20 observations per Petri dish (ten observations per recording period). Aggregation behaviour in the presence of shelters was quantified by the average number of animals under the occupied shelters (hereafter, 'shelter sharing') at a given observation. Sheltering behaviour was represented by the number of animals under shelter (hereafter, 'sheltering') at a given observation. To quantify aggregation behaviour in the absence of shelters, we measured the distance between all pairs within the group for every observation and calculated the mean of these distances; hence, there was only one number per group for every observation describing group cohesion (hereafter, 'distance'). The distance between individuals was measured manually using the software IMAGEJ (Schneider *et al.*, 2012). Note that individual-level (non-averaged) distance data could also be analysed, but adding the extra level of hierarchy to the statistical model made the model problematic. However, we ran also this model and it yielded qualitatively similar results to the analysis of averaged data; hence, we report only the results from the former model run on averaged data.

STATISTICAL ANALYSES

For analysis of distance and shelter sharing, we ran separate linear mixed models (LMMs) by using the packages *lme4* (Bates *et al.*, 2015) and *lmerTest* (Kuznetsova *et al.*, 2016) available in the RSTUDIO interface v.1.2.5001 (R Developmental Core Team, 2020; RStudio Team, 2020). All models were parameterized with population, treatment (light vs. dark), recording period (beginning vs. end period of the 150 min assays) and their interactions as fixed effects. Given that the interpretation of three-way factorial interactions is highly problematic, we included only the two-way interactions. The order of the trial (first vs. second 150 min run within a day) was treated as a nuisance variable, and we included it in the models as a single fixed effect. The shelter sharing probability is obviously affected by the number of individuals sheltering at a given observation, even if refuge use is random regarding the presence of conspecifics under the given shelter. Therefore, the number of individuals sheltering was also added to the model run on shelter

sharing as a fixed effect for correction. Furthermore, we excluded observations with fewer than two individuals hiding (68.5% of the observations) from the model run on shelter sharing, because these observations do not provide information on shelter sharing. Group (i.e. the five individuals per Petri dish) identity was also added to the models as a random effect to control for the non-independence of the data. In these models, fixed effects were tested by Wald's χ^2 tests and random effects by likelihood ratio tests. Sheltering (ordinal data) was analysed using a generalized linear mixed model (GLMM) with negative binomial distribution and log link function; fixed and random effects were the same as in the previous models.

We extracted the estimated marginal means from the models using the *emmeans* package (Lenth, 2019). We also report the proportion of explained variance by the fixed factors (marginal R^2) and by both fixed and random factors (conditional R^2) available in the *MuMIn* package (Barton, 2009).

RESULTS

The LMM on distance revealed significant population \times treatment and population \times recording period interactions (Table 1). However, the only strong pattern was among populations: individuals from the Molnár János Cave and Götés Lake aggregated significantly less than individuals from Malom Lake and Dunakeszi Moor (Fig. 1A, B). The population \times treatment interaction patterns were inconsistent, suggesting that the cave population aggregated more in the light, whereas the surface populations aggregated more in the dark. The population \times recording period interaction indicated an inconsistent trend, whereby populations with generally higher aggregation decreased whereas populations

Table 1. Result of a linear mixed model on aggregation in the absence of shelters in *Asellus aquaticus*

| Model term | χ^2 (d.f.) | P-value |
|--|-------------------|-------------------|
| Fixed effects | | |
| Population | 482.49 (4) | < 0.001 |
| Treatment | 4.05 (1) | 0.04 |
| Recording period | 0.52 (1) | 0.47 |
| Order of trial | 4.89 (1) | 0.03 |
| Population \times treatment | 16.84 (1) | < 0.001 |
| Population \times recording period | 13.81 (3) | 0.003 |
| Treatment \times recording period | 0.43 (1) | 0.51 |
| Random effects | | |
| Group | 269.27 (1) | < 0.001 |

Significant effects are shown in bold.

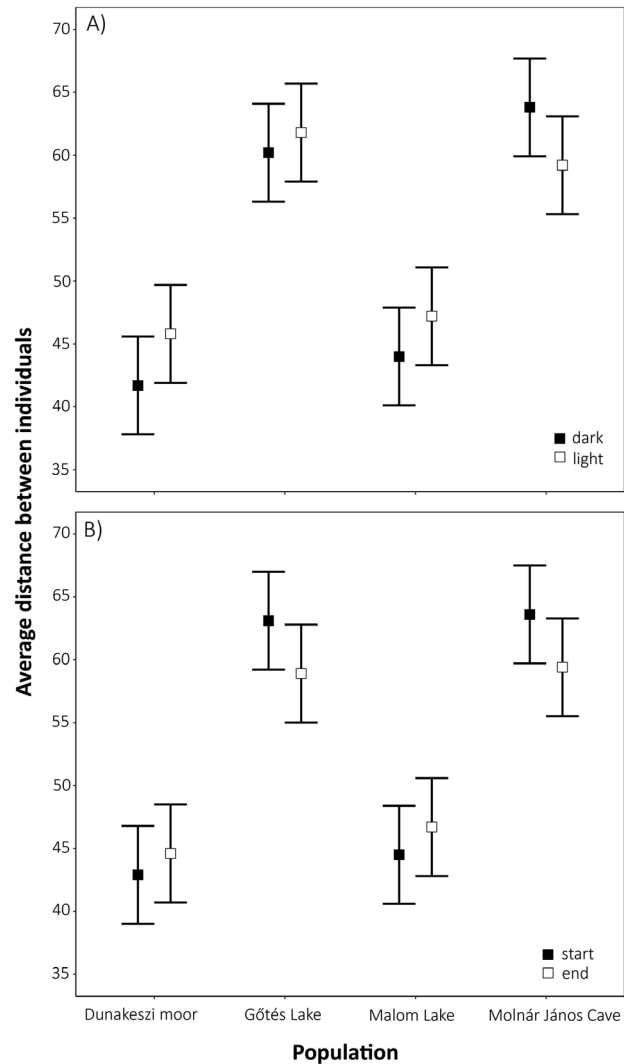


Figure 1. Aggregation in the absence of shelters (i.e. average distance between individuals) in the four tested populations of *Asellus aquaticus*. A, aggregation in the presence vs. absence of light. B, aggregation in the first vs. second recording period. Estimated marginal means \pm SE are shown.

with generally lower aggregation increased aggregation along the observation. The order of the trial was also significant, although it explained only a small fraction of variation (Table 1): individuals aggregated less in the second round of the experiment than in the first (data not shown). The fixed effects explained 23.4% of the total variance, and the full models explained 49%. For the non-significant effects, see Table 1.

Results of the LMM on shelter sharing revealed significant population \times treatment and population \times recording period interactions (Table 2). The population \times treatment interaction was based on

Table 2. Result of a linear mixed model on shelter sharing in *Asellus aquaticus*

| Model term | χ^2 (d.f.) | <i>P</i> -value |
|--------------------------------------|------------------|-------------------|
| Fixed effects | | |
| Population | 57.55 (4) | < 0.001 |
| Treatment | 0.78 (1) | 0.38 |
| Recording period | 6.03 (1) | 0.01 |
| Order of trial | 2.29 (1) | 0.13 |
| Number of individuals hiding | 6.27 (1) | 0.01 |
| Population × treatment | 11.79 (3) | 0.008 |
| Population × recording period | 13.56 (3) | 0.004 |
| Treatment × recording period | 0.31 (1) | 0.58 |
| Random effects | | |
| Group | 6.24 (1) | 0.01 |

Significant effects are shown in bold.

Malom Lake animals showing increased aggregation as a response to light, Götés Lake individuals showing a weaker opposite response, and the remaining populations showing no response (Fig. 2A). The population × recording period interaction revealed that all individuals apart from those from the Dunakeszi Moor tended to reduce aggregation throughout the experiment, with Molnár János Cave individuals showing the strongest response (Fig. 2B). The number of individuals hiding had an obvious effect: when more individuals were under shelter, shelter sharing increased (data not shown). The fixed effects explained 14.6% of the total variance, and the full models explained 21.3%. For the non-significant effects, see Table 2.

The GLMM on sheltering revealed significant population × treatment and population × recording period interactions (Table 3). The population × treatment interaction showed that all populations tended to shelter more in the light treatment than in the dark treatment, but this reaction was particularly strong in the Malom Lake population (Fig. 3A). The population × recording period interaction revealed that sheltering was similar across the four populations at the beginning of the experiment, whereas towards the end of the experiment the surface populations sheltered more and the cave population sheltered less (Fig. 3B). The population differences were also clear, in that cave-adapted individuals sheltered less than their surface conspecifics, especially if we focused only on the behaviour of cave individuals in the dark, which is natural for them (Fig. 3A, B). The order of the trial was also significant (Table 3): individuals sheltered more in the second round of the experiment than in the first (data not shown). The fixed effects explained 7.6% of the total variance, and the full models explained 18.5%. For the non-significant effects, see Table 3.

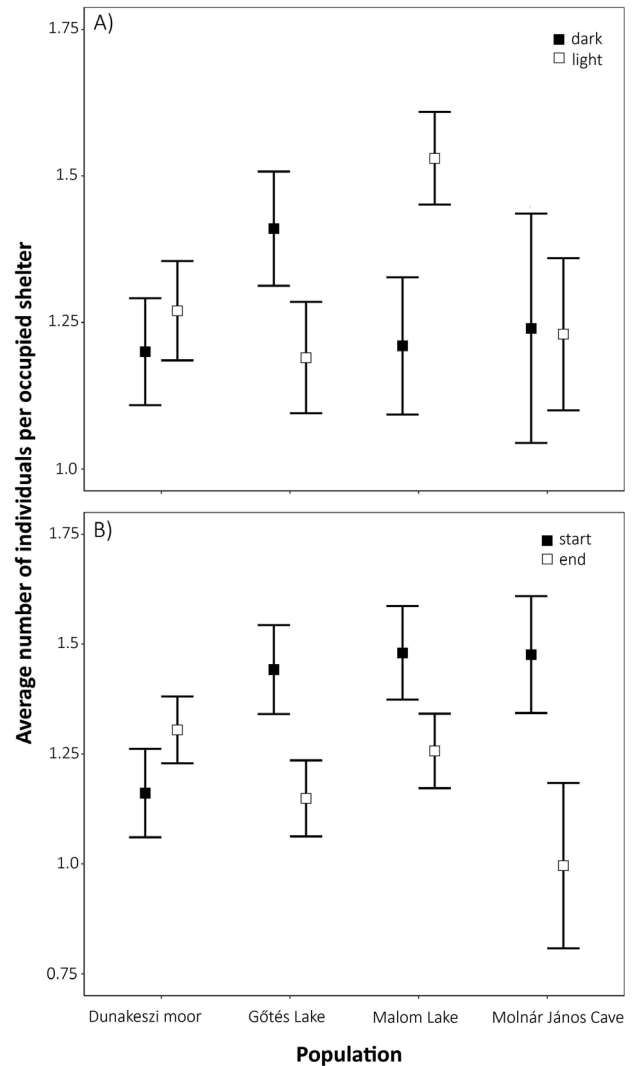


Figure 2. Shelter sharing (i.e. average number of individuals per occupied shelter) in the four tested populations of *Asellus aquaticus*. A, aggregation in the presence vs. absence of light. B, aggregation in the first vs. second recording period. Estimated marginal means ± SE are shown.

DISCUSSION

Aggregation and sheltering behaviours can be seen as two potential forms of evolutionary adaptation that give animals multiple advantages, including increased defence against predators. In the present study, we tested whether adaptation to a special, predator-free cave environment resulted in decreased aggregation and sheltering and how cave-adaptation affects behavioural responses to changing light conditions in the widely distributed habitat generalist *A. aquaticus*, a small freshwater isopod that successfully colonizes caves. We tested these hypotheses in a common

Table 3. Result of a generalized linear mixed model on sheltering in *Asellus aquaticus*

| Model term | χ^2 (d.f.) | P-value |
|--------------------------------------|------------------|-------------------|
| Fixed effects | | |
| Population | 18.88 (4) | < 0.001 |
| Treatment | 51.76 (1) | < 0.001 |
| Recording period | 12.41 (1) | < 0.001 |
| Order of trial | 12.85 (1) | < 0.001 |
| Population × treatment | 20.87 (3) | < 0.001 |
| Population × recording period | 38.59 (3) | < 0.001 |
| Treatment × recording period | 3.32 (1) | 0.07 |
| Random effects | | |
| Group | 50.27 (1) | < 0.001 |

Significant effects are shown in bold.

garden experiment based on three surface and one cave-adapted population (showing troglomorphic phenotype and being genetically isolated from surface populations $\geq 60\,000$ years ago; Pérez-Moreno *et al.*, 2017). The results are mixed, in that patterns of sheltering behaviour supported our predictions, whereas population divergence in aggregation and light-induced plasticity in general were unexplained by habitat type.

AGGREGATION

Contrary to our initial prediction, we found no systematic differences in aggregation behaviour among surface- and cave-dwelling *A. aquaticus* in an environment without shelters. However, we found high between-population variation, with two surface populations showing a considerably higher tendency for aggregation than the cave population and the third surface population (Gótés Lake). It is possible that the Gótés Lake population experiences lower than expected predation pressure (we have no detailed fauna list with density estimates from the studied populations) or that some other environmental factor is responsible for the population variation. For instance, it is possible that in Gótés Lake more aggressive male *A. aquaticus* are favoured than in the other surface populations, resulting in the reported patterns. Aggregation behaviour might also be connected to functions other than predator avoidance. For instance, aggregation could reduce the time spent on searching for mating partners and provide an increased number of mating opportunities (Beauche & Richard, 2013); hence, local population densities or a variation in operative sex ratio might affect this behaviour. According to a previous study, aggregation might also be related to feeding behaviour (Heupel & Simpfendorfer, 2005). However, based on

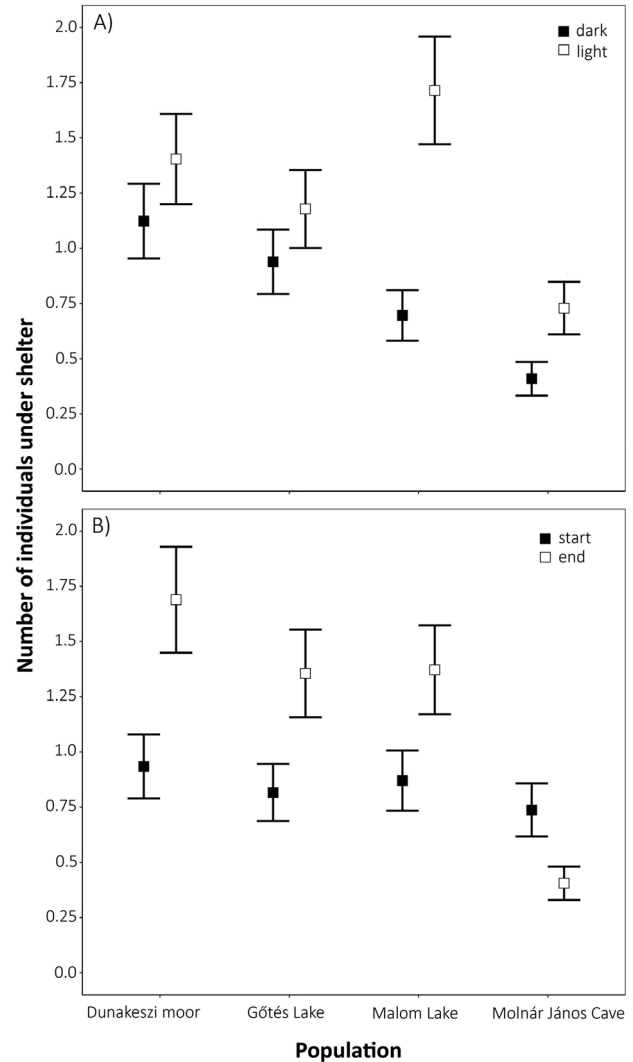


Figure 3. Sheltering (i.e. number of individuals under shelter) in the four tested populations of *Asellus aquaticus*. A, sheltering in the presence vs. absence of light. B, sheltering in the first vs. second recording period. Back-transformed estimated marginal means \pm SE are shown.

our knowledge, *A. aquaticus* is a detritivorous species feeding on various living and dead plant material in its surface habitats and on endogenous bacterial mats in Molnár János Cave (Herczeg *et al.*, 2020); hence, it is improbable that aggregation yields any foraging benefits for the species.

Animals aggregate not only in the open or during activity, but also often under shelters during inactivity (Devigne *et al.*, 2011). In a previous study on the common rough woodlouse (*Porcellio scaber* Latreille, 1804), in which aggregation under shelters is generally observed and individuals show strong thigmotaxis, researchers found the same dynamics of aggregation in the presence or absence of shelters

(Devigne *et al.*, 2011; Broly *et al.*, 2012). It has also been shown experimentally that when groups of rough woodlice in bright arenas were offered two dark shelters, almost 80% of individuals in a group aggregated under one shelter (Broly & Devigne, 2011; Broly *et al.*, 2012). Hence, sheltering is often non-random; individuals can prefer shelters that are already occupied or choose shelters with larger aggregations. We expected similar patterns regarding aggregation under shelters than without shelter, assuming that aggregation under shelters might have relevance against predators. However, there were no clear population trends between the cave population and the surface populations.

Regarding the light treatment, considering (1) the night activity of surface *A. aquaticus* (Andrikovics, 1981), (2) expected higher predation pressure during daylight for the surface populations, and (3) light avoidance of the cave population, we predicted higher aggregation in the light than in the dark treatment, with the effect being strongest in the cave population. When we tested for aggregation without shelters, the results contradicted this prediction. Despite the significant population \times light treatment interaction, the patterns seem somewhat inconsistent. The cave population aggregated more in the light than in the dark, whereas the surface populations tended to aggregate more in the dark than in the light or showed no response. Hence, there were some indirect signs of the expected light avoidance in the cave population. However, the surface populations behaved in an unexpected way, and we can only speculate about the reasons. For instance, it is plausible that some social activity might take place in darkness. Surface *A. aquaticus* are assumed intuitively to have diurnal activity, and they are active during the light period. However, Andrikovics (1981) showed that the trappability of the species is three times higher during night than during daylight when using passive traps, suggesting higher movement activity during night. Previous experiments of ours reproduced comparable results in laboratory settings (Ge H., unpublished observations). Hence, the species seems to show activity around the clock. Although it is possible that some sort of increased social activity results in higher aggregations during night, such activity among male individuals is unknown. Another potential explanation is increased male–male aggression during the day, but again, we have no data about it. Finally, it is possible that predation pressure is, contrary to our expectations, higher during the night than during daylight. However, there are no data about the relative changes in predation pressure during a day. To answer this question, future research is required on the ecology and behaviour of this species in the wild.

In the case of aggregation under shelters, there was a weak trend for stronger aggregation in the dark in Gótés Lake, but we found a strong pattern only in Malom Lake, where individuals aggregated under the shelters much more strongly in the light than in the dark treatment. During the 20th century, guppies (non-native in Hungary) were introduced to the Malom Lake. To our knowledge, there are no native fish species present, and we never observed large insect predators (e.g. dragonfly larvae, Dytiscidae) in the lake. As a consequence, guppies are under negligible predatory risk, and their density in the lake is high. We repeatedly observed groups of guppies feeding on *A. aquaticus*. Given that guppies are diurnal, *A. aquaticus* might suffer much higher predation pressure during the day, explaining the increased aggregation observed in the light treatment.

SHELTERING

We predicted that the cave-adapted population, evolving with a lack of predation, would use shelters less than the surface populations under presumably different, but definitely significant predation risk. Previously, Fišer *et al.* (2019) showed that shelter-seeking behaviour exists in *A. aquaticus*, but their results about differences between cave and surface populations were inconsistent, because only one cave population showed the expected decrease in shelter use. Here, the prediction was supported, because *A. aquaticus* from the Molnár János Cave sheltered less than the surface populations. Given that we studied only one cave population, the results cannot be generalized, but they show that the reduced shelter use in one cave population in the study by Fišer *et al.* (2019) is not an exception.

We also predicted, based on expected higher diurnal than nocturnal predation pressure in the surface populations and strong light avoidance in the cave population, that (1) sheltering would be higher in light than in dark conditions, and (2) the difference would be more pronounced in the cave than in the surface populations. Our findings support the first prediction, but not the second. All populations sheltered more in the light treatment. Interestingly, the reaction norms were similar in all populations except the Malom Lake, which showed an elevated response. This pattern is congruent with our findings regarding aggregation under shelters, where we also reported an elevated response to light in Malom Lake. We believe that the explanation is the same: elevated shelter use and formation of larger aggregations under shelters in light are adaptations to the high predation pressure induced by the diurnal guppies in Malom Lake.

HABITUATION PATTERNS

All the behaviours above discussed were recorded at both the beginning and the end of the given observation period. We assumed that the beginning (being placed in the cylinder or an abrupt change in light conditions) of the experiment was perceived as stressful by the focal animals; hence, they should behave differently in comparison to the end of the experiment after 90 min had elapsed without disturbance. According to Blumstein (2016): ‘The process of habituation leads to decreased responsiveness to a repeatedly presented stimulus, making it less likely that individuals will respond to harmless stimuli’. In contrast, a reverse mechanism, known as sensitization, intensifies the behavioural response to constant stimulation (Bee, 2001; Stamps *et al.*, 2012). If we treat being in a novel environment/situation as a permanent stimulus, the behavioural change during our experiment can be seen as a form of habituation (e.g. Herczeg *et al.*, 2019). Hypothesizing that cave *A. aquaticus* adapted to the lack of predation will be less sensitive, we predicted that the cave population would express quicker/stronger habituation. Although we detected a significant population × recording period interaction in all studied behaviours, the results were mixed. Regarding aggregation in the absence of shelters, the significant population variation in habituation revealed a weak pattern: populations with higher aggregation tended to decrease whereas populations with lower aggregation tended to increase aggregation throughout the observation period. Whether this pattern can be seen as support for sensitization in some and habituation in the other populations (*sensu* Blumstein, 2016) warrants further targeted studies. In aggregation under shelter, the Molnár János Cave population showed the strongest habituation by decreasing aggregation behaviour throughout the experiment. This can be seen as a pattern supporting our prediction. However, when testing the same question in sheltering behaviour, we found that the surface populations changed their behaviour more during the experiment by increasing sheltering (sensitization), whereas the cave population showed only a small decrease (habituation). Although our prediction was supported only in part, we believe that population variation in habituation/sensitization governed by differences in predation pressure is an interesting idea worthy of further pursuit.

CONCLUSION

Taken together, we tested differences in aggregation and sheltering behaviours between surface and cave-adapted populations of *A. aquaticus*. We predicted that cave *A. aquaticus* adapted to the lack of predation

and permanent darkness would show decreased aggregation and sheltering and stronger light-induced plasticity (photophobia) than surface *A. aquaticus*. We obtained mixed results. Only the results about sheltering behaviour supported the predictions. However, we detected various population differences in aggregation and light-induced plasticity in general, which were not explained by habitat type. The results suggest that population divergence in these traits might be explained by factors other than the ones differing systematically between cave and surface habitats. Besides further laboratory studies including more populations and also females, understanding this system warrants extensive field surveys to reveal the relevant factors in environmental variation. More behavioural tests will also be needed for the separation of the roles of sociability and aggression in the variation of aggregation behaviour.

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DATA AVAILABILITY

Data are available at Figshare: <https://doi.org/10.6084/m9.figshare.14673306>

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