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RESEARCH ARTICLE

Increased parasite load is associated with reduced metabolic rates and escape responsiveness in pumpkinseed sunfish

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ABSTRACT

Wild animals have parasites that can compromise their physiological and/or behavioural performance. Yet, the extent to which parasite load is related to intraspecific variation in performance traits within wild populations remains relatively unexplored. We used pumpkinseed sunfish (*Lepomis gibbosus*) and their endoparasites as a model system to explore the effects of infection load on host aerobic metabolism and escape performance. Metabolic traits (standard and maximum metabolic rates, aerobic scope) and fast-start escape responses following a simulated aerial attack by a predator (responsiveness, response latency and escape distance) were measured in fish from across a gradient of visible (i.e. trematodes causing black spot disease counted on fish surfaces) and non-visible (i.e. cestodes in fish abdominal cavity counted post-mortem) endoparasite infection. We found that a higher infection load of non-visible endoparasites was related to lower standard and maximum metabolic rates, but not aerobic scope in fish. Non-visible endoparasite infection load was also related to decreased responsiveness of the host to a simulated aerial attack. Visible endoparasites were not related to changes in metabolic traits or fast-start escape responses. Our results suggest that infection with parasites that are inconspicuous to researchers can result in intraspecific variation in physiological and behavioural performance in wild populations, highlighting the need to more explicitly acknowledge and account for the role played by natural infections in studies of wild animal performance.

KEY WORDS: Black spot disease, Ecophysiology, Fast-start escape response, Infection intensity, Respirometry, Whole-organism performance

INTRODUCTION

Experimental biologists studying wild animals often assume that their subjects are healthy and performing to the best of their ability (e.g. Wilson et al., 2015). However, at any given moment, animals are host to a range of parasites or pathogens that can compromise their physiological and behavioural performance, with significant ecological repercussions (Poulin et al., 1994; Marcogliese, 2004; McElroy and de Buron, 2014; Binning et al., 2017; Timi and Poulin, 2020). For example, infection by the protozoan *Ophryocystis elektroscirrha* causes 14% shorter flight durations and 19% shorter flight distances in monarch butterflies, *Danaus plexippus*, impairing their ability to successfully migrate (Bradley and Altizer, 2005). The problem of infection is not unique to animals caught in the wild. The microsporidium *Pseudoloma neurophilia*, a common infection in laboratory populations of zebrafish, *Danio rerio*, alters fish shoaling behaviour and startle responses (Spagnoli et al., 2015, 2017). As a result, parasites may be an important, yet overlooked, driver of intraspecific trait variation in both wild and laboratory animal populations.

The pervasiveness of parasites in both terrestrial and aquatic systems has been repeatedly highlighted in the ecological literature (Poulin and Morand, 2000; Kuris et al., 2008; Caballero et al., 2015). Similarly, their physiological and behavioural effects on animal hosts can be dramatic. For instance, trophically transmitted parasites can affect host predator-avoidance or risk-taking behaviours to facilitate transmission to their final host (Kuris, 2003; Blake et al., 2006; Parker et al., 2015). In killifish *Fundulus parvipinnis*, individuals infected with larval trematodes swim to the surface, jerk and shimmer more often than uninfected fish, rendering them 31 times more susceptible to predation by birds (Lafferty and Morris, 1996). Although parasites generally have a detrimental effect on host performance capacity (i.e. the ability of an organism to carry out ecologically relevant tasks; McElroy and de Buron, 2014), infection can also impact hosts in counter-intuitive ways. For example, high loads of the muscle-dwelling myxozoan *Kudoa inornate* are related to faster burst-swimming speeds and gait transition speeds in spotted seatrout, *Cynoscion nebulosus* (McElroy et al., 2015). Thus, the effects of parasite infection on individual performance capacity can be difficult to predict.

Performance capacity, including aerobic metabolic performance, can determine individual success in activities such as foraging, locomotion, reproduction and predator avoidance (Bennett, 1980; Arnold, 1983). Aerobic metabolic performance is tightly linked to an organism's ability to take up oxygen and can be estimated by measuring an animal's oxygen consumption rate ($\dot{M}_{O_2}$) as a proxy of whole-organism metabolic rate (Claireaux and Lefrançois, 2007; Chabot et al., 2016a). Two important physiological traits can be used to describe the upper and lower bounds of an animal's ability to metabolize oxygen. The maximum metabolic rate (MMR) and standard metabolic rate (SMR) are defined, respectively, as the maximum and minimum amount of energy metabolized aerobically by an organism (Hulbert and Else, 2000). In ectotherms, SMR is the minimal amount of energy needed for maintenance at a given temperature and is estimated by measuring

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$\dot{M}_{O_2}$ in a non-reproductive, resting and post-absorptive state (Chabot et al., 2016b). MMR can be estimated by measuring an organism's $\dot{M}_{O_2}$ during or shortly after exhaustive exercise (Norin and Clark, 2016; Rummer et al., 2016). The difference between these two estimates, the absolute aerobic scope (AS), represents an animal's ability to perform functions above those required for basic maintenance, including mounting an immune response, digesting, moving, growing and reproducing (Claireaux and Lefrançois, 2007). Parasites that interfere with any aspect of energy demand or physiology might affect the upper and lower bounds of an animal's AS, and therefore its capacity to carry out various physiological or behavioural tasks. Notably, activation of the immune system during infection may lead to an increase in the host's SMR, and therefore reduce its AS (Eraud et al., 2005; Bashir-Tanoli and Tinsley, 2014). Alternatively, parasite infection has also been found to decrease SMR when parasites are located in – or cause tissue damage to – metabolically active organs (Caballero et al., 2015; Ryberg et al., 2020). Similarly, parasites that affect tissues such as the gut, liver or skeletal muscles could impair MMR if they affect the ability of the animal to direct blood flow to these tissues (Coleman, 1993; Gentile and King, 2018). Host anaerobic performance may also be impaired during infection. In response to a predator attack, fish often perform a sudden burst of anaerobically powered swimming, known as a fast-start escape response (Domenici and Blake, 1997). Parasitic infection could alter both behavioural (responsiveness, response latency) and kinematic (escape distance, swimming speed, acceleration) components of fast-start escapes. For instance, a recent study reported that experimental infection with a gnathiid isopod ectoparasite in juvenile Ambon damselfish, *Pomacentrus amboinensis*, increased their escape latency to a simulated predator attack by 32% (Allan et al., 2020).

Assessing the effects of parasites on individual-level physiological and behavioural host performance traits necessitates a proper quantification of parasite load (i.e. the number of parasites in a host, a.k.a. infection intensity). There are many challenges associated with quantifying infection: for example, it is time consuming and often requires detailed knowledge of parasite taxonomy. These reasons may explain why researchers tend to use individual infection status (infected versus non-infected) rather than their actual loads (i.e. number of parasites in a host) to study the effect of infection on performance. Few studies have explicitly quantified the relationship between host physiological or behavioural performance in wild populations across a gradient of natural parasite infection (but see Ruehle and Poulin, 2019; Ryberg et al., 2020; Sun et al., 2020). Moreover, parasites may be internal and thus can only be counted post-mortem. As a result, parasites are not routinely considered in studies on wild animals (Dougherty et al., 2016). This oversight is unfortunate given that some parasites may occur with high prevalence in wild populations and have important ecological roles (Timi and Poulin, 2020). One means by which some researchers have got around this problem is by focusing on the presence of visible infections that are easy to identify. For example, Happel (2019) used photos uploaded to the public database iNaturalist (www.inaturalist.org) to explore the biogeography of black spot disease in fishes across North America. Black spot disease is caused by infection with the metacercaria of digenean trematodes and can easily be identified and quantified non-invasively through the presence of conspicuous black spots on a fish's surfaces. Heavy black spot loads in juvenile bluegill sunfish (*Lepomis macrochirus*) causes changes in oxygen consumption rates, body condition and total body lipid content,

reducing overwinter survival to nearly 0% for fish with more than 50 black spots (Lemly and Esch, 1984). Such externally visible and quantifiable infections provide an unparalleled opportunity to consider effects of parasites on wild animals, and thus in experimental research using these wild animals. Wild animals are often simultaneously co-infected with several parasite species (Bordes and Morand, 2011). The effects of co-infection can be synergistic or antagonistic, and thus difficult to account for (Cox, 2001; Viney and Graham, 2013). However, identifying whether 'visible' parasites, such as those causing black spot disease, are related to infection load with other 'non-visible' parasites may provide researchers with a simple and useful means of accounting for some types of co-infections in their studies.

Fish are useful model systems for assessing the relationships among infection and behavioural/physiological performance traits. In particular, sunfishes (*Lepomis* spp.), which are small freshwater fish native to and abundant in Eastern North America, have been used as model species in behavioural, ecological and kinematic studies for decades (Brett and Sutherland, 1965; Lemly and Esch, 1984; Tytell and Lauder, 2008; Gerry et al., 2012; Crans et al., 2015). Sunfishes are also hosts to a range of parasites (Margolis and Arthur, 1979). In particular, trematodes causing black spot disease are common in many populations of sunfishes (Chapman et al., 2015). The trematodes that cause black spot disease have a complex life cycle requiring two intermediate hosts, typically a snail and a fish, with a piscivorous bird or mammal as the final host (Hunter and Hunter, 1938). Larval trematode cercaria emerge from the snail and encyst under the fish's skin, in fins and muscle, forming black spots approximately 21 days after infection (Hunter and Hunter, 1938; Hugghins, 1959; Berra and Au, 1978). Pumpkinseed sunfish, *Lepomis gibbosus*, are hosts to many other endoparasites (e.g. cestodes, including *Proteocephalus* spp.; other trematodes, including yellow grub, *Clinostomum marginatum*), which can be counted and identified post-mortem (Margolis and Arthur, 1979).

To better understand the role of parasite infection on host performance traits, we explored the relationship between parasite infection and aerobic metabolic performance as well as fast-start escape performance in wild-caught, naturally infected pumpkinseed sunfish, *L. gibbosus*. First, we assessed whether visible infections can be used as a proxy for overall endoparasite burden, and thus costs, in hosts, by separately quantifying visible (i.e. trematode metacercaria causing black spot disease) and non-visible (i.e. other cestode and trematode endoparasites) infections in fish. Next, we examined the relationship between parasite load and aerobic metabolism (MMR, SMR, AS) as well as escape performance (responsiveness, response latency, distance travelled) in wild-caught fish. Although we were interested in testing for a relationship between visible and non-visible infections, we had no *a priori* prediction as to the direction of this relationship. Following the overall tendency for parasites to decrease host performance (McElroy and de Buron, 2014), we also predicted that aerobic metabolism and escape performance would be negatively related to greater parasite load in fish hosts.

MATERIALS AND METHODS
Fish collection and housing
A total of 42 naturally parasitized pumpkinseed sunfish, *Lepomis gibbosus* (Linnaeus 1758), of similar size (mean±s.d. total length 8.5±0.7 cm, body mass 10.24±2.46 g) were captured with minnow traps and seine nets in Lake Cromwell near the Université de Montréal's Station de biologie des Laurentides (SBL, QC, Canada; 45.98898°N, −74.00013°W) in July 2019. Individuals of this size at

this location are typically between 2 and 4 years of age (scale-based age determination; E.C., unpublished data). Fish were transported to the SBL laboratory facilities within 1 h of capture and received a hydrogen peroxide treatment (2.5 ml of 3% H_2O_2 per litre of freshwater) for 30 min to remove ectoparasites, fungus or surface bacteria. Fish were then transferred to a 600 l flow-through holding tank (215×60×60 cm, length×width×height) supplied with water pumped from nearby Lake Croche (45.99003°N, −74.00567°W) and held under a 12 h:12 h light:dark cycle. Water was particle-filtered, oxygenated and UV-sterilized before entering the holding tanks at a rate of 0.14–0.68 m^3 h^{-1}, allowing a full water replacement every 1–4 h (flow rate adjustments were made to maintain the water temperature near 21°C; actual range: 19–21°C). Water temperature and oxygen levels were monitored twice daily (OxyGuard, Handy Polaris, Farum, Denmark) and excess food and debris were siphoned daily. Fish were left in the holding tank for 24 h before each individual was measured [wet mass (g), total length (TL) and standard length (SL) (mm)]. Each fish was identified with a unique three-colour code using visual implant elastomer tags (VIE; Northwest Marine Technology) implanted on each side of the dorsal fin with a 29 gauge needle. Throughout all procedures, fish were manipulated in individual water-filled plastic bags to minimize air exposure and stress. All fish were fed to satiation twice daily (08:30 h and 18:30 h) with a mix of bloodworms and commercial fish pellets (Nutrafin Bug Bites, Cichlid Formula) and were habituated for 3–5 days before the onset of experiments during which behaviour was monitored to ensure the well-being (use of refuges and dominance) of the animal as well as their intake of food. After this habituation period, during which fish fed and swam freely, the fish underwent respirometry trials to estimate oxygen consumption rates. This study was conducted with approval from the Université de Montréal's animal care committee (Comité de déontologie de l'expérimentation sur les animaux; certificate number 19-034).

Respirometry trials

Metabolic traits (MMR, SMR, AS) were estimated as rates of oxygen uptake ($\dot{M}_{O_2}$: mg O_2 h^{-1}) using intermittent flow respirometry. Two identical, separate experimental water baths (78×33×38 cm, length×width×height, 80 l) each contained four resting chambers made of Perspex cylinders (16×6 cm, length×diameter). The chambers were opaque with a transparent viewing window located on top. Each chamber was connected to a closed water circuit (491 ml; volume includes recirculation tubes) with a recirculation pump (to achieve adequate water mixing) to which a fibre-optic oxygen probe (Firesting 4-channel oxygen meter, PyroScience GmbH, Aschen, Germany) was connected. Dissolved oxygen levels were measured every 3 s. The four chambers were connected to a flush pump operated by a digital timer programmed to turn on for 4 min and off for 6 min. This created a 10 min loop comprising a 4 min period of water replacement and oxygenation and a 6 min period where the chambers were sealed with no outside exchange of water. A third water bath where temperature was regulated via an aluminium coil pumping chilled water (Thermo Fisher Scientific, EK20 immersion cooler) was used to keep water temperature in the chambers near 21°C (actual range: 20.8–21.7°C). Water replacement in this bath was carried out using filtered lake water (same as for the holding tanks) pumped through a UV-sterilizer. Background oxygen consumption rates (background $\dot{M}_{O_2}$) were estimated in each empty chamber for 30 min before and after each respirometry trial. The respirometry chambers, tubing, pumps and

water baths were cleaned every 3 days with a mix of warm water and 3% hydrogen peroxide (H_2O_2) and left to dry outside in direct sunlight.

Fish were fasted for 24 h prior to all respirometry experiments to ensure they were in a post-absorptive state; the absence of faeces in the respirometry chambers at the end of trials suggested that this fasting period was sufficient to avoid metabolic activity associated with digestion (Clark et al., 2013; Chabot et al., 2016b,c). Each trial started with a 3 min chase protocol followed by 1 min of air exposure, a common method for estimating MMR in fishes that are poor endurance swimmers (Roche et al., 2013; Rummer et al., 2016). A fish was transferred to a circular chase arena (48×41 cm, height×diameter, 67 l) using a water-filled plastic bag. The fish was then chased by hand for 3 min. When the fish began to fatigue, the experimenter would lightly pinch the fish's tail to force swimming until it no longer swam. The fish was then removed from the arena and held out of the water for 1 min. It was then placed into a respirometry chamber, which was immediately (<15 s) sealed for 10 min to estimate MMR. Once all eight fish had been chased and the 10 min measurements completed, control of the system was switched to the automatic timers running the 10 min loops as described above for the next 18–20 h, during which oxygen consumption rates of fish stabilized, and SMR could be estimated. Oxygen levels remained above 80% in the chambers during all trials. Once a respirometry trial was over, fish were removed from the chambers and returned to their holding tank to recover for 5 days before the escape response trials. This protocol follows best practices for collecting and reporting respirometry data as described in Killen et al. (2021).

Escape response trials

Escape response experiments were conducted to estimate a fish's reaction to a simulated aerial predator attack. These experiments were performed between 08:30 h and 17:00 h, on fish that had been fasted for 12–20 h, to prevent them from regurgitating food during a trial and to maximize the energy available for swimming and recovery. The escape response arena and experimental protocol were based on designs and procedures described in Binning et al. (2014) and Roche (2021). Briefly, fish were introduced into the escape response arena in a water-filled plastic bag to minimize air exposure. The arena was a 60×60×30 cm (length×width×height) acrylic clear bottom tank under which a mirror was suspended at a 45 deg angle to film the escape response from below. The escape response arena was filled with the same water as the holding tanks to a height of 8 cm, which limited vertical movements by the fish while permitting the full extension of their dorsal and pelvic fins. The water temperature in the arena was maintained at 21°C and changed every hour to control temperature and oxygen levels (>95% air saturation). Prior to the experiments, fish were left undisturbed in the arena for 10 min to acclimate. We used a mechano-acoustic stimulus located in the far-left corner of the arena to simulate an aerial attack. A weighted stimulus (iron bolt, 2.6 cm long) was released by an electromagnet and fell through an opaque PVC tube (22 cm long and 4 cm wide) suspended 1 cm above the water surface to avoid visual stimulation of the fish (Binning et al., 2014; Marras et al., 2011). Fish were stimulated when they were static (i.e. not swimming), had the stimulus in their field of view and were a maximum of 10 cm from the stimulus. Each individual was subjected to three trials, with a 10 min interval between trials to allow recovery (see Jornod and Roche, 2015). Escape responses were filmed at 240 Hz with a high-speed camera (EX-FH100, Casio). Fish were euthanized following the escape response

experiments with an overdose of eugenol solution and placed in a freezer at −18°C until they were dissected.

Fish dissection

The number of black spots was assessed by counting the number of cysts on the body surface and on all fins visible on the left side of each individual; that number was used as a proxy for black spot infection level, but in reality fish harboured approximately double that black spot number (Ferguson et al., 2010). Experimental fish harboured a varying number of black spots (6–273 metacercaria quantified on the left side of the fish only). Fish body cavity, liver, digestive tract, muscles and gills were dissected and inspected for internal parasites under a dissecting scope. Internal parasites were counted and identified to the species level by morphological features. Two species of internal parasites were identified: *Proteocephalus ambloplites* was the most prevalent and abundant species (prevalence: 93%, 0–153 parasites per fish), and was mostly found in the liver and body cavity; *Clinostomum marginatum* was less prevalent (prevalence: 26%, 0–7 parasites per fish) and found mostly encysted in the gills and muscle. Hereafter, internal parasites refer to the parasite load for these two parasite species combined. To correct a fish's mass for the number of internal parasites it harboured, we weighed approximately 20 individuals of each species of internal parasite and then divided this mass by the number of parasites to obtain an estimate of the mass of one individual parasite. This process was repeated 5 times with different internal parasites. We averaged these five estimates for each internal parasite species to get a mean individual parasite mass, and then corrected the mass of each fish by the number of parasites of each type it contained (parasite-corrected fish mass; hereafter fish body mass; Lagrue and Poulin, 2015). Metacercaria causing black spots were not weighed as their collective mass was too small to be accurately estimated (±0.000001 g), and probably had no influence on overall fish mass.

Data extraction and analysis

Respirometry data

All oxygen consumption rates ($\dot{M}_{O_2}$) were extracted using the package *respR* (Harianto et al., 2019) in R v. 3.6.1 (http://www.R-project.org/). Dissolved oxygen measurements (DO; in mg l^{-1}) were converted to metabolic rate ($\dot{M}_{O_2}$; in mg h^{-1}) using the following equation:

$$\dot{M}_{O_2} = \left[\frac{\Delta DO}{\Delta t}\right] \times V \times 3600 \,, \tag{1}$$

where $\Delta DO/\Delta t$ is the slope of the oxygen decrease over time (in s) and V corresponds to the volume of water in the closed system (chamber and pump and tubing of the recirculation loop) corrected for the volume of the fish in litres (assuming a density of 1 kg l^{-1}).

Metabolic rate estimates ($\dot{M}_{O_2}$; mg O2 h^{-1}) were calculated from the slope obtained from the linear regression between oxygen concentration and time, accounting for the volume of the respirometer subtracting fish volume (assuming a density of 1 g ml^{-1}). Background $\dot{M}_{O_2}$ was subtracted from the $\dot{M}_{O_2}$ measurements assuming a linear increase in bacterial respiration from the start to the end of the trial. SMR was estimated from measurements taken ~10 h after the onset of the trial (the moment at which $\dot{M}_{O_2}$ stabilized to a minimum level) until sunrise. The lowest 0.2 quantile of slopes after the habituation period (minimum number 29 slopes, maximum number 59 slopes) was used to estimate SMR with the *fishMO2* package (https://rdrr.io/github/denis-chabot/fishMO2/man/fishMO2-

package.html; Chabot et al., 2016b); the mean R^2 of slopes for all fish was 0.99. We used the *respR* package (Harianto et al., 2019) to estimate MMR with a rolling regression that determines the highest rate of change in oxygen over 60 s in the 10 min measurement following the chase and air exposure protocol (the first 30 s were excluded to allow proper mixing after the respirometers were sealed). Absolute AS was calculated as the difference between MMR and SMR (Halsey et al., 2018). Metabolic rates were estimated for 39 fish; data from 3 individuals were excluded because of irregularities in the $\dot{M}_{O_2}$ readings due to an air leak.

Escape response data

We analysed the behavioural components of escape responses using VLC media player (VideoLAN, Paris, France). Responsiveness was assessed over the three trials: for each trial, we recorded whether a fish responded to the stimulus (i.e. performed a C-start following contact of the stimulus with the water). Escape latency (in s) was calculated from the number of frames between the first contact of the stimulus on the water and the first head movement of the fish initiating an escape response. As fish did not respond to the stimulus in all trials, we assessed response latency by recording the best performance (shortest time to respond) of an individual across the three trials (Domenici, 2010). Best performance for the distance travelled (D_{esc}, see below) was also used for the analysis. Stage 1 of the fast-start response started at the first head movement of the fish, followed by stage 2, which was defined as the first reversal movement of the head and ended once the fish's body straightened during the contralateral contraction resulting in a half tail beat (Domenici and Blake, 1997; Eaton et al., 2001). Lolitrack 5 (Loligo Systems, Copenhagen, Denmark) was used to track a fish's centre of mass (CoM) and extract the three following variables: (1) escape distance (D_{esc}): distance covered in a fixed amount of time, (2) maximum speed (U_{max}) and (3) maximum acceleration (A_{max}). Following the onset of stage 1, all variables were measured over 5 ms, which corresponds to the mean duration of stages 1 and 2 for all fish. We used ImageJ (National Institutes of Health, Bethesda, MD, USA) to estimate (4) a fish's distance to the stimulus prior to the stimulus touching the water (i.e. the straight-line distance between the fish's CoM and the centre of the stimulus), and (5) the fish's orientation relative to the stimulus [i.e. the angle formed by (a) the linear segment relating the fish's CoM to the centre of the stimulus and (b) the linear segment relating the fish's CoM to its snout; Jornod and Roche, 2015]. These last two measurements (4 and 5) were included in the models to verify whether the fish's position relative to the stimulus influenced escape responsiveness, latency or D_{esc}. We did not examine the effect of parasites on maximum speed and acceleration to reduce the number of statistical tests and as they are the first and second derivative of distance which is analysed in this study. See Table S4 for summary statistics of variables observed during the fast-start experiment.

Statistical analysis

All data were analysed in R v. 3.6.1 (http://www.R-project.org/). Pearson's correlation coefficient was used to test for a relationship between visible and non-visible infections in pumpkinseed sunfish. We included 44 fish collected at the same time and with the same collection methods from another study. General linear models (LM; lm function in R) were used to model the effect of parasite load on metabolic traits (MMR, SMR and AS). The number of internal parasites, number of black spots, fish body mass (parasite-corrected fish mass), the interaction between the number of internal parasites and fish body mass, and the interaction between the number of black

4

spots and fish body mass were included as predictors in all three models of metabolic traits. Collinearity between fixed factors in the models was assessed using the variance inflation factor (VIF; vif function in *car* package; Fox and Weisberg, 2011). The number of black spots and fish body mass were correlated (n=42; Pearson's correlation r=0.38, P=0.01), but the VIF terms for these predictors were low (2 at most), so we kept both in all of our models (Legendre and Legendre, 2012). As fish body mass and fish total length were highly correlated (n=42; Pearson's correlation r=0.96, P<0.001), only one of the two was used as a predictor in each model; fish body mass was used in models with metabolic traits as response variables, and TL was used in models with measures of escape performance as response variables.

A general linear model was used to quantify the effect of parasite load on response latency. Response latency was log10 transformed to meet model assumptions. The number of internal parasites, number of black spots, fish total length, distance, and angle of the fish relative to the stimulus, the interaction between the number of internal parasites and fish total length as well as the interaction between the number of black spots and fish total length were included as fixed effects in the model.

A general LM was used to quantify the effect of parasite load on D_{esc}. The number of internal parasites, number of black spots, fish TL, distance and angle of the fish relative to the stimulus, the interaction between the number of internal parasites and fish TL as well as the interaction between the number of black spots and fish TL were included as fixed effects in the model.

We used a generalized linear mixed-effects model (GLMM) with a binomial error distribution (logit link) using the package *lme4* (Bates et al., 2014) to quantify the effect of parasite load on fish responsiveness during escape response experiments. Fish ID was included as a random effect. The number of internal parasites, number of black spots, fish TL, distance and angle of the fish relative to the stimulus, and the interaction between the number of internal parasites and the number of black spots were included as fixed effects. Covariates in all models were z-transformed using the scale function in R. The angle of the fish relative to the stimulus was sine transformed following Roche (2021). Model assumptions were assessed visually with diagnostic plots and were met for all models (we used functions in the package *DHARMa* for GLMMs; https://CRAN.R-project.org/package=DHARMa): the residuals of all models were normal; no relationship was observed between the residuals and the observed variable and no deviation from the 1:1 line in q–q plots.

RESULTS

The number of black spots found on a fish's left side ranged from 6 to 273 (median: 56.5), and the number of internal parasites was between 0 and 153 (median: 15). Internal parasite counts include *P. ambloplites* and *C. marginatum* (see Materials and Methods, 'Fish dissection'). We found no relationship between the number of internal parasites and the number of black spots present in a fish (n=86, Pearson's correlation r=0.12, P=0.24; Fig. S1). Therefore, visible and non-visible loads were treated as separate variables in analyses.

Aerobic metabolic performance

MMR ranged from 2.3 to 7.5 mg O2 h^{-1} while SMR ranged from 0.42 to 2.9 mg O2 h^{-1}. There was a significant positive relationship between all three metabolic traits estimated and fish body mass (Table 1), and no significant interactions between parasite load (black spot and internal) and fish body mass for any

Table 1. Relationship between the three metabolic traits estimated, fish body mass and parasite load

Response	Predictors	d.f.	F-value	P-value	R^2
MMR (mg O2 h^{-1})	Black spots	1, 33	0.82	0.37	0.67
	Internal	1, 33	5.15	**0.03**	
	Mass	1, 33	59.20	**<0.001**	
	Black spots×mass	1, 33	3.38	0.08	
	Internal×mass	1, 33	0.38	0.54	
SMR (mg O2 h^{-1})	Black spots	1, 33	0.44	0.51	0.38
	Internal	1, 33	7.75	**0.009**	
	Mass	1, 33	12.64	**<0.001**	
	Black spots×mass	1, 33	0.53	0.47	
	Internal×mass	1, 33	0.88	0.35	
AS (mg O2 h^{-1})	Black spots	1, 33	0.54	0.47	0.59
	Internal	1, 33	2.27	0.14	
	Mass	1, 33	47.02	**<0.001**	
	Black spots×mass	1, 33	2.80	0.10	
	Internal×mass	1, 33	0.13	0.73	

Test statistics obtained from linear models (LM) of maximum metabolic rate (MMR), standard metabolic rate (SMR) and aerobic scope (AS) as a function of black spots, internal parasites (Internal), fish body mass (Mass), the interaction between black spots and mass (Black spots×mass), and the interaction between internal parasites and mass (Internal×mass) in pumpkinseed sunfish (n=39). Statistically significant results are indicated in bold. See Table S1 for test statistics for the relationship between parasite load and metabolic traits for fish with 0–50 parasites and Tables S2 and S3 for model estimates without interactions.

of the metabolic traits estimated (Table 1). None of the three metabolic traits estimated was related to black spot load (Fig. 1A, C,E); however, both MMR and SMR were negatively related to internal parasite load (Fig. 1B,D): fish with a higher number of internal parasites had a lower MMR and SMR (Table 1). There was no relationship between internal parasite load and AS (Table 1). The number of internal parasites ranged from 0 to 50 for all fish except for one individual harbouring 153 internal parasites. When this individual was excluded from the analysis, both MMR and SMR were still negatively related to internal parasite load (Table S1 and Fig. S2A,B). However, AS decreased with internal parasite load when this individual was excluded (Table S1 and Fig. S2C).

Escape behaviour

There was no relationship between response latency to an aerial attack and parasite load (black spot or internal) (LM: n=30, black

Table 2. Relationship between response latency to an aerial attack, parasite load, fish length, and distance and angle of the fish relative to the stimulus

	d.f.	χ^2	P-value	Estimate	R^2
Black spots	1	3.7	0.05	1.07	0.04
Internal	1	4.62	**0.03**	−1.07	0.05
Distance	1	10.67	**0.001**	−3.48	0.20
Angle	1	0.27	0.6	0.14	0.01
TL	1	10.43	**0.001**	−13.74	0.10
Black spots×TL	1	0.12	0.73		
Internal×TL	1	0.03	0.85		

Generalized linear mixed-effects model (GLMM) estimates for the effect of black spot load, internal parasite load, distance and angle of the fish from the stimulus and total length (TL) on responsiveness of pumpkinseed sunfish. Estimates are from the model without the interactions. Marginal R^2 for the model=0.35. Statistically significant results are indicated in bold.
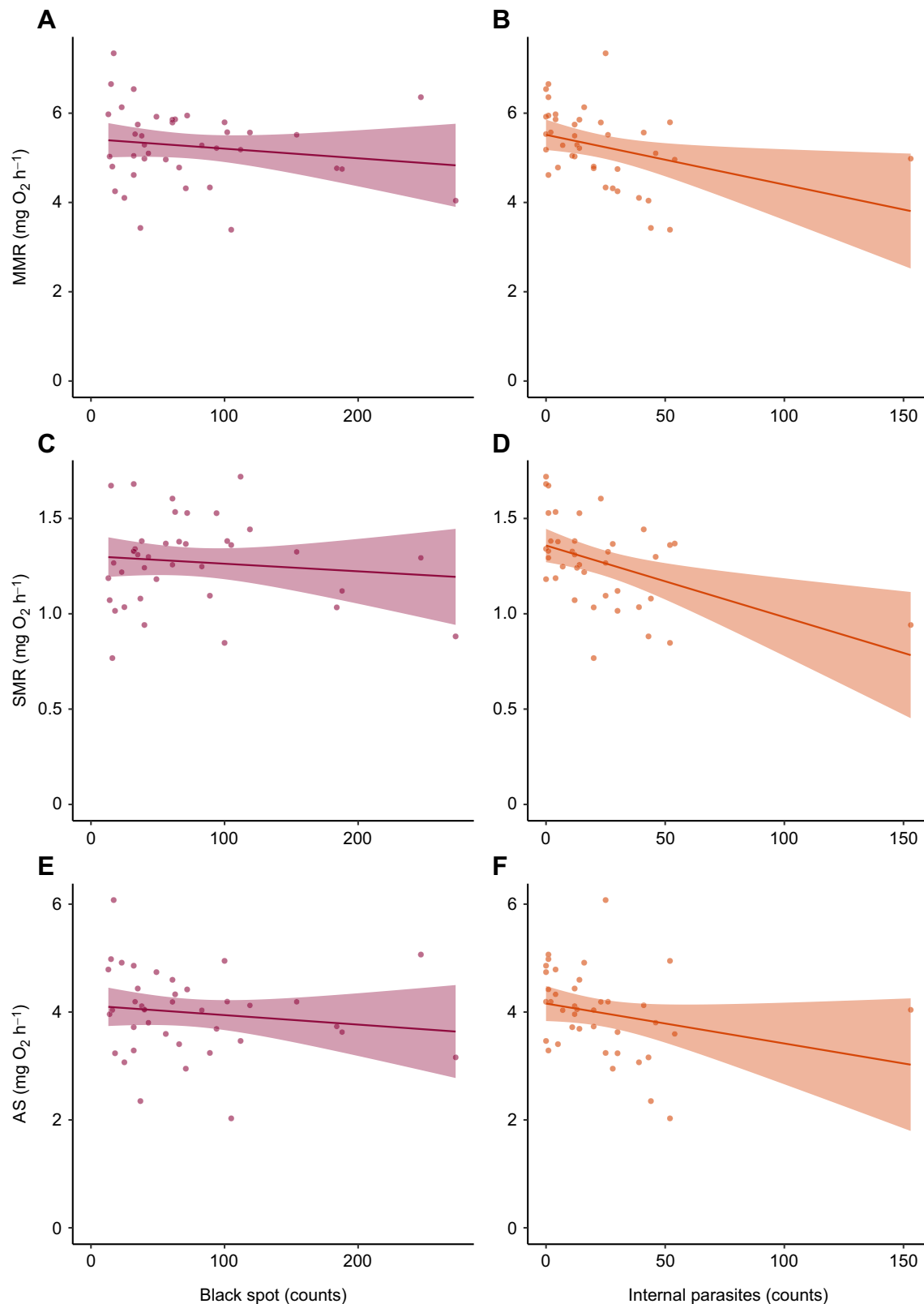
Fig. 1. Relationship between host metabolic traits and parasite load. Mass-adjusted metabolic traits (A,B: maximum metabolic rate, MMR; C,D: standard metabolic rate, SMR; E,F: aerobic scope, AS) as a function of number of black spots (left) and number of internal parasites (right) in pumpkinseed sunfish (*n*=39). Points represent individual fish. The shading around the regression lines represents 95% confidence intervals. Mass-adjusted metabolic rates are metabolic rates (MMR and SMR) adjusted to a common body mass (10.4 g) by adding the residuals of a regression of log metabolic rate versus log body mass to the fitted model value for the average body mass of all fish in the study (see Fig. S1 for the relationship between parasite load and metabolic traits for fish with 0–50 parasites).
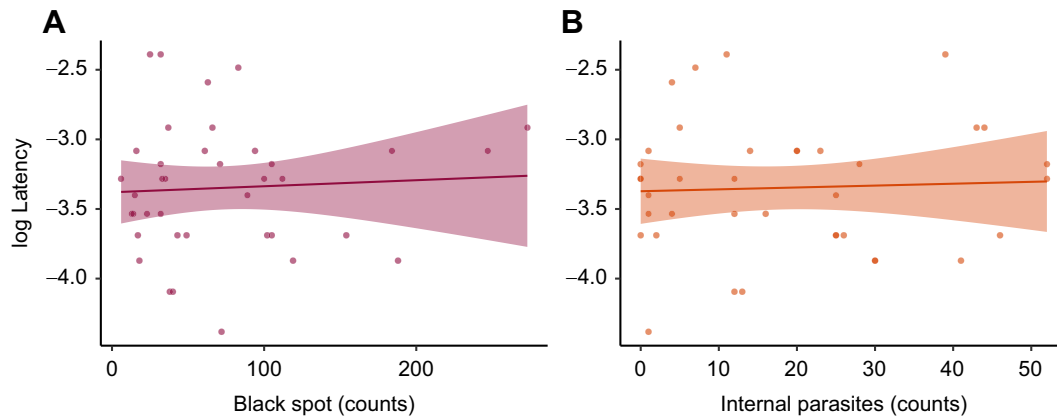
Fig. 2. Relationship between response latency and parasite load. Influence of (A) black spots and (B) internal parasites on escape latency (in s) in pumpkinseed sunfish ($n=38$). Points represent individual fish. The shading around the regression lines represents 95% confidence intervals.

spot: $F=0.13$, $P=0.72$; internal: $F=1.05$, $P=0.31$; Fig. 2), TL (LM: $n=30$, $F=0.002$, $P=0.96$), or distance and angle of the fish relative to the stimulus (LM: $n=30$, distance: $F=0.07$, $P=0.79$; angle: $F=3.73$, $P=0.07$). None of the interactions between black spots and TL ($P=0.09$) or internal parasites and TL ($P=0.35$) were significant.

There was no significant relationship between D_{esc} and parasite load (black spot or internal) (LM: $n=35$, black spot: $F=1.32$, $P=0.26$; internal: $F=0.48$, $P=0.26$), host TL (LM: $n=35$, $F=4.14$, $P=0.05$) and distance or angle of the fish relative to the stimulus (LM: $n=30$, distance: $F=0.99$, $P=0.33$; angle: $F=0.26$, $P=0.61$). None of the interactions between black spots and TL ($P=0.21$) or internal parasites and TL ($P=0.26$) were significant.

There were no significant interactions between any of the measured variables in the model with responsiveness to the stimulus as the response variable (Table 2). There was no relationship between fish responsiveness and black spot load (Fig. 3A, Table 2). However, there was a significant negative relationship between fish responsiveness to an aerial attack and fish length (GLMM: $n=42$, $\chi^2=10.43$, $P<0.001$) and a significant effect of the distance of the fish from the stimulus on responsiveness (Table 2). Larger fish responded less often than smaller conspecifics and fish further from the stimulus responded less to the simulated aerial attack. There was also a significant negative relationship between fish responsiveness

and the number of internal parasites (GLMM: $n=42$, $\chi^2=4.62$, $P=0.03$). Heavily infected fish responded less often to a simulated aerial attack than those with a lower level of infection (Fig. 3B). However, this relationship seemed to be driven by two heavily infected individuals (107 and 153 internal parasites, respectively). When these individuals were removed from the analysis, this relationship was no longer present (GLMM: $n=40$, $\chi^2=0.006$, $P=0.94$; Fig. 3C).

DISCUSSION

Our results highlight the importance of considering parasite load when studying the physiological and behavioural performance of wild animal populations. We found that metabolic rate estimates (MMR and SMR), as well as responsiveness to a simulated predator attack decreased along a gradient of non-visible internal parasite infection in pumpkinseed sunfish. This is one of the first studies investigating the impact of parasite load on aerobic metabolic and escape response performance in adults across two different types of infection (i.e. externally visible black spot infection versus non-visible internal infection). Our results suggest that experimental studies interested in animal performance may be missing an important driver of intraspecific trait variation by not taking natural parasite infections into account.
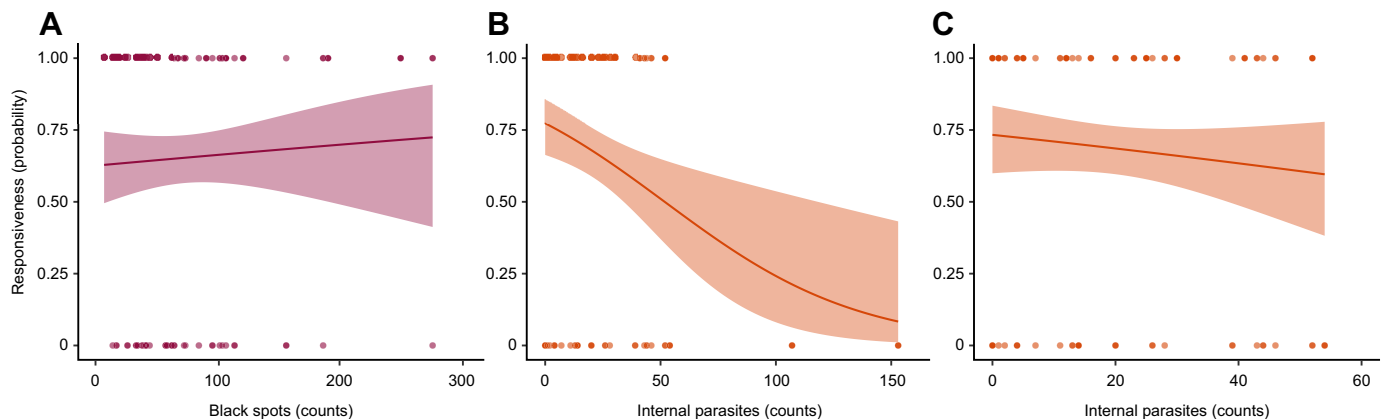

Fig. 3. Relationship between responsiveness to a simulated aerial attack and parasite load. (A) Effect of black spots, (B) internal parasites and (C) internal parasites excluding the two most heavily infected individuals on the proportion of trials eliciting a fast-start during escape response experiments in pumpkinseed sunfish ($n=42$). Points represent escape response measurements (up to 3 per fish). The shading around the regression lines represents 95% confidence intervals.

Aerobic metabolic performance

Aerobic metabolic performance traits measured in pumpkinseed sunfish were not related to black spot load. Other studies have also found no noticeable effect of black spot infection on various aspects of host performance capacity. For example, black spot infection did not impact the critical thermal limit or the body condition of three cyprinid species (Hockett and Mundahl, 1989). Similarly, Vaughans and Coble (1975) found no effect of black spot number on the length–mass relationship, temperature tolerance or susceptibility of yellow perch, *Perca flavescens*, to predation. Black spot formation is the result of the host's immune system responding to trematode metacercaria encysting in the host tissues, usually the muscle or fins (Berra and Au, 1978). However, once encysted, metacercaria have very low metabolic costs and, thus, are unlikely to have long-lasting direct effects on the host's metabolic traits once the infection is visible (Lemly and Esch, 1984). However, Lemly and Esch (1984) found that the oxygen consumption rates of bluegill sunfish increased approximately 1 month following experimental infection with the black spot-causing trematode *Uvulifer ambloplitis*. This corresponds to the average development time (21 days) of the parasite in this host and reflects the time period during which the parasite is likely to extract an energetic toll. Oxygen consumption rates returned to pre-infection levels 2 months after experimental infection (i.e. 1 month after the formation of visible cysts), suggesting that the metabolic costs of these infections are short lived (Lemly and Esch, 1984). A recent meta-analysis also found that the host stress response to parasites is higher early in an infection (O'Dwyer et al., 2020). One rationale for exploring the effects of black spot trematodes on fish performance capacity was to assess whether this visible infection could be used as a proxy for overall infection load and costs, given the ease with which black spots can be counted on their hosts. Unfortunately, we did not find a strong relationship between these visible infections and internal parasite load (Fig. S1), meaning that there is no shortcut to quantifying overall parasite load when assessing the impact of infection on individual performance traits.

Although we found no trend between black spot trematodes and aerobic metabolic traits, there were strong negative relationships between the intensity of the internal parasite load and metabolic rates. Indeed, we found that both MMR and SMR were lower when internal parasite load was high. This trend was probably driven by infection with *P. ambloplites*, which was much more prevalent and abundant in our population than *C. marginatum* (93% versus 26% prevalence; 0–153 versus 0–7 parasites per fish, respectively) and more likely to cause extensive tissue damage. *Proteocephalus ambloplites* tapeworms infect sunfish through the ingestion of infected crustaceans (first intermediate host) such as copepods and cladocerans (Bangham, 1927). The cestode larvae then make their way through the fish's intestinal walls to the body cavity, where they derive energy and nutrients from host tissues including the liver and gonads (Daly et al., 2006). As such, *P. ambloplites* can cause substantial damage to the organs of its intermediate and final hosts (piscivorous fishes) (Esch and Huffines, 1973; Mitchell et al., 1983). Conversely, *C. marginatum* encysts in the fish's skin, gills and muscle, and can cause physical damage at the site of encystment as a consequence of its relatively large size (3–8 mm) (Lane and Morris, 2000). Although the taxonomy and distribution of *Clinostomum* and *Proteocephalus* species have been relatively well studied (Osborn, 1911; Mackie et al., 1983; Muzzall and Peebles, 1998; Caffara et al., 2011; Zimik et al., 2019), little is known about their effects on any of their hosts' physiology or behaviour. Our study is among the first to document decreases in physiological and behavioural performance in fishes with high loads of these parasites, which is surprising given their high prevalence and widespread distribution throughout North America.

SMR represents the minimum rate of energy expenditure required to sustain life and sets the floor for an animal's aerobic metabolic performance (Chabot et al., 2016b). Our results show that parasite infection can be associated with reductions in SMR. Although some studies suggest that parasites tend to increase host energy demands through immune stimulation and maintenance costs (Hvas and Bui, 2022), infection can conversely lead to metabolic suppression in hosts either through a reduction in organ or tissue (e.g. muscle) mass or through a decrease in the function of organs associated with energy metabolism (Santoro et al., 2013; Mehrdana et al., 2014; Ryberg et al., 2020). Although a lower SMR can be advantageous in scenarios where food or oxygen is limited (Killen et al., 2016), reduced SMR associated with high parasite loads is more likely to be a pathological consequence of infection; as much of an individual's SMR is used to maintain internal organ function, damage caused by parasites can reduce organ function and, thus, SMR (Hulbert and Else, 2000; Seppänen et al., 2008; Behrens et al., 2014; Ryberg et al., 2020). For example, Eastern Baltic cod, *Gadus morhua*, infected with high levels of the liver nematode *Contracaecum osculatum* displayed lower SMR, reduced albumin to globulin ratio, and reduced liver lipid content, suggesting that the metabolic function of this organ is compromised at high parasite loads (Ryberg et al., 2020). Similarly, *P. ambloplites* were mostly found in our fish's liver, which was often damaged when infection loads were high. This suggests a causal effect of *P. ambloplites* infection on host aerobic metabolic performance in these sunfish – as the parasite causes direct damage to the fish's liver, which is a metabolically important organ, the observed decrease in SMR with increasing parasite load is probably caused by this infection. Experimental infections are needed to establish a causal link between infection and decreased host performance in this system.

MMR sets the ceiling for aerobic metabolic performance and is associated with increased performance during energetically demanding activities and in high-energy environments (Eliason et al., 2011; Binning et al., 2014; Norin and Clark, 2016). Our results show that internal organ and tissue damage caused by endoparasites probably reduces both MMR and SMR in hosts. Studies across taxa report decreases in host MMR with parasite infection (e.g. Careau et al., 2012; Bruneaux et al., 2017; Hvas et al., 2017). Importantly, a decrease in MMR is also often associated with a decrease in AS (Norin and Clark, 2016). We did not observe a decrease in AS with increasing internal parasite load across the entire range recorded (0–153 internal parasites). Nevertheless, removing the most infected fish still resulted in an observable negative relationship between metabolic traits (MMR, SMR and AS) and internal parasite load over a range of 0–50 internal parasites. Reduced AS can result in less capacity for growth, reproduction and, potentially, survival of heavily infected individuals (Metcalfe et al., 2016). These relationships, and the potential ecological consequences in parasitized individuals, need to be explored more thoroughly.

Escape behaviour

Responsiveness to a simulated aerial attack was negatively correlated with internal parasite load, but not black spot trematodes. When startled, many fish species perform a characteristic C-start escape response, which is an important determinant of an individual's survival during a predator attack (Domenici et al., 2011). Escape

distance and latency to respond to this attack are all considered important parts of this reaction (Domenici et al., 2011), and can all be impacted by infection (Allan et al., 2020). Yet, we found no relationship between parasite infection and response latency in our adult sunfish. Other studies on adult fish have also found no effect of parasites on escape performance. In bridled monocle bream, *Scolopsis bilineata*, parasitized by *Anilocra* isopod ectoparasites, response latency, maximum velocity, maximum acceleration and cumulative distance travelled did not differ between infected and non-infected fish (Binning et al., 2014). Similarly, Ruehle and Poulin, (2019) failed to detect a significant reduction in escape performance in infected common bully, *Gobiomorphus cotidianus*, even when host vision was affected. In contrast, Flink et al., (2017) observed a negative relationship between parasite intensity and the reaction to an avian attack in round gobies, *Neogobius melanostomus*, infected with eye flukes, *Diplostomum* spp. Indeed, the overall effect of infection on host escape behaviour remains unclear.

Although the kinematic components of an animal's escape response offer useful predictors of an individual's escape performance, escape responsiveness is arguably the most important determinant of survival in the face of a threat (Domenici, 2010): an individual that does not react to an attacking predator has almost no chance of survival, regardless of how fast it can escape. In our study, the two most heavily infected individuals never responded to our simulated aerial attack. Although the negative relationship we observed between responsiveness and parasite load is driven by the escape performance of these two individuals, our results remain ecologically relevant: we collected very few other heavily infected individuals, possibly because these individuals are selectively removed from the population through predation. Over-dispersion of parasites within hosts, whereby a few individuals harbour most of the parasites in a population, is a well-documented ecological phenomenon (Anderson and Gordon, 1982). Although many factors can explain such patterns, including increased susceptibility and/or tolerance to infection in some individuals, our results suggest that performance reduction in heavily infected individuals may also play a role. If heavily infected individuals are predated upon at higher rates than uninfected or lightly infected individuals, in part as a result of decreased responsiveness, we would expect to sample fewer of these individuals in a given population. This phenomenon would also facilitate trophic transmission and therefore be beneficial to the parasite life cycle.

Other considerations

Host life stage can play a large role in individual responses to parasites. For example, juvenile chipmunks, *Tamias striatus*, show a 7.6% increase in resting metabolic rate in response to infection by botfly larvae, resulting in a ~5 g body mass loss over summer (Careau et al., 2010), whereas no effect of infection was observed in adults (Careau et al., 2012). It is possible that younger individuals are more affected by stressors, including infection, because more energy is required for growth and development (Careau et al., 2010; Allan et al., 2020). Older hosts also typically harbour more parasites than younger ones, probably because parasites are recruited faster than they die in hosts, especially in the case of encysted parasites such as those causing black spot disease (Hawlena et al., 2006). The fact that we did see strong relationships between internal infections and our performance measures, even in our adult fish, is a further reminder of the important impact that parasites can have on their hosts, and their contribution to otherwise unexplained intraspecific variation in performance often observed in natural populations (Timi and Poulin, 2020).

Our study explicitly quantified the infection load of both externally visible and internally non-visible parasites. However, the process of counting and identifying endoparasites is difficult and time consuming, and is often not included in the context of studies on wild populations (but see Ryberg et al., 2022). When infection is considered, the host's infection status (i.e. infected or not) is typically the variable of interest as it is often easier to assess than parasite load. This binomial categorization can be relevant and related to the impact of parasites on some host populations, especially in the case of large endoparasites or ectoparasites (Fogelman et al., 2009; Jolles et al., 2020). However, parasite load can be more important than infection status for understanding the physiological, behavioural and ecological effects of parasites on their hosts (Poulin, 2019; Timi and Poulin, 2020). For instance, killifish, *Fundulus parvipinnis*, infected with larval trematodes face higher rates of predation by birds along an infection gradient (predation rates in uninfected hosts: 0.02%, lightly infected hosts: 22%, heavily infected hosts: 80%) (Lafferty and Morris, 1996). As there are generally fewer highly parasitized individuals in natural host populations (Crofton, 1971; Shaw et al., 1998), it can be difficult to accurately estimate the effect of high parasite loads on populations because these individuals can be hard to sample and test in the lab. Heavily infected individuals may also be more susceptible to environmental stressors, which could potentially lead to selective mortality based on infection status during extreme events such as freezing or heat waves (Lemly and Esch, 1984; Bruneaux et al., 2017; Greenspan et al., 2017). The effects of concomitant stressors such as temperature and parasite load have rarely been tested in natural populations and this remains an area in need of further research, especially given projected future global changes.

Perspectives and conclusion

Hosts and parasites co-exist in a constant arms race where each player influences the evolutionary trajectory and selection on performance traits of the other. Yet, within host populations, parasites can also act as important constraints on individual performance, with impacts on survival. Our results suggest that parasite load is an important, often overlooked driver of intraspecific performance trait differences in host populations. Experimental infections are needed to confirm the causal relationship between infection load and performance traits in fish hosts. We expect experimental infection with black spot and/or internal parasites would result in similar performance trait impairments to those documented here. The fact that we were unable to link externally visible black spot infection with non-visible internal parasites is potentially problematic for experimental biologists as these visible infections are thus a poor proxy of overall infection load. Also, non-visible internal infections, which seem to be related to the highest performance costs, are less likely to be taken into consideration by experimental biologists. While we acknowledge that sacrificing individuals to quantify endoparasite infection is not always feasible or desirable in the context of experimental work on wild animals, we encourage researchers to consider alternative ways of controlling for this potential confounding effect, such as treating experimental animals with anti-parasite treatments like praziquantel (Bader et al., 2019) prior to testing their performance, after confirming that such treatments themselves do not impact the traits to be measured.

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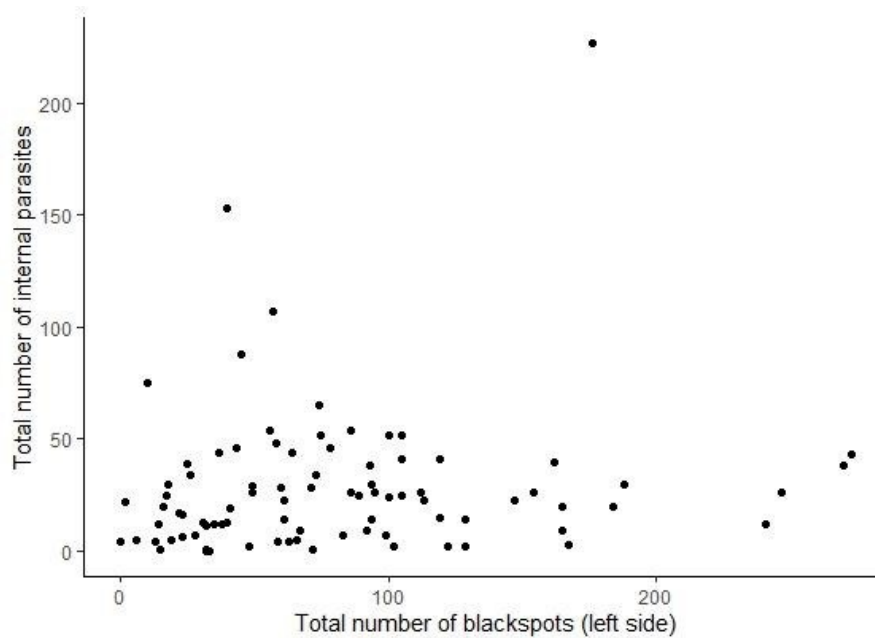
Fig. S1. Total internal parasite load versus the total number of black spots on the left side of pumpkinseed sunfish (*Lepomis gibbosus*) collected from Lake Cromwell (n=86). Representing all the fish sampled during the same period for this study and for preliminary tests.

There was no significant correlation between the number of black spots and number of internal parasites in fish collected from Lake Cromwell (N= 86, Pearson's correlation r = 0.12, P=0.23) (Fig. S1). This sample includes 86 fish dissected for a separate project and not analyzed here.
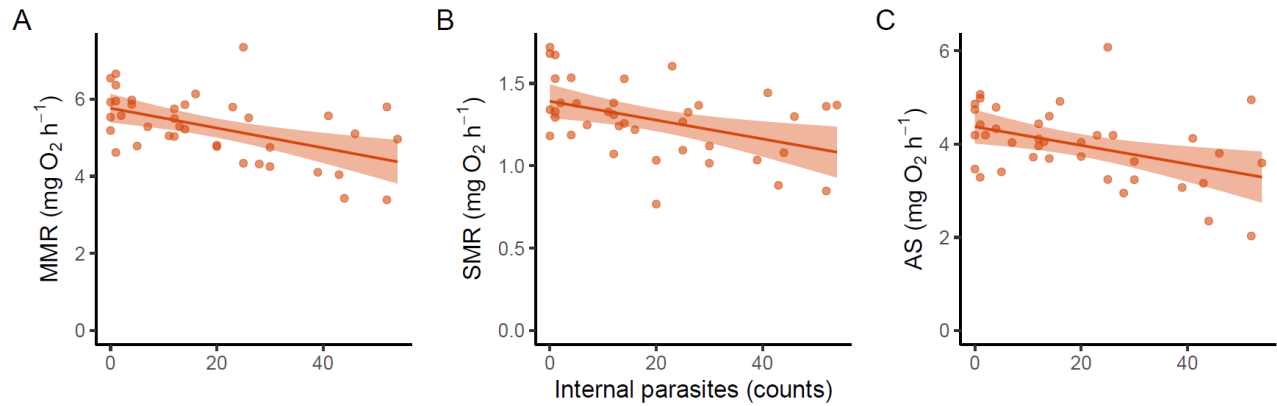
Fig. S2. Effect of parasites on metabolic traits without one heavily infected individual. Mass-adjusted metabolic rates (MMR, SMR, AS) as a function of parasite load for internal parasites without the heavily infected individual. (A) Influence of internal parasites on MMR. (B) Influence of internal parasites on SMR. (C) Influence of internal parasites on AS (n= 38).

Table S1. Test statistics obtained from linear regression models of MMR, SMR and AS as a function of black spot, internal parasites (Internal), fish body mass (Mass), and interactions between black spot number and mass (BS*mass), and internal parasites and mass (Int*mass) in sunfish from Lake Cromwell without the one individual with 153 internal parasites. (n= 38). Statistically significant results are in **bold.** See Table S3 for model estimates without interactions.

Response	Predictors	DF	F-value	P-value	R^2
MMR	Black spot	1, 32	0.18	0.68	0.69
(mgO_2h^{-1})	Internal	1, 32	13.16	**0.001**	
	Mass	1, 32	72.67	**<0.001**	
	Black spot * mass	1, 32	3.2	0.083	
	Internal * mass	1, 32	0.024	0.88	
SMR	Black spot	1, 32	0.21	0.65	0.28
(mgO_2h^{-1})	Internal	1, 32	6.93	**0.013**	
	Mass	1, 32	13.18	**0.001**	
	Black spot * mass	1, 32	0.42	0.52	
	Internal * mass	1, 32	0.37	0.55	
AS	Black spot	1, 32	0.08	0.78	0.62
(mgO_2h^{-1})	Internal	1, 32	8.01	**0.0079**	
	Mass	1, 32	55.70	**<0.001**	
	Black spot * mass	1, 32	2.56	0.12	
	Internal * mass	1, 32	0.12	0.73	

Table S2. Model estimates from fixed factors obtained using the summary function in R from models (Table S1) without interactions of pumpkinseed sunfish (*Lepomis gibbosus*) without the one individual with 153 internal parasites (n=38). (R^2a: adjusted R^2)

	SMR		MMR		AS	
	Estimates	R^2a	Estimates	R^2a	Estimates	R^2a
Intercept	0.81	0.31	1.38	0.68	0.57	0.61
Internal	-0.024		-0.092		-0.068	
Mass	-0.15		-0.69		-0.54	
Black spot	0.62		4.62		4.0	

Table S3. Model estimates from fixed factors obtained using the summary function in R from models (Table 1) without interactions (n=39).

	SMR		MMR		AS	
	Estimates	R^2a	Estimates	R^2a	Estimates	R^2a
Intercept	0.80	0.31	1.30	0.61	0.50	0.55
Internal	-0.04		-0.18		-0.14	
Mass	-0.14		-0.40		-0.27	
Black spot	0.60		4.50		3.90	

Table S4. Summary statistics of the variable observed during the fast-start experiment. Mean for all individuals, maximum, minimum performance for the variable observed for all fish, standard deviation (SD) and standard error (SE) for response latency (Latency), escape distance (D_{esc}), maximum speed (U_{max}), and maximum acceleration (A_{max}).

	Mean	Max	Min	SD	SE
Latency	0.05	0.15	002	0.03	0.005
D_{esc}	2.63	4.75	0.35	1.11	0.18
U_{max}	16.93	26.38	6.37	4.99	0.84
A_{max}	318.21	688.64	118.42	145.0	24.51