



# Food preference and food type innovation of surface- vs. cave-dwelling waterlouse (*Asellus aquaticus*) after 60 000 years of isolation

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Received: 2 November 2020 / Revised: 19 November 2021 / Accepted: 23 November 2021 / Published online: 15 December 2021  
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## Abstract

Behavioural innovativeness is important for colonising new habitats; however, it is also costly. Along the colonisation event of a simple, stable and isolated habitat offering only new food sources, one could hypothesize that the colonising individuals are more innovative than the average in their source population, showing preference to the new resource, while after colonisation, the adapted population will lose its innovativeness and become specialised to the new resource. To test this hypothesis, we compared food preference and food type innovation of a cave-dwelling waterlouse (*Asellus aquaticus*) population (genetically isolated for at least 60 000 years) to three surface-dwelling populations, also sampling individuals that have recently entered the cave ('colonists'). In the cave, the only food sources are endogenous bacterial mats, while surface populations feed on various living and dead plant material together with their fungal and bacterial overgrowth. We assayed all populations with the familiar and unfamiliar food types from the natural habitats and two novel food types not occurring in the natural habitats of the species. We found that all populations preferred surface to cave food and consumed the unnatural novel food types. Surface populations avoided cave food and colonists spent the most time with feeding on surface food. We conclude that the cave population maintained its preference for surface food and did not lose its food type innovativeness. We suggest that adapting to the special cave food was a major challenge in colonising the cave.

## Significance statement

Behavioural innovativeness is a key trait for adapting to environmental changes or to colonise new habitats. However, it has developmental and maintenance costs due to the high energy need of the necessary sensory and neural organs. Therefore, we asked whether behavioural innovativeness decreases after colonising an isolated, stable and highly specialised habitat. By comparing food type innovativeness of surface-dwelling populations of waterlouse (*Asellus aquaticus*) to a population that has colonised a cave at least 60 000 years ago, we found that the high innovativeness towards unnatural food was retained in the cave population. Further, all populations preferred surface food (decaying leaves), with surface populations almost completely avoiding cave food (endogenous bacteria mats). We suggest that (i) food type innovativeness is evolutionary rigid in our system and (ii) the cave food was rather an obstacle against than a trigger of cave colonisation.

**Keywords** Adaptation · Behavioural flexibility · Behavioural innovation · Behavioural plasticity · Colonisation · Food preference

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Communicated by J. C. Choe.

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## Introduction

Phenotypic plasticity is a genotype's ability to develop/express alternative phenotypes in different environments (West-Eberhard 2003). Behaviour is perhaps the most plastic phenotypic trait, with developmental (environmental variation triggers alternative developmental trajectories during ontogeny) and activational (environmental variation triggers

underlining networks for an immediate effect) plasticity both being present (Snell-Rood 2013). Behavioural plasticity has obvious fitness benefits, but it is not without costs. Phenotypic plasticity, in general, has various costs and limits, which can be broadly categorised into (i) energetic costs of developing and maintaining the costly sensory and neural machinery used in acquiring and processing environmental stimuli and (ii) energetic and ecological (i.e. increased exposure to predation, lost foraging or mating opportunities) costs of expressing the plastic response (e.g. DeWitt et al. 1998). From the energetic aspect, behavioural plasticity is mainly affected by the former (Snell-Rood 2013), while the ecological costs obviously depend on the behaviour in question and the ecological situation. The fact that the costs of developing and maintaining neural tissue are supported (e.g. Aiello and Wheeler 1995; Kotschal et al. 2013) suggests that costs of capacity for behavioural plasticity are likely to be important (Herczeg et al. 2019).

Behavioural innovation, an individual's new behaviour formerly absent in the population (Reader 2003; Reader and Laland 2003), is important in handling novel situations and thus a key trait in colonising new habitat types (Mayr 1965; Morse 1980). It can be seen as a form of activational behavioural plasticity, with the same costs as detailed above. The evolution of behavioural innovation has been attracting great scientific interest. Evolutionary inference was drawn almost exclusively from phylogenetically corrected interspecific comparative analyses and revealed a number of positive links between innovativeness, brain size, cognitive abilities and colonisation success (e.g. Sol and Lefebvre 2000; Reader and Laland 2002; Sol et al. 2002, 2005a,b; Lefebvre et al. 2004; Overington et al. 2009). Further, habitat generalists, who need to process more environmental information than specialists, were shown to have larger brains, better cognitive abilities and higher innovativeness than specialists (Daly et al. 1982; Ratcliffe et al. 2006; Overington et al. 2011). However, such comparative studies are inherently correlative, making the separation of correlation from causation impossible (e.g. Gonda et al. 2013). For more direct inference about adaptive evolution, intraspecific population comparisons are recommended, where phenotypic variation, its heritable component and selection acting on the heritable phenotypic variation can all be estimated (e.g. Gonda et al. 2013; Herczeg et al. 2020). Such population comparison based evolutionary studies on behavioural innovativeness are scarce at best (Reader 2003), even with regard to the pioneering steps of comparing innovativeness between populations adapted to different habitats (but see Herczeg et al. 2020). Foraging success is a key component of survival, and thus foraging innovations might have a key role in adapting to environmental change or colonising new habitats. There are two main types of foraging innovations: technical innovation (inventing a new method) and food type innovation

(utilising a previously unknown resource without modifying the foraging technique) (Overington et al. 2009). The former is relevant mainly for higher vertebrates (mammals and birds), while the latter can be relevant for all animal taxa. In their comparative study, Overington et al. (2009) found a positive link between food type innovation and brain size in birds, but also showed that technical innovations explain significantly higher variation in brain size than food type innovations. We are aware that food type innovation in invertebrates might not involve cognition or intelligence in the sense these terms used for birds and mammals; however, we still expect it to be linked to neural and sensory performance. We also note that 'food type innovation' (consuming previously unknown food) is similar to one aspect of exploration behaviour, avoidance/acceptance of novel food (Dingemanse et al. 2007). Hence, it can be seen as a measure of a personality trait (Réale et al. 2007). However, for the sake of consistency with Overington et al. 2009 and our preliminary study (Herczeg et al. 2020), we treat the ability of consuming previously unknown food items as a simple form of innovation.

Colonisation of caves by surface populations includes adapting to a major change in environment. There are different hypotheses available to explain it. Simply put, animals either colonise caves to avoid harsh surface conditions (climate change, predation) or to exploit new resources provided by caves (Howarth 1980, 1987; Peck and Finston 1993; Danielopol and Rouch 2005; Culver and Pipan, 2009; Romero 2009). In both cases, colonisers are expected to show high innovativeness, especially when the most important resource, food, is completely novel in the colonised cave. Comparative studies on birds have repeatedly suggested that innovative species are better colonisers than species with more rigid behaviour (e.g. Sol and Lefebvre 2000; Sol et al. 2002); however, we are not aware of any intraspecific tests of this idea.

In the present paper, we utilize a unique study system of the waterlouse (*Asellus aquaticus*) to look for indicators of local adaptation in food preference and food type innovation. *A. aquaticus* is a widely distributed habitat generalist freshwater isopod feeding on various living and dead plant materials including their bacterial and fungal overgrow (e.g. Moore 1975; Graça et al. 1993; Bloor 2011). The key of our model system is a special population that successfully colonised the thermal water filled Molnár János Cave, where the only available food source is endogenous bacteria forming mats (Herczeg et al. 2020; for details about the bacterial composition, see Anda et al. 2017). This particular cave population has been genetically isolated from the surface populations for at least 60 000 years (Pérez-Moreno et al. 2017) and shows the typical troglomorphic adaptations like depigmentation and eye-degeneration. By comparing food preference (surface vs. cave food) and food type innovation

towards unnatural food of this cave population to recent colonisers (surface morph found in the cave) and three surface populations from the vicinity, we tested hypotheses about the evolutionary change in specialisation and innovativeness. We hypothesised that (i) colonisation is mainly driven by individuals with high innovativeness and pre-existing preference for cave food, while (ii) adaptation to the isolated, stable and simple cave habitat includes the specialisation towards bacterial mats (strong preference for bacterial mats and reduced or lost food type innovativeness). In a preliminary study (Herczeg et al. 2020), we showed that the cave population did not lose its ability to identify and consume surface food, and our data hinted that they might even prefer that. However, in the preliminary study, test animals could not choose directly between the food types and were not tested against food that is absent from all natural populations. Further, recent colonisers were not included. Here, by running direct food preference tests between cave (bacterial mats) and surface (decaying poplar leaves) food types, and testing the study populations against unnatural food sources (commercial crayfish food and raw cucumber), we could explicitly test our hypotheses.

## Material and methods

### Study system and sampling

Detailed description of the Molnár János Cave is given in Herczeg et al. (2020). Briefly, this is a hypogean (water forming the cave is not coming from the surface) cave filled with thermal water. There is no exogenous food in the cave, only endogenous bacteria forming mats. Therefore, this cave's community is most likely based on organic matter produced by chemoautotrophic bacteria living in complete darkness, similarly to the Romanian Movile Cave (Sarbu et al. 1996). The cave community is extremely simple, and there are no predators of *A. aquaticus* present. The cave is connected to a surface pond called Malom Lake formed by the water outflow right at the cave entrance, and there is no physical barrier between the habitats. Despite this fact, the troglomorph population (showing eye degeneration and depigmentation) in Molnár János Cave is genetically isolated from the surrounding surface populations (including Malom Lake) for at least 60 000 years (Pérez-Moreno et al. 2017). It is noteworthy that the cave's outflow is strong, hence, passive dispersal to the cave is highly unlikely.

We sampled individuals from five populations: Molnár János Cave, Malom Lake (47.518277° N, 19.035999° E), surface ecomorphs (i.e. pigmented individuals with normal eye-development) found in the cave, and two surface populations from the vicinity, Gőtés Lake (47.354357° N, 19.22980° E) and the Dunakeszi Peat-moor (47.615613° N,

19.126392° E) between 16 and 18th May 2019. We considered collected individuals larger than 4 mm as adults (Hasu et al. 2007; Bloor 2010). Molnár János Cave could only be sampled with cave-diving techniques; the other populations had easy access. Gőtés Lake and Dunakeszi Peat-moor populations experience temperature and light fluctuations typical to the region. These populations were chosen randomly to represent typical surface habitats. Malom Lake has constant water temperature similar to the cave (ca. 23–24 °C) and is subjected to light fluctuations typical to the region. Surface ecomorphs found in the cave, coming from Malom Lake, are not necessarily in the process of colonising the cave; they might as well occur in the cave without reproduction. However, for simplicity, we will refer to them as 'recent colonisers'. They probably do not form a population, but for simplicity, we will refer to the compared groups (the four populations and the recent colonisers) as 'populations'.

All animals were transported immediately after capture to the aquacultural facilities of the Eötvös Loránd University (Budapest, Hungary) and housed individually in 90×25 mm (diameter and height, respectively) plastic Petri dishes, with emery-paper-coarsened bottoms aiding the animals' normal movement (Fišer et al. 2019). Cave and recent coloniser individuals were kept in constant darkness, while surface populations in a 16-h light:8-h dark daily light cycle in custom-built chambers (for details, see the next section). The temperature in the lab was set to 23–24 °C, which is the constant water temperature in the Molnár János Cave and Malom Lake and is within the natural range of the other surface populations at this time of the year. Water collected from the natural habitats were used for keeping and testing the animals throughout the laboratory period.

The collected individuals participated in other behavioural tests before the assays (same for all tested individuals). There was some mortality following collection and transportation, and we could use the following number of adult individuals in the tests reported here: Molnár János Cave, 7 males (M)/4 females (F); recent colonisers, 7 M/4 F; Malom Lake, 12 M/10 F; Gőtés Lake, 11 M/13 F; Dunakeszi Peat-moor, 12 M/14 F. In our preliminary study (Herczeg et al. 2020), we found no sex-effect in various analyses of various behavioural traits connected to feeding. Hence, considering the somewhat low sample size, we pooled males and females for the analyses.

### Behavioural assays

We used custom-built chambers for both keeping the study animals under the above-detailed light settings and video-recording their behaviour. The chambers had the following dimensions: 100 cm length×55 cm width×105 cm height. The chambers' sides and top were covered by black plastic sheets to block any incoming light. The chambers were

equipped with two light sources. On the top, we installed LEDs imitating daylight (4500 K, CRI > 90), while on the bottom, we installed infrared LEDs (920 nm). The lights could be switched on/off from the outside. Daylights were used to produce the planned daily light regime, while infrared LEDs were used to make the video-records in either light or dark. On the bottom of the chambers, we placed an opal plexiglass sheet to diffuse the infrared light evenly and to provide a platform for the Petri dishes. In all chambers, we mounted four webcams (Logitech C920 FullHD; Logitech, Lausanne, Switzerland) to the top that were modified for infrared video-recording. We used the OBS Studio software (OBS Studio Contributors) to capture videos (5 frames per second, HD [1280 × 720] resolution). Since the cave population and the colonizers were tested in constant darkness, while surface populations were tested in daylight, we could not fully randomise test animals across the chambers. Therefore, we tested cave and coloniser individuals in one chamber (randomised within chamber) and the surface populations in two chambers (randomised between and within chambers). The chambers were fully identical, produced in one batch and placed next to each other in the same room. We are aware that our design is not perfectly randomised for the above-detailed reasons, but we are confident that the reported patterns are not resulting from this setup.

The feeding tests took place between 3rd and 6th June on 4 consecutive days. The tests started approximately at 11.00 h on each day. The animals did not receive food before the tests (16–18 days, depending on the day of capture) to ensure they are hungry and eager to feed. In our preliminary study (Herczeg et al. 2020), we used shorter no-food acclimation period, but 30% of test animals did not feed at all that way, so we decided to extend the acclimation period. In the present study, more than 80% of the tested individuals fed in the tests.

Assays were done in the given population's natural light regime (recent colonisers were tested in dark). We ran two types of tests. First, we ran food preference tests offering surface and cave food types simultaneously to the focal animals. Second, we ran food type innovation tests by offering food items *A. aquaticus* do not meet in either habitat. In all tests, we provided food in similar quantities (by eye), which were significantly more than what an *A. aquaticus* individual could consume during an hour.

For the food preference test, we placed two rubber rings (diameter: 5 mm, height: 1 mm) into the Petri dishes 1 h before the tests to provide the different food items in a standardised position and to prevent food dislocation and mixing. We offered decaying poplar (*Populus* sp.) leaves (surface food type) and bacteria mats from the Molnár János Cave (cave food type). Right before video-recording, we placed the food items into the rubber rings. Then we started the recording, which lasted for 60 min. Every individual was

tested twice, on the first and third days. After the tests, the rubber rings and all remaining food were removed.

The food type innovation tests were done on the second and fourth days. In the first round, we offered the pet food 'Hikari Crab Cuisine' (Kyorin Food Industries, Ltd., Japan) designed for crustaceans, while in the second round, animals were provided with pieces of raw cucumber. We used similar rubber rings (one per Petri dish) and recording protocol as described above for providing the given food item.

In both test types, we extracted three variables from the videos (Herczeg et al. 2020, names changed slightly): 'presence of feeding' (binary variable describing whether an individual did or did not feed on the given food type during the observation), 'feeding duration' (total time spent with feeding on the given food type, measured in seconds) and 'feeding bouts' (number of feeding events on the given food type). A feeding event started when the focal individual started to manipulate the food and ended when the focal individual left the food (was more than one body length distance away from the food). Only tests where the given individual did feed was included in analyses of feeding duration. While we did not quantify movement activity, we observed during the video-analyses that animals could explore the small Petri dishes used for the tests easily.

## Statistical analyses

For testing food preference, we ran generalized linear mixed models (GLMMs). Presence of feeding was a binary variable (the focal individual did or did not feed). To analyse it, we built GLMM with binomial distribution and logit link function. We added 'population' (the four sampled populations and the recent colonisers as a separate group), 'food type' (surface vs. cave) and the population × food type interaction as fixed effects. To control for habituation to the test setup, we added the centred order of trials (hereafter, 'order') as a single fixed effect. We added individual identity as a random intercept. Feeding duration and feeding bouts were analysed in GLMMs with negative binomial distribution and log link function, with similar model structure as described above.

For testing food type innovation, we ran generalized linear models (GLMs) separately for the assays with pet food and raw cucumber. Presence of feeding was analysed in GLMs with binomial distribution and logit link function, while feeding duration and feeding bouts in GLMs with negative binomial distribution and log link function. In these models, we added 'population' (the four sampled populations and the colonisers as a separate group) as a fixed effect.

We decided about the best distribution and link function for the models based on the Q-Q plots of the model residuals. We applied Wald's Chi-square tests to test the fixed effects and likelihood ratio tests for the random effects. We followed Zuur et al. (2009) for calculating *P* values for the

likelihood ratio tests. For post hoc comparisons, we used the false discovery rate (FDR) method. We ran the models in R 3.6.2 (R Core Team 2019). We built the GLMMs and GLMs with the R packages *lme4* and *lmerTest* (Bates et al. 2015; Kuznetsova et al. 2016) and ran the FDR tests with the *lsmeans* package (Lenth 2016).

## Results

### Food preference

Out of 94 individuals, 77 individuals fed on at least one food type, and out of the 188 feeding tests, there were 114 with feeding recorded. For raw data distributions, see Electronic Supplementary Material (ESM; Figs. 1-4). All three GLMMs (testing presence of feeding, feeding duration and feeding bouts, respectively) revealed significant population, food type and population  $\times$  food type interaction effects (Table 1). All populations were more likely to feed on surface food than on cave food, the difference is less pronounced in Malom Lake and Molnár János Cave (Fig. 1a). Recent colonisers from Malom Lake to Molnár János Cave showed similar trends to the two ‘normal’ surface populations, with somewhat higher presence of feeding in general, while the two ‘normal’ surface populations seemed to avoid cave food (Fig. 1a). All populations spent more time feeding on surface food than on cave food (Fig. 1b). However, the difference was less pronounced in Molnár János Cave individuals than the other populations, while recent colonisers expressed the highest difference, spending almost twice as much time feeding on surface food than the other populations (Fig. 1b). Further, only Molnár János Cave individuals spent considerable time with consuming cave food, the others avoided it almost completely (Fig. 1b, ESM Fig. 2). All populations tended to have more feeding bouts on surface food than on cave food, the difference being the smallest in Molnár János Cave individuals (Fig. 1c).

Habituation was significant only for feeding duration: individuals spent less time feeding with time (Table 1). The

individual effect was significant for feeding duration and feeding bouts (Table 1).

### Food type innovation

Out of 91 individuals tested in both tests (91 tests with pet food and 91 tests with cucumber; 182 tests altogether), 75 individuals fed on the pet food, and 72 fed on the cucumber. For raw data distributions, see Electronic Supplementary Material (ESM; Figs. 1-4). All GLMs revealed similar patterns: population divergence was only detectable in feeding duration irrespective of food type (Table 2). Recent colonisers had the highest feeding durations but differed significantly only from the surface population with the lowest feeding duration in both food types (Fig. 2). It is important to emphasise that all populations did consume both novel food types, with no apparent differences between the cave and surface populations (Fig. 2). Further, both presence of feeding and feeding duration was higher on pet food or cucumber compared to natural surface or cave food (Figs. 1, 2; ESM Figs. 1-4).

## Discussion

Behavioural innovation is a key factor in successfully colonising new habitats, adapting to environmental change or to better exploit available resources (e.g. Mayr 1965; Morse 1980; Reader and Laland 2003; Lefebvre et al. 2004). Since behavioural innovation (which can be seen as a form of behavioural plasticity) is expected to be costly due to the energetic needs of the necessary sensory and neural machinery (DeWitt et al. 1998; Snell-Rood 2013; Herczeg et al. 2019), its evolutionary loss is predicted in simple, stable and isolated environments. However, the evolution of behavioural innovativeness was typically addressed in an interspecific comparative framework so far (e.g. Sol et al. 2002, 2005a,b; Overington et al. 2009, 2011). Here, we tested the above prediction in an intraspecific context, comparing food preference and food type innovativeness of generalist surface *A. aquaticus* populations feeding on various live and dead

**Table 1** Results of the generalised linear mixed models on food preference. Significant effects are in bold font. Results are visualised in Fig. 1

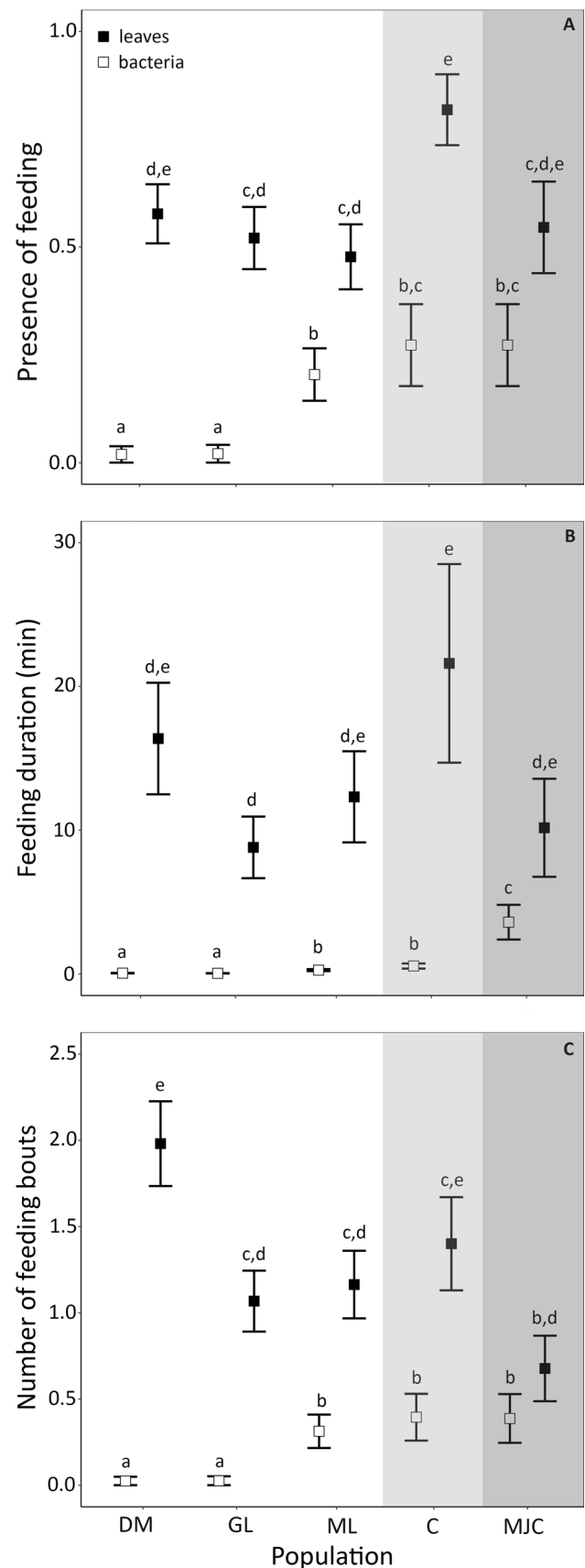
Model term	Presence of feeding		Feeding duration		Feeding bouts	
	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value
<i>Fixed effects</i>						
Population	<b>17.33 (4)</b>	<b>0.002</b>	<b>39.4 (4)</b>	<b>&lt;0.001</b>	11.26 (4)	<b>0.02</b>
Food type	<b>50.7 (1)</b>	<b>&lt;0.001</b>	<b>28,362 (1)</b>	<b>&lt;0.001</b>	50.13 (1)	<b>&lt;0.001</b>
Population $\times$ food type	<b>12.4 (1)</b>	<b>0.02</b>	<b>14,180.3 (4)</b>	<b>&lt;0.001</b>	17.66 (1)	<b>0.002</b>
Habituation	0.15 (1)	0.7	<b>10,225.6 (1)</b>	<b>&lt;0.001</b>	3.4 (1)	0.07
<i>Random effect</i>						
Individual	<0.001 (1)	0.49	<b>107,483.5 (1)</b>	<b>&lt;0.001</b>	36.33 (1)	<b>&lt;0.001</b>

**Fig. 1** Food preference variation between the studied *Asellus aquaticus* populations. DM=Dunakeszi Peat-moore (surface, unconnected to the cave;  $N=26$ ); GL=Götés Lake (surface, unconnected to the cave;  $N=24$ ); ML=Malom Lake (surface, connected to the cave;  $N=22$ ), C=(recent colonist, from ML to MJC;  $N=11$ ); MJC=Molnár János Cave (genetically isolated cave population;  $N=11$ ). Least squares means  $\pm$  standard errors are shown (back-transformed to the original scale). **A** Presence of feeding, **B** feeding duration, **C** feeding bouts. White indicates surface type individuals, light grey indicates recent colonists, while dark grey indicates cave-adapted individuals. Statistical results are shown in Table 1. Letters above whiskers denotes the results of the FDR post hoc comparisons

plant materials to a cave population feeding exclusively on bacterial mats developing in the cave. Our most salient findings are twofold. First, we showed that cave *A. aquaticus* did not lose its food type innovativeness; further, we proved that it actually prefers the surface food over cave food. Second, we showed that surface individuals strongly avoided the cave food type and recent cave colonisers spent the most time feeding on surface food, suggesting that adapting to cave food was a major challenge during the course of colonising the cave. Below, we discuss the results about food preference between the natural food sources and feeding behaviour against unnatural food sources.

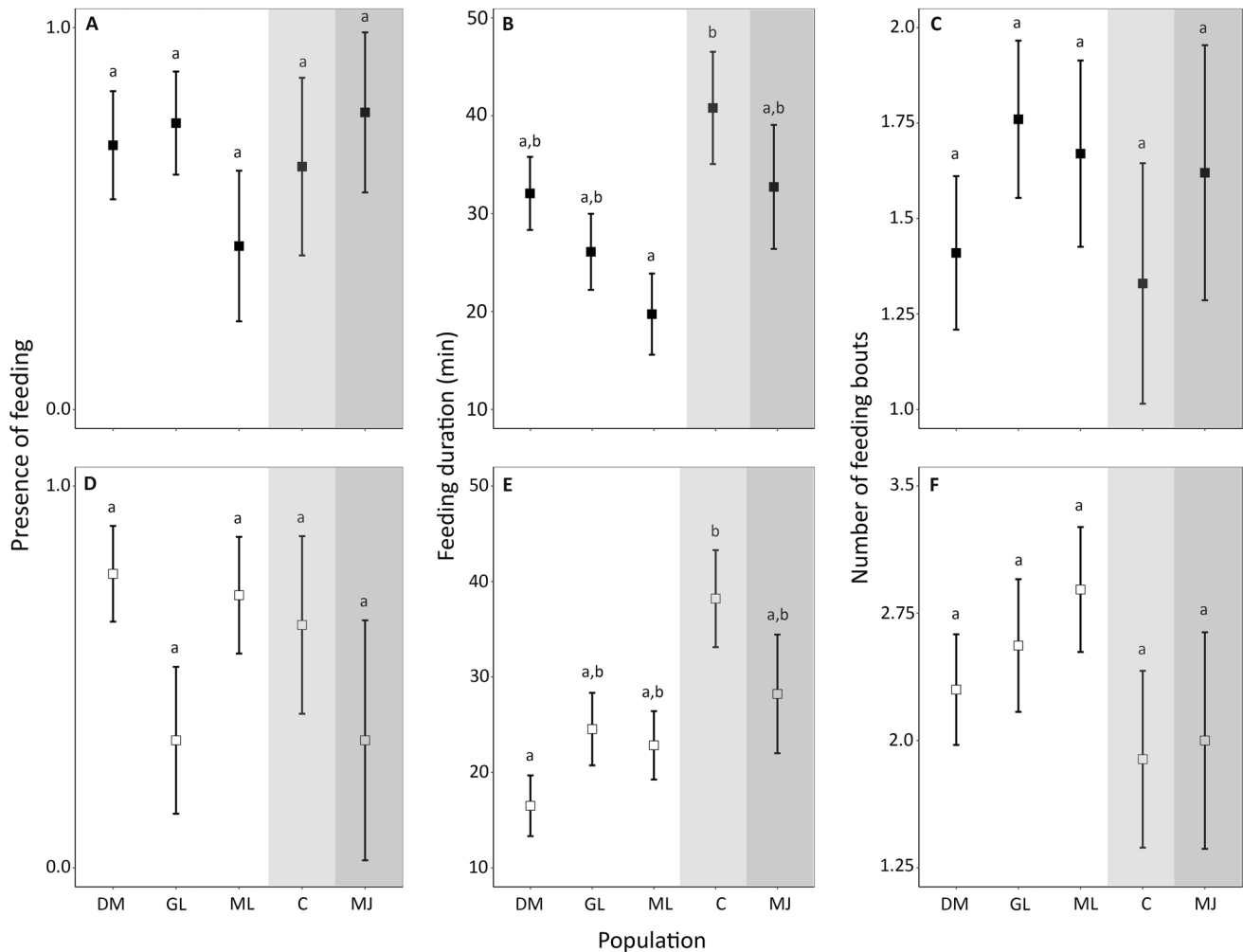
### Preference between cave vs. surface food

*A. aquaticus* is a widespread surface freshwater generalist, feeding on a wide variety of food (Moore 1975; Graça et al. 1993; Bloor 2011) and occupying a wide variety of habitats (Prevorčnik et al. 2009). We hypothesized that more than 60 000 years spent in the isolated cave habitat (Pérez-Moreno et al. 2017) with only bacterial mats as food resulted in food specialisation and thus predicted that cave *A. aquaticus* have high preference for cave food with low ability to consume surface food. Our results contradicted the above expectations. All three behavioural variables tested showed clearly that cave *A. aquaticus* identified and consumed surface food and differences in feeding duration proved that they actually preferred it over cave food. Hence, cave-adapted *A. aquaticus* maintained its preference for surface food. The patterns (see also the next section) also suggest that cave-adapted *A. aquaticus* remained a generalist feeder, despite being forced by the environment to feed on only one food source. The above findings are partly in agreement with our preliminary study (Herczeg et al. 2020). However, in the previous study, focal animals were tested against cave vs. surface food separately, supporting the innovativeness of the cave population, with results suggesting stronger preference towards surface food in cave than in surface populations. The direct preference tests in the present study clearly reject the latter conclusion, with the weakest preference being detected in the cave population. This is in line with the findings of Mösslacher and Creuté des Châtelliers (1996), who found



**Table 2** Results of the generalised linear models on food type innovation. Significant effects are in bold font. Results are visualised in Fig. 2

Model term	Novel food 1 (crab cousine)						Novel food 2 (raw cucumber)					
	Presence of feeding		Feeding duration		Feeding bouts		Presence of feeding		Feeding duration		Feeding bouts	
	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value	$\chi^2$ (df)	<i>p</i> value
<i>Fixed effects</i>												
Population	2.38 (4)	0.67	<b>10.81 (4)</b>	<b>0.03</b>	4.36 (4)	0.36	4.99 (4)	0.29	<b>13.93 (4)</b>	<b>0.007</b>	3.35 (4)	0.5



**Fig. 2** Food type innovation variation between the studied *Asellus aquaticus* populations. DM=Dunakeszi Peat-moore (surface, unconnected to the cave;  $N=26$ ); GL=Gótés Lake (surface, unconnected to the cave;  $N=24$ ); ML=Malom Lake (surface, connected to the cave;  $N=21$ ), C=(recent colonist, from ML to MJC;  $N=11$ ); MJC=Molnár János Cave (genetically isolated cave population;  $N=9$ ). Least squares means  $\pm$  standard errors are shown (back-transformed to the

original scale). Upper row of graphs (A, B, C), commercial crayfish food; lower row of graphs (D, E, F), raw cucumber. A, D Presence of feeding, B, E feeding duration, C, F feeding bouts. White indicates surface type individuals, light grey indicates recent colonists, while dark grey indicates cave-adapted individuals. Statistical results are shown in Table 2. Letters above whiskers denotes the results of the FDR post hoc comparisons

that surface *A. aquaticus* from Austria fed more on surface food than *A. aquaticus* from the Movile Cave system (Romania), which is another cave where the only available food is bacterial mats. This discrepancy between our present and

preliminary results draws attention to the danger of discussing preference based on comparing separate tests and supports the importance of direct preference tests.

We also expected that the generalist surface populations have preference for surface food with the ability to switch to cave food. This prediction was partly rejected. While surface *A. aquaticus* did prefer surface food, they hardly consumed any cave food, even though they showed innovativeness towards unnatural food types (see the next section). Further, recent colonisers spent the most time with feeding on surface food, implying that they were more food-deprived than the other populations. Therefore, cave adaptation was highly unlikely to be a result of exploiting the new food resource, and we suggest that it was rather driven by escaping from the harsh surface conditions (Danielopol and Rouch 2005; Culver and Pipan, 2009; Romero 2009). While the lack of predators in the cave might support the idea that *A. aquaticus* colonised the cave to avoid predation, we speculate that since the colonisation of Molnár János Cave happened 60 000–140 000 years ago (Pérez-Moreno et al. 2017), it is more plausible that the cave colonisation is a result of *A. aquaticus* seeking for a thermal refugium during a glacial event within Pleistocene. At any rate, it seems that the only food offered by Molnár János Cave to *A. aquaticus* was rather an obstacle than a trigger during colonisation. We have no exact information about the nutritional content of bacterium mats or decaying leaves; however, a previous study testing the effect of different food sources on the growth-rate of surface type *A. aquaticus* reports that individuals fed with Canadian waterweed (*Elodea canadensis*) or decaying Oak (*Quercus* sp.) leaves grew faster than conspecifics fed with algae or *Sphaerotilus natans* bacteria (Marcus et al. 1978). This indirectly suggests that the nutrition content of leaf litter might be substantially higher than bacterium mats. Further, bacterium mats in Molnár János Cave might have compounds that are directly avoided, like high concentration of potentially toxic elements accumulated on the extracellular polymeric substance of the microbial mats (Dobosy et al. 2016; Enyedi et al. 2019).

### Food type innovation towards unnatural food types

Results from the present (see the previous section) and our preliminary study (Herczeg et al. 2020) show that cave-adapted *A. aquaticus* has no problem in identifying surface food as food, and further, these results prove that even after at least 60 000 years of isolation in the Molnár János Cave with only endogenous bacterial mats as food, the cave population prefers surface food. Even though we are certain that surface food does not enter our studied hypogean cave (e.g. Erőss et al. 2006; see also Herczeg et al. 2020 for more details), for a targeted comparison of food type innovativeness between generalist surface populations and the cave population, we tested how they react to unnatural food types. We predicted generalist surface populations to be more innovative than the cave population. Again, our prediction was

rejected. There was no apparent difference between the cave and the surface populations in their willingness of consuming commercial crayfish food or raw cucumber. These and the food preference results clearly demonstrate that the Molnár János Cave population did not lose or reduce its food type innovativeness and the generalist feeding strategy of surface populations did not change during the course of local adaptation to the cave environment either. Interestingly, most populations were more likely to feed and spent more time feeding on the unnatural food types, than with the surface (and obviously, cave) food types. However, we can only speculate about the reasons behind this; it is equally possible that crayfish food or raw cucumber is of higher quality than the natural food types or that it takes more time for *A. aquaticus* to process them. We note that in a preliminary analysis, we found no detectable morphological differences in the mouthparts between cave and surface populations (unpublished data).

How can we explain the lack of evolutionary change? Our hypothesis about the evolutionary reduction of costly behavioural innovativeness following the colonisation of a simple, stable and isolated environment is well-grounded. First, the high developmental and maintenance costs of neural tissue is well supported (Aiello and Wheeler 1995; Kotschal et al. 2013). Second, any forms of phenotypic plasticity, including activational behavioural plasticity, need costly neural and sensory machinery (DeWitt et al. 1998; Snell-Rood 2013). Third, habitat generalism was found to be positively correlated with brain size and innovativeness (Ratcliffe et al. 2006; Overington et al. 2011). Finally, behavioural innovativeness, which we see as a form of behavioural plasticity, was found to be positively correlated with brain size (Reader and Laland 2002; Sol et al. 2005b; Overington et al. 2009). Further, habitat-dependent population variation in behavioural phenotypic plasticity has been shown, supporting the contention that behavioural plasticity can be a trait under selection (de Meester 1993; Salonen and Peuhkuri 2007; Herczeg and Välimäki 2011). Still, after our preliminary (Herczeg et al. 2020) and present results, it is clear now that our hypothesis is not supported in this study system. There are several potential explanations. For instance, one could think that the studied cave population did/could not genetically adapt to the cave environment for some reasons, and the reported genetic isolation from the surface populations (Pérez-Moreno et al. 2017) only reflects drift. However, the typical cave adaptations of *A. aquaticus*, depigmentation and eye reduction (with known genetic basis, Protas et al. 2011) can be seen in the Molnár János Cave population, so this idea is unlikely. Alternatively, other proximate constraints (developmental, physiological) could have prevented the evolutionary reduction of the sensory and neural machinery needed for



innovativeness. However, this is also unlikely considering the marked eye degeneration seen in our population. Finally, one can question whether food type innovativeness is costly at all. It is hard to give an unequivocal answer, but if we accept that behavioural innovativeness or even simply the ability to separate edible from non-edible matter depends on the costly central nervous system and sensory apparatus, there is no reason to not expect a decrease in innovativeness after tens of thousands of generations of *A. aquaticus* living in the isolated and extremely simple cave habitat providing only one food type. The question of how could our cave *A. aquaticus* population (i) remain food generalist, (ii) prefer surface food and (iii) show food type innovativeness similar to its surface conspecifics warrants further investigations.

Comparative interspecific studies repeatedly revealed that colonisation success is positively linked to behavioural innovativeness (e.g. Sol and Lefebvre 2000; Sol et al. 2002). Similar patterns can be expected in intraspecific or even in intrapopulation levels: in our case, one would expect that the most innovative individuals from a population will colonise new habitats with markedly different environmental conditions, including food sources. In our model system, where Malom Lake is directly connected to the Molnár János Cave, surface ecomorphs found in the cave can be seen as individuals from a surface population that are ‘attempting’ to colonise the cave. Whether they will be successful or not is indifferent from this aspect. Note that the strong outflow from Molnár János Cave to Malom Lakes makes accidental/passive dispersion to the cave unlikely. Recent colonisers spent more time feeding on commercial crayfish food and tended to spend more time with raw cucumber (FDR test;  $P = 0.067$ ) than the average Malom Lake specimen (actually, they spend the most time with feeding on the unnatural food from all populations), which supports the hypothesis that the most innovative individuals from a population are likely to invade new, markedly different habitats. Animal personality, i.e. consistent behavioural differences between individuals within a population over time and across different ecological contexts (Wilson et al. 1994; Gosling 2001; Dall et al. 2004), can be found in a wide array of taxa (Smith and Blumstein 2008), and personality-dependent dispersal is also known from different species (Cote et al. 2010). Therefore, it is intuitively logical that between-individual variation in innovativeness (consistent or not) affects colonisation patterns. However, since recent colonisers were found to spend the most time with feeding on surface food as well, we cannot exclude the simple explanation that these individuals of surface origin were simply deprived in the cave, where only the potentially suboptimal bacterial mats were available for them.

## Conclusions

Taken together, we found that the *A. aquaticus* population in Molnár János Cave — being genetically isolated from the surface populations for at least 60 000 years — did not change the generalist feeding strategy seen in surface populations. It preferred the surface food type despite being adapted to an isolated habitat where the only available food is endogenous bacterium mats. Further, its food type innovativeness towards commercial crayfish food and raw cucumber was similar to those of the surface populations: all populations consumed these food items readily. Therefore, we conclude that despite the evident cave-adaptations like depigmentation and eye reduction, food generalism and the capacity for food type innovativeness is evolutionary rigid in our system. So far, we can only speculate about the reason for this pattern; proximate constraints against evolutionary change or negligible costs of food type innovativeness might be responsible. Surface *A. aquaticus* (pigmented, with fully developed eyes), colonising Molnár János Cave from Malom Lake recently, showed particularly high feeding activity both on surface food in the food preference tests and on the unnatural food sources in the innovations tests. Further, the two surface populations unconnected to the cave avoided cave food almost exclusively. Finally, the isolated cave-adapted population preferred surface food over cave food. These patterns suggest that cave food is rather an obstacle against, than a trigger of colonising the cave. We suggest that the colonisation of the Molnár János Cave was more likely the result of avoidance of the harsh surface environment (predation, climate change), than exploitation the new food resource provided by the cave.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00265-021-03109-x>.

**Acknowledgements** We are highly indebted to Kata Kerekes and Hajriz Berisha for their help during the experiment. We also thank two anonymous reviewers for their valuable comments.

**Author contribution** All authors designed the study; GeH, VN, and GB collected data and performed the experiments; GeH and GáH analysed the data; GáH wrote the manuscript with the substantial contribution of GeH, GB and VN; all authors reviewed the manuscript and gave final approval for publication.

**Funding** Open access funding provided by Eötvös Loránd University. This research was funded by the National Research, Developmental and Innovation Fund for international cooperation (#SNN-125627 for GáH). GeH also gained support from the Postdoctoral research grant of the National Research, Development and Innovation Fund (#PD-132041).

**Data availability** The database is provided as Electronic Supplementary Material.

## Declarations

**Ethics approval** All applicable international, national, and institutional guidelines for the care and use of animals were followed. Experiments were performed according to the guidelines of the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, Sect. 243/1998), which conforms to the regulation of animal experiments by the European Union. Individuals from the Molnár János Cave were collected under the permission of Pest County Bureau, Department of Nature and Environment Protection, Hungary (PE/KTF/14718–3/2016).

**Conflict of interest** The authors declare no competing interests.

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