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

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Abstract

Aggregation (gathering together) and sheltering (hiding in cover) are basic behaviours that might reduce the risk of predation. However, both behaviours have costs, like increased competition over resources and high prevalence of contact-spread parasites (aggregation) or lost opportunities for foraging and mating (sheltering). Therefore, adaptive 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. Since the cave environment is constantly dark, we also tested for population variation in light-induced behavioural plasticity by applying light treatments. 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 were unexplained by habitat type. Cave individuals habituated (decreased sheltering), while surface individuals showed sensitization (increased sheltering). We suggest that population variation in sheltering is driven by predation, while variation in aggregation must be driven by other, unaccounted environmental factors, similarly to light-induced behavioural plasticity. Based on habituation/sensitization patterns, we suggest that predation-adapted populations are more sensitive to disturbance related to standard laboratory procedures.

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 optimisation to the prevailing environment, geographic 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. For instance, the effect of varying predation pressure on behaviour has been proven in common frog (*Rana temporaria*) tadpoles (Van Buskirk & Arioli, 2005), nine-spined stickleback (*Pungitius pungitius*) (Herczeg, Gonda, & Merilä, 2009a) or guppy (*Poecilia reticulata*) (Magurran & Seghers, 1991, 1994). Such environmentally driven population divergence in behaviour has been proven to be genetically based in several species (see Breden, Scott, & Michel, 1987; Brown, Burgess, & Braithwaite, 2007; Herczeg, Ab Ghani, & Merilä, 2013; Laine *et al.*, 2014).

Aggregation (gathering together) is a basic behaviour exhibited by many animals (Parrish & Hamner, 1997; Parrish & Edelstein-Keshet, 1999), 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 terrestrial species avoiding desiccation (Brockett & Hassall, 2005), it could improve feeding efficiency (Heupel & Simpfendorfer, 2005) and it could provide a mechanism of defence from predators (Broly et al., 2013). However, aggregation behaviour may also have costs, such as higher vulnerability to contact-spread parasites, increased competition for resources and conspicuousness of the group towards predators (Pitcher, 1986; Romey, 1995). Hence, decreased aggregation is expected in populations where the benefits of the behaviour are low. For instance, the negative covariance between predation pressure and the social costs of aggregation was proven experimentally (Herczeg, Gonda, & Merilä, 2009b). While many studies were focusing on aggregation behaviour (schooling, shoaling, flocking, herding) in vertebrates, it is also well-known in invertebrates (Hassall & Tuck, 2007; Kullmann *et al.*, 2008; Tanaka & Nishi, 2008; Broly *et al.*, 2012).

Sheltering (hiding under cover) is one of the most straightforward antipredator behaviours, when it is not applied against some other environmental effects, like too harsh sunlight. Sheltering behaviour has obvious benefits, but it also incurs costs due to lost opportunities connected to alternative beneficial behaviours, like mate-searching or foraging (e.g. Cooper & Frederick, 2007; Sih, 1992). We know from previous studies that sheltering behaviour successfully reduces the risk of predation (Cooper & Frederick, 2007; Kullmann et al. 2008). Further, a previous study showed that a marine isopod, *Idothea balthica*, traded shelter for food (Vesakoski, Merilaita, & Jormalainen, 2008). The antipredatory role of aggregation and sheltering together with their predation pressure related population variation was supported in many vertebrate taxa (e.g. fish; Magurran & Seghers, 1994; Griffiths, Armstrong, & Metcalfe, 2003; Heupel & Simpfendorfer, 2005; amphibians: Watt, Nottingham, & Young, 1997; DeVito, 2003; reptiles: Gardner *et al.*, 2016; birds: Carrascal, Alonso, & Alonso, 1990; Forsman *et al.*, 1998; Goodenough *et al.*, 2017; mammals: Fryxell, 1991; Treves, Drescher, & Ingrisano, 2001).

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 strongly buffered against daily, seasonal and yearly environmental variation, further, as being physically separated from each other, they represent natural replicates 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, Culver, & Pipan, 2019; but see Culver, 1975), in fact, predator avoidance was previously suggested as a main factor behind cave-colonisation in several insect species (Rivera *et al.*, 2002; Juan & Emerson, 2010), as well as in Mexican tetra, *Astyanax mexicanus* (Romero, 1985). Further, 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 longer history of cave-adaptation, the loss or reduction of anti-predatory responses is expected. In line with this, empirical results show that predator recognition is maintained in cave-dwelling populations of Pyrenean newt (*Calotriton asper*), a recent colonist, while such behaviour is lost in the highly adapted Olm (*Proteus anguinus*) (Manenti *et al.*, 2020). Despite of the consensus on the importance of difference in predation pressure between surface and subterranean habitats, how between-population environmental variation affects behavioural traits, such as aggregation and sheltering together is rarely tested at best. Previously, it was shown that in two cave-dwelling populations of Atlantic molly, *Poecilia mexicana*, fish exhibit reduced shoaling behaviour (Plath & Schlupp, 2008) and the same pattern was endorsed from multiple cave-adapted populations of *A. mexicanus* (Kowalko *et al.*, 2013). However, loss of vision might be a more important factor behind such behavioural differences in these fishes than the lack of predation (Kowalko *et al.*, 2013).

Here, we studied population variation in aggregation and sheltering behaviour of common water louse, *Asellus aquaticus*. In a common garden experiment, we tested aggregation behaviour with and without shelter, and shelter use in general in a highly specialised cave population (no predation, permanent darkness) and three surface populations (various predators present, daily light fluctuations. We also applied light treatments (presence/absence) under common garden settings. Aggregation would hardly increase foraging efficiency of a detritivorous species feeding on a wide variety of stationery 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 studeied cave, see Herczeg et al., 2020), and the potential importance of aggregating for mating was ruled out by involving only males in the study. Hence, we expect that the main environmental driver of the evolution of aggregation behaviour in our study system is the presence / 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, but predation. Therefore, we predicted that predation-adapted surface *A. aquaticus* populations are 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 are less straightforward. While vision of cave-adapted *A. aquaticus* is highly reduced, they are still sensing light (see Protas, Trontelj, & Patel, 2011; Pérez-Moreno, Balázs, & Bracken-Grissom, 2018; Re *et al.*, 2018) and showing negative phototaxis (Fišer *et al.*, 2016). Assuming that surface *A. aquaticus* populations are under higher predation risk during light than at night, we predicted all populations to show higher aggregation and shelter use when tested in light, the response being the strongest in cave-adapted individuals.

Material and Methods

Study system

A. aquaticus is widespread in a wide variety of surface freshwater habitats across the Western Palearctic (Verovnik, Sket, & Trontelj, 2005) and successfully colonized caves in Central Europe on several independent occasions (Verovnik *et al.*, 2005; Verovnik, Prevorčnik, &

Jugovic, 2009). Similarly to other cave-dwelling species, cave adapted *A. aquaticus* exhibit the typical troglomorphic adaptations, like 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 genetically isolated from surface populations (including the Malom Lake population, see below) for at least 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 (personal observation). This latter notion is supported by results from Erőss et al. (2006) and Bodor et al. (2015), whose results indicate no hydraulic connection between the Rózsadomb recharge area and the discharge area of the Boltív Spring (connected to the Molnár János Cave – Malom lake system). The cave's outflow 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 surface light, 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; personal observation). The remaining two surface populations, Gőtés Lake (47.59556 ° N, 19.04142° E) and Dunakeszi Peat-moor (47.615613° N, 19.126392° E) are subject to natural surface light regime and temperature fluctuations typical to the region. These surface populations are members of a diverse community consisting competitor and predator species, *A. aquaticus* is known to be a food source for fish and larval dragonflies in surface habitats (Harris, Karlsson Green, & Pettersson, 2013), while the cave-dwelling population experience low biotic complexity with a small number of competitors and absence of predators in a stable and predictable environment.

Collecting and housing the experimental animals

Adult animals were collected on 30 October 2019. Samples were collected by hand sorting with mash net except the subterranean animals from the Molnár János Cave where a modified Sket-bottle was used (Chevaldonné *et al.*, 2008) and cave diving was necessary. 2019 had an exceptionally warm autumn in the region (record 19.7 ºC daily high temperature in Budapest on 21 October), hence, water temperature at the surface habitats was similar to the temperature of thermal water at Malom Lake and Molnár János Cave. After collection, animals were immediately transported to the facilities of the Biological Institute of Eötvös Loránd University (Budapest, Hungary). As mentioned above, we used only males to rule out the potential importance of aggregating for mating. Populations were randomly divided into two subgroups and individuals were housed together in transparent plastic containers (volume: 5 L, dimensions: 32cm × 21cm × 11cm, length × width × height, respectively). Individuals were kept in these containers during the whole experiment (except behavioural tests, see below). Water collected at the source habitats were used to fill the containers, water was regularly refilled. We also provided small stones as shelters. Containers were placed in custom made, light-controlled chambers (see below). Surface populations were acclimated to a daily light cycle (10h light: 14h dark; controlled by a timer), while the cave-dwelling population was acclimated to complete darkness and all handling processes were done under red light. The temperature in the lab varied between 23-24 °C. Animals did not receive food during the acclimation period and experiments.

Experimental setup

To video-record the animals’ behaviour 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: LEDs imitating daylight (4500 K, CRI > 90) at the top and infrared LEDs (920 nm) at the bottom. This infrared wavelength is out of the visible range of the animals (Dember & Richman, 2012). The chambers were closed from sides with non-transparent black plastic boards so 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 per second at HD resolution (1280 x 720).

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 all Petri dishes was coarsened with emery paper to enable animals’ normal movement (Fišer *et al.*, 2019). These Petri dishes were housing the tested groups (see below). Five shelters, made of red glass were placed into the arenas (red glass reduces light intensities and filters the spectrum of light; Devigne et al., 2011). The shelters were quadratic in shape (3 cm wide), each of them was placed equal distances from each other inside the arenas. One side of the shelter was raised slightly with a tiny piece of glass so the animals could crawl under and receive thigmotactic stimuli (Fišer et al. 2019).

Animals within population were randomly divided into six groups (five individuals in each group, sampled from the two holding tanks per population randomly). The groups were placed into small removable cylinders (diameter = 28 mm) located in the centre of the Petri-dishes. The 24 Petri-dishes were randomly divided between two recording chambers. After ca. three minutes of acclimation, the cylinders were removed and the video-recording has started. Each group was tested both in the presence and absence of light, treatments having different order in the recording chambers. After 150 min of recording, light regimes were changed in the recording chambers and a second recording-period of 150 min started. Infrared light was on for all tests, as it was needed for video recording. Aggregation in the absence of shelters were tested on 5 November 2019. The experimental set-up was similar as in the previous experiment, except that individuals' behaviour was assessed in the absence of shelters.

For the video analyses, the first and the last 30 minutes of recording were used (hereafter: recording period) to see how behaviour changed along the experiment. 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 proportion of animals under shelter (hereafter 'sheltering') at a given observation. Each behavioural variable was recorded in every third minute within these recording periods, which left us 20 observations per Petri-dish altogether (10 observations per recording perios). To quantify aggregation behaviour in the absence of shelters, we measured the distance between all pairs within the groups for every observation and then calculated the mean of these distances, thus, there was only one number per group for every observation describing group cohesion (hereafter: 'distance'). For measuring the distances between individuals, we used ImageJ software (Schneider, Rasband, & Eliceiri, 2012). Note that individual (non-averaged) data could be analysed too, but adding the extra level of hierarchy to the model would make the model problematic. However, we ran this model too and it yielded qualitatively similar results to the analysis of averaged data, and thus we only report the latter.

Statistical analyses

For analysing distance, shelter sharing and sheltering, we ran separate linear mixed models (LMMs) by using the packages *lme4* (Bates *et al.*, 2015) and *lmerTest* (Kuznetsova, Brockhoff, & Christensen, 2016) available in the RStudio interface 1.2.5001 (RStudio Team, 2020). All models were built with population, treatment (light vs. dark), recording periodt (beginning *vs.* end period of the experiment) and their interactions as fixed effects. Because interpreting three-way factorial interactions is highly problematic, we included only the two-way interactions. Order of trial (first *vs.* second run within a day) was treated as a nuisance variable and we included it in the models a single fixed effect. For the model on shelter sharing, the number of individuals hiding was also added as a fixed effect for correction. In this model, we excluded observations where less than two individuals were hiding. Group identity was also added to the models as a random effect to control for the non-independence in the data. In these models, fixed effects were tested by Wald's chi-square tests and random effects by likelihood ratio tests. Sheltering (proportion data) was arcsine transformed (following Sokal & Rohlf, 2012) to stabilize variances. 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 × treatment and population × recording period interactions (Table 1). However, the only strong pattern was among populations:

individuals from the Molnár János Cave and Gőtés Lake aggregated significantly less than individuals from Malom Lake and Dunakeszi Moor (Fig 1 a, b). The population × treatment interaction patterns were rather weak and inconsistent, suggesting that the cave population aggregates more in light, while the surface populations aggregate more in dark than in light. The population × recording period interaction showed weak trends suggesting that populations with higher aggregation increased, while populations with lower aggregation decreased aggregation along the observation. Order of trial was also significant (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, while the full models explained 49% which can be seen as sufficient explanatory power for behavioural variables. For the non-significant effects see Table 1.

Table 1. Result of Linear Mixed Model on aggregation behaviour in the absence of shelters in *Asellus aquaticus*. Significant effects are in bold font.

Model term	χ^2 (df)	*P*
Fixed effects		
population	**510.17 (4)**	**< 0.001**
treatment	1.04 (1)	0.31
recording period	1.78 (1)	0.18
order of trial	**4.22 (1)**	**0.04**
population × treatment	**18.52 (1)**	**< 0.001**
population × recording period	**12.83 (3)**	**0.005**
treatment × recording period	0.43 (1)	0.51
Random effects		
Group	**269.27 (1)**	**< 0.001**

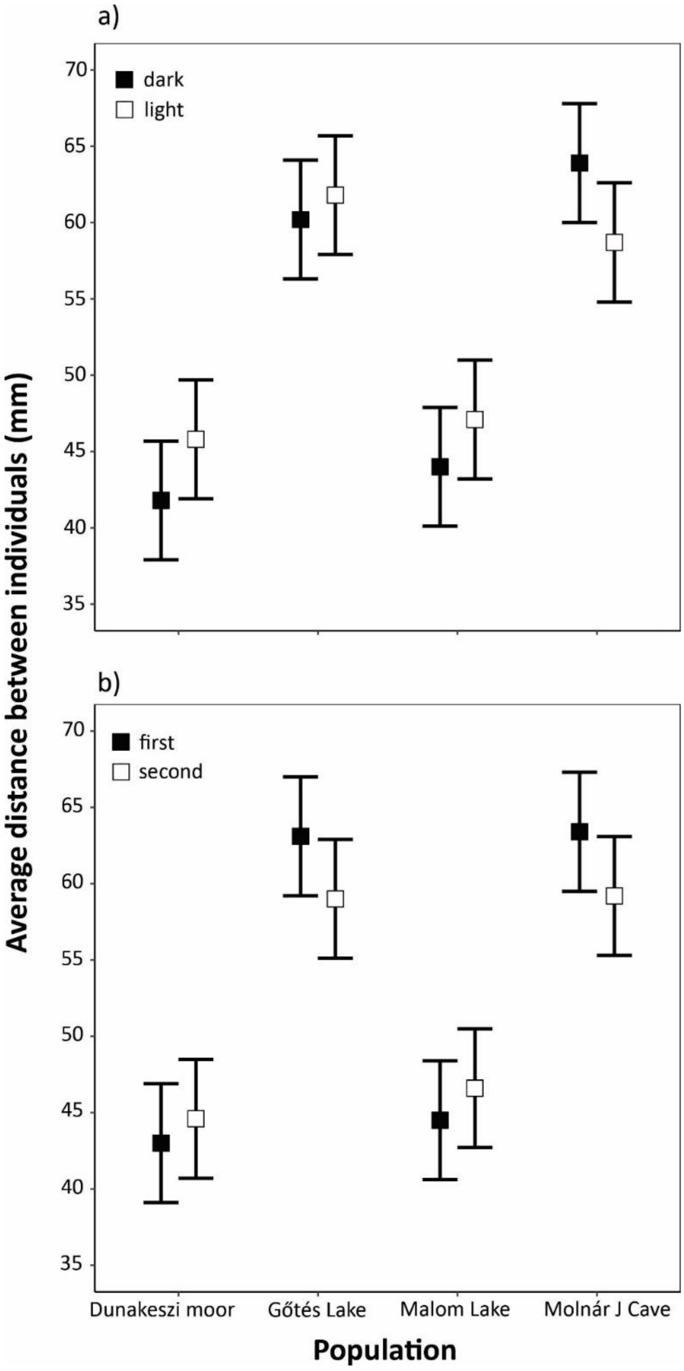

Fig.1. Aggregation in the absence of shelters in the four tested populations of *Asellus aquaticus*. a) aggregation in the in the presence vs. absence of light. b) aggregation in the first vs. second recording period. Least square means ± standard errors are shown.

Results of LMM on shelter sharing revealed significant population × treatment and population × recording period interactions (Table 2). The population × treatment interaction is based on

Malom Lake animals showing increased aggregation as a response to light, Gőtés Lake individuals showing a weaker opposite response, while the remaining populations showing no response (Fig. 2a). The population × recording period interaction revealed that all but the Dunakeszi moor individuals tended to increase aggregation along the experiment, Molnár János Cave individuals showing the strongest response (Fig. 2b). Number of individuals hiding had the obvious effect: when more individuals were under shelter, shelter sharing increased (data not shown). The fixed effects explained 14.6% of the total variance, while the full models explained 21.3% which can be seen as sufficient explanatory power for behavioural variables. For the non-significant effects see Table 2.

Table 2. Result of Linear Mixed Model on aggregation behaviour under shelters in *Asellus aquaticus.* Significant effects are in bold font.

Model term	χ^2 (df)	*P*
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**

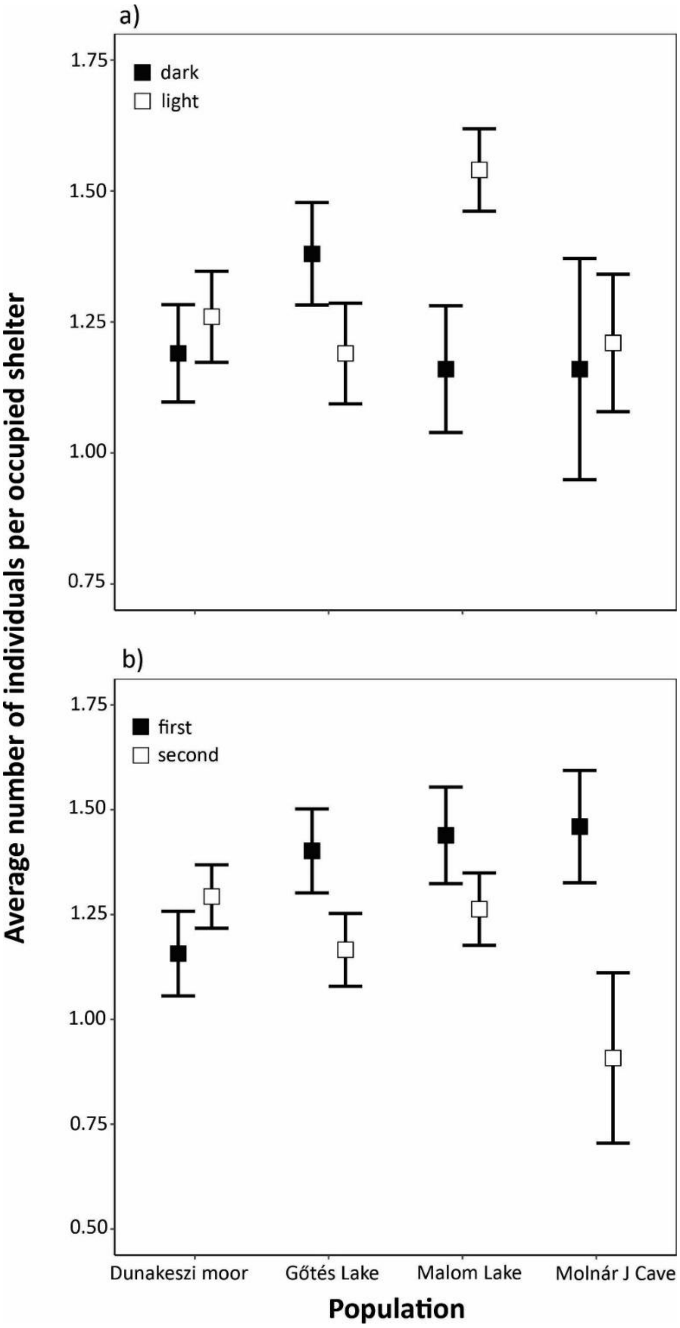

Fig.2. Aggregation under shelters in the four tested populations of *Asellus aquaticus*. a) aggregation in the in the presence vs. absence of light. b) aggregation in the first vs. second recording period. Least square means ± standard errors are shown.

LMM on sheltering revealed significant population × treatment, population × recording period and treatment × 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, while towards the end of the experiment surface populations sheltered more and the cave population sheltered less (Fig. 3b). The treatment × recording period interaction indicated that the increase in sheltering along the experiment was stronger in the light, than in the dark treatment (data not shown). The population trends were also clear, cave-adapted individuals sheltered less than their surface conspecifics (Fig. 3a, b). Order of 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 23.3% of the total variance, while the full models explained 34.6% which can be seen as sufficient explanatory power for behavioural variables. For the non-significant effects see Table 3.

Table 3. Result of Linear Mixed Model on sheltering behaviour in *Asellus aquaticus*. Significant effects are in bold font.

Model term	χ^2 (df)	*P*
Fixed effects		
population	**278.31 (4)**	**< 0.001**
treatment	**81.88 (1)**	**< 0.001**
recording period	**42.44 (1)**	**< 0.001**
order of trial	**16.92 (1)**	**< 0.001**
population × treatment	**33.00 (3)**	**< 0.001**
population × recording period	**69.56 (3)**	**< 0.001**
treatment × recording period	**9.18 (1)**	**< 0.001**
Random effects		
Group	**87.65 (1)**	**< 0.001**

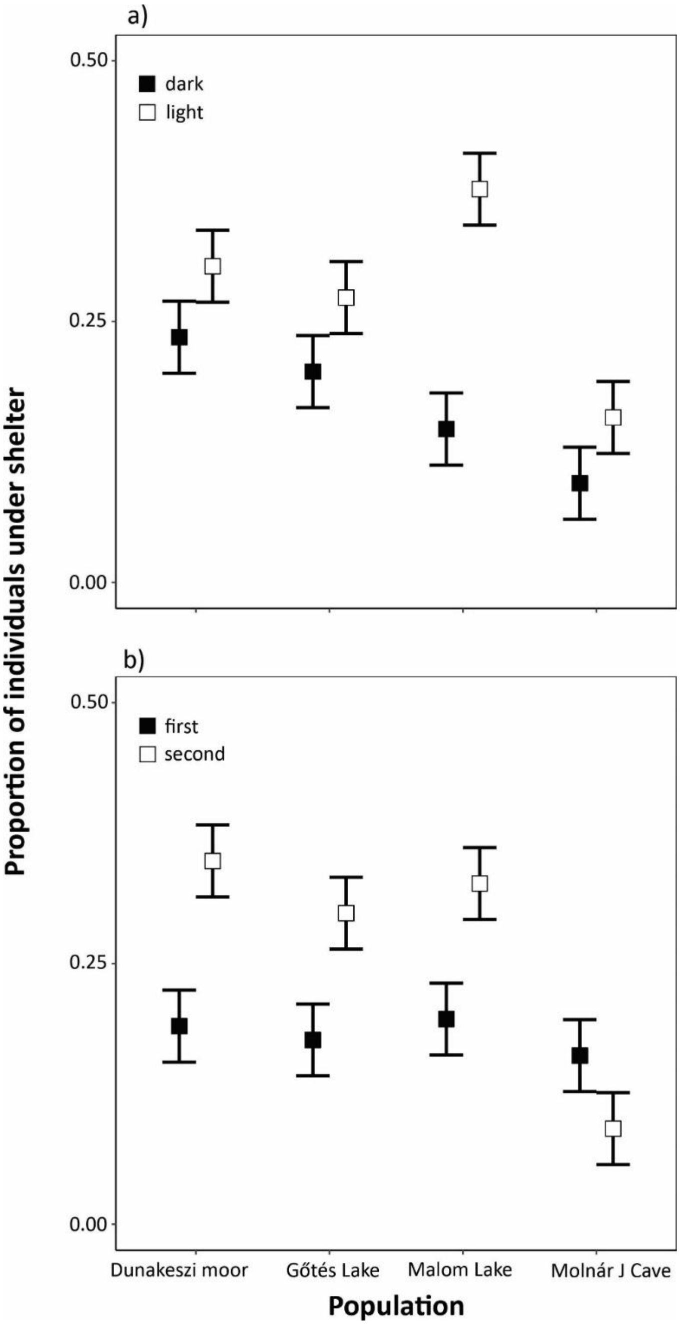

Fig.3. Sheltering in the four tested populations of *Asellus aquaticus*. a) sheltering in the presence vs. absence of light. b) sheltering in the first vs. second recording period. Least square means ± standard errors are shown.

Discussion

Aggregation and sheltering behaviours could be seen as two potential forms of evolutionary adaptation that gives animals multiple advantages, including increased defence against predators. In the present study, we tested hypotheses about how the predator-free cave environment select for 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 colonises caves. We tested these hypotheses in a manipulative common garden experiment based on three surface and one cave-adapted (showing troglomorphic phenotype and being genetically isolated from surface populations at least 60,000 years ago; Pérez-Moreno et al., 2017) populations. The results are mixed, in some cases supporting our predictions, in others rejecting it, while some unexpected patterns got also revealed.

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, two surface populations showing considerably higher tendency for aggregation than the cave population and the third surface population (Gőtés Lake). It is possible that the Gőtés Lake population experiences lower than expected predation pressure (we have no detailed fauna list with density estimates from the studied populations), or some other environmental factor is responsible for the population variation. For instance, it is possible that in Gőtés Lake, more aggressive male *A. aquaticus* are favoured than in the other surface populations, resulting in the reported patterns. Note that aggression and sociability are different personality traits (sensu Réale et al., 2007), hence, they can potentially evolve independently. Aggregation behaviour might also be connected to other functions than predator avoidance. For instance, aggregation could reduce the time spent on finding mating partners and provide increased number of mating opportunities (Beauche & Richard 2013) and thus this behaviour might be affected by local population densities or variation in operative sex ratios. According to a previous study, aggregation might be related to feeding behaviour (Heupel & Simpfendorfer 2005). However, based on 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 and references therein), thus, it is improbable that aggregation yields any foraging benefits for the species.

Animals are not only aggregating in the open or during activity but they also often aggregate under shelters, during inactivity (Devigne *et al.*, 2011). In a previous study on common rough woodlouse (*Porcellio scaber* Latreille, 1804), where aggregation under shelters is generally observed and individuals are showing 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 experimentally shown that when groups of rough woodlouse 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, shelter use is often non-random, individuals can prefer shelters that are already occupied, choosing shelters with larger aggregations. We expected similar patterns regarding aggregation under shelters than without shelter, assuming that aggregation under shelters might have a relevance against predators. However, there were no clear population trends between the cave population and the surface populations.

Regarding the light treatment, considering the (i) night activity of surface *A. aquaticus* (Andrikovics, 1981), (ii) expected higher predation pressure during daylight for the surface populations and (iii) the negative phototaxis of the cave population, we predicted higher aggregation in the light than in the dark treatment, the effect being the strongest in the cave population. When we tested for aggregation without shelters, the results contradicted the prediction. We note that despite the significant population × light treatment interaction, the actual patterns seem somewhat weak. While the cave population aggregated more in light than in dark, the surface populations rather tended to aggregate more in dark than in light or showed no response. Hence, there are some indirect signs of the expected negative phototaxis 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* is intuitively assumed to have diurnal activity and they are indeed 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 these results under laboratory settings (unpublished). Hence, the species seems to show activity all around the clock. However, even though it is possible that some sort of increased social activity results in higher aggregations during night, but such activity among male individuals is yet 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 – is higher during the night than during daylight. However, there is no data about the relative changes in predation pressure during a day. To answer this question, future research is to be done on the species' ecology and behaviour in the wild.

In the case of aggregation under shelters, there was a weak trend for stronger aggregation in the dark in Gőtés Lake, while we found a strong pattern only in Malom Lake, where individuals aggregated under the shelters much stronger in the light than in the dark treatment. During the 20th century, guppies (non-native in Hungary) were introduced to the Malom Lake, and to our knowledge, there are no native fish species present and we never observed large insect predators (e.g. dragonfly larvae, Dytiscidae, etc.) 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*. As guppies are diurnal, *A. aquaticus* might suffer extremely higher predation pressure during the day, which explains the increased aggregation observed in the light treatment.

Sheltering

We predicted that the cave-adapted population, evolving under the lack of predation, will 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, as only one cave population showed the expected decrease in shelter use. Here, the prediction was supported, as *A. aquaticus* from the Molnár János Cave sheltered less than the surface populations. Obviously, we studied only one cave population, hence the results cannot be generalised, but it shows that the reduced shelter use in one cave population in Fišer et al.’s (2019) study is not an exception.

We also predicted, based on similar grounds that is explained in the previous subchapter, that (i) shelter use will be higher under light than under dark conditions and (ii) the difference will 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 but 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 forming larger aggregations under shelters in light are adaptations to the high predation pressure induced by the diurnal guppies in Malom Lake.

Habituation patterns

All above discussed behaviours were recorded both in the beginning and at 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 is perceived as stressful by the focal animals and thus they should behave differently compared to the end of the experiment after 90 minutes elapsed without disturbance. According to Blumstein (2016), ‘Habituation is a process that leads to decreased responsiveness to a stimulus with repeated presentation and is often adaptive in that it makes it less likely that individuals will respond to harmless stimuli’. In contrast, a reverse mechanism, known as sensitization, intensifies behavioural response to constant stimulation (Bee, 2001; Stamps, Briffa, & Biro, 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 will express quicker/stronger habituation. Even though we detected a significant population × recording period interaction in all studied behaviours, the results are mixed. Regarding aggregation in the absence of shelters, the significant population variation in habituation revealed a weak pattern: populations with higher aggregation tended to increase, while populations with lower aggregation tended to decrease aggregation along the observation period. Whether this pattern can be seen as support for sensitisation 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 along 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 (sensitisation), while the cave population showed only a small decrease (habituation). Even though our prediction was not supported, we believe that population variation in habituation/sensitisation governed by differences in predation pressure is an interesting idea worth pursuing in the future.

Conclusions

Taken together, we tested how does the adaptation to the predator-free, permanently dark cave environment affect aggregation and sheltering behaviours and their light-induced plasticity in *A. aquaticus*. We predicted that cave *A. aquaticus* will show decreased aggregation and sheltering, and stronger (negative) light-induced plasticity than surface *A. aquaticus*. We got mixed results. Only results about sheltering behaviour supported the predictions. However, we detected various population differences in aggregation, and light- induced plasticity in general, which were unexplained by habitat type. We conclude that population divergence in aggregation is explained by other factors than the ones differing systematically between cave *vs.* 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 be also needed for the separation of the roles of sociability and aggression in the variation of aggregation behaviour.

Acknowledgements

We are thankful for Hajriz Berisha and Viktória Nyitrai for their help during the field work and the experiments. The project was funded by the National Research, Development and Innovation Office for international cooperation (SNN 125627). GeH also gained support from the Postdoctoral research grant of the National Research, Development and Innovation Office (PD 132041). GB was supported by the ÚNKP-20-4 New National Excellence Program of the Ministry of Innovation and Technology from the source of the National Research, Development and Innovation Office.

Data availability

Data will be deposited in Figshare upon publication

Conflict of interest

The authors declare no conflict of interest

Author contributions

All authors designed the study; GeH, SS and GB collected the data; GeH and SS analysed the data with a substantial contribution from GH; GeH drafted the manuscript with substantial a contribution from SS and GH; all authors reviewed the manuscript and gave final approval for publication

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