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Abstract. Parasites commonly manipulate host behaviour to increase transmission success between hosts. While most behavioural changes comprise slight alterations to host activity patterns and habitat use, some represent impressive alterations to routine behaviour which, while having direct positive effects on parasite transmission, compromise host survival. Here, we report conspicuous risky behaviour in an African annual killifish, *Nothobranchius furzeri*, infected by metacercariae of a strigeid trematode, *Apatemon* sp., residing in their cranial cavity. We demonstrate a striking contrast in the spatial and temporal behavioural responses of fish from populations naturally infected with *Apatemon* sp. and fish from two control populations with either a similar baseline parasite fauna but lacking *Apatemon*, or an overall low-level of infection. During routine activity, fish from *Apatemon*-infected populations positioned themselves just below the water surface, while other fish spent most of their time near the bottom. During a simulated avian attack, killifish from *Apatemon*-infected populations jumped above the water surface, moved in an uncoordinated manner, and rotated in the upper water layer, while fish from the control populations rapidly escaped into deeper water and ceased moving. The same self-exposing behaviour (jumping out of the water and lying on floating lily pads for extended periods) was also observed under natural conditions. Such behaviour greatly facilitates location of *Apatemon*-infected host fish by avian definitive hosts, especially in turbid pools. Moreover, the nothobranchiid killifish host's own life history, i.e. an extremely short lifespan limited to several months, may represent an important driver in the evolution of behavioural manipulation.

Key words: *Nothobranchius furzeri*, *Apatemon*, manipulation hypothesis, anti-predator behaviour, intermediate host, Trematode

Introduction

A range of parasites are able to affect their host's phenotype by altering host morphology, physiology and/or behaviour (Poulin 1995, Poulin & Thomas 1999, Cézilly et al. 2010, Lafferty & Shaw 2013). For parasites transferred to the definitive host through predation, any alteration in the behaviour of the infected host that favours parasite transmission between hosts represents an increase in parasite fitness (Holmes & Bethel 1972, Barber et al. 2000, reviews in Thomas et al. 2005, Cézilly et al. 2010). Such behavioural changes range from slight alterations to existing traits to completely new activities (Lefèvre et al. 2009). Thus, host manipulation by a parasite can be defined as any alteration in host phenotype that has fitness benefits for the parasite. Such behavioural alterations may

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be caused in two basic ways. The first is indirect and results in an energy cost, caused primarily by parasites that grow significantly while in the host's body, utilizing energy that would otherwise be used by the host (Moore 2002, Lafferty & Shaw 2013). However, most parasites use more sophisticated means to shape the behaviour of both invertebrate and vertebrate hosts by directly targeting the host's neural, endocrine, neuromodulatory and immunomodulatory systems, leading to measurable changes in host behaviour (Beckage 1993, Adamo 2002, 2013, Moore 2002, Lefèvre et al. 2009).

Manipulation of host behaviour has been documented in most of the major lineages of parasitic organisms (van Houte et al. 2013). Parasites that manipulate host behaviour occupy a range of sites in their hosts, especially the body cavity, muscles and central nervous system (CNS). The CNS is the key organ system for coordinating host behaviour and parasites located in the CNS can manipulate the host by either direct damage or through more subtle manipulation (Lafferty & Shaw 2013). Compared to the impressive ability of parasite species to infect specific host organs and their subsections, the ability to selectively attack specific brain regions appears modest (Adamo 2012). Trematodes are known to infect the widest range of sites within their intermediate hosts, including several species occupying the CNS of vertebrates (Lafferty & Shaw 2013). Larval trematodes (metacercariae) are known to affect intermediate hosts by manipulating host traits in such a way as to take advantage of final host foraging behaviour to aid transmission. In doing so, they may affect schooling behaviour (Radabaugh 1980), swimming activity (Brassard et al. 1982, Coleman 1993, Shirakashi & Goater 2002), feeding efficiency and time exposed (Crowden & Broom 1980), predator avoidance (Poulin 1993) or escape response (Seppälä et al. 2005). As one example, trematodes located in the host's CNS increase vulnerability to predators through conspicuous behaviour and microhabitat selection (Lafferty & Morris 1996, Lafferty & Shaw 2013).

Trematodes of the genus *Apatemon* Szidat, 1928 are cosmopolitan, trophically transmitted parasites with a three-host life cycle including freshwater molluscs, fish as intermediate hosts, and aquatic birds as definitive host. Metacercariae of *Apatemon* spp. are known to infect a wide spectrum of fish hosts and their location within the host varies

greatly, ranging from the abdominal cavity, the most common site of infection, to the pericardial cavity, eyes or brain (Blair 1976, Bell et al. 2002, Zhokhov et al. 2008, Blasco-Costa et al. 2016, Nezhybová et al. 2017). *Apatemon* sp. infecting African killifish and representing new African lineage of this genus, are located exclusively in the host's brain (Nezhybová et al. 2019); unlike other *Apatemon* species that tend to occupy the brain cavity only in heavy infections (Zhokhov et al. 2008, Blasco-Costa et al. 2016). However, to date no conspicuous behavioural changes have been reported in fish intermediate hosts parasitised by *Apatemon* species encysting in the brain.

Killifish of the genus *Nothobranchius* (Cyprinodontiformes, Nothobranchiidae) are small fishes living under extreme conditions (annually desiccating pools) throughout the east African savannah (Wildekamp 2004). At the onset of the rainy season, as temporary pools fill with rainwater, the fish hatch from embryos buried in the sediment, whereupon they grow, reproduce and, depending on the size of the pool, die as the pool dries up, a cycle that may last only several weeks (Vrtílek et al. 2018). The unique biology of annual killifish is predicted to impose significant selection pressure on their parasite fauna, and especially on larval parasite stages that must transfer to subsequent hosts. Since sexual reproduction of *Apatemon* trematodes is only possible in the intestine of fish-eating birds, any effect on individual host anti-predator behaviour, such as an escape response, would have a substantial influence on rates of parasite transmission.

We experimentally tested microhabitat selection and behavioural responses of naturally infected killifish from four localities. Fish from the first two localities were infected with *Apatemon* sp. metacercariae in the cerebral cavity, along with several other parasite taxa. Fish from the third locality hosted a similar parasite fauna but with *Apatemon* sp. absent, thereby separating the effect of *Apatemon* and other parasite taxa in highly infected populations (highly parasitised control population, Control HP). Fish from the fourth locality were infected with just one parasite species at low abundance, representing a low-infection population with negligible impact on host traits (low parasitised control population, Control LP). Based on field observations, we predicted a decrease in anti-predator behaviour (particularly in fish affected by *Apatemon* infection), which would

Table 1. List of parasite species collected during parasitological surveys at the four study localities in Mozambique (i.e. non-experimental fish, Nezhybová et al. 2017), showing proportion of infected fish, prevalence (*P* − in %) and mean number of parasites from all fish examined (abundance − A). Metacercariae of *Apatemon* sp. in bold. mtc = metacercaria, trematode larval stage; larv = larval stage of nematode or cestode. Ten individual fish were screened for parasites at each locality. "-" – represents absence of parasite species.

	Location in/on host	APATEMON 1		APATEMON 2		Control HP		Control LP	
		P (%)	A	*P* (%)	A	*P* (%)	A	*P* (%)	A
Diplostomidae sp.1 (mtc)	Muscle	60	67.7	100	258.5	100	123.8	-	-
Diplostomidae sp.2 (mtc)	Muscle	70	13.6	100	18.8	90	15.3	-	-
Apatemon **sp. (mtc)**	Cerebral cavity	**60**	**3.9**	**10**	**0.3**	-	-	-	-
Echinostomatidae (mtc)	Gills	10	0.5	-	-	-	-	-	-
Strigeidae sp.1 (mtc)	Muscle	-	-	-	-	-	-	100	3.5
Strigeidae sp.2 (mtc)	Abdominal cavity	-	-	-	-	10	0.1	-	-
Digenea sp. (mtc)	Muscle	70	7.8	-	-	30	3.3	-	-
Camallanidae sp. (larv)	Intestine	40	4.5	40	1.0	10	0.3	-	-
Gnathostomatidae sp. (larv)	Muscle	50	6.3	50	1.9	10	0.1	-	-
Cestoda sp. (larv)	Abdominal cavity	-	-	-	-	70	3.I	-	-

increase a parasite's chances of being transmitted to a definitive avian host. To test this prediction, we measured fish spatial distribution in undisturbed aquaria, accounting for either increased (located near the water surface) or decreased (located near the bottom) chance of contact with an avian predator. We also tested response time and anti-predator behaviour following a simulated avian attack (surface foraging) in order to identify mode and speed of fish reaction response (Lafferty & Shaw 2013).

Material and Methods

Fish collection

Field collection was conducted as part of a study monitoring the metazoan parasites of killifish in Mozambique (Nezhybová et al. 2017). Using a dip net, ten fish per locality were haphazardly captured from several localities in south-east Mozambique at the end of the rainy season in March 2013 and subjected to parasitological examination (Table 1, Nezhybová et al. 2017). During fieldwork, unusual fish behaviour was observed at two localities. When removing fish from the aquaria in which they were stored prior to dissection, several individuals jumped out of the water and made distinct uncoordinated movements. This behaviour was recorded and the fish dissected as described in Nezhybová et al. (2017). Strikingly, all fish jumping out of aquaria contained large metacercariae in their cerebral cavity; the absence of this parasite was never observed in conspicuously behaving hosts. Therefore, fish exhibiting such behaviour

(i.e. containing metacercariae of *Apatemon* sp.) were collected for further behavioural experiments.

For behavioural experiments, 25 naturally infected *Nothobranchius furzeri* Jubb, 1971 were originally collected from each of four pre-screened localities (termed Med2, Med3, Med4 and Dry2 in Nezhybová et al. 2017) reflecting different levels of parasite infection (APATEMON 1, APATEMON 2, Control HP, Control LP, respectively). By the start of the experiment, however, available numbers had been reduced to 10, 19, 20 and 14 fish, respectively, due to mortality which occurred during and shortly after the fish transportation. While fish from the APATEMON 1, 2 and Control HP sites were infected with a similar parasite community, *Apatemon* metacercariae were present in fish cranial cavities at APATEMON localities but absent at Control HP (Table 1). Other metacercariae were present in the muscles of infected fish. Fish from the Control LP site were free of parasites, except for metacercariae of another strigeid trematode in the muscles (Table 1). The fish were transferred to the Czech Republic where the experiments were conducted.

Behavioural experiments

The spatial distribution of fish in an undisturbed aquarium was measured four to five weeks after import. Individual fish were removed from the home aquarium using a fine net and immediately transported to an experimental aquarium (300 × 500 × 450 mm, water depth 300 mm). The water column was divided into four vertical sectors (the bottom

third of the aquarium, the middle third, the upper third and the surface) that were visibly marked on the wall of the aquarium. After allowing the fish to settle for 10 minutes, its spatial position in the water column was recorded every 20 seconds for 4 minutes (total score 12).

Several days (8-18) after the undisturbed spatial distribution experiment, a simulated avian attack was performed on the same fish to assess escape response, response time and vertical distribution after disturbance. After acclimatisation, a model "attacking heron" was released and allowed to "attack" the water surface, followed by its immediate retraction. After 1 to 2 minutes, the attack was repeated (total of three attacks per fish). At the end of the trial, the fish was removed and returned to its home aquarium. The immediate response of the fish to the stimulus was recorded using a Nikon Coolpix 510 digital video camera (30 frames s^{-1}) positioned in front of the tank, allowing subsequent analysis of escape response and determination of vertical position. Fish position was determined every 5 seconds for 1 minute after the first, second and third attacks (12 records of fish position for each phase).

Four distinct response types were recognised after a simulated avian attack: 1) freeze, 2) slow swim, 3) dash and 4) jump (Table 2). For each response, with the exception of the freeze reaction, response time was measured as the number of frames (1 frame = 0.033 s) between the point at which the avian model contacted the water surface and the active fish response (i.e. dash, swim or jump).

Following behavioural experiments, presence of *Apatemon* in the experimental fish was confirmed through successful infection of domestic ducklings (definitive host) by six infected fish (APATEMON populations), allowing us to acquire the adult stage of *Apatemon* sp. trematodes for further research (Nezhybová et al. 2019).

Statistical analysis

A "vertical score" was used to describe fish vertical position. Fish distance from the surface in each of 12 observations was expressed as either at the surface (value of 0), in the upper third (1), in the middle third (2) or in the bottom third (3). Vertical score was calculated as the sum of these twelve values, ranging from 0 (fish present at the surface all the time) to 36 (fish present in the bottom third all the time). The proportion of each fish's vertical score against the maximum possible vertical score (x out of 36) was used as a binomially distributed response variable in statistical models.

Fish body size was strongly related to sex (males larger than females; logistic regression, df = 1 and 49, $P < 0.001$, proportion of explained variability = 0.52; Table S1), and, therefore, was not included in the analysis. The effect of population (APATEMON 1, APATEMON 2, Control HP, Control LP), sex (male, female) and population:sex interaction on fish starting position was tested using a generalised linear model (GLM, binomial distribution; Table S2).

Generalised linear mixed models (GLMM, binomial distribution) were used to test shifts in position after attack first (i.e. difference between fish position after attack first and starting position) and after the last attack (difference between fish position after attack third and starting position). In these models, treatment (starting position, position after attack), population and treatment:population interaction were used as fixed predictors (Table S3) and individual as a random factor. Appropriate measures were undertaken when detecting overdispersion in binomial models (using a beta distribution in the GLM and introducing an observation-level random effect to GLMMs; Elston et al. 2001).

The effect of population, number of previous attacks and their interaction on the probability of occurrence of each of the four response types was tested using a multinomial logistic model (MLM; Table S5) that

Table 2. Type of response by killifish after a simulated predator attack.

| Response type	Description
'Dash'	Fish quickly and directly escapes from the point of attack or quickly escapes in a series of bursts; movement does not last more than 0.5 s
'Slow swim'	Fish swims slowly away from the point of attack; movement lasts 0.5-3 s
'Freeze'	Fish does not move for at least 3 seconds following attack
'Jump'	Fish does not swim away from the point of attack, but jumps above the water surface, performs uncoordinated movements or rotates its body
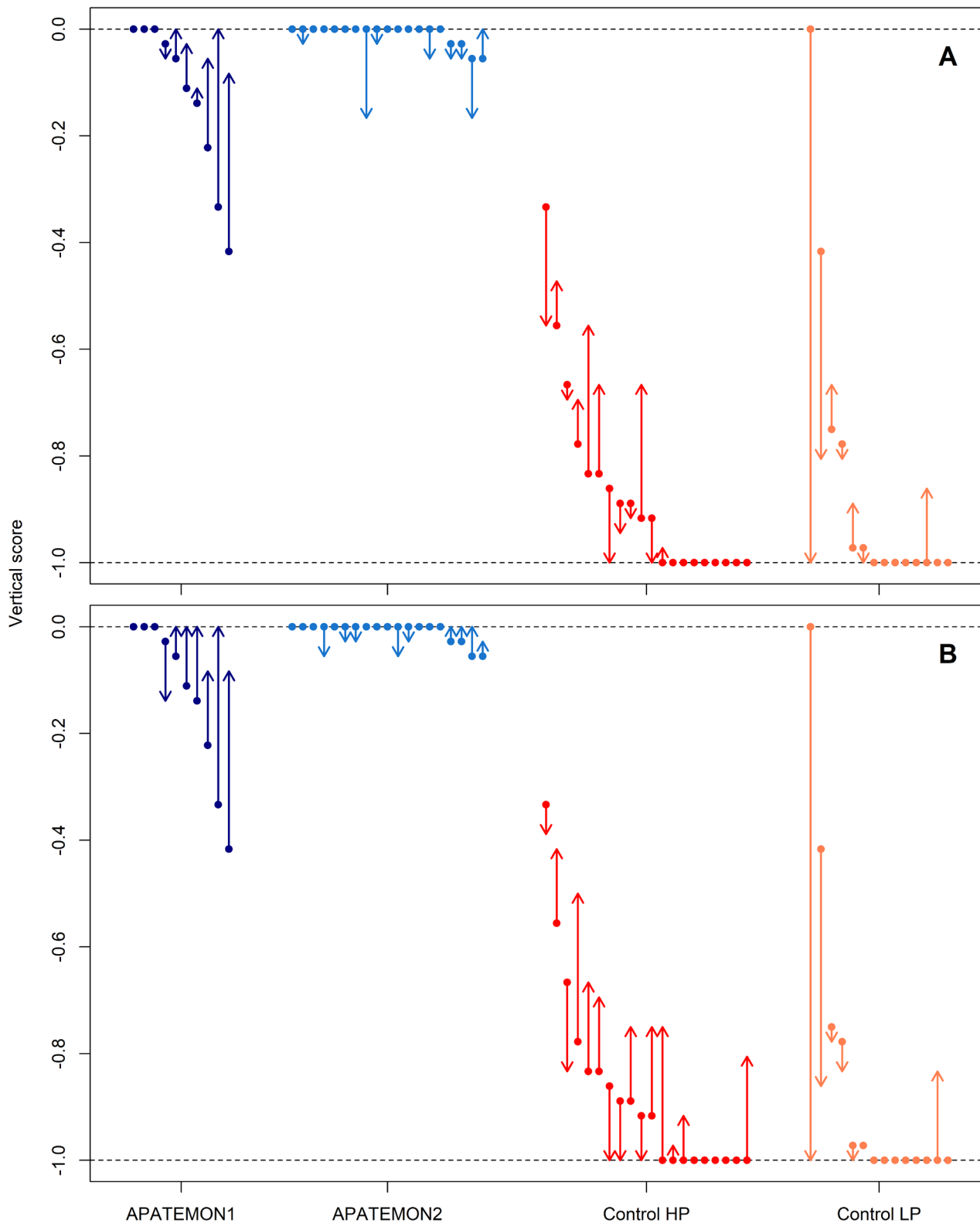
Fig. 1. Shift in fish vertical score before (full points) and after disturbance (arrow heads), i.e. after the first attack (panel A) and after all three attacks (panel B). Vertical score expressed as a proportion from the maximal value, ranging from 0 to 1, and multiplied by −1 for visualization to display fish at the water surface at the top.

included a series of binomial models testing for the effect of predictors on each possible combination of categorical response variable level pairs, allowing for repeated measurements on an individual

(Croissant 2019). As the response time was non-normally distributed, with some measurements returning negative values (i.e. fish responded before the model reached the surface), response
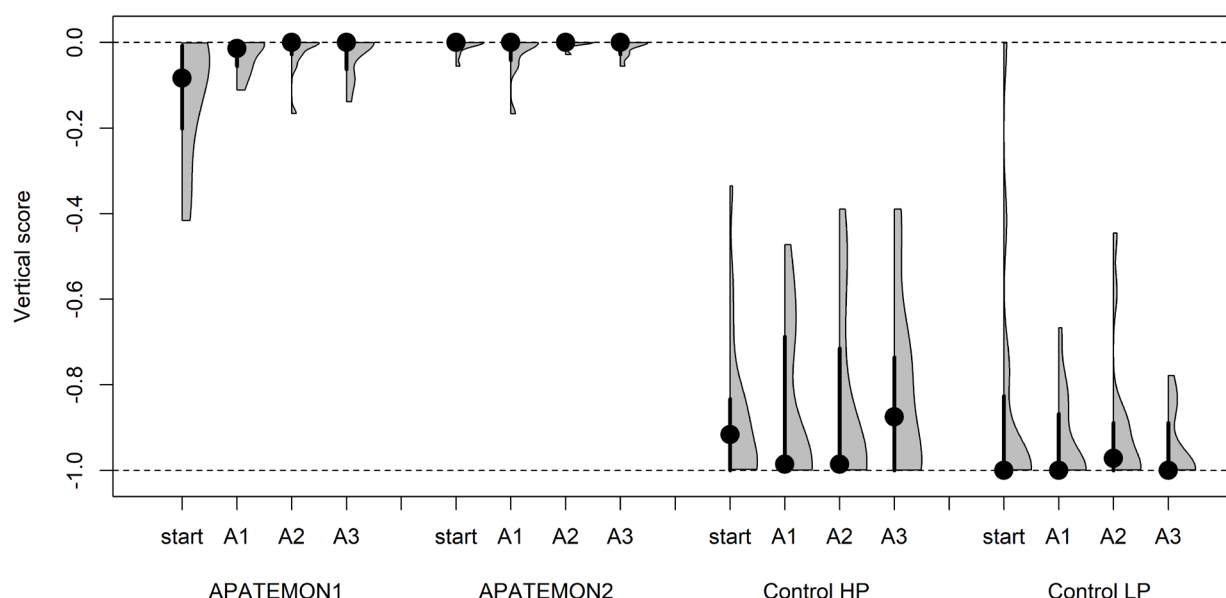
Fig. 2. Vertical score, representing relative distance of the fish from the water surface, expressed as a proportion of the maximum value (ranging from 0 to 1) and multiplied by −1 in order to display surface-dwelling fish at the top. Data collected prior to the simulated attack (Start), and after three successive attacks (A1, A2, A3) are shown. Grey areas represent relative density along the vertical score, full points are median values and solid lines represent inter-quartile ranges.

time was standardised for analytical purposes as $\ln(t − m + 1)$, with m describing the minimum response time measured (i.e. $t = −12$ frames). The effect of population, number of previous attacks and their interaction on the standardised response time was analysed using a linear mixed model (LMM), with individual as a random factor (Table S4). LMMs were validated using a visual check of model residuals (normality, lack of patterns).

For each model, a backward stepwise selection procedure was used to select the best (final) model (Zuur et al. 2009). This procedure was used in order to obtain a single model interpretation. Selection was based on a comparison of AICc (i.e. Akaike information criteria with correction for finite sample sizes) for each pair of nested models. Models with ΔAICc of < 2 were considered as equivalent (Burnham & Anderson 2002), with the simpler model preferred in the selection process (results of log-likelihood tests comparing the two models were used as a supplementary criterion and are presented in the results and tables).

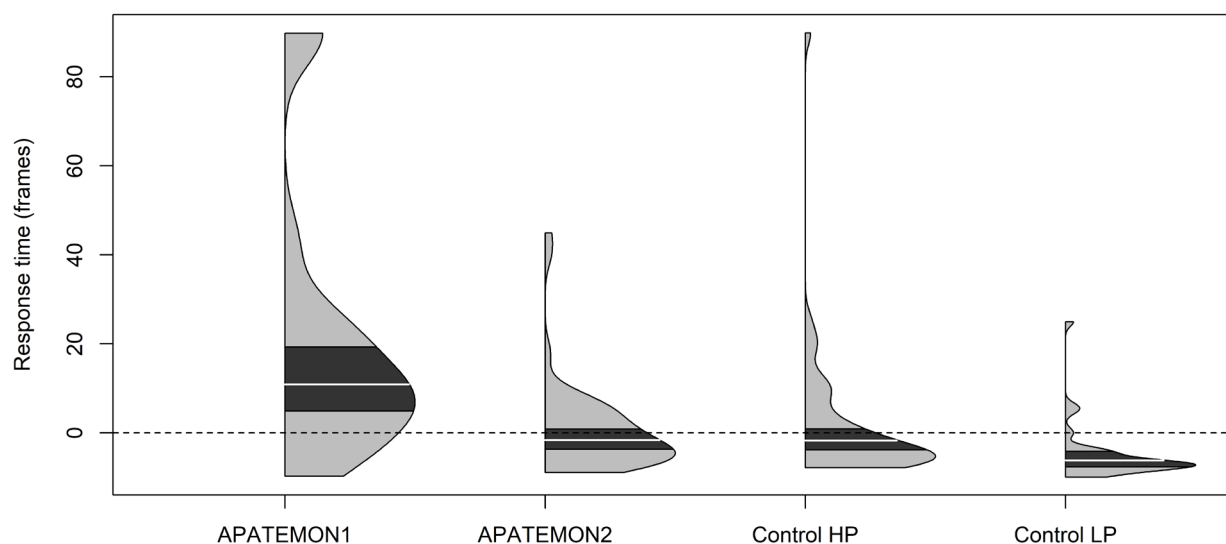All analyses were conducted using R statistical software version 3.5.2 (R Core Team 2018), using

Fig. 3. Response time to a simulated attack for each population. Grey areas represent distribution of response time, while white lines (and dark grey areas) are populations-specific means (and their 95% confidence intervals).
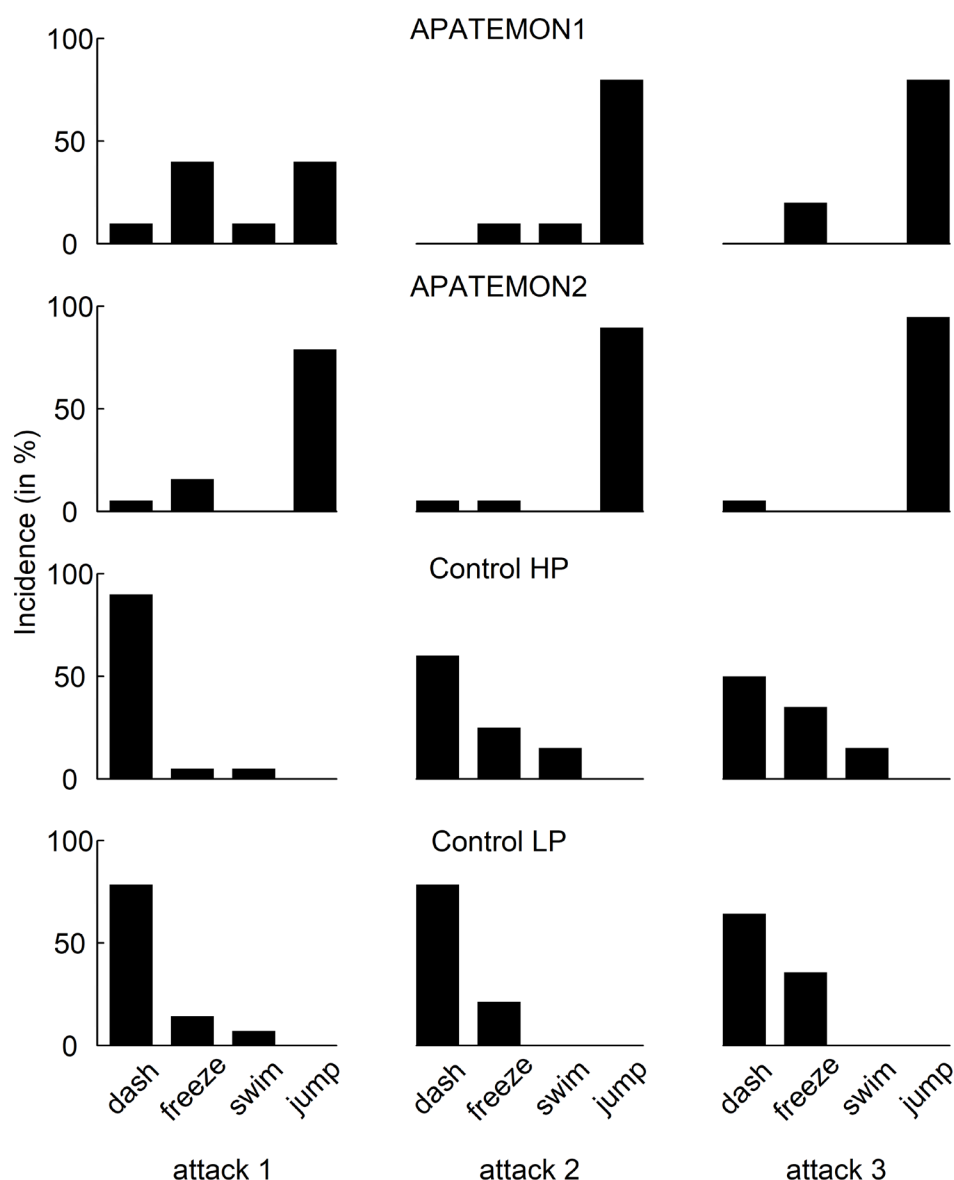
Fig. 4. Population-specific incidence of four types of response to each of three subsequent simulated predator attacks.

the base (R Core Team 2018), betareg (Cribari-Neto & Zeileis 2010), lme4 (Bates et al. 2015), mlogit (Croissant 2019) and MuMIn packages (Bartoń 2018).

Results

Vertical distribution without disturbance

Fish vertical position was affected by population identity only, with fish from the APATEMON 1 and APATEMON 2 populations staying significantly closer to the surface than the control populations (GLM; n = 24 and 30 for APATEMON 1, and 33 and 41 for APATEMON 2 population, all $P < 0.001$; Table S2), with no difference between the two control groups (GLM; n = 34, $P = 0.883$) or between the two *Apatemon*-infected populations (GLM, n = 29, $P = 0.124$). Most of the fish from both APATEMON populations spent the whole observation period near the surface, while most fish from the control populations stayed in the bottom third of the aquarium.

Vertical distribution after disturbance

While some fish apparently responded to the simulated attacks by changing their vertical position (Fig. 1), the differences between the two APATEMON populations and the two control populations remained clear after all three simulated avian attacks (Fig. 2). The shift from starting vertical position and both position after the first attack and position after the third (last) attack differed significantly between the populations (GLMM, both n = 88, both $P < 0.001$). Fish from the APATEMON populations tended to stay closer to the surface after the third attack (GLMM both
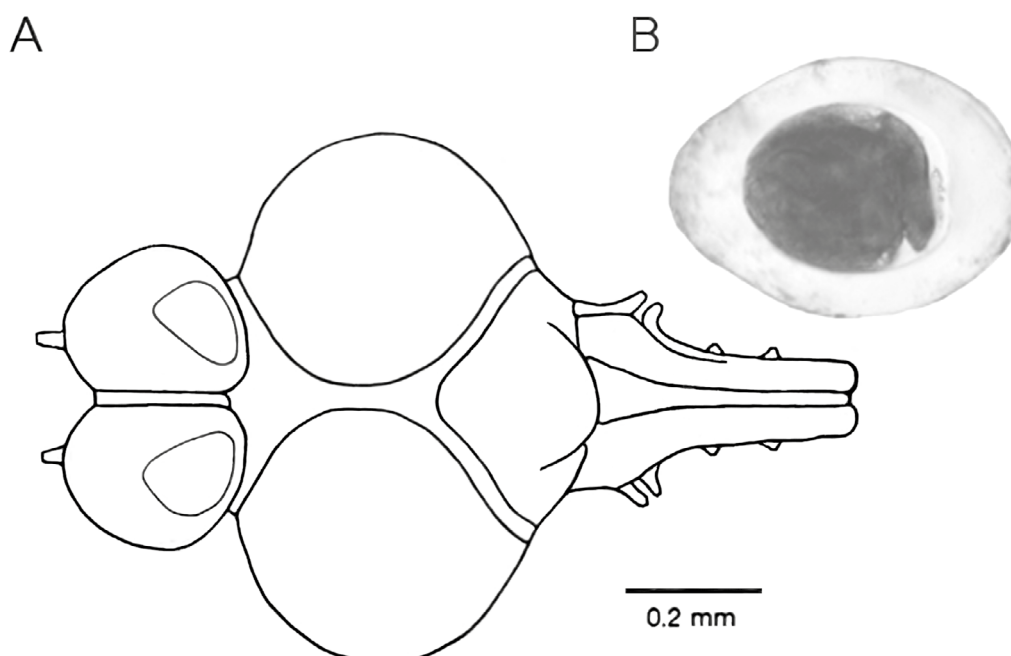
Fig. 5. Schematic representation of the relative proportions of (A) the fish host's (*Nothobranchius furzeri*) brain (redrawn from D'Angelo 2013) and (B) metacercaria of *Apatemon* sp. Both figures to equal scale.

n = 20, both $P < 0.001$). Fish from the APATEMON 2 population slightly, but significantly, lowered their position in the water column after the first attack (GLMM, n = 38 $P = 0.004$; Fig. 1a), but returned to a position that was not significantly different from the starting position after all three attacks (GLMM, n = 38, $P = 0.590$; Fig. 1b). Fish from Control HP population shifted from an almost strictly bottom position after the attacks, with the shift being significant after all three attacks but not after the first attack (GLMM, both n = 40, $P = 0.002$ and 0.287). While some fish from Control HP population reached almost the mid-column position (Fig. 1), the majority of fish stayed close to the bottom and none of the fish reached the surface (Figs. 1, 2). Although there were no significant differences between vertical position before and after disturbance in fish from Control LP population (GLMM, both n = 28, $P = 0.537$ and 0.412), clear individual differences were visible: while fish closer to the surface moved to the bottom position after the first attack and remained there after all the attacks, some fish relaxed their original bottom position to some extent (Fig. 1).

Response time

Response time varied significantly between populations (LMM; n = 155, $P < 0.001$), with no effect of number of previous attacks (LMM; n = 155, $P > 0.05$ for both the 'attacks' term and 'attacks:population' interaction; Table S4). Fish from the APATEMON 1 population were significantly slower in their responses compared with all other populations (LMM; n = 76, 70 and 55, all $P < 0.001$), with the Control LP population being significantly faster in their response than both the APATEMON 2 and the Control HP populations (LMM; n = 79, $P = 0.004$ and 0.005; Fig. 3, Table S4).

Escape response

Differences in killifish behavioural response to a simulated avian attack were mainly apparent in fish from populations infected by *Apatemon* metacercariae. Both inter-population differences (MLM, $P < 0.001$) and number of previous attacks (MLM, $P = 0.008$) affected the type of response (Table S5). Jumping behaviour was only observed in the APATEMON 1 and APATEMON 2 populations (Fig. 4). The probability of the freeze or active fish responses was unaffected by population identity and number of attacks (partial MLM tests, all $P > 0.05$; Fig. 4). The probability of the dash response significantly decreased with increasing number of attacks, resulting in an increased incidence of a freeze response in control populations and jumping behaviour in both APATEMON populations (partial MLM tests, all $P < 0.05$, Fig. 4).

Discussion

Behavioural response to a simulated predator attack was markedly different between African killifish originating from populations with *Apatemon* sp. metacercariae present in the cerebral cavity and

two control populations that were either infected with a similar parasite fauna but lacking *Apatemon* infection, or largely lacking parasites. Fish from two *Apatemon*-infected populations spent most time near the water surface and showed a different response to fish from the two control populations, and responded to simulated avian attack more slowly than fish from the low infection site. All alterations in anti-predator behaviour observed in the *Apatemon*-infected populations appear to increase the probability of the fish being captured by piscivorous birds, thus enhancing trophic transmission of the parasite (Lafferty & Morris 1996). We acknowledge that we did not dissect experimental fish after behavioural trials and their infection status was extrapolated from that of wild fish (which were examined in the field). Most notably, the status of *Apatemon* infection was based on the selection of individuals that performed conspicuous behaviour during their collection and confirmation that those individuals where indeed always infected by *Apatemon* sp. in cranial cavity on a subsample of 10 individuals.

Fish from the APATEMON populations spent most time near the water surface during periods with no disturbance, and this behaviour became even more apparent following simulated predator attacks (Fig. 2), activity that would significantly increase the risk of predation (Kramer et al. 1983). In contrast, fish from the two control populations occupied the lower part of the aquarium, despite the fact that several of the parasite taxa infecting these fish also require a definitive bird host for maturation. In a wide range of fishes, modification of microhabitat choice is frequently associated with infection of diplostomid metacercariae invading the CNS and peripheral sensory organs. Infected fish typically swim closer to the water surface, rendering them more visible to avian predators (Crowden & Broom 1980, Radabaugh 1980). The mechanisms causing swimming at the surface differ among parasite taxa, being related to buoyancy in cestode plerocercoids (Smith & Kramer 1987) or affected vision in eye fluke trematodes (Crowden & Broom 1980). Mechanical pressure on the brain tissue can affect various neuron-regulated processes, such as vision, respiration or locomotion. As the mechanism underpinning this conspicuous behaviour was not investigated in this study, any of these factors may potentially contribute to the behaviour observed in fish from *Apatemon*-infected populations.

The location that parasites occupy within a host can be an important factor in the mechanism of host behaviour modification (Moore 2002, Lafferty & Shaw 2013). Parasites occupying the CNS show relatively low specificity for particular brain regions and may infect areas not currently considered as involved in parasite-induced behavioural changes (Adamo 2013). The metacercariae of *Euhaplorchis californiensis* Martin, 1950 infecting the North American killifish *Fundulus parvipinnis* Girard, 1854 are often located in fish brain tissue in high numbers (several thousands). This parasite modulates serotonin and dopamine metabolism, which is essential for locomotion control (Shaw et al. 2009), resulting in conspicuous behaviour that increases susceptibility to predation by avian definitive hosts (Lafferty & Morris 1996). Compared to *E. californiensis*, which encysts on the pial surface of the brain (Shaw et al. 2009), metacercariae of *Apatemon* sp. are positioned between the host's skull and brain, at no specific location within the cerebral cavity (Nezhybová et al. 2019). Moreover, the metacercariae are relatively large (approx. 500 μm diameter) compared to the fish brain (Fig. 5), suggesting a direct mechanical effect on the host. Considering the size of the fish host (32-55 mm; Table S1), even a low number of 0.5 mm metacercariae might be expected to increase pressure in the cranial cavity, with possible detrimental effects on the brain tissue. Unfortunately, without a detailed study focused on the neurobiological mechanisms induced by the parasite, we were unable to identify the principal mechanism of behavioural alteration and distinguish physical damage to brain tissue from secretion of behaviour modifying molecules.

Once a predator strikes, fish have multiple strategies to maximise their chances of escape. Avoidance behaviour consisting of dashing towards the bottom or escape to cover (active avoidance) and inhibition of movement or 'freezing', preferably under cover (passive avoidance), are thought to be primary avoidance responses in many fish species (Schall & Pianka 1980, Höglund et al. 2005). Indeed 'dash', involving quick and direct escape or quick escape in a series of bursts from the point of interference (Fig. 1), was the most commonly observed response in fish from populations without *Apatemon* metacercariae (Control HP, Control LP). This response is likely to be effective as the movement is unpredictable and can confuse

predators (Schall & Pianka 1980). On the other hand, 'jump' and 'freeze' response modes were most frequently observed in the *Apatemon*-infected populations, with the fish also responding much more slowly compared to fish from Control LP population (Fig. 2), allowing sufficient time for an initially unsuccessful predator to perform a secondary attack. A delayed response to a simulated predator attack, observed particularly in fish from APATEMON 1 population, also indicated decreased fish activity. Consistent with our results, Ness & Foster (1999) found that sticklebacks naturally infected by the cestode *Schistocephalus solidus* (Müller, 1776) appeared to move sluggishly and were less likely to respond to simulated attacks compared to uninfected fish. A marked decrease in the activity of infected fish was also recorded in sheepshead minnow *Cyprinidon variegatus* Lacepède, 1803 infected with the trematode *Ascocotyle pachycystis* Schroeder & Leigh, 1965 (Coleman 1993), and guppies *Poecilia reticulata* Peters, 1859 infected with *Diplostomum spathaceum* (Rudolphi, 1819), this outcome being correlated with an increase in susceptibility to predation (Brassard et al. 1982).

Jumping out of the water, uncoordinated movement and rolling of the body in the upper water layer were all typical responses to simulated avian attack in fish from the *Apatemon*-infected populations. In contrast, fish from the control populations responded to simulated predation by escape and protracted hiding. Under natural conditions, disturbed fish in *Apatemon*-infected populations jumped from the water and were frequently stranded on floating water lily pads, greatly facilitating location by predators at the sampling sites (V. Nezhybová, R. Blažek; pers. observ.). Infection-associated modifications in host swimming, described as being "conspicuous" to human observers (hence, probably to other predators), are likely to be important mechanisms facilitating detection and selection by predators, thereby significantly increasing the probability of parasite transmission to a possible definitive host (Lafferty & Morris 1996). For example, erratic circular swimming behaviour has been documented in minnows *Phoxinus phoxinus* (Linnaeus, 1758) infected with brain-encysting *Diplostomum phoxini* (Faust, 1918) (Rees 1955). Metacercariae of *D. phoxini* aggregate at specific brain regions controlling the host's anti-predator response (Barber & Crompton 1997, Dezfuli et al. 2007), with all fish hosting > 600 metacercariae in the brain and neurocranium displaying conspicuous behaviour. In comparison, prominent behaviour, including uncoordinated movement near the surface and jumping, has been observed in killifish infected by just four *Apatemon* sp. metacercariae (V. Nezhybová, pers. observ.). While this effect may reflect the larger size of *Apatemon* metacercariae compared with those of *D. phoxini*, the mechanisms causing behavioural changes may also be different. Irrespective of mechanism, such behaviour likely increases the probability of detection of a fish by predators.

Owing to the ephemeral character of killifish habitats, typified by high turbidity limiting fish detection and the short lifespan of the fish host (Reichard et al. 2009), any increase in the probability of transmission to the definitive host could be beneficial to the parasite. Many parasitic species have evolved the ability to control their host's behaviour order to enhance their transmission (Moore 2013). Occupation of the fish cerebral cavity has previously been reported for metacercariae of a few *Apatemon* species, but with no behavioural alterations detected thus far (Zhokhov et al. 2008, Blasco-Costa et al. 2016). However, in these cases the fish hosts (i.e. cyprinid, cichlid or galaxiid fishes) have a longer lifespan than nothobranchiid killifish, in which lifespan is limited to just a few weeks or months (Vrtílek et al. 2018). As a result, there may be unusually strong selective pressure on the parasite for rapid transmission to its definitive host before the killifish dies due to habitat desiccation. In this case, the host's life history may represent an important driver in the evolution of behavioural manipulation. All parasites found in the killifish populations used in this study were at larval stages, most of them being allogenic (i.e. using birds or other terrestrial animals as definitive hosts) trematode metacercariae. Thus, conspicuous host behaviour enhancing the probability of transmission to suitable final hosts could be equally beneficial for these other parasites, making co-infection with *Apatemon* beneficial for their own transmission.

To conclude, our study provides evidence of conspicuous risky behaviour in *N. furzeri* killifish from a locality with presence of the newly described African lineage of the trematode *Apatemon* sp. While the use of fish from different populations with diverse natural parasite infections is considered a limitation to our experimental design (raising the possibility that factors other than

parasites contributed to the behaviour observed), there are three indicators that lead us to assign the presence of *Apatemon* sp. metacercariae in the brain cavity as the most probable factor causing the change in behaviour. First, during field investigations all fish exhibiting unusual behaviour hosted metacercariae of *Apatemon* sp., while this parasite was never observed in normally behaving individuals from the same population. Second, after completing behavioural experiments, several fish from the *Apatemon*-infected population exhibiting conspicuous behaviour were used for infection of domestic ducklings in order to acquire the adult stage of *Apatemon* sp. trematodes. This procedure proved successful (Nezhybová et al. 2019), confirming presence of the parasite in experimental fish (though intensity of infection was not determined). Third, the contrasting behaviour we observed was comparable across two *Apatemon*-infected and two control, *Apatemon*-free host populations, strengthening our proposal that *Apatemon* sp. rather than other factors caused a conspicuous behavioural response to simulated predator attack. However, we also acknowledge that direct causality can only be confirmed through experimental infection.

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Supplementary online material

Table S1. Number (N) of fish used in the behavioural experiments. SL = fish standard length in mm.

Table S2. Model selection steps for prediction of fish vertical position using GLM. Models with ΔAICc < 2 were considered as equivalent and a simpler model was preferred in these cases. *P* represents the *P*-value of the term to be removed in the next step.

Table S3. Simplified model selection statistics for predicting shifts in fish vertical position after simulated predator attacks, i.e. between starting position and that after the first attack (a) and between starting position and that after all attacks (b), using GLMM. Models with ΔAICc < 2 were considered as equivalent and a simpler model was then preferred. *P* represents the *P*-value of the term to be removed in the next step. All models included the term "individual" as a random factor.

Table S4. Model selection steps for predicting time from response using LMM. Models with ΔAICc of < 2 were considered as equivalent, with the simpler model preferred. *P* represents the *P*-value of the term to be removed in the next step. Attacks stand for number of previous attacks. Term "individual" was included as random in each model.

Table S5. Model selection steps for predicting the incidence of four distinct response types (jump, dash, swim, freeze) to simulated predator attacks, using MLM. Models with ΔAIC of < 2 were considered as equivalent, with the simpler model preferred. *P* represents the *P*-value of the term to be removed in the next step. Attacks stand for number of previous attacks.

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