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Haemosporidian infections influence risk-taking behaviours in young male blackcaps, *Sylvia atricapilla*

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Animal behaviour becomes essential to the dynamics of parasitism if some behaviours favour pathogen transmission or increase exposure to infection. Infections may also influence host behaviour when health and future fitness are compromised, which predicts infected individuals will adaptively change risk-taking behaviours. We studied whether haemosporidian infection influences exploratory, foraging and antipredator behaviours of male young blackcaps before their first migration. The study was conducted in captivity using subjects of a medication experiment with the antimalarial drug primaquine, which had temporarily cleared parasite blood stages of treated individuals 1 month before. In an initial exploration test in a cage unknown to the birds, infected birds started exploring earlier than uninfected ones. Risk-taking behaviours were further assessed in a sequence of tests starting with the opening of new feeders to induce a startle response, and continuing with simulations of increased predation risk. We first challenged birds with acoustic cues of predation risk by playing recorded conspecific alarm calls, using heterospecific song as a control for the reaction to sound. Then, we challenged birds with visual cues of risk, showing them a taxidermic sparrowhawk and a bottle as a control for the reaction to an unspecific threat. Uninfected birds showed appropriate sentinel behaviour, turning around more frequently in the presence of the sparrowhawk compared to the bottle, while infected birds tended to behave similarly when faced with both stimuli, a behavioural difference that was driven by individuals with single parasite infections. Throughout the trial, infected medicated individuals tended to alarm-call more often than infected unmedicated individuals, a weak effect of medication which was not observed in parasite-free birds. Our results show that haemosporidian infections can influence blackcap risk-taking behaviours and support the view that host behavioural repertoires are broadly associated with parasitism.

Decisions that animals make when breeding, feeding or facing predators are aimed at maximizing their fitness (Lima & Dill, 1990), but the same behaviour can have both advantages and disadvantages. For example, being bold and exploring quickly may ensure priority access to good foraging or breeding patches, at the cost of increasing exposure to natural enemies like predators and parasites (Carter et al., 2010; Jones & Godin, 2010). Furthermore, coping behaviours, that is, animals' reactions to challenging or threatening situations such as the exploration of novel environments or predation risk, are energy demanding (Koolhaas et al., 1999) and may be influenced by a trade-off between current and future fitness (Wolf et al., 2007). Riskier behaviours may be promoted to improve fitness when individual life expectancy is reduced (Dingemanse et al., 2009; Nicolaus et al., 2012). Hence, predators, parasites or food availability are ecological agents constraining behavioural alternatives, because they might influence life expectancy by influencing either individual health or the resources on which it relies (Réale et al., 2010).

Parasitism plays a major ecological role through its relationships with behaviour (Barber & Dingemanse, 2010) and life history traits (Poulin, 2007). By depleting the host's resources, parasites impair its condition (Bonneaud et al., 2004) and consequently its ability to perform costly behaviours (Spencer et al., 2005). However, parasite infections may be not only the cause but also the consequence of certain behaviours. Some individuals may be more exposed to infections than others if their social, dispersal or exploratory behaviours increase parasite encounters (Boyer et al., 2010) or if their physiological response to stressors such as predation risk weakens their immune function (Navarro et al., 2004). Hence, host behaviour is a key factor influencing parasite spread, host competence or susceptibility to infection (Barron et al., 2015; Hawley et al., 2011).

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Avian haemosporidians are widespread vector-borne parasites that affect most bird species, typically developing into chronic infections that follow seasonal dynamics with temporal recrudescence of parasites in circulating erythrocytes and periods of latency in the host's internal tissues (Valkiūnas, 2005). Several studies have found that chronic infections may reduce survival and breeding success of birds (Asghar et al., 2015; Merino et al., 2000). However, how these parasites influence behavioural traits has received less attention, with contrasting results for different species. For example, infected individuals increased risk-taking behaviours compared to uninfected ones in house sparrows, *Passer domesticus*, and nightingales, *Luscinia megarhynchos* (García-Longoria et al., 2015; Marinov, Zehtindjiev, et al., 2017), decreased them in yellow wagtails, *Motacilla flava* (Marinov, Marchetti, et al., 2017) and did neither in collared flycatchers, *Ficedula albicollis* (Garamszegi et al., 2015). These results suggest that the influence of haemosporidian infection on risk-taking behaviours might be species specific or context dependent. To shed light on the issue, studies should ideally be done in common environments to minimize condition-dependent outcomes other than those associated with parasites, combining experimental manipulation of parasite load with controlled simulation of increased risk.

We tested whether haemosporidian parasites influence coping behaviours in first-year male blackcaps, in exploratory, foraging and predation risk challenges. We manipulated perceived predation risk by playing conspecific alarm calls and showing a predator, where acoustic and visual cues of predation risk simulated increasing levels of threat. Individual infection was determined in an experiment conducted shortly before the behavioural tests, where parasites were temporarily removed from the bloodstream in some birds with an antimalarial drug (Arriero et al., 2018). Although the medication experiment was not specifically designed to assess the effect of parasites on blackcap behaviours (Arriero et al., 2018), it provided valuable context to our study by experimentally altering individual trajectories of host–parasite interactions before we measured behaviours. Thus, not only could we compare in a controlled environment the risk-taking behaviours of individuals that were infected or not, or hosted none, one or more parasite types, but we could also investigate whether temporarily supressed parasitaemia has carry-over effects on risk-taking behaviours measured later in life. Similar antimalarial treatments have improved reproductive performance (Merino et al., 2000) and life expectancy of infected birds (Asghar et al., 2015; Martínez-de la Puente et al., 2010).

Parasites reduce life expectancy and consequently an animal's residual reproductive value (Poulin, 2007). Therefore, state-dependent personality models predict increased risk taking with infection (Réale et al., 2010). We predicted that infected individuals that were temporarily relieved of blood parasites would show fewer risk-taking behaviours and greater antipredator responses than unmedicated, infected birds, as would uninfected birds. Furthermore, we predicted a gradual escalation of these behaviours from parasite-free to multiple-infected individuals, as coinfection increases the cost of parasitism compared to single infection.

METHODS

Ethical Note

Birds were captured with mist-nets; we played blackcap song recordings to increase capture rate. We put young individuals into individual transport cages (20.5 × 11 cm and 17 cm high) with food spread around the cage and a sponge soaked in water for hydration. We deemed individuals tolerant to captivity if they ate normally shortly after capture (Bocetti, 1994). Birds were transported to the aviary in the cages which were covered with a cloth (Bocetti, 1994; Fair et al., 2010). We monitored all individuals each day, recording the amount of food eaten, the quantity, colour and consistency of faeces and the presence of any anomalous behaviour. Birds were handled by observers with at least 8 years of bird-handling experience. The research was carried out with approval from the Committee on Animal Testing of the Complutense University of Madrid (licence n° 103/2012) and Comunidad de Madrid, which authorized all handling and sampling procedures. Birds were captured in their housing cages with a small net and transported in a cotton bag to the test room, which was located in the same building. We used a web camera to monitor all the behavioural trials from an adjacent room without interfering with the test. After the behavioural tests, birds were returned unharmed to the aviary where they were offered fresh dates to motivate immediate feeding. Most birds quickly ate and no anomalous behaviour was observed. Blackcaps were released 1–2 days later at the site of capture, and they increased their body mass by 0.74 ± 0.45 g during this period.

Field Sampling and Antimalarial Treatment

The blackcap is a songbird species common and widespread throughout Europe, with abundant background information about its biology and haemosporidian parasite interactions (Aymí et al., 2020). We used male subjects from a previous parasite manipulation experiment aimed at examining individual physiological variation involved in resistance and tolerance mechanisms against disease (Arriero et al., 2018). Young blackcaps captured in summer 2012 in central Spain were kept indoors in individual cages (45 × 30 cm and 40 cm high), with natural photoperiod, ambient temperature and access to fresh water. Birds were fed ad libitum with a varied diet including mealworms, fresh fruits and commercial food for insectivorous birds (Patée con insetti, Raff). Water was changed and the cages cleaned of faeces and food remains daily, and feeders, water troughs and other cage components were deep-cleaned weekly (Fair et al., 2010). Young birds (born in spring 2012) were selected to homogenize individual histories of host–parasite interaction, ensuring that all infected birds had acquired parasites during the postfledging life stage in the same place, with little opportunity for parasite clearance and reinfection altering individual infection trajectories. Six days after capture, individuals were given an antimalarial treatment (an oral daily dose of 0.04 mg of primaquine, SIGMA, St Louis, MO, U.S.A.; henceforth PQ) or water during 14 consecutive days, which resulted in four groups in relation to infection status (infected or not) and treatment (medicated or not). Medication efficacy was assessed by analysing blood samples collected at three times: capture, at the end of the treatment and 21 days post-treatment. Parasitaemia was suppressed by primaquine: of 30 infected birds, 18 were medicated and their parasitaemia was much lower than that of unmedicated individuals at the end of the treatment, with seven of these birds having no parasites detectable by qPCR at that time. However, recrudescence was observed in 16 of the 18 medicated birds and four of 12 unmedicated birds, and infection intensity of medicated and unmedicated individuals converged to similar levels 21 days post-treatment (see Appendix 1, Fig. A1). Physiological changes during the experiment are described in Arriero et al. (2018). Behavioural tests were conducted in September, 29.91 ± 1.48 (range 25–33) days post-treatment. Our previous experiment included 46 males and 18 females (Arriero et al., 2018), which would reduce within-cell sample sizes should we include sex as a classification factor. Therefore, we did not measure behaviours of females and released them immediately. Sacrificing the analysis of sexual differences in behaviour not to compromise the reliable estimation of infection by treatment effects with small sample sizes

prevented us from generalizing our conclusions to all individuals of the species.

Infection Status and Parasitaemia

Infection was determined by a combination of molecular and microscopic techniques. We extracted total DNA from blood using a DNeasy Blood & Tissue Kit (Qiagen), which was used to test for parasite presence in blood using different nested PCR protocols that amplify the MalAvi DNA barcode, a fragment of 479 base pairs in the cytochrome *b* gene of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* parasites (Bensch et al., 2009). The method involved a preamplification PCR in a total volume of 25 μl, with 25 ng of total genomic DNA, 1.25 mM of dNTP, 1.5 mM $MgCl_2$, 10 μM of preamplification primers and 0.5 units of AmpliTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The thermal profile involved a denaturation step of 94 °C for 3 min, 20 amplification cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C and a final extension step for 10 min at 72 °C. We used 1 μl of the PCR product as the template for the second PCR, with the same reaction conditions during 35 amplification cycles. Several negative and positive controls were included in each PCR plate (negative controls never produced positive results). We visualized 4 μl of the final PCR product on a 2% agarose gel stained with GelRed Nucleic Acid Stain. We started screening samples with primers HaemNF1/HaemNR3 in the preamplification step, followed by HaemF/HaemR2 to amplify *Haemoproteus* and *Plasmodium*, or HaemFL/HaemRL for *Leucocytozoon* (Hellgren et al., 2004). PCR products were precipitated with ammonium acetate and ethanol and sequenced with a dye terminator AmpliCycle sequencing kit and an ABI PRISM 3700 sequencing robot (Applied Biosystems). When we could not sequence the products obtained with these primer sets due to poor amplification, alternative primers Plas1F/HaemNR3 (preamplification) and 3760F/HaemJR4 (Pérez-Rodríguez et al., 2013) were used with the same PCR conditions, which in some cases helped to retrieve parasite DNA sequences.

To assess the efficacy of the medication treatment, we quantified parasite loads in blood samples using real-time quantitative PCR (qPCR) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). We used primers 343F and 496R to amplify 154 nucleotides of parasite RNA-coding mitochondrial DNA (Fallon et al., 2003), and quantified total DNA using primers sfsr3Fb and sfsr3Rb to target an ultraconserved nuclear sequence of birds (Asghar et al., 2015). Reactions were done in 10 μl volumes including 2.5 μl DNA template (1 ng/μl), 2.5 μl primers (1.2 μM) and 5 μl of FastStart Universal SYBR Green Master (Rox, Roche Diagnostics, Indianapolis, IN, U.S.A.). Thermal cycles included an initial incubation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 57 °C for 1 min. Each DNA sample was run in duplicate, and the average value was used for further analysis. Standard curves were produced by diluting the samples in four 10× dilution steps (2.5–0.0025 ng of DNA per well). Standard curves were around 95% qPCR efficiency. We did not analyse the relationship between infection intensity and behaviour because parasitaemia is very dynamic and intensity estimates obtained 9 days before behavioural tests could not reliably capture between-individual variation in current parasite load (parasitaemia measured by qPCR changed during the 21 days post-treatment, leading to low individual repeatability: $r_i = 0.031$, $P = 1$ for birds not treated with primaquine).

We visually searched for parasites in Giemsa-stained smears taken from each blood sample, inspecting ca. 2000 erythrocytes at 1000× magnification with a Leica 2500 DM light microscope. PCR methods are more sensitive at detecting infections than microscopy, but a combination of both methods is always advisable (Valkiūnas et al., 2006). All microscopy positives in our study were confirmed with molecular methods, and no PCR negative blood samples turned positive by microscopy.

Individuals that tested positive in any blood sample, either by nested PCR, qPCR or microscopy, were considered infected. We classified infections as single or multiple based on the minimum number of unique parasites identified during the whole history of parasite detections of each individual. Parasite lineages were considered distinct if they differed from other lineages by at least 1 bp at the MalAvi DNA barcode (Pérez-Tris et al., 2007). For analyses involving multiple status of infection, we considered that birds with mixed sequence signals in sequencing electropherograms (Pérez-Tris & Bensch, 2005), and birds with at least two parasite lineages retrieved from blood samples (even if samples taken at different times produced unique sequences), harboured multiple infections. We considered that birds had single infections when the same unique parasite was observed in all blood samples. In these analyses, we excluded three of 30 infected birds with too low a parasite load to allow us to sequence the parasite in all samples, which made determining multiple status of infection unreliable.

Behavioural Tests

Test room set-up

Birds were individually placed in a novel cage (67.5 × 34.0 cm and 35.0 cm high) located in the centre of a homogeneous 250 × 176.5 cm test room inside an indoor facility (Appendix 2, Fig. A2). The cage was divided in half by an opaque plastic sheet with a 15 × 9 cm open window in its centre, with a perch on the lower side of the window. Two perches were located in each section, in a mirrored position, to facilitate bird movement. A wooden feeder with a movable lid painted in red and white stripes was placed in each corner of the cage. This colour pattern was new to the birds. A 35 × 42 cm window visible from both sides of the cage was placed on two opposite walls of the test room. The windows could be opened by the observer by moving a curtain made of the same fabric as the test room, to reveal either a taxidermic sparrowhawk, *Accipiter nisus*, that represented a major blackcap predator (Mason, 1995), or a bottle as a control of the reaction to any potential threat. These two stimuli, presented on different windows to each bird, allowed us to distinguish responses to disturbance from responses to a predator (Fransson & Weber, 1997; Gentle & Gosler, 2001). The positions of the sparrowhawk and the bottle were randomized between trials. Three cameras (one overhead and two lateral) were placed outside the cage to record bird activities during the tests. All behavioural trials were carried out in the morning (allowing us to test two to five birds per day) and individuals were tested in random order with respect to infection and treatment after approximately 1 h of fasting to encourage foraging (mean ± SD = 78.49 ± 12.85 min). The same observer (C.R.) collected all the behavioural data from the videos and was blind to the experimental design.

Test sequence

We put the bird in the test cage in the dark, using a red light headlamp to handle the bird safely without initiating the test conditions too early. A 5 min open-field test was initiated at time 0, when the light was turned on and the bird found itself in a new environment, as both the cage and the background were unknown to it. The behaviours recorded during this time were considered exploratory (Réale et al., 2007). Four minutes after the end of exploration, a sequence of tests with increasing risk started with the opening of the feeders aimed to challenge birds with the opportunity to feed from an unknown source, continued with playback of conspecific alarm calls as a sign of increased risk perceived

from information from other individuals, and ended with exposure to a predator model as a threat directly perceived by the individuals (see the sequence and timing of all events in Fig. 1). The opening of the feeders induced a startle response, and therefore foraging-related behaviours may be considered indicative of an individual's risk assessment. Seven minutes later, we assessed the bird's responses to acoustic cues and then to visual cues. The acoustic stimulus consisted of playing back an alarm call, a low-pitched meow that blackcaps produced when we entered the aviary for housekeeping and induced immediate freezing and silence of all birds for a few seconds (henceforth conspecific alarm call). The blackcap alarm call lasted 1.3 s and was recorded in the aviary with a Marantz CP430 stereo three-head portable cassette recorder, using an Optimus CM810 super cardioid condenser microphone and TDK CDing-II tape, and digitized in MP3 format. A 0.7 s phrase from a song recording (Roché, 1993) of a Bonelli's warbler, *Phylloscopus bonelli*, a common sound in the area where the blackcaps had grown up, was used as a control stimulus, with a 10 min interval between the two stimuli played in random order. Vocal recordings were played using a SONY CMT-NEZ50 High Fidelity system, setting volume at level 16 with a SS-CNEZ50 loudspeaker located close to the cage out of the bird's sight (Appendix 2, Fig. A2). This set-up reproduced the test stimuli at volumes that were recorded by camera C2 (Appendix 2, Fig. A2) between −44.59 dBFS (decibels relative to full scale) for Bonelli's warbler song and −34.81 dBFS for the blackcap alarm call, as measured with the RMS function in Audacity software (https://audacity.es/, version 2.4.2). These loudness levels were within the range of the calls produced by the blackcaps recorded in our videos (for example −32.57 to −45.27 dBFS measured from one individual). Ten minutes after the second acoustic stimulus, the visual component of the trial started. Birds were presented with a taxidermic sparrowhawk for a minimum of 25 s to simulate a visually perceived predation risk or a plastic bottle that was used as a control stimulus (range 25–44 s, average ± SD = 29.39 ± 3.57 s; variation was due to speed of operation of the curtain). The stimuli were presented 10 min apart and the order of presentation was randomized. We used the same stimuli (conspecific and heterospecific vocal recordings and sparrowhawk and bottle) to challenge all birds. Between the end of exploration and the opening of the feeders, a song playback test was performed displaying conspecific or heterospecific (chaffinch, *Fringilla coelebs*) songs as a part of a concurrent study.

Behavioural variable recording

The timeframe during which behaviours were measured varied with the test (Fig. 1). For exploration, we measured behaviours during the first 5 min of the bird's stay in the test cage. For acoustic and visual challenges, immediate responses were assessed by measuring behaviours during the timeframe the stimulus was active (0.7–1.3 s for acoustic cues and 25 s for visual cues). Delayed responses were assessed by comparing 2 min of behaviours after the end of the acoustic or visual stimuli with the reference behaviour measured during the previous 2 min.

For the video analysis, the cage was divided into 15 different positions to locate all bird movements (Appendix 2, Fig. A3). On each section we distinguished perch 1, perch 2, floor, ceiling and three lateral cage walls. The 15th position was the perch on the window that connected both sections. Similar to other studies (Dingemanse et al., 2002; Verbeek et al., 1994) we recorded the time, frequency and location of all movements between different positions and within-position behaviours (body turns) to infer variables associated with exploratory or antipredator behaviour. We also tried to identify subtle and overt antipredator behaviours displayed immediately after acoustic conspecific alarm or visual predation presence stimuli. Birds usually alarm-call in risky situations (Hollén & Radford, 2009), and blackcaps are readily spotted in the field by loud 'tac' calls, which are contact vocalizations that birds produce at a higher rate when disturbed by rivals or predators (Aymí et al., 2020; Grim, 2008). Blackcaps never produced meow alarm calls during our tests, but some individuals produced 'tac' calls, which were also annotated throughout the experiment. We identified two overt antipredator behaviours when the sparrowhawk model was present: crown raising and alarm calling. Nevertheless, we could only analyse crown raising in this context, because only six individuals produced alarm calls when faced with the sparrowhawk.

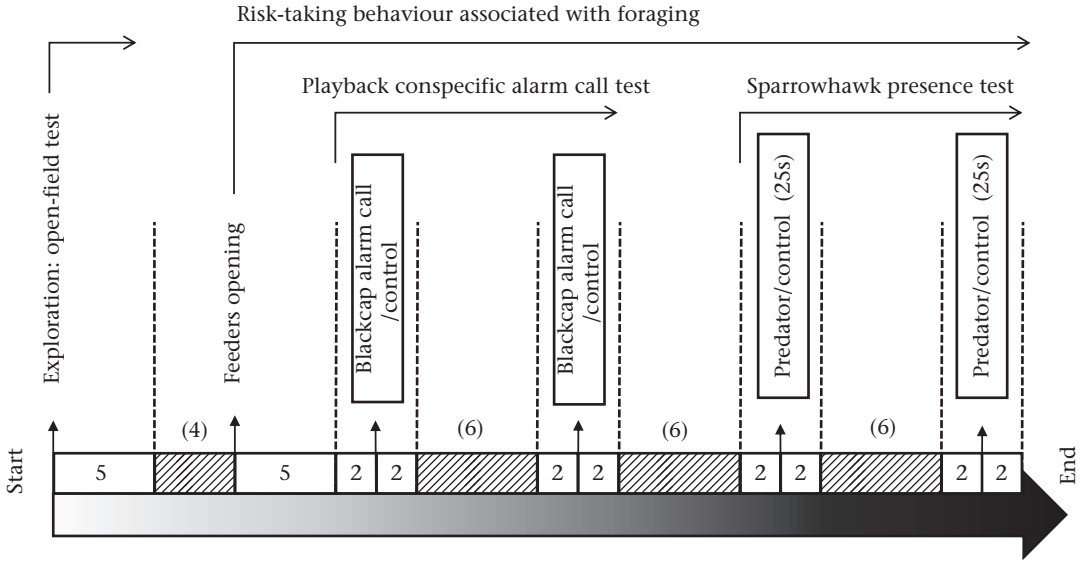

Figure 1. Sequence and timeframe of each behavioural test, from the first open-field test to assess exploration to the last sparrowhawk presence test to assess predator risk assessment. Vertical arrows indicate the activation of the different stimuli associated with each test; open boxes represent timeframes (5 min/2 min) during which behavioural responses to each test were measured and hatched boxes represent intervals (4 min/6 min) between tests. Horizontal arrow at the bottom indicates that tests were increasing in risk (from entering a new environment to seeing a predator) along the experimental trial.

Latency. During exploration, we used the time to first change of position from time 0 as 'initial exploration latency' and the first time birds crossed to the other section of the cage through the window as 'latency to cross'. To assess speed of exploration we also recorded the time to move to newly visited cage positions with a maximum of six positions, including initial latency (13 of 40 birds did not explore six different positions during the exploration test). Otherwise, we used the time from the presentation of each stimulus (acoustic and visual) until the first change of position as 'initial antipredator latency'. As we aimed to induce freezing behaviour in birds with the low-pitched conspecific alarm call, we did not measure initial antipredator latency of any bird that did not move during the 2 min prior to the acoustic stimulus (final $N = 27$). For individuals that did not move after a test we used the maximum time in which behaviours were measured during the corresponding test.

Activity. Factor scores from a principal component analysis (PCA, see Appendix 3) were used as an index of activity to summarize initial latency, number of movements, number of body turns and number of different positions during the timeframe of each test. We could not count latency in delayed behaviours for visual predation stimuli.

Relative body turns. In birds with lateral eyes, turning the head or the whole body may increase the field of vision (Fernández-Juricic, 2012; Tellería et al., 2001), and sentinel behaviours have been described that consist of surveying the area by turning around on a prominent perch (McQueen et al., 2017; Yasukawa & Cockburn, 2009). Blackcaps in our experiment frequently displayed body turns while perched or moving between perches. To assess the motivation to turn around as a proxy of vigilance behaviour independent of individual activity level, we used the residuals of a linear regression of the number of body turns on the total number of movements.

Alarm calls. We counted alarm calls produced by the bird during the experiment. For five of 3379 call-like sounds we needed to visualize spectrograms with the software Audacity to tell real calls from noise. As few individuals alarm-called ($N = 16$) we only analysed whether a bird did or did not produce alarm calls.

Risk-taking variables associated with foraging. We recorded whether birds ate or did not eat and, for birds that did eat, the latency to eat and the number of pecks from the opening of the feeders until the end of the trial.

Statistical Analyses

We analysed the influence of haemosporidian infection on each behaviour with general linear models (GLM) or generalized linear models with binomial error and logit link function depending on whether the behavioural variable was continuous or dichotomous (see Table 1 for tests used in the analyses of each variable). We analysed categorical variables with empty cells in the frequency table with exact logistic regression based on a Markov chain Monte Carlo algorithm (King & Ryan, 2002), using the R package elrm (Zamar et al., 2007).

Table 1
Summary of statistical analyses and sample size for each behavioural parameter assessed in each test

Behaviour (dependent variable)	Model	*N*
Exploration of new environment		
Activity level	General linear model	39
Relative body turns	General linear model	39
Log (initial latency)	General linear model	39
Log (latency to cross)	General linear model	39
Speed of exploration	Repeated measures ANOVA; within-subject factor = order of the newly visited cage positions	39
Foraging on a new food source		
Eating (yes/no)	Binomial regression model	43
Latency to eat for eating birds	General linear model	25
Log (pecks) in eating birds	General linear model	24[a]
Risk perceived from audible conspecific alarm call		
Freezing behaviour	Binomial regression model/exact logistic regression model	43
Activity (yes/no)	Generalized mixed model with binomial logit link with bird identity as random factor; within-subject factor = type of stimulus and moment	43[b]
Activity level	Repeated measures ANOVA; within-subject factor = type of stimulus and moment	25[b,c]
Relative body turns	Repeated measures ANOVA; within-subject factor = type of stimulus and moment	43
Log (initial latency)	Repeated measures ANOVA; within-subject factor = type of stimulus and moment	27[c,d]
Visible predator threat (sparrowhawk presence)		
Crown raising	Binomial regression model/exact logistic regression model	42
Activity level	Repeated measures ANOVA; within-subject factor = type of stimulus (+ moment for delayed responses)	42
Relative body turns	Repeated measures ANOVA; within-subject factor = type of stimulus (+ moment for delayed responses)	42
Log (initial latency)	Repeated measures ANOVA; within-subject factor = type of stimulus	42[c]
Alarm calling during the risk-taking trial		
Alarm calling (yes/no)	Binomial regression model/exact logistic regression model	42

The general formula of the most complex fixed part of the models included the terms infection, treatment and their interaction. Within-subjects or random effects are described when applicable. Sample size differed between tests due to technical issues during the trial, or because some behaviours could only be measured in a subset of birds (e.g. number of pecks of the birds that ate).

[a] A technical failure at the end of the test interrupted recording behaviour for one of the birds that ate.

[b] High frequency of individuals remaining immobile during the playback conspecific alarm call test made bird activity distribution too skewed to meet general linear model assumptions. Therefore, we dichotomized the variable (active or inactive), and activity level was only assessed as a continuous variable in active birds.

[c] Sample size was further reduced due to the influence of the order of stimuli (Appendix 4).

[d] Birds that moved in the 2 min prior to the stimulus.

In all models we included the infection status, treatment (PQ or water) and their interaction as fixed factors. If the interaction between infection and treatment was not significant, we tested simple effects. Infection status was tested in different models as the binary status of infection (infected or not) or considering the multiple status of infection, with three levels: uninfected, single infection or multiple infection. Post hoc comparisons were performed when the main effect or interactions were significant using Bonferroni (for restricted contrast in repeated analyses) or Tukey (all pairwise comparison) corrections to account for the increase in likelihood of type I error with multiple tests. We also applied the false discovery rate (FDR) correction for multiple hypotheses testing (Benjamini & Hochberg, 1995) when several behaviours were tested with data obtained from the same behavioural test (exploration, foraging, conspecific alarm call and sparrowhawk presence) and with our two approaches to the status of infection, binary or multiple. Nevertheless, corrections may increase the probability of type II error (Garamszegi, 2006; Nakagawa & Cuthill, 2007), particularly so in experimental studies that minimize sample sizes for ethical reasons, a circumstance that we took into account when we interpreted our results. In tests involving within-individual measurements, repeated-measures analysis of variance (ANOVA) was applied with the R afex package (Singmann et al., 2022) and ges parameters (generalized eta squared, η^2_G) are reported to indicate the effect size of the main effect and interactions (Bakeman, 2005). To assess speed of exploration we included the sequence of newly visited cage positions from the first to the sixth as the levels of a within-subject factor. In conspecific alarm call tests and sparrowhawk presence tests, type of stimulus (risk or control) and moment (before or after, only for delayed responses to acoustic or visual cues) were included as levels of a within-subject factor. To assess whether the probability of showing context-appropriate antipredator responses varied with infection and treatment, we dichotomized freezing behaviour in the conspecific alarm call test, and crown-raising behaviour in the sparrowhawk presence test. In these tests, risk and control stimuli helped to distinguish between appropriate and inappropriate responses given the actual levels of threat simulated by risk and control stimuli. Thus, while responding to control stimuli may be biologically meaningful if heterospecific singing or the presence of a novel object convey information about potential threats, in terms of modulating antipredator responses according to actual threat levels these behaviours are more akin to not responding. Therefore, in these tests birds scored 1 if they showed antipredator responses only to the conspecific alarm call or the predator, respectively. They scored 0 if they did not respond to either stimulus, responded to the control alone or responded similarly to both stimuli.

We first assessed the efficacy of the acoustic or visual stimuli used to elicit behavioural responses including type (risk or control) and order of stimuli (first or second) as within-subject and between-subject factors, respectively (see Appendix 4). We only analysed the influence of haemosporidian infection on effective behaviours, that is, those responses that varied between the risk and control stimuli. No delayed response was observed to any of the stimuli (Appendix 4). When the effect of the order of stimuli was significant, we tested which order elicited effective behaviours to include only these samples in subsequent analyses. In these cases, we could not test the interaction between treatment and infection due to low sample size.

We computed the scores of a PCA with tarsus and wing length as a measure of structural body size (Brown, 1996). Then, we calculated body condition as the residuals of body mass against body size. Body condition was not significantly related to any bird behaviour measured in the study (all Pearson correlations with $P \geq 0.06$; this value corresponds to the correlation with the number of pecks); therefore, we did not include body condition as a covariate in the analyses.

Ecological or evolutionary consequences of variation in behavioural traits is better understood when it affects individual performance (Réale et al., 2007). Birds were weighed at the beginning and at the end of each trial using a 0.01 g precision digital balance (KERN CM 60-2N). We conducted an ANOVA for differences in the percentage of initial body mass lost after the sequence of behavioural tests between birds that ate or did not eat. The residuals of this analysis were used as a measure of the capability of individuals to maintain energy reserves under risk. We used general linear models to study whether the loss of body mass depended on the level of expression of behaviours that were significantly related to infection status.

All analyses were performed with R 4.1.2 software (R Core Team, 2021) using several packages (a complete list of R packages used is provided in the analysis scripts available at Zenodo repository, https://doi.org/10.5281/zenodo.7176704). Variables that did not meet normality assumptions were transformed in accordance with the type of data. When outliers were observed, their influence was assessed by repeating the analyses with or without the outliers. Sample sizes differed between analyses because not all tests were successful.

RESULTS

Infection Status and Medication Treatment Efficacy

Of 43 tested birds, 30 were infected (18 PQ/12 water) and 13 were uninfected (seven PQ/six water). Eleven birds had single infections (seven PQ/four water) and 16 were coinfected (eight PQ/eight water). For three cases with low parasite load we could not retrieve DNA sequences; these were not included in the coinfection analyses.

Exploratory Behaviour

We assessed exploratory behaviour of 40 birds. We excluded from the analyses one individual that did not move during the exploration (results did not change qualitatively but the residuals of the model improved). Infected birds had lower initial latency than uninfected individuals regardless of the treatment they received (Fig. 2, Appendix 5, Table A1). Single- and multiple-infected birds showed similar behaviour (Fig. 2), although the overall effect of multiple status of infection did not reach statistical significance after FDR correction (Appendix 5, Table A1).

Latency to reach newly visited positions from the first to the sixth varied with the binary status of infection (infection*latency to reach new positions: $F_{2.22,80} = 6.55$, $\eta^2_G = 0.044$, $P_{FDR} = 0.02$). Bonferroni-corrected post hoc analyses revealed that infected birds reached the first two positions quicker (both $P_{Bonferroni} < 0.01$) but differences were not statistically significant afterwards (Fig. 3). When the analysis was conducted to test the effect of multiple infections, the interaction became not significant after FDR correction ($F_{4.18,66.85} = 2.97$, $\eta^2_G = 0.046$, $P_{FDR} = 0.12$). We did not detect any significant effect of infection or treatment on latency to cross, relative body turns or activity (Fig. 2, Appendix 5, Table A1).

Foraging

Twenty-five birds ate during the trials (mean latency to eat ± SD = 1409 ± 619.52 s). Bird infection was not related to the probability of eating (Appendix 5, Table A2), latency to eat or the

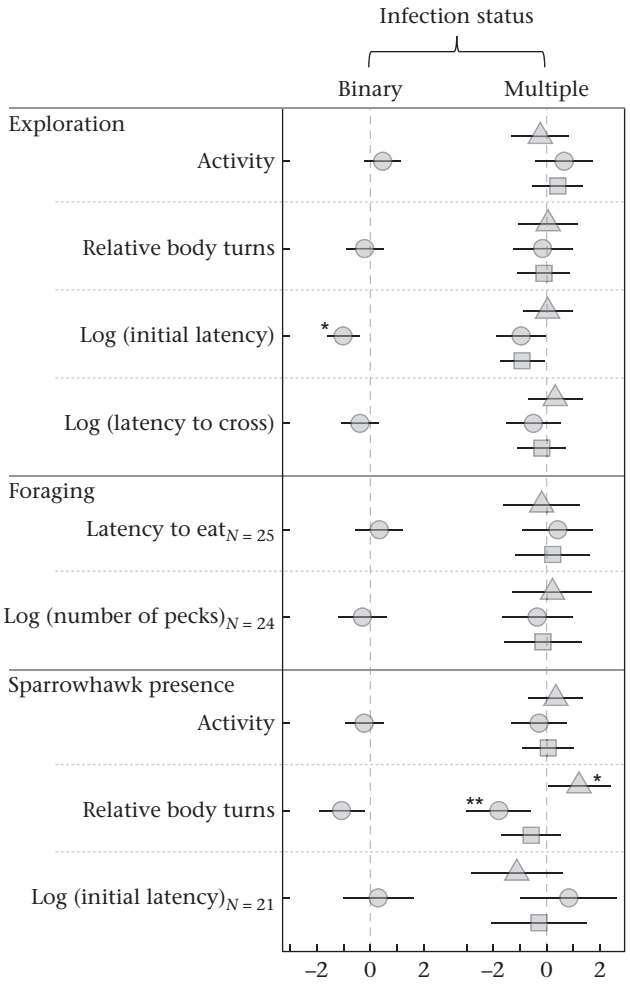

Figure 2. Standardized estimates ± 95% confidence intervals of relationships between continuous behavioural variables and haemosporidian infection. Binary infection status: uninfected or infected; multiple infection status: uninfected, single infection or multiple infection. Shapes represent different comparisons: triangles: multiple versus single infection; circles: single versus no infection; squares: multiple versus no infection. In all pairwise comparisons, the reference level is the one with the lowest infection (e.g. infected–uninfected). All interactions between infection variables and medication treatment were not significant and for behavioural traits where a subset $_N$ of individuals was analysed, the interaction was not tested due to low sample size. Significant variables after FDR correction are shown in bold. ** $P < 0.01$; * $P < 0.05$.

log of the number of pecks (Fig. 2). The low sample size of birds that ate prevented us from testing interactions between treatment and infection in this subset of birds (Appendix 5, Table A2).

Playback of Conspecific Alarm Call Test

Ten of 43 individuals responded by freezing when exposed to an acoustic stimulus, eight of which responded to the conspecific alarm call and two to the Bonelli's warbler's song (Appendix 4). We did not find evidence of a relationship between infection status and appropriate antipredator freezing (estimate$_{exact} = -1.15$, 95% confidence interval, CI = −3.07 to 0.72, $P = 0.17$). There was an almost significant effect of treatment with only one bird treated with water freezing to the conspecific alarm call alone (estimate$_{exact} = -1.94$, 95% CI = −5.69 to 0.20, $P = 0.05$). Birds did not change other behaviours measured during the trial as a response to conspecific alarm calls and in some cases the subset of individuals responding was too small to allow statistical tests (Appendix 4).

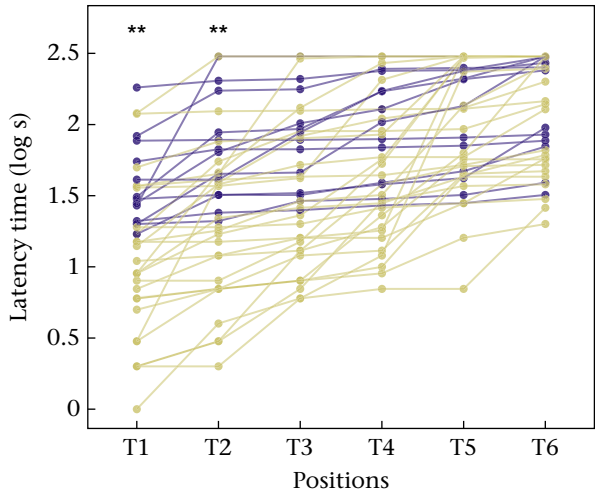

Figure 3. Time to move (log s) to the first six newly visited cage positions (T1–T6) for birds that were uninfected (blue) and infected (ochre). **Bonferroni-corrected $P < 0.01$. Each line represents an individual.

Sparrowhawk Presence Test

Thirty of 42 individuals showed an appropriate crown-raising response to the presence of a predator, erecting their crown only to the sparrowhawk (Appendix 4). Appropriate crown raising did not vary with binary infection (estimate$_{exact} = -0.40$, 95% CI = −2.30 to 1.24, $P_{FDR} = 0.91$), medication treatment (estimate$_{exact} = -0.11$, 95% CI = −1.60 to 1.51, $P_{FDR} = 1.0$) or the interaction between infection and treatment (estimate$_{exact} = -2.03$, 95% CI = −infinite to 0.62, $P = 0.12$; Appendix 5, Table A3). When the multiple status of infection was considered, a very weak interaction with treatment became not significant after FDR correction (Table A3). Nevertheless, post hoc differences between treatments were not significant within any infection level (lowest $P_{Bonferroni} = 0.18$ for multiple infected birds).

Blackcaps increased relative body turns as an antipredator strategy according to the status of infection and irrespective of the type of treatment received (Appendix 5, Table A3). Nevertheless, post hoc comparisons of the levels of multiple status of infection revealed that single-infected individuals differed from both uninfected and multiple-infected individuals (Figs 2, 4). The presence of the sparrowhawk induced a significant increase in relative body turns in coinfected individuals ($t = 3.13$, $P_{Bonferroni} = 0.01$), similar to the increase observed in uninfected birds ($t = 4.65$, $P_{Bonferroni} < 0.001$). However, single-infected birds reacted similarly to the predator and the control ($t = -0.65$, $P_{Bonferroni} = 1$; Fig. 4).

Initial latency to move and activity in presence of the predator were not related to infection (Fig. 2, Appendix 5, Table A3). Activity did not vary significantly with treatment after FDR correction in the model analysing multiple status of infection ($P_{FDR} = 0.11$, Appendix 5, Table A3). Medicated individuals reacted more actively to the sparrowhawk than to the bottle while not treated individuals reacted similarly to both stimuli (post hoc test: $t_{PQ} = 4.36$, $P_{Bonferroni} < 0.001$, $t_{water} = 0.81$, $P_{Bonferroni} = 0.84$).

Alarm Calls

Sixteen of 42 individuals produced alarm calls during the whole risk-taking assessment timeframe. We observed a weak interaction between the binary status of infection and medication which lost significance after FDR correction of P values (treatment*binary status of infection: LRT = 4.02, $P_{FDR} = 0.07$). The trend was towards

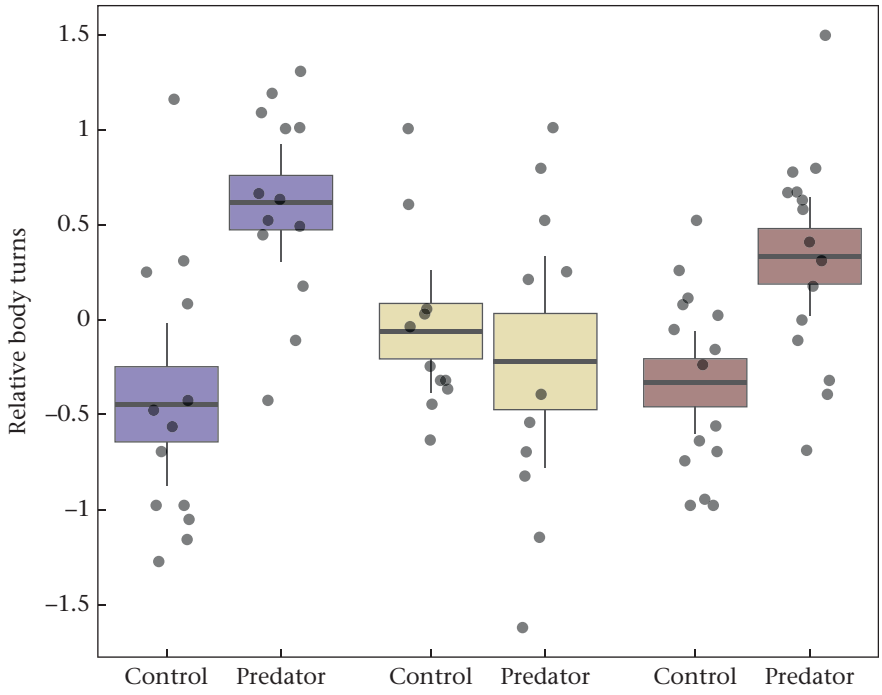

Figure 4. Comparison of relative body turns among blackcaps with different levels of multiple status of infection (blue = uninfected; ochre = single infection; red = multiple infection) in response to the presence of a taxidermic sparrowhawk (predator) or a bottle (control). Box plots show the mean and standard error, while whiskers represent the 95% confidence intervals. Points represent observed values.

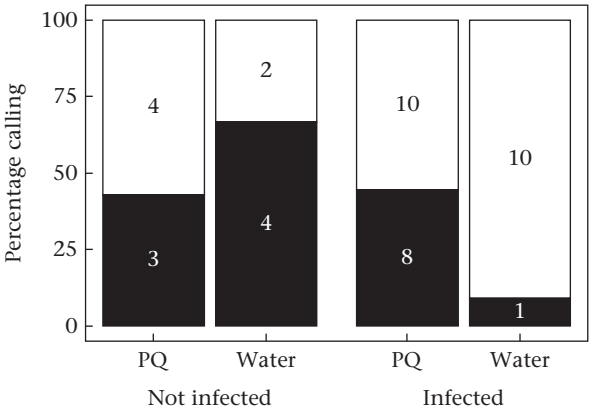

Figure 5. Frequency of uninfected and infected blackcaps producing (black) or not (white) alarm calls in each treatment (water or primaquine, PQ). Numbers inside the bars indicate sample sizes.

infected birds that were not medicated being less likely to alarm-call than those that had been medicated (LRT = 4.49, $P_{Bonferroni} = 0.07$), while no significant effect of treatment was detected for uninfected individuals (LRT = 0.75, $P = 0.39$, $P_{Bonferroni} = 0.78$; Fig. 5).

We found a weak effect of multiple status of infection (LRT = 7.01, $P_{FDR} = 0.07$) which was not affected by treatment (main effect LRT = 0.98, $P_{FDR} = 0.32$; treatment*multiple status of infection $estimate_{exact} = -1.81$, 95% CI = −5.68 to 0.47, $P = 0.10$). Nevertheless, differences in the post hoc test were not significant (all $P_{Bonferroni} \geq 0.09$ for the comparison between multiple-infected and uninfected birds).

Behaviours Associated with Infection and Host Energy Reserves

Birds that did not eat throughout the trial lost more weight (mean ± SD = 2.72 ± 0.55%) than those that ate (2.05 ± 0.84%; $F_{1,41} = 8.54$, $P = 0.006$). Controlling for this effect, the loss of body mass during the trial was negatively related to the initial exploratory latency (estimate ± SE −0.53 ± 0.19, $P_{FDR} = 0.02$) and to the relative body turns in the presence of the sparrowhawk (−0.48 ± 0.10, $P_{FDR} < 0.001$). There were no differences in mass lost between individuals relative to their tendency to raise the crown against a predator ($F_{1,39} = 1.62$, $P_{FDR} = 0.27$) or to alarm-call during tests ($F_{1,39} = 0.22$, $P_{FDR} = 0.63$), regardless of the presence of parasites (all interactions with infection status with $P \geq 0.24$).

DISCUSSION

Haemosporidian infection in young male blackcaps was related to the way birds explore new environments and display antipredator behaviours, mostly with defensive alarm calls and modulation of the antipredator response according to actual levels of risk. We found that some behaviours were related to the infection history of the host. Thus, a temporary reduction in parasitaemia appeared to have carryover effects later in life, promoting some antipredator behaviours such as alarm calling, although this effect was weak. Thus, unmedicated individuals with parasites tended not to alarm-call, which suggests a potential influence of parasites on predator–prey interactions via infection-dependent propensity to display mobbing behaviour or to share information on predator presence (Grim, 2008). Independent of the treatment they received, birds with parasites started exploring earlier than uninfected ones and were slower to reach different positions. In addition, singly infected birds did not increase relative body turns when confronted with a predator, although birds harbouring multiple infections did show appropriate antipredator sentinel behaviour in this context. These activities were energetically costly since birds that began exploring earlier and exhibited fewer body turns for their activity lost more weight.

Individuals showed different exploratory patterns depending on the presence of parasites. The influence on initial latency was independent of the treatment received and consequently may be

explained either by higher exposure to parasites of the earliest explorers or by parasite manipulation of the host promoting exploration (Barber & Dingemanse, 2010; Réale et al., 2007). Higher infection rates in active exploratory individuals have been found for other species although causality has rarely been elucidated (Boyer et al., 2010; Dunn et al., 2011; Ezenwa et al., 2016). On the other hand, both experimental infection (Mukhin et al., 2016) and antimalarial treatment (Cauchard et al., 2016) have shown that haemosporidian parasites reduce host activity in accordance with the typical sickness symptoms.

When the sparrowhawk was presented, we found complex associations between infection status and bird behaviours, where birds infected with one parasite lineage reacted similarly to risk and control stimuli, while multiple-infected birds showed more appropriate antipredator responses, reacting to the predator like uninfected individuals did. Dealing with infection under predation risk may involve a trade-off between immune function and antipredator behaviour (Navarro et al., 2004). The response of animals to predators is triggered by costly physiological mechanisms (Romero, 2004) and strengthening immune function in infected individuals may compromise antipredator mechanisms (Adelman et al., 2017; Roncalli et al., 2018). Blackcaps in our experiment invested in their immune system to control parasitaemia (Arriero et al., 2018) which could have been costly. Surprisingly, the ability to modulate relative body turns when faced with the predator was reduced in individuals with single infections compared to multiple-infected ones, which behaved the same as uninfected birds. Contrary to our results, other studies have reported a stronger influence of haemosporidian coinfection on risk-taking behaviours in other species (Dunn et al., 2011; Marinov, Marchetti, et al., 2017). This apparent discrepancy may arise because the community composition of host–parasite assemblages can be very influential in virulence, and dilution or amplification effects have been either reported for coinfections (Johnson & Hoverman, 2012; Marzal et al., 2008). For example, a recent study on red-winged blackbirds, *Agelaius phoeniceus*, found, in line with our results, a damping effect of haemosporidian coinfections in resistance versus tolerance investment, suggesting competition between parasites as the causal mechanism (Schoenle et al., 2019).

Haemosporidian parasite infections seemed to compromise the ability or motivation of young male blackcaps to alarm-call in risky situations, with only one of 11 unmedicated infected individuals doing so. Alarm calling is a costly antipredator behaviour which may honestly signal individual condition discouraging predator attacks (Kavaliers & Choleris, 2001; Laiolo et al., 2004) and other studies have confirmed that birds' vocalizations may be compromised by infection (Laiolo et al., 2007; Spencer et al., 2005). Although we cannot determine the proximate physiological causes of this pattern, our results support the notion that temporary parasite reduction during early life influences antipredator behaviours of infected individuals later in life.

Recognizing predation risk and responding appropriately to the level of risk may be key for individuals to survive (Langenhof & Komdeur, 2018), especially in juveniles that suffer high mortality to predation (Alatalo et al., 2008). If infection promotes inappropriate antipredator behaviours in blackcaps, predation could be higher for infected birds. The latter idea agrees with ecoevolutionary studies proposing the existence of low parasite prevalence in high predation contexts due to higher mortality of sick individuals (Møller & Nielsen, 2007). In addition, blackcaps that started exploration earlier and were less sensitive to predator presence lost more weight, which reveals a potential physiological cost of some of the behaviours that were related to infection status (Abbey-Lee et al., 2016; Pérez-Tris et al., 2004).

Short-term reduction of parasites in the blood after treatment with primaquine could not be sufficient to relieve all sickness-associated behaviours in the long term, as parasitaemia later increased. Our behavioural test was performed almost 30 days after medication, a period in which most infected medicated birds had parasites in the blood again and all infected individuals showed a seasonal decrease in their parasite load (Arriero et al., 2018). Therefore, the case for causation is likely to be strongest for behavioural responses that may represent long-lasting effects of parasites on their hosts. Our results suggest the existence of carryover effects of parasites in alarm-calling behaviour. Possible explanations include overlapping of the time when parasitaemia was lowered with sensitive periods for the development of the vocal repertoire (Brainard & Doupe, 2002), or more complex patterns involving a trade-off between physiological mechanisms of tolerance and energetically demanding antipredator responses (Navarro et al., 2004). In addition, ad libitum feeding of the birds might have contributed to improving the nutritional condition of infected individuals, reducing our capacity to detect other sickness-associated behaviours (Cornet et al., 2014). Finally, it should not be forgotten that sometimes the same behavioural traits are both cause and consequence of infection (Blanchet et al., 2009), although our experimental design did not allow us to always ascertain when behaviour was driving infection.

We are aware that some of our results are to be interpreted with caution, because our multiple approaches increased the probability of detecting effects by chance, and significance corrections made to retain results that are robust to false discovery questioned the statistical support of some of the relationships found. However, we deemed these results informative in our experiment, where sample size is necessarily small for ethical reasons, and different approaches such as distinguishing between binary or multiple status of infection increase insight. We observed recurrent associations between host behaviour and parasites, and none of the significant relationships we found was against predictions, which suggests that by correcting *P* values we increased type II error rates (Garamszegi, 2006; Nakagawa & Cuthill, 2007). All in all, our study found a broad array of relationships between haemosporidian infections and risk-taking behaviours in young male blackcaps. It also provides the basis to postulate that the history of parasite infection, that is, variation in parasitaemia earlier in the life of the individuals, was important, as shown by the effect of an antimalarial treatment on blackcap behaviours measured once the parasitaemia levels of medicated birds had increased again. Altogether, these results contribute to broadening knowledge of the links between host behavioural repertoires and parasitism, providing novel hypotheses for further research on the ecological and physiological mechanisms implicated.

Author Contributions

Conceptualization: C.R. and J.P.; Methodology: C.R., J.P. and A.R.; Formal analysis: C.R.; Investigation: C.R., J.P., A.R. and E.A; Data curation: C.R.; Writing – original draft preparation: C.R.; Writing – review and editing: C.R., J.P., A.R. and E.A.; Visualization: C.R.; Project administration: CR; Funding acquisition: J.P. and E.A.

Data Availability

All data and R code necessary to reproduce the results and figures of the paper have been stored at Zenodo repository, https://doi.org/10.5281/zenodo.7176704.

Declaration of Interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix 1. Efficacy of medication treatment

We analysed the efficacy of the medication treatment with the antimalarial primaquine in the 30 infected male blackcaps used to assess how infection was related to risk-taking behaviours. Intensity of parasites was assessed by qPCR following the procedures described in the Methods. Before treatment, infected birds did not differ significantly in their parasitaemia levels ($F_{1,28} = 2.09$, $P = 0.16$). Immediately after the birds were treated with primaquine, they lowered parasite load (log mean ± SD $= 0.00284 \pm 0.00471$) more than control birds (log mean ± SD $= 0.0634 \pm 0.0832$; $F_{1,28} = 9.66$, $P = 0.004$; Fig. A1). Almost all birds decreased parasitaemia according to the natural dynamics of the infection, but only medicated infected birds had no detectable parasites by qPCR at the end of the treatment, an outcome observed for seven of the 18 medicated individuals (estimate$_{exact} = -2.19$, 95% CI −infinite to 0.16, $P = 0.02$). Twenty-one days later, parasitaemia recrudescence was often observed (medicated birds: 16 of 18 birds; unmedicated birds: four of 12 birds; exact logistic regression estimate$_{exact} = 2.66$, 95% CI 0.64 to 6.24, $P = 0.004$). At that time, there were no significant differences in parasitaemia between primaquine and water treatments ($F_{1,28} = 0.48$, $P = 0.49$).

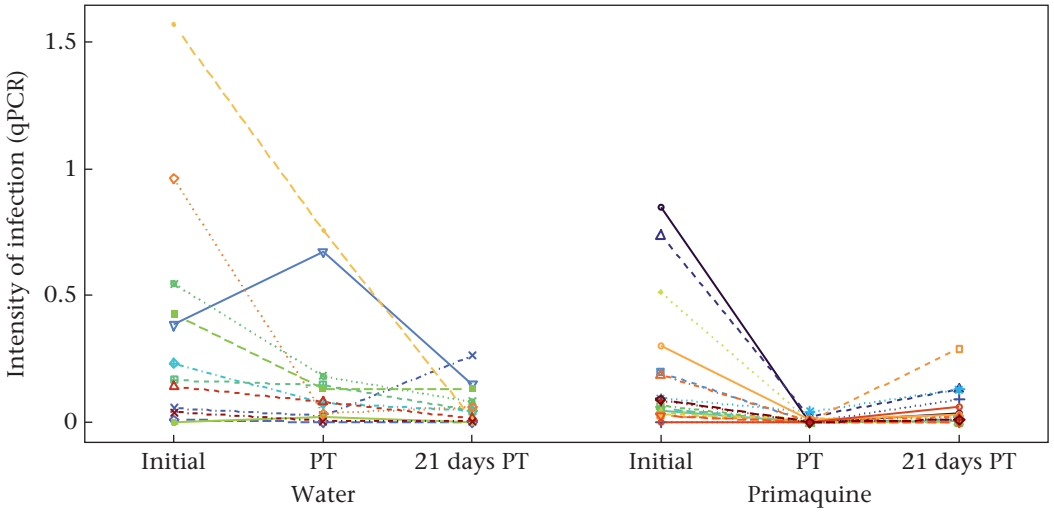

Figure A1. Efficacy of the antimalarial treatment with primaquine in young male blackcaps infected by haemosporidian parasites. Individual variation in intensity of infection in control (water) and primaquine-medicated birds is shown at the beginning of the study, right after the 14-day treatment (post-treatment, PT) and 21 days after the end of the treatment. Different symbols and colours represent different individuals.

Appendix 2. Description of the test room and test cage

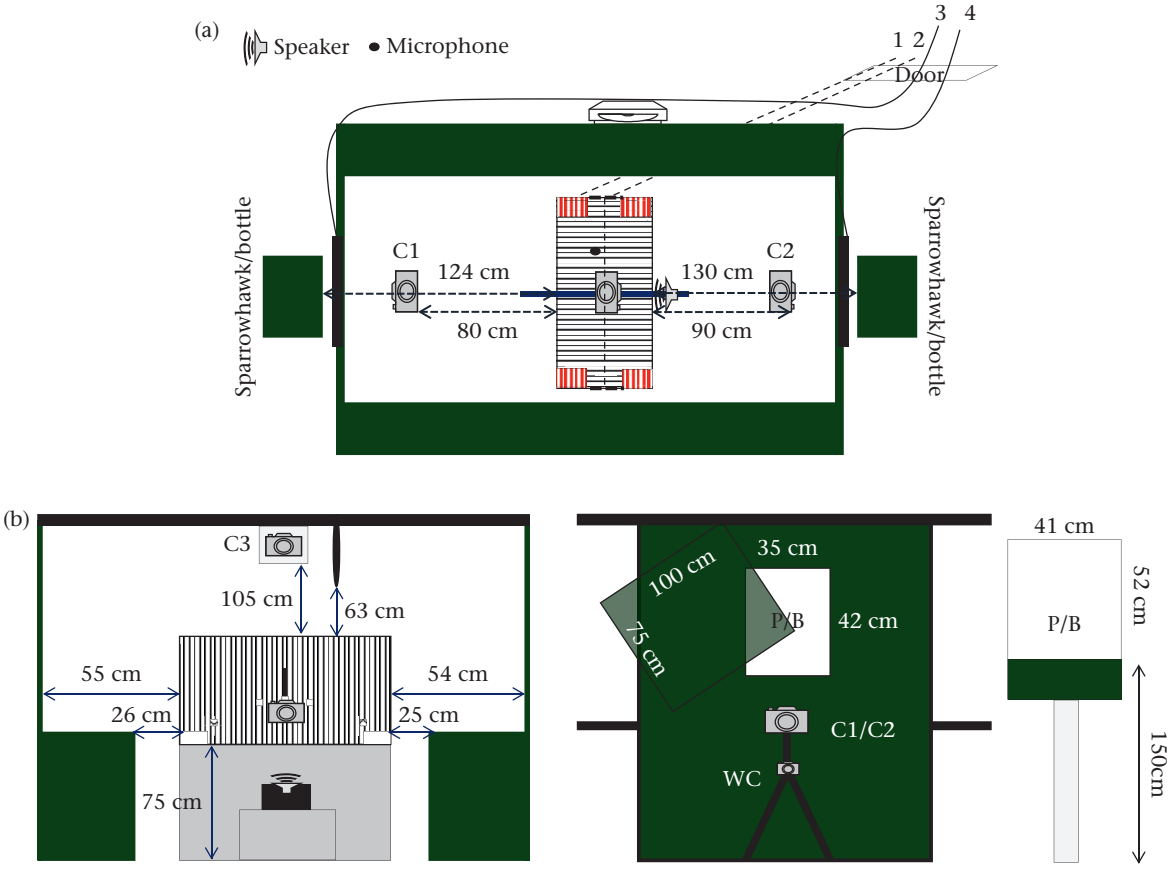

Figure A2. (a) Top and (b) side views from the behavioural room. C1/C2/C3 are the three cameras to record bird activities during the tests and WC is the web camera that helps to monitor the trials from an adjacent room without interfering with the experiment. In (a), dashed lines 1 and 2 represent the triggers that open the feeders and continuous lines 3 and 4 those that open the curtains to show the visual stimuli. P = sparrowhawk; B = bottle. The test room had walls made of green plastic sheeting to provide a homogeneous background and avoid external stimuli.

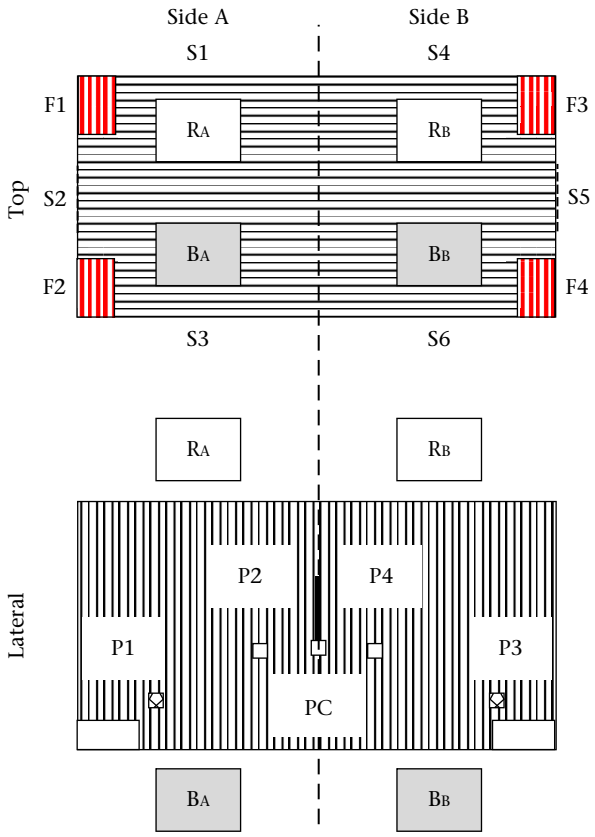

Figure A3. Diagram of the division of the test cage. F = feeders; S = cage side; R = cage roof; B = cage bottom; P = perch; PC = central perch (on the window ledge). Subscripts A and B are used to differentiate sides A and B.

Appendix 3. Results of PCA of behavioural variables

Variables were standardized and log- or sqrt-transformed when necessary to improve normality. Factor scores were used as an index of activity.

Exploratory behaviour

From the PCAs, we obtained one factor that explained 59% of the variance. This factor was positively related to the square root of the number of movements (factor loading: 0.92), the number of body turns (0.64) and the number of different positions visited (0.79). It was negatively related to the logarithm of the initial latency (−0.70). Therefore, PC1 scores reflected the latency and frequency of movements, and we considered it a measure of the exploratory activity of the birds.

Playback conspecific alarm call test

The scores of the PC1 with behaviours measured as a response to acoustic stimuli were positively related to the logarithm of the number of movements (0.99), the square root of the number of body turns (0.93) and the logarithm of the number of different positions visited (0.93), summarizing 90% of the variance. This PC1 was used as a measure of activity levels in response to acoustic stimuli.

Sparrowhawk presence test

The scores of the PC1 with behaviours measured as a response to the presence of the visual predator stimulus was negatively related to the logarithm of initial latency (−0.88), and positively related to the square root of the number of movements (0.94), the square root of the number of body turns (0.36) and the square root of the number of different positions visited (0.90), accounting for 65% of the variance. This PC1 was used as a measure of activity levels in response to visual stimuli.

A second PCA was used to compute an index of activity levels as a delayed response to the presence of a visual predator stimulus. The PC1 was positively related to the square root of the number of movements (0.97), the square root of the number of body turns (0.86) and the square root of the number of different positions visited (0.82) accounting for 78% of the variance.

Appendix 4. Assessment of efficacy of the tests

To correctly interpret the observed results, bird responses must meet two prerequisites: the order of presentation of the stimuli did not influence bird responses and there were differences according to the type of stimulus: risk or control.

Playback conspecific alarm call test

The order of the acoustic stimulus influenced blackcaps' response for activity level ($F_{1,21} = 6.47$, $P = 0.02$) and latency ($F_{1,25} = 4.19$, $P = 0.05$; other variables like freezing behaviour, likelihood of being active and relative body turns: $P \geq 0.39$). Further post hoc analyses revealed that blackcaps significantly decreased activity ($F_{1,10} = 6.46$, $P = 0.03$, $\eta^2_G = 0.172$; $t_{\text{blackcap}} = -3.05$, $P = 0.01$, $t_{\text{bonelli}} = 0.97$, $P = 0.35$) and delayed their response ($F_{1,13} = 6.35$, $P = 0.03$, $\eta^2_G = 0.086$; $t_{\text{blackcap}} = 4.71$, $P < 0.001$, $t_{\text{bonelli}} = 0.95$, $P = 0.36$) when presented with the conspecific call only when this was played second, while there were no differences when the

blackcap conspecific call was played first (all $P \geq 0.48$). Nevertheless, the sample size of individuals where the conspecific call was played second was too low to allow for acoustic analyses (11 and 14 for activity and latency, respectively).

Birds were more likely to freeze in response to a conspecific call than to the Bonelli's warbler song (LRT = 4.33, $P = 0.04$; only 10 individuals froze, eight of which responded to conspecific alarm calls and two to the control stimulus). Blackcaps did not differ in their likelihood of being active or in relative body turns according to the type of stimulus (all $P \geq 0.64$). Birds were less likely to be active after any sound was played (moment*type of stimulus: LRT = 0.01, $P = 0.93$; moment: LRT = 6.97, $P = 0.008$). There were no differences in relative body turns within individuals after any stimulus (moment*type of stimulus: $F_{1,41} = 0.21$, $P = 0.65$; moment: $F_{1,41} = 3.60$, $P = 0.07$).

Sparrowhawk presence test

Immediate response to predator presence

The order in which the sparrowhawk and the bottle were presented only influenced the immediate latency response ($F_{1,40} = 4.33$, $P = 0.04$; all other variables: $P \geq 0.14$). There were significant differences in latency between the sparrowhawk and the bottle appearance only when the sparrowhawk appeared second ($F_{1,20} = 11.95$, $P = 0.002$; when the sparrowhawk appeared first: $F_{1,20} = 0.39$, $P = 0.54$).

When the sparrowhawk was presented, blackcaps were more likely to raise their crown (LRT = 79.23, $P < 0.001$; 30 of 42 individuals erected their head crown only with the sparrowhawk), decreased initial latency (when the sparrowhawk appeared second as described above: $t = -3.46$), and increased activity levels ($F_{1,41} = 11.30$, $P = 0.002$, $t = 3.36$) and relative body turns ($F_{1,41} = 15.47$, $P < 0.001$, $t = 3.93$), compared with their behaviours in the presence of the bottle.

Difference in response after and before predator stimulus

The order of the stimulus did not influence delayed activity ($F_{1,40} = 0.58$, $P = 0.45$) and delayed relative body turns ($F_{1,40} = 2.63$, $P = 0.11$). We did not detect delayed antipredator responses for either activity (interaction type of stimulus*moment: $F_{1,41} = 1.19$, $P = 0.28$, $\eta^2_G = 0.005$) or relative body turns ($F_{1,41} = 41.87$, $P = 0.18$, $\eta^2_G = 0.004$).

Appendix 5. Additional tables

Table A1
Results of a GLM analysing the relationships between binary or multiple infection status and risk-taking behaviours measured during the open-field exploration test

Exploratory behaviours	Binary infection status						Multiple infection status					
	Infection			Treatment			Infection			Treatment		
	df	*F*	P_{FDR}	*df*	*F*	P_{FDR}	*df*	*F*	P_{FDR}	*df*	*F*	P_{FDR}
Log (initial latency)	1,36	11.85	**0.02**	1,36	1.44	0.51	2,32	4.81	0.10	1,32	0.59	0.64
Log (latency to cross)	1,36	1.30	0.51	1,36	0.11	0.82	2,32	0.73	0.64	1,32	0.62	0.64
Activity level	1,36	1.91	0.51	1,36	1.07	0.51	2,32	1.23	0.51	1,32	1.08	0.51
Relative body turns	1,36	0.39	0.64	1,36	0.37	0.64	2,32	0.06	0.94	1,32	0.05	0.87

Significant result is highlighted in bold. Statistical significance was computed after false discovery rate correction for multiple hypotheses testing (P_{FDR}). All infection *treatment interactions were not significant (all $P \geq 0.19$).

Table A2
Results of GLM and binomial regression models analysing the relationships between binary or multiple status of infection and foraging behaviours

Foraging behaviour	Binary infection status						Multiple infection status					
	Infection			Treatment			Infection			Treatment		
	df	*F* (LRT)	P_{FDR}	*df*	*F* (LRT)	P_{FDR}	*df*	*F* (LRT)	P_{FDR}	*df*	*F* (LRT)	P_{FDR}
Eating (yes/no)	1	(1.00)	0.79	1	(0.12)	0.79	1	(2.85)	0.79	1	(0.15)	0.79
Latency to eat for birds that ate[a]	1,22	0.61	0.79	1,22	0.17	0.79	2,20	0.32	0.79	2,20	0.10	0.79
Log (pecks)[b]	1,21	0.48	0.79	1,21	0.20	0.79	2,19	0.23	0.79	2,19	0.09	0.79

Numbers in parentheses correspond to likelihood ratio tests (LRT) in binomial models; otherwise, *F* statistics are shown. In the assessment of the likelihood of eating all interactions between infection parameter and treatment were not significant (all $P \geq 0.15$). Statistical significance was computed after false discovery rate correction for multiple hypotheses testing (P_{FDR}).

[a] Effects estimated for the subset of birds that ate ($N = 25$); in these analyses interactions could not be tested due to low sample size.

[b] One bird was not included in the analysis of the number of pecks because it could not be monitored throughout the sparrowhawk presence test due to a technical issue.

Table A3
Results of binomial regression model and repeated measures ANOVA analysing the relationships between binary or multiple status of infection and antipredator responses to the presence of a potential predator

	Binary infection status								Multiple infection status							
	Infection				Treatment				Infection				Treatment			
	df	*F* (est)	P_{FDR}	η^2_G	*df*	*F* (est)	P_{FDR}	η^2_G	*df*	*F* (est)	P_{FDR}	η^2_G	*df*	*F* (est)	P_{FDR}	η^2_G
Crown raising		(−0.40)	0.91			(−0.11)	1.00		(1)							
Log (latency to move)[a]	1,18	0.22	0.91	0.006	1,18	1.38	0.48	0.03	2,16	1.49	0.48	0.09	2,16	2.19	0.40	0.07
Activity level	1,39	0.42	0.87	0.003	1,39	2.75	0.32	0.02	2,35	0.38	0.91	0.006	1,35	5.09	0.11	0.04
Relative body turns	1,39	6.64	0.10	0.08	1,39	0.001	1.00	<0.001	2,35	6.74	**<0.05**	0.15	1,35	0.00	1.00	<0.001

Numbers in parentheses correspond to estimate parameters in exact logistic probability models (est); otherwise, *F* statistics are shown. Significant result is highlighted in bold. Statistical significance was computed after false discovery rate correction for multiple hypotheses testing (P_{FDR}). η^2_G represents effect size of the main effects. Infection*-treatment interaction: $estimate_{exact} = -2.35$, 95% confidence interval = −6.28 to −0.12, $P = 0.02$, $P_{FDR} = 0.10$.

[a] Effects estimated for the subset of birds in which latency to move was measured ($N = 21$).