

Behavioural alterations in surface and cave populations of isopod crustacean *Asellus aquaticus* by *Acanthocephalus anguillae*

Research Paper

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Corresponding author:

R. Kostanjšek;
 Email: rok.kostanjsek@bf.uni-lj.si

G. Benko , Ž. Fišer  and R. Kostanjšek 

University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Abstract

Acanthocephalans are obligatory endoparasites that often alter the phenotype of their invertebrate intermediate host to facilitate trophic transmission to their final vertebrate host. *Acanthocephalus anguillae*, a widespread parasite of European freshwater fishes and isopod *Asellus aquaticus*, was recently discovered also in Postojna-Planina Cave System (Slovenia) parasitising olms (*Proteus anguinus*) and cave populations of *A. aquaticus*. This setting offers a unique opportunity to investigate potential fine-tuning of parasitic manipulations to the specifics of the highly divergent subterranean environment where some common phenotypic alterations lose functionality, but others might gain it. We measured three behavioural traits: movement activity, shelter-seeking, and response to light of infested and uninfested isopods from surface and cave populations. All behaviours were quantified from 1-h video-recordings via video-tracking isopod's movement in empty or custom modified (half-sheltered/half-illuminated) Petri dishes. Infested isopods of both populations spent significantly less time sheltering and were significantly less photophobic than uninfested ones, whereas the activity of isopods was not altered. However, we observed almost no cave-specific responses upon infestation in the two altered behaviours. It seems phenotypic alterations are not particularly fine-tuned to the subterranean environment and its hosts, and likely still reflect the parasite's surface origin.

Introduction

Manipulating the appearance and behaviour of the host is an efficient strategy for parasite transmission. In addition to well known, even notorious examples of 'snails with pulsating eyes', 'zombie ants', 'fearless rodents', and 'suicidal crickets' (Hughes *et al.*, 2011; Adamo, 2012), host manipulation is common among parasites with complex developmental cycles (Cezilly *et al.*, 2010). Host manipulation is particularly prevalent in parasites that use natural food chains for their transmission from the infected intermediate host to the definitive host by altering the phenotype of the former in a way of increased probability of its encounter and predation by the latter (Fayard *et al.*, 2020).

Parasite-induced changes in the host are considered part of the extended phenotype (*sensu* Dawkins, 1982) of the parasite and are the subject of evolution. Because of the clear benefit to the fitness of the parasite, manipulations of the intermediate host are often considered adaptations that are the result of alleles of the parasite selected for their effects (Poulin, 2010). Manipulated hosts often exhibit a range of phenotypic changes, a phenomenon known as multidimensionality or infestation syndrome (Benesh *et al.*, 2008; Cezilly & Perrot Minnot, 2010; Thomas *et al.*, 2010). The advantage for the parasite is obvious: multiple traits that facilitate trophic transmission significantly enhance the likelihood of such an event compared to a single trait. However, multidimensionality increases the complexity of assessing the relative importance of certain manipulated traits in enhancing parasite transmission from intermediate to definitive hosts (Bakker *et al.*, 2017). In addition, some host alterations may be mere by-products of infestation resulting from common biochemical processes and may have no effect on transmission or even reduce its probability (Cezilly & Perrot Minnot, 2010). On the other hand, host manipulations that correspond to 'purposive design' (*sensu* Poulin, 1995) and facilitate transmission may not be simple or true adaptations of the parasite. Several alternative mechanisms have been proposed to explain this. First, manipulation by the parasite may also increase host fitness, resulting in natural selection also acting on host alleles. According to the mafia hypothesis (Zahavi, 1979), the host exhibits a maladaptive trait because its fitness would be even lower without this trait. A well-known example is bird brood parasites such as cuckoos. The passerine host accepts the cuckoo's egg because its own brood and nest are otherwise exposed to a high risk of predation or destruction by the cuckoo. According to the host compensation hypothesis (Lefevre *et al.*, 2008), the infected host makes phenotypic changes to reduce the fitness costs caused by the parasite. An example of this is a parasite inducing energy depletion in the host. The host compensates by increasing its activity and foraging, which improves its average fitness but also

makes it more susceptible to predation. Secondly, the adaptive nature of host manipulations has recently been challenged by the immune system hypothesis (Cezilly *et al.*, 2010), which postulates that manipulations are the cause and not the consequence of trophic transmission of parasites. This hypothesis suggests that infestation triggers the host immune system, which could inadvertently affect various behaviours and appearance due to its close association with the nervous system and pigmentation.

It is obvious that the proximate and ultimate mechanisms of host manipulation are intricate, and that it is rarely possible to prove them unambiguously. However, if the manipulated traits are adaptive, they should respond to changes in the biotic and abiotic conditions of the environment. An important environmental determinant of the effectiveness of a manipulated trait, and thus the nature of its modification, is the predator–prey interaction between the final and intermediate host. From the parasite's perspective, this interaction can vary in several ways. The perception and foraging behaviour of the definitive host and the defense strategies of the intermediate host are finely tuned by the environment. In lightless habitats, for example, predators cannot rely on vision and must use other senses to locate prey. In a habitat with a dark background or substrate, dark pigmentation of prey would be cryptic, whereas the same phenotype would expose prey to predation in habitats with a bright background or substrate. In addition, some parasites may utilize more than one final and/or intermediate host species, and if their predator–prey interactions differ, the effectiveness of parasite manipulations will also change. Consequently, we might expect a change in the intensity, frequency or type of adaptive manipulations if any aspect of the predator–prey interaction changes. Such patterns are not predicted if the manipulated traits are not adaptive.

Finding appropriate host–parasite models providing new insights into the adaptive potential of manipulative parasites by addressing reciprocal interactions between ecological and evolutionary processes (i.e., 'eco-evo dynamics') behind manipulative parasitism in hosts with multidimensional response remains challenging (Perrot-Minnot *et al.*, 2023). Preferably, these models would encompass a host–parasite association with well-described host-manipulation potential, a limited number of host traits that can potentially be altered by a manipulative parasite, and the possibility of observation of these traits under different environmental conditions.

Herein, we present an intriguing model system that could prove useful in answering these questions. The model system consists of an acanthocephalan parasite and two ecomorphs of its isopod crustacean intermediate hosts inhabiting surface and subterranean waters. *Acanthocephalus anguillae* (Müller, 1780) is a common parasite of the digestive tracts of European freshwater fishes, utilizing the freshwater isopod crustacean *Asellus aquaticus*, also known as a common water louse, as its intermediate host. Besides waterlice populations colonizing debris-rich surface waters across Europe (Sket, 1994), troglodytic *A. aquaticus* populations exist in groundwater throughout the continent, most of them in the Dinaric Karst (Protas *et al.*, 2023). Being strictly bound to subterranean environment, these *A. aquaticus* populations also referred to as cave ecomorphs, have evolved several adaptations including loss of pigmentation, eye reduction, and elongated appendages (Turk *et al.* 1996; Prevorčnik *et al.* 2004; Konec *et al.* 2015; Balázs *et al.* 2021; Protas *et al.*, 2023), which enables their morphological distinction from the surface ecomorph. In contrast, the distinction between the surface and cave populations of *A. anguillae* is much less clear. A population of *A. anguillae* was recently described in the *A. aquaticus* cave ecomorphs from Postojna-Planina Cave System

(Slovenia) (hereafter PPCS) and in the digestive tract of the European cave salamander (*Proteus anguinus*), also known as the olm (Amin *et al.*, 2019). Sharing identical COI, ITS, 28S, and 18S rRNA between parasite isolated from cave *A. aquaticus* and the olm, and the latter being by far the most numerous vertebrate in the underground waters, the olm was proposed as the definitive host of *A. anguillae* in the PPCS (Amin *et al.*, 2019), although sexual reproduction of the parasite in the olm has not been proven directly. At the same time, identical sequences of selected nuclear markers and a negligible genetic distance in the COI gene sequence between *A. anguillae* from the PPCS and *A. anguillae* from European eels (*Anguilla anguilla*) in Germany indicate close relationships between geographically distant populations of *A. anguillae* (Amin *et al.*, 2019). Despite the supposedly constant presence of *A. anguillae* within cave ecomorphs in PPCS, it remains unclear whether this represents an isolated cave population of the parasite capable of completing its reproduction cycle in its cave hosts, or whether its presence in cave hosts may be attributed to constant influx of *A. anguillae* eggs released from surface definitive hosts (cyprinid fishes). In this case, ingestion of the parasite by the olm would represent its reproductive dead end and a population sink of the local surface population of *A. anguillae*.

In surface and cave environments, *A. anguillae* finds clearly different abiotic and biotic conditions, which leads to different predator–prey interactions. In surface waters, cyprinid fish are the final host of *A. anguillae*, and their foraging is mainly driven by visual cues. Infested intermediate hosts are less photophobic and darker pigmented (Lyndon, 1996) than uninfested ones, which presumably increases the likelihood that they will be preyed upon, thus facilitating transmission of the parasite to the final host. Cave habitats are lightless, nutrient-limited, with buffered daily and seasonal environmental fluctuations and simpler communities with lower population densities (Culver & Pipan, 2019). Among abiotic conditions, constant darkness represents the most significant disruption to existing predator–prey interactions, rendering manipulations of photophobia and pigmentation ineffective. In addition, cyprinid fish are rare, occasional visitors to caves, where their role as apex predators is replaced by the olm. The olm is an obligate cave-dwelling species with a number of remarkable adaptations to its unique environment (Kostanjšek *et al.*, 2023). It has an exceptionally sensitive non-visual sensory system, including olfaction (Dumas & Chris, 1998; Tesarova *et al.*, 2022; Uiblein & Parzefall, 1993), mechanoreception, electroreception, and magnetoreception (Bulog & Schlegel, 2000; Schlegel & Bulog, 1997; Schlegel *et al.*, 2009). The olm relies on these sensory systems during active foraging, a behaviour that is preferred over sit-and-wait foraging when prey is invisible and scarce (Uiblein *et al.*, 1992; Manenti *et al.*, 2024). To increase the susceptibility of the intermediate host to olm predation, *A. anguillae* would need to manipulate traits detectable by non-visual senses. Taken together, if host manipulations of *A. anguillae* are adaptive, they should be fine-tuned to the specific predator–prey interaction in the cave environment to ensure successful transmission between hosts. Such fine-tuning or phenotypic adjustment could be achieved by altering allelic composition or phenotypic plasticity. To investigate the hypothesis that host manipulations are adaptations, we performed a comparative analysis of three behaviours commonly altered by acanthocephalans in uninfested and *A. anguillae*-infested surface and cave populations of the intermediate host *A. aquaticus*. Movement activity releases visual, mechanical and olfactory signals into the environment, making more active prey more susceptible to predation by an active predator. Searching for and staying in thigmotactic shelters, such as

under leaves and stones or in narrow crevices (hereafter referred to as “shelter-seeking”), protects the prey from predators. Both increased movement activity and reduced shelter-seeking would enhance parasite transmission in both surface and cave environments. However, these behavioural manipulations should be more critical in caves, as vision manipulations are ineffective in the lightless environment. Therefore, we predicted a greater change in these two behaviours in infested cave hosts compared to infested surface hosts, relative to their uninfested counterparts. Behavioural response to light, particularly photophobia, is another antipredatory strategy, with infested hosts usually being less photophobic. In the lightless cave environment dominated by a non-visual predator, this manipulation is ineffective. Thus, we predicted a lesser or no change in response to light in infested cave hosts compared to infested surface hosts, relative to their uninfested conspecifics. Patterns deviating from these predictions could indicate that these behavioural manipulations are non-adaptive but could also result from several phenomena specific to our model system, such as a sink of parasite population in the cave with the olm acting as a dead-end host.

Material and methods

Animal collection and acclimation

Experimental animals were obtained from one surface and one cave population of the *Asellus aquaticus* species complex. Surface isopods were collected from a ditch at Planina Polje (N45.83227, E14.25832) at the end of May 2020. Cave isopods were collected from the adjacent Pivka Channel of Planina Cave (coordinates of the cave entrance: N45.81990, E14.24567) at the beginning of July 2020. This cave population is referred to as Old Subterranean Pivka (Protas *et al.*, 2023) and exhibits typical traits associated with cave life (i.e., complete depigmentation, eye reduction, and elongation of certain appendages). We could not sample surface and cave populations at the same time because of the differing seasonal patterns (by our unpublished observation) of acanthocephalan infestation within each population. However, we collected the animals within the shortest possible time interval to minimize potential seasonal effects on isopods' phenotype.

After collection, isopods were transferred to a cave laboratory (total darkness, 11–13 °C) at the Department of Biology, Biotechnical Faculty, University of Ljubljana. All further procedures related to acclimatization and experiments were conducted in this controlled environment. Initially, adult isopods (> 5 mm) were examined under a Leica StereoZoom S9E stereomicroscope to identify individuals infested by acanthocephalans and to determine their sex based on gonopod morphology. Because of the possibility of errors in identifying parasite presence in their live hosts, each isopod underwent dissection after the experiments to confirm its infestation status. As *A. aquaticus* hosts different acanthocephalan species, each parasite was morphologically examined to ensure *Acanthocephalus anguillae* was the specific parasite present. Animals were grouped based on ecomorph, sex, and infestation status and left to acclimate for 4–5 days in common containers. Next, each isopod was transferred into a Petri dish (90 × 15 mm) with 80 mL of dechlorinated tap water and kept there for another week of acclimation. To facilitate animals' normal locomotion, Petri dish bottoms were abraded with P150 grain sandpaper, as recommended by Fišer *et al.* (2019). During the acclimatization period, isopods fed *ad libitum* on decomposing black alder (*Alnus glutinosa*) leaves.

In their natural habitats, surface and cave isopods experience distinct lighting conditions: a day/night cycle for surface isopods and constant darkness for cave isopods. Acclimating each ecomorph to its natural light setting would hinder interpreting experimental outcomes as potential differences could be due to plasticity or genetic factors. Conversely, exposing either ecomorph to unnatural light conditions could prompt short-term behavioural changes (Emmer *et al.*, 2018). An ideal experimental approach would involve acclimating both ecomorphs to their respective natural and unnatural lighting conditions. However, the limited number of infested cave isopods made this impossible. Therefore, we opted to acclimate both ecomorphs solely to darkness for two primary reasons. First, we anticipated a more pronounced impact of the totally unnatural diurnal light cycle on cave isopods' behaviour compared to the effect of semi-natural constant darkness on surface isopods. Second, previous research on surface isopods from Planina Polje found no statistically significant difference in their behavioural response to light between individuals acclimated to the diurnal cycle and those acclimated to darkness (Fišer, 2017).

Experimental design, setup, and procedures

We evaluated three behavioural traits: movement activity, shelter-seeking and response to light. The behavioural traits were assessed twice for each individual within surface and cave ecomorphs of both infestation statuses and sexes. Cave isopods were assayed approximately 1 month after the surface isopods (see the previous section). Sample sizes for each group varied from 24 to 47 individuals (for details, see Table 1). We assessed all behavioural traits by analyzing 1-h videos recorded using a setup similar to studies conducted by Berisha *et al.* (2023), Herczeg *et al.* (2020, 2022), and Horváth *et al.* (2021, 2023). The recording chamber was designed with daylight-mimicking LEDs (color temperature = 4500 K, color rendering index > 90) positioned at the top and infrared (IR) LEDs (920 nm) at the bottom. The daylight LEDs emitted approximately 5 μmol of PAR photons per m⁻² s⁻¹ and were switched on only during recording response to light. The IR LEDs were on throughout all recordings. They were covered with opal plexiglass to uniformly diffuse light, also serving as a surface for placing Petri dishes containing experimental animals. Neither light source raised the water temperature in the Petri dishes during the length of the recording. Three webcams (Logitech C920 FullHD), modified for improved recording in IR light, simultaneously captured the behaviour of a maximum of 36 individuals, each webcam covering 12 individuals. It took five sessions to record one behavioural trait for all individuals. Within each recording session, uninfested and infested isopods of both sexes were equally included and randomly arranged across the recording panel. The videos were recorded at FullHD resolution (1920 × 1080 dpi) and 5 frames per second using Bonsai 2.3.0 (Lopes *et al.*, 2015). During the experiment preparation, we utilized dim red light (approximately 630 nm) from the Black Diamond Spot Headlamp as it was previously demonstrated that both ecomorphs do not respond to it behaviourally (Fišer *et al.*, 2016, Fišer, 2017). Behavioural traits were recorded sequentially in the following order: movement activity, shelter-seeking, and response to light, with an acclimation period of 3–4 days between each assessment. To measure movement activity, animals were recorded in their home Petri dishes, from which food and feces were removed. Shelter-seeking behaviour was assessed following the method outlined by Fišer *et al.* (2019). A plastic shelter designed for thigmotactic responses covered half of a bottom-grinded Petri dish (90 × 25 mm) at an

Table 1. Sample size and estimates of central tendency and dispersion for movement activity, shelter-seeking, and response to light in eight groups of *Asellus aquaticus* defined by ecomorph, acanthocephalan infestation, and sex.

	Movement activity			Shelter-seeking			Response to light		
	N ₁ / N ₂	Me	IQR	N ₁ / N ₂	Me	IQR	N ₁ / N ₂	Me	IQR
Surface uninfested (F)	25 / 25	0.41	0.24	24 / 25	0.50	0.15	25 / 24	0.33	0.13
Surface uninfested (M)	30 / 26	0.38	0.17	30 / 26	0.50	0.11	27 / 25	0.33	0.20
Surface infested (F)	42 / 41	0.38	0.20	41 / 41	0.60	0.13	42 / 41	0.50	0.12
Surface infested (M)	30 / 29	0.45	0.24	30 / 28	0.60	0.20	29 / 28	0.56	0.08
Cave uninfested (F)	35 / 32	0.11	0.21	33 / 30	0.57	0.24	33 / 29	0.12	0.26
Cave uninfested (M)	43 / 37	0.11	0.13	41 / 36	0.58	0.27	39 / 35	0.16	0.22
Cave infested (F)	30 / 29	0.08	0.13	30 / 30	0.71	0.28	29 / 28	0.38	0.30
Cave infested (M)	47 / 47	0.15	0.23	46 / 46	0.89	0.35	47 / 47	0.35	0.24

Abbreviations: F, females; IQR, interquartile range; M, males; Me, median; N₁ – sample size for the first recording, N₂ – sample size for the second recording.

angle suitable for accommodating isopods of varying sizes. This setup divided each dish into sheltered and exposed halves. Both shelter-seeking and movement activity were recorded in the dark. To evaluate the response to light, the animals' home Petri dishes had one half covered with an opaque black plastic cap, creating illuminated and dark halves. In both shelter-seeking and light response assessments, each individual was initially confined to either the exposed or illuminated half of the Petri dish using a plastic ring barrier for 10 min (Fišer *et al.*, 2016, 2019). This ensured exposure to the adverse stimulus and allowed the isopods to recover from handling stress. After recording the response to light, the isopods underwent a 9-day acclimation period. Subsequently, the same procedure was repeated, and each surviving individual was eventually assessed twice for each behavioural trait.

At the end of the experiments, we measured the body size of each isopod using body length (from the head's start to the end of the pleotelson) as a proxy, rounded to the nearest millimeter. All isopods were then dissected to confirm their infestation status and identify the parasite species (see the previous section).

Video and image analysis

Movement activity recordings were video-tracked using Bonsai 2.3.0 (Lopes *et al.*, 2015). The resulting movement trajectories were processed using custom scripts in R 4.2.1 (R Development Core Team 2022) to calculate total path (cm), time moving (proportion), average speed (cm/s), and maximum instantaneous speed (cm/s). Shelter-seeking and response to light videos were analysed using BORIS 7.10 (Friard & Gamba, 2016). Each transition between the halves of the Petri dishes, be it between sheltered and exposed halves or between illuminated and dark halves, was logged. Following this, we computed the proportion of time spent by the isopods at the exposed (outside shelter) and illuminated halves of the Petri dishes, respectively.

Data analyses

To eliminate the potential impact of animal handling before each recording session, we cut off the first 10 min (50 min remained) for movement activity data, and the first 15 min (45 min remained) for shelter-seeking and response to light data. In the latter two, we cut off more as the disturbance of animals was greater. Infrequent

instances when individuals crawled out of their Petri dishes during recording were excluded from the analysis. After dissection, 25 individuals were found to be infested with more than one cystacanth. Additionally, despite starting the experiments with only non-gravid females, 19 surface and 14 cave females developed a marsupium full of eggs sometime during the process. As preliminary analyses indicated no discernible effect of infestation intensity or gravidity on observed behaviours, we retained these individuals in the final analysis. Typically, each individual was recorded twice for each behaviour. In our final analysis, we used the averaged data for each individual. Isopods that were recorded only once for a certain behaviour due to escape or mortality were still included in the analysis, but they were assigned a lower weight (0.5 vs. 1) in the regression models. The four variables describing movement activity (total path, time moving, average speed, and maximal instantaneous speed) were mutually highly correlated and showed the same general pattern (Figures S1-S3). In the final analyses of movement activity, we selected the proportion of time moving as a proxy because it was on the same scale (proportions) as the other two behavioural variables and allowed us to use the same type of statistical models.

All three behavioural response variables (movement activity, shelter-seeking, and response to light) were expressed as proportions and were not normally distributed. We used median and interquartile range to show summary statistics for each of the eight groups defined by ecomorph, infestation status, and sex. Data were further analysed by fitting a beta regression model to each of the three response variables. Models included three two-level factors, i.e., ecomorph (surface, cave), infestation status (uninfested, infested), and sex (female, male), as well as all their interactions. The experimental setup used to measure shelter-seeking and response to light caused that more active individuals inclined towards a 0.5 value of the response variables (Figures S4-5). To control this effect, we included the count of transitions between the Petri dish's halves (a proxy for activity) as a covariable in models for shelter-seeking and response to light. We used a fixed dispersion parameter when modeling movement activity, whereas a variable dispersion parameter was used in models for shelter-seeking and response to light. Applying post hoc pairwise comparisons we tested for differences between ecomorphs of the same infestation status and sex (4×), between infestation statuses of the same ecomorph and sex (4×), and between sexes of the same ecomorph and

infestation status (4×). As shelter-seeking and response to light were statistically significantly affected by infestation (see Results), we tested also whether ecomorphs of the same sex (2×) and sexes of the same ecomorph (2×) differ in the magnitude of change caused by infestation (Table S1). Models were fitted via the 'betareg' function from the 'betareg' package (Cribari-Neto & Zeileis, 2010) in R 4.2.1 (R Development Core Team, 2022). The statistical significance of model terms was estimated using the function 'lrtest' in the 'lmtest' package (Zeileis & Hothorn, 2002). Post hoc pairwise comparisons were done in package 'emmeans' (Lenth, 2023), and *p*-values were adjusted via the 'fdr' method (Benjamini & Hochberg, 1995). All plots were drawn using package 'ggplot2' (Wickham, 2016).

Parasite infestation can collectively affect various traits in the intermediate host, manifesting in an infestation syndrome. Traits constituting this syndrome are expected to be correlated. To assess this, we conducted Spearman rank correlations between traits that were altered in infested isopods. For the surface ecomorph, these were shelter-seeking and response to light, while for the cave ecomorph, these were shelter-seeking and response to light (see Results). Movement activity was unaltered by infestation in both ecomorphs, so we excluded it from correlation analyses. In both ecomorphs, infested and uninfested isopods of both sexes were tested separately. The correlation coefficients and their corresponding *p*-values were independently calculated for each of the eight observed groups. All correlation analyses were conducted using the 'cor.test' function from the 'stats' package in R 4.2.1 (R Development Core Team, 2022) and *p*-values were adjusted via the 'fdr' method (Benjamini & Hochberg, 1995). To assess the consistency (repeatability at the individual level) we conducted Intraclass Correlation Coefficient (ICC) analysis for all behavioural traits. The ICC values and their corresponding *p*-values were independently calculated for each of the eight observed groups. All correlation analyses were conducted using the 'icc' function from the 'irr' package in R 4.2.1 (R Development Core Team, 2022) and *p*-values were adjusted via the 'fdr' method (Benjamini & Hochberg, 1995).

Results

Table 1 provides estimates of central tendencies and dispersion in eight distinct groups defined by ecomorph, infestation status, and sex for each of the three observed behaviours: movement activity, shelter-seeking, and response to light.

Table 2. Effect of ecomorph, infestation status, sex, and their interactions on movement activity, shelter-seeking, and response to light in *Asellus aquaticus* as deduced from beta regression models.

Model term	Movement activity		Shelter-seeking		Response to light	
	χ^2	<i>p</i> -value	χ^2	<i>p</i> -value	χ^2	<i>p</i> -value
Ecomorph	168.49	<0.001	8.754	0.068	33.215	<0.001
Infestation_status	4.013	0.404	47.114	<0.001	95.387	<0.001
Sex	5.632	0.228	11.703	0.020	6.817	0.146
Ecomorph*Infestation_status	0.173	0.917	5.263	0.072	0.599	0.741
Ecomorph*Sex	1.468	0.480	5.536	0.063	0.220	0.896
Infestation_status*Sex	3.142	0.208	3.864	0.145	3.927	0.140
Ecomorph*Infestation_status*Sex	0.190	0.663	1.501	0.221	0.117	0.733
No. of transitions	/	/	8.523	0.004	2.902	0.088

An asterisk indicates an interaction of variables. Statistically significant effects are bolded.

Movement activity

Movement activity, measured as the proportion of time the isopods spent moving, was statistically significantly affected only by ecomorph, whereas infestation status, sex, and all interactions were statistically non-significant (Table 2, Fig. 1). Surface isopods were more active than cave isopods. The former moved about 41% of the time, whereas the latter moved only about 11% of the time (Table 1). Post hoc pairwise comparisons revealed this overall outcome was consistent for both infestation statuses and sexes (Table S1, Figure 1). Besides, the response of surface isopods was more variable compared to the cave isopods. The same general pattern was also found in the other three variables related to movement activity (i.e., total path, average speed, and maximum instantaneous speed) (Figures S1-S3).

Shelter-seeking

Shelter-seeking, measured as the proportion of time the isopods spent on the exposed (outside shelter) half of the Petri dish, was statistically significantly affected by infestation status and sex, whereas ecomorph and all interactions were statistically non-significant (Table 2, Fig. 2). Infested isopods sheltered less than uninfested isopods. The former spent about 70% of the time outside the shelter, whereas the latter spent only about 54% of the time unsheltered (Table 1). Post hoc pairwise comparisons showed this general pattern was roughly consistent across both ecomorphs and sexes (Table S1, Fig. 2). Pairwise comparisons also revealed that the main effect of sex (females sheltered about 4% more than males) was due to the extraordinarily high proportion of time spent exposed by infested cave males, as the only statistically significant difference was between these and surface infested females. The relevance of the main effect of sex on shelter-seeking is thus questionable. On the other hand, cave infested males also sheltered statistically significantly less (about 29%) than surface infested males. Although it did not result in statistically significant interactions between ecomorph and infestation status or ecomorph, infestation status, and sex (Table 2). Further, the response of cave isopods was more variable compared to the surface isopods. The covariable (the count of transitions between the Petri dish's halves), included to account for the effect of different movement activity between ecomorphs and individuals on shelter-seeking, was statistically significant (Table 2, Fig. S4).

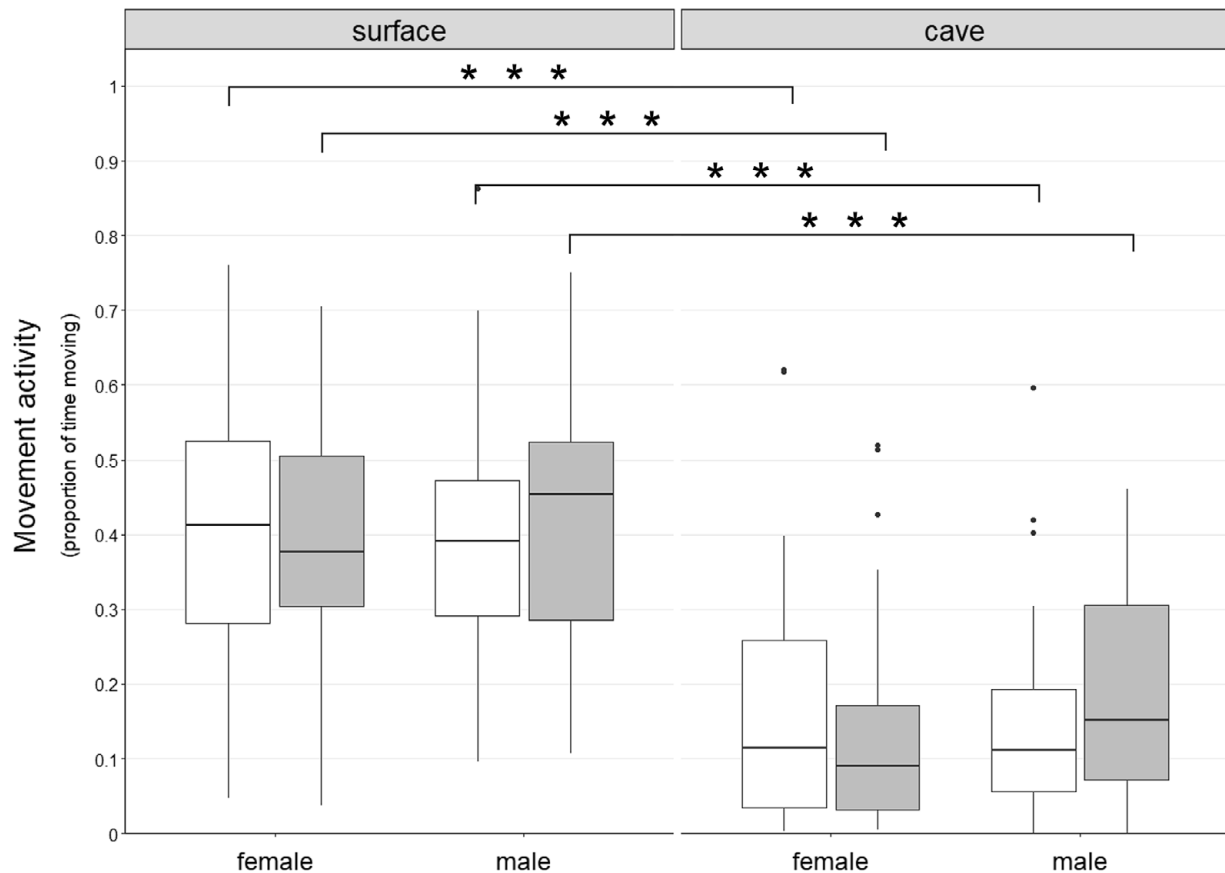


Figure 1. Movement activity (expressed as proportion of time moving) in the eight groups of *Asellus aquaticus* defined by ecomorph (surface, cave), infestation status (infested, uninfested), and sex (female, male). Infested and uninfested isopods are represented with grey and white boxplots, respectively. Statistically significant differences between groups are marked with asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Response to light

Response to light, measured as the proportion of time the isopods spent at the illuminated half of the Petri dish, was statistically significantly affected by ecomorph and infestation status, whereas sex and all interactions were statistically non-significant (Table 2, Fig. 3). Surface isopods avoided light less than the cave isopods. The former spent about 43% of the time on the illuminated half, whereas the latter spent only about 25% of the time there (Table 1). Also, infested isopods avoided light less than uninfested isopods. The former spent about 45% of the time on the illuminated half, whereas the latter spent only about 24% of the time there (Table 1). Post hoc pairwise comparisons revealed these general patterns were consistent across both sexes (Table S1). Additionally, pairwise comparisons showed a statistically significant difference between surface infested females and males, with the latter spending about 6% of the time more in the light. Besides, the response of cave isopods was more variable compared to the surface isopods. The covariable (the count of transitions between the Petri dish's halves), included to account for the effect of different movement activity between ecomorphs and individuals on shelter-seeking, was statistically non-significant (Table 2, Fig. S5).

Trait correlations

We tested correlations only between traits that were altered in infested isopods. Because movement activity of surface and cave

isopods was not affected by parasite infestation, we tested correlations between shelter-seeking and response to light. In both ecomorphs, infested and uninfested isopods of both sexes were tested separately. Overall, the traits were not statistically significantly correlated (Table S2). The only statistically significant result was the positive correlation between shelter-seeking and response to light in surface uninfested males ($\rho = 0.61$, $p = 0.001$). Individuals that spent less time under the shelter were also less photophobic.

Discussion

Subterranean habitats provide an exceptional model system for studying complex ecological and evolutionary principles on relatively simple communities composed of organisms adapted to highly selective environment. Studies of the transition of various animal groups from surface to subterranean environments have, among others, provided valuable insights into the process of adaptation and improved our understanding of mechanisms behind speciation (Protas & Jeffery 2012). At the same time, and despite the ability of parasites to adapt to hosts in highly selective environments (Aleuy & Kutz, 2020), records of their presence in subterranean environments are scarce. *Acanthocephalus anguillae*, parasitizing olm and cave *Asellus aquaticus* in the PPCS may represent the first known endoparasite restricted to a subterranean environment (Amin *et al.*, 2019; but see Nickol & Whittaker, 1978), which makes it a unique model for studying parasite's potential to

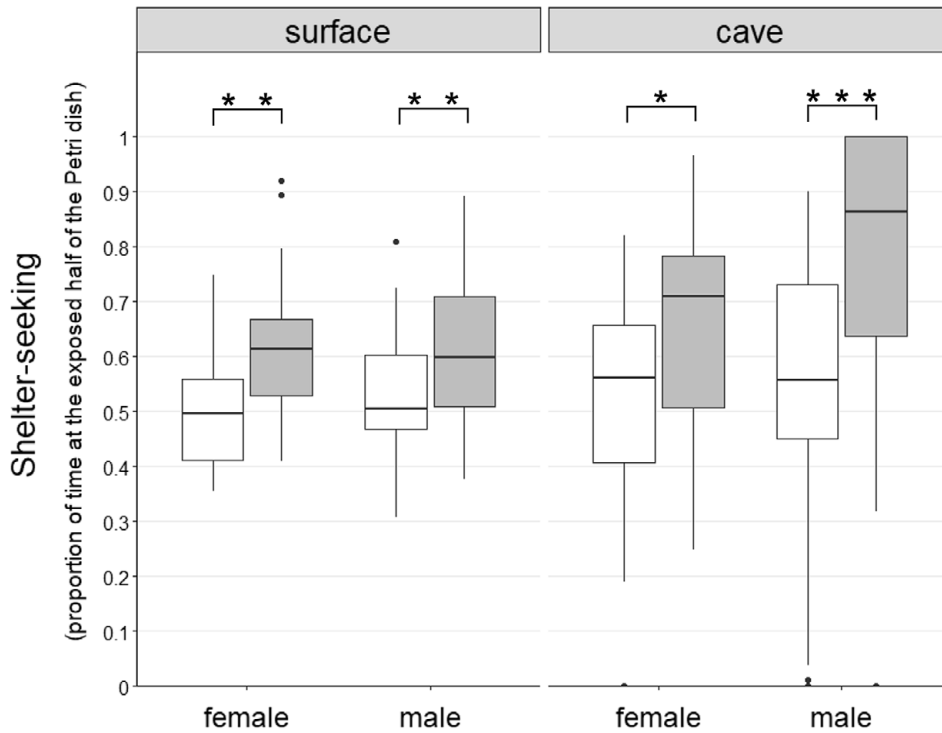


Figure 2. Shelter-seeking (expressed as proportion of time spent at the exposed half of the Petri dish) in the eight groups of *Asellus aquaticus* defined by ecomorph (surface, cave), infestation status (infested, uninfested), and sex (female, male). Infested and uninfested isopods are represented with grey and white boxplots, respectively. Statistically significant differences between groups are marked with asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Additional statistically significant differences not shown in the plot include 'surface infested (M) - cave infested (M)' and 'cave infested (F) - cave infested (M)' (see Table S1).

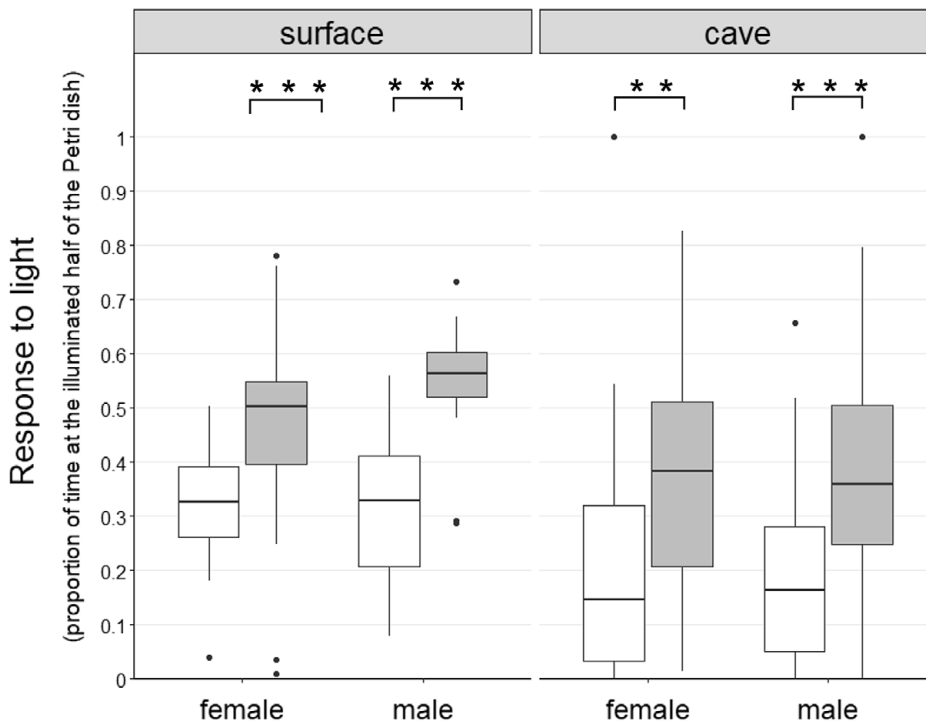


Figure 3. Response to light (expressed as proportion of time spent at the illuminated half of the Petri dish) in the eight groups of *Asellus aquaticus* defined by ecomorph (surface, cave), infestation status (infested, uninfested), and sex (female, male). Infested and uninfested isopods are represented with grey and white boxplots, respectively. Statistically significant differences between groups are marked with asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Additional statistically significant differences not shown in the plot include 'surface uninfested (F) - cave uninfested (F)', 'surface uninfested (M) - cave uninfested (M)', 'surface infested (F) - cave infested (F)', 'surface infested (M) - cave infested (M)' (see Table S1).

Table 3. Behavioural modifications in the shared intermediate host (*Asellus aquaticus*) by two closely related European acanthocephalans from the genus *Acanthocephalus*

	<i>Acanthocephalus lucii</i>	<i>Acanthocephalus anguillae</i>	
Final host (preferred)	<i>Perca fluviatilis</i>	<i>Squalius cephalus</i> , <i>Barbus barbus</i>	<i>Proteus anguinus?</i>
Intermediate host	<i>Asellus aquaticus</i> (surface)	<i>Asellus aquaticus</i> (surface)	<i>Asellus aquaticus</i> (cave)
Activity	Not altered	Not altered*	Not altered*
Shelter-seeking	Reduced	Reduced*	Reduced*
Photophobia	Not altered	Reduced	Reduced*
Substrate colour preference	Not altered	Not altered	Not relevant

Entries marked with asterisks represent new findings from this study (Benesh *et al.*, 2008, 2009; Lyndon, 1996; Pilecka-Rapacz, 1986).

adapt its host-manipulating strategies in response to a selective environment.

Despite acanthocephalans are well-recognized manipulative parasites (Fayard *et al.*, 2020; Perrot-Minnot *et al.*, 2023), the current knowledge on the modification of their intermediate hosts is understudied because of the diversity of potentially manipulated traits in the host and the complexity of the physiological processes behind them. Consequently, studies of host manipulation in European species of the genus *Acanthocephalus* include only *A. anguillae* (Lyndon, 1996) and *A. lucii* (Benesh *et al.*, 2008, 2009; Seppälä *et al.*, 2008). To place our results in the context of previous behavioural studies, we have summarized the most important observations in Table 3.

Shelter-seeking behaviour is one of the most common antipredator behaviours in waterlice (Cooper & Frederick, 2007; Horváth *et al.*, 2021) and therefore a frequent target of host manipulation strategies for acanthocephalans (Benesh *et al.*, 2008; Park & Sparkes, 2017; Perrot-Minnot *et al.*, 2014). Our observations showed a reduction of sheltering in parasitized surface and cave *A. aquaticus* ecomorphs. This observation confirms previous reports on other European species in the genus *Acanthocephalus* (Table 3) and suggests reduced sheltering behaviour as a common strategy in the genus (Park & Sparkes, 2017). Although the effect of infestation was not significantly different between the ecomorphs, infested cave males sheltered significantly less than infested surface males. This is the only result in our study that may indicate an adaptation of *A. anguillae* to a subterranean environment and the olm as the final host. At the same time, the possibility of increased susceptibility of cave ecomorphs to parasite manipulation as a result of host-related factors cannot be excluded. The first host-related factor could be an increased susceptibility and response to parasite's manipulative strategies described in naive, unadapted hosts (Franceschi *et al.*, 2010), which would infer an absence of coevolution between acanthocephalans and cave ecomorphs, and presence of *A. anguillae* in the underground waters as a relatively recent evolutionary event. Another possibility for increased susceptibility to parasite as a host-related factor would be a reduced ability of the cave host to suppress the parasites. This could evolve as a consequence of a reduced investment of cave organisms in the immune system in response to the lower abundance of parasites in the subterranean environment (Peuß *et al.*, 2020). Because the

host's immune defense against parasites can be additionally suppressed by androgens (Zuk & McKean, 1996; Klein, 2004), this could also explain the tendency to greater reduction of sheltering in males compared to females observed in our study. The observed discrepancy in infested males also justifies experimental setup with males and females as separated groups, although results on sex-dependent response to acanthocephalan infestation in previous studies are not unanimous. Manipulation of *Asellus aquaticus* by *A. lucii* for example did not show any differences in the intensity of manipulations between host sexes (Benesh *et al.*, 2008, 2009), whereas differences in activity and body size were observed between sexes of North American isopod *Caecidotea intermedius* infested with *A. dirus* (Park & Sparkes, 2017). Finally, surface and cave ecomorphs exhibited similar shelter-seeking behaviour, which is in contrast to studies of Fišer *et al.* (2019) and Horváth *et al.* (2021) who found that cave individuals shelter significantly less than surface ones. This mismatch might be due to differences in the experimental design, protocol, and choice of populations among these studies.

Infection with *A. anguillae* also significantly decreased photophobia of surface and cave waterlice. This phenotypic alteration of the host by *A. anguillae* has been shown to be effective in surface ecosystems (Lyndon, 1996), whereas its benefits in subterranean habitats are highly unlikely and this manipulation is expected to be lost or reduced during adaptation. It could be beneficial to parasites infesting cave ecomorphs that reside near or occasionally migrate to the cave entrance because it increases the probability that they will be preyed upon by surface vertebrate hosts. However, the population of cave ecomorphs used in our study exclusively inhabits deeper parts of the PPCS (Sket, 1994). The rate of decreased photophobia did not differ significantly between infested ecomorphs, so there is no evidence supporting adaptation of this host manipulation in the cave environment. At the same time, increased photophobia was observed in uninfested cave ecomorphs compared to uninfested surface ecomorphs, which may be attributed to natural selection in cave populations of waterlice favoring avoidance of surface habitats with suit of conditions adverse for the cave adapted waterlice (Breder & Rasquin, 1947).

In contrast to shelter-seeking and response to light, the alteration of movement activity of infected waterlice as a parasite strategy for enhanced transmission to the final host by predation is not so unambiguous. Either increase or decrease in activity might be considered advantageous for the parasite because the former would increase the possibility of detection and encounter with the predator, whereas the latter would enhance its catchability (Fayard *et al.*, 2020). As waterlice are slow-moving and their vertebrate predators display active foraging both at the surface and in the cave, we predicted an increase of movement activity upon infestation. However, infestation by *A. anguillae* had no significant effect on any of the three proxies used for movement activity (time in motion, traveled distance, movement speed) in neither surface nor cave ecomorphs. This is congruent with previous observations of surface *A. aquaticus* infested with *A. lucii* (Benesh *et al.*, 2008) (Table 3). The lack of a cave-specific response of the intermediate host infestation effect suggests that this trait has not been fine-tuned to the predator-prey interaction in the cave environment. However, the lack of infestation effect on movement activity could also be an experimental artefact. Individuals of many species, including waterlice show increased activity after being introduced to a new environment such as a test arena (Fišer, personal observation). Such a pattern can last for several hours before activity returns to normal levels. Measurements during this elevated phase

may not detect differences from normal levels, especially if they are small. Finally, cave ecomorphs of both sexes and infestation statuses were less active than their surface counterparts, which may be explained by the advantage of an energy-conservation strategy in an oligotrophic subterranean environment (Hervant *et al.*, 1997). At the same time, Berisha *et al.* (2023) have found that *A. aquaticus* from caves with abundant food and lacking predators were more active than their neighbouring surface populations (Berisha *et al.* 2023). In that view, the difference between the ecomorphs observed in our study suggests that olm predation is also a considerable factor for cave waterlice in the PPCS.

When discussing adaptation, it is important to consider the potential correlation of modified traits and the possibility that these traits are altered in a coordinated manner to enhance parasite transmission (Benesh *et al.*, 2008; Cezilly & Perrot Minnot, 2010; Thomas *et al.*, 2010). Assessing the adaptability of each trait separately can be misleading because correlated traits provide an opportunity for unfavorable changes to persist due to common underlying mechanisms. For example, reduced photophobia might persist in a subterranean environment if it is intrinsically linked to shelter-seeking behaviour, even if its benefits are unlikely. In our study, the altered shelter-seeking traits and response to light in infected isopods were not correlated (Table S2), providing no evidence for shared mechanisms, which is consistent with studies by Benesh *et al.* (2008) and Park and Sparkes (2017) and suggests that the traits are altered independently. In this scenario, we would expect each alteration to be subject to natural selection for energetic efficiency and potentially eliminated in a highly selective environment. Nevertheless, because of the low consistency (repeatability at the individual level) of our measurements (Table S3), our study cannot definitively determine whether common or independent mechanisms are behind the observed changes. This low repeatability undermines the reliability of our correlation results and calls for future studies to focus on improving the consistency of behavioural measurements to better understand these mechanisms and gain clearer insights into the adaptive strategies of parasite manipulation.

Overall, our results provide little support for the hypothesis that host manipulations by *A. anguillae* are adaptive. The behaviours examined (movement activity, shelter-seeking, and response to light) showed similar patterns when infesting both surface and cave hosts and were not specific to the distinct predator–prey interactions in these environments. This similarity may suggest that these traits are non-adaptive and may have arisen as by-products of the host immune response, as suggested by Cezilly *et al.* (2010). The lack of correlation between the studied behaviours further supports this assumption. However, several factors specific to our model system suggest alternative explanations that do not completely exclude the possibility that these behaviours are adaptive in surface environments. First, gravid parasite females have not yet been confirmed in the olm, which might be a dead-end host. If this is the case, the *A. anguillae* population in PPCS would represent a sink of local surface population unable to adapt. Second, the population sizes of both intermediate and final hosts are much larger on the surface than in the cave, which should also apply to the parasite. With a constant high gene flow from the surface to the cave, adaptation would be a challenge even under strong natural selection. Furthermore, even if gene flow is highly restricted and the parasites are isolated in the cave, sufficient time is required for adaptations to evolve. Currently, there is insufficient genomic data available to shed light on the structure and history of parasite populations. Third, it is possible that predator–prey interactions

in the two environments are not as different as anticipated, and that surface-adapted manipulations might work well in caves without the need for fine-tuning. Finally, and perhaps more likely, the behaviours tested may not be critical for successful transmission of the parasite in the cave-specific predator–prey interaction. Significant differences might exist in behaviours more strongly related to mechanical and chemical stimuli. For example, infested cave isopods might be significantly more attracted to sudden water disturbances or odors compared to their surface counterparts. They might emit more mechanical signals through enhanced exploratory behaviour, feeding, or respiration (aquatic isopods breathe via pleopod beating) or chemical signals. Increased sociability could lead to aggregations that emit more mechanical and chemical stimuli compared to solitary animals. Theoretically, all these manipulations could make cave isopods more susceptible to olm predation, and it seems justified to test them in the future.

The present work represents the first behavioural study of parasite-induced phenotypic alteration in a subterranean environment. Our study also demonstrates the potential of parasitic associations in a selective subterranean environment to overcome the challenges of existing models for studying the adaptation of host manipulation strategies in surface habitats (Thomas *et al.*, 2011). For example, a constant subterranean environment eliminates seasonal variation in manipulation intensity. Phenotypic alterations for enhancement of parasite transmission by predation are reduced to those that can be detected by non-visual sensory systems of the final host (e.g., fixed total depigmentation of cave intermediate hosts eliminates the effectiveness of altered appearance from the manipulative arsenal). Last but not least, the reduction of the set of multiple final hosts to a single available species, the olm, prevents the interpretation of the observed phenotypic alterations of the intermediate host as backup strategies to exploit potential alternative hosts or to avoid inappropriate hosts.

Supplementary material. The supplementary material for this article can be found at <http://doi.org/10.1017/S0022149X24000592>.

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Competing interest. None.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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