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PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Intensity-dependent energetic costs in a reciprocal parasitic relationship

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Abstract

Parasitic infections elicit host defences that pose energetic trade-offs with other fitness-related traits. Bitterling fishes and unionid mussels are involved in a two-way parasitic interaction. Bitterling exploit mussels by ovipositing into their gills. In turn, mussel larvae (glochidia) develop on the epidermis and gills of fish. Hosts have evolved behavioural responses to reduce parasite load, suggesting that glochidia and bitterling parasitism are costly. We examined the energetic cost of parasitism on both sides of this relationship. We used intermittent flow-through respirometry to measure (1) standard metabolic rate (SMR) of individual duck mussels *Anodonta anatina* (a common bitterling host) before and during infection by embryos of the European bitterling *Rhodeus amarus*, and (2) SMR and maximum oxygen uptake (MO_2max) of individual *R. amarus* before and during infection with glochidia of the Chinese pond mussel *Sinanodonta woodiana* (a mussel species that successfully infects bitterling). As predicted, we observed an increase in mussel SMR when infected by bitterling embryos and an increased SMR in glochidia-infected bitterling, though this was significantly mediated by the time post-infection. Contrary to our predictions, glochidia infection did not impair MO_2max and the number of glochidia attached to gills positively (rather than negatively) correlated with MO_2max. The results suggest that tolerance is the prevailing coping mechanism for both fish and mussels when infected, while resistance mechanisms appear to be confined to the behavioural level.

Keywords Acheilognathinae · Coevolution · Evolutionary arms race · Host–parasite relationship · Unionidae

Introduction

Parasitism is a ubiquitous life-history strategy whereby one species exploits another. To counter the costs imposed by a parasite, hosts mobilize defences. Defence mechanisms are likely to face energetic trade-offs with other fitness-related traits (Sheldon and Verhulst 1996). Indeed, mounting an immune response is energetically costly, with immunologically challenged individuals displaying increased resting metabolic rates (Ots et al. 2001; Martin et al. 2002), and coping with parasite infection demands greater energy uptake (Knutie et al. 2016). Since resources are limited, less energy will be available for other fitness-related functions, such as growth (Barber et al. 2011), reproduction (French et al. 2009) and locomotor performance (Husak et al. 2016). Therefore, a general assumption is that parasites have a negative impact on host fitness via energetic trade-offs (Robar et al. 2011; Knutie et al. 2017).

Bitterling fishes (Acheilognathinae) and unionid mussels (Unionidae) are involved in a two-way host–parasite relationship. Bitterling are obligate brood parasites of mussels (Wiepkema 1961) and unionid mussels possess a larval stage (glochidium) that is obligate parasite of fish, including bitterling (Barnhart et al. 2008; Douda et al. 2017a). Female bitterling oviposit into the gills of host mussels where embryos reside for approximately 1 month before emerging as active juveniles (8–10 mm large) (Aldridge 1999). Bitterling eggs (2.5–3 mm along the longest axis) lodge in the interlamellar spaces and water tubes of the mussel gill. Upon hatching (2 days), embryos anchor themselves in the mussel gills using specialized structures, such as yolk sac projections and modification of the skin surface (Kim and Park 1985; Aldridge 1999). Bitterling embryos can compromise

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mussel gill functions (feeding and gas exchange) by damaging the ciliated epithelium and disrupting water flow across the gill (Stadnichenko and Stadnichenko 1980). Embryos also directly compete with the host mussels for oxygen and nutrients (Spence and Smith 2013) and impede their growth (Reichard et al. 2006). To avoid these costs, mussels can decrease the success of oviposition (Reichard et al. 2010) and expel bitterling eggs and embryos (Smith et al. 2000; Mills and Reynolds 2003) by rapid closure of the valves (Kitamura 2005).

Glochidia (size < 400 µm), parasitic larval stages of unionid mussels, are discharged into the water column and must attach on fish host, typically on the fins and gill filaments (Barnhart et al. 2008). Once attached, glochidia must be encysted in host tissue to complete their metamorphosis into juveniles (Arey 1932; Dudgeon and Morton, 1984), which lasts several days to weeks (Barnhart et al. 2008). The encystment causes localized tissue swelling and can cause fusion of gill filaments and lamellae. This reduces the surface area for gas exchange and can lead to impaired respiratory performance manifested as higher basal ventilation rates (Crane et al. 2011), lower critical swimming speed (Taeubert and Geist 2013), or longer time to reach basal ventilation rates after peak exercise (Thomas et al. 2014). High glochidia loads can even cause host mortality (Howerth and Keller 2006; Taeubert and Geist 2013). The activation of both non-specific (Meyers et al. 1980) and specific immune defences have been observed in glochidia-infected fish (Dodd et al. 2006). While antibody response against glochidia has not been documented in bitterling, bitterling have evolved behavioural adaptations to minimize the risk of exposure (Rouchet et al. 2017) and are capable of shedding attached glochidia (Douda et al. 2017a; Modesto et al. 2018).

The bitterling–mussel relationship evolved in East Asia (Chang et al. 2014), where unionid mussels frequently eject bitterling eggs and embryos (e.g. Kitamura 2005; Reichard et al. 2007; Kitamura et al. 2012) and bitterling are largely devoid of glochidia infections (Dudgeon and Morton 1984; Douda et al. 2017a). We have previously detected a positive correlation between resting metabolic rate of Chinese pond mussel (*Sinanodonta woodiana*) and the number of Chinese rose bitterling (*Rhodeus ocellatus*) embryos incubating on the gills of mussels (Methling et al. 2018), suggesting that bitterling embryos do impose an energetic cost on their host. In turn, glochidia load may also affect host energetics. Increased standard metabolic rate (Filipsson et al. 2017) and swimming costs (Slavík et al. 2017) as well as decreased feeding activity (Österling et al. 2014) and growth rates (Ooue et al. 2017) were demonstrated in fish hosts. However, whether glochidia infection imposes energetic cost on bitterling is not known.

Bitterling presence in Europe is natural but relatively recent (Bohlen et al. 2006; Chang et al. 2014). The European bitterling (*Rhodeus amarus*) uses all European unionids as hosts for their embryos (Smith et al. 2000). European mussels rarely eject bitterling embryos (Reichard et al. 2010) and their glochidia fail to parasitize bitterling (Huber and Geist 2019), leaving European bitterling as a parasite of European unionid mussels that avoids hosting their glochidia. *Sinanodonta woodiana* is a recent East Asian invader to European unionid mussel communities (Konečný et al. 2018) that effectively ejects all *R. amarus* embryos while its glochidia successfully parasitize *R. amarus* (Reichard et al. 2012, 2015). This contrast in the ability of native European mussels and invasive *S. woodiana* to parasitize *R. amarus* enables energetic quantification of the host–parasite relationship between bitterling fish and unionid mussels despite their effective defences, though we acknowledge that our study tests general predictions for the bitterling–mussel association rather than the coevolutionary relationship between the two species.

Specifically, we test the hypothesis that hosting bitterling embryos is associated with an elevated resting metabolic rate in mussels and, likewise, that glochidia infections are associated with an elevated resting metabolic rate in bitterling. In addition, we test the effect of glochidia infection on the maximum oxygen uptake rate of bitterling, predicting that glochidia will cause a decrease in the maximum oxygen uptake rate by impairing gill function. We predicted that the costs of parasite infections are positively related to the intensity of infection. To test these predictions, we first measured oxygen uptake at rest (standard metabolic rate, SMR) in the duck mussel *Anodonta anatina* hosting embryos of the European bitterling *R. amarus* and compared it with the oxygen uptake of non-parasitized mussels, using a before–after design on control and experimentally parasitized individuals. We also measured SMR and maximum oxygen uptake (MO_2max) in *R. amarus* before and after infection with *S. woodiana* glochidia.

Materials and methods

Animals and husbandry

Rhodeus amarus and *A. anatina* were collected in April 2018 from the River Kyjovka (fish) and adjacent oxbow lakes (mussels) and transported to the Institute of Vertebrate Biology, Brno, Czech Republic, where they were housed outdoor in large 1700-L fiberglass tanks. Fish tanks were equipped with artificial plants to provide shelter. A mussel was added to each tank in which fish were housed prior to their use to stimulate fish reproductive activity. All tanks were exposed to natural daylight and water temperature varied between 14 and 22 °C. Fish were fed twice each day with a mix of bloodworm and *Daphnia* while mussels

fed on abundant phytoplankton. Ten days prior to the pre-treatment measurement of bitterling respiration, bitterling ($N=48$) were moved indoors and housed in a 240 L tank for temperature acclimation (21–24 °C). Four days prior to pre-treatment measurements, bitterling were housed in pairs (one male, one female) in 15-L tanks to allow individual identification. Indoor tanks were exposed to natural daylight. Water temperature ranged between 24 and 25 °C between pre-treatment and post-treatment measurements.

Respirometry

Metabolic rates of individual mussels and bitterling were estimated indirectly by measuring oxygen consumption rates using intermittent flow-through respirometry (Steffensen 1989). Respirometry was performed in an air-conditioned darkened room. Respirometers were placed in a 100-L holding tank. The holding tank was continuously supplied with aged water from a 100-L reservoir passed through a sponge filter and UV lamp to minimize microbial respiration. Water temperature was maintained at 22.0 ± 0.1 °C (mussels) and 24.0 ± 0.1 °C (fish). The respirometers were fitted with two sets of tubing: one recirculation loop and inlet/outlet for periodically flushing the respirometer with water from the holding tank. Chamber oxygen partial pressure (pO_2) was measured with an OXY-4mini (PreSens, Germany) fibre optic O_2 transmitter, placed in the recirculation loop and recorded by the AutoResp4™ software (Loligo Systems, Denmark).

For *A. anatina*, four individuals (two control and two treatment) were collected from the outdoor tank in the morning and their shells were gently brushed to remove epibionts. Shell length, width and height were recorded to estimate mussel volume and each mussel was individually marked. Mussels were transferred to the respirometers and MO_2 was measured over the following 24 h. Chambers were periodically flushed for 4 min., followed by a closed 1-min wait period to reach steady state, followed by a 5-min closed measuring period. For *R. amarus*, two pairs (two males and two females) were transferred to individual respirometers after recording their wet mass. Respirometers were wrapped in dark coating so that fish were not able to see each other. MO_2 was measured over the following 23 h to estimate standard metabolic rate (SMR) using the same procedure as described for mussels. The following morning, fish were removed from the respirometer, transferred to small 1.5-L circular tank, where they were chased for 3 min and exposed to air for 30 s (netting) in order to elicit maximum metabolic rate (MO_2max) (Clark et al. 2013). Fish were immediately returned to the respirometer (30–40 s before the beginning of a measurement period) and MO_2max was estimated as the first MO_2 measurement after the chasing protocol.

Experiment 1: exposure to spawning bitterling

Forty-eight mussels (shell length 67.7–91.3 mm) were semi-randomly (size matched) assigned to either control ($N=24$) or treatment ($N=24$) group. After pre-treatment MO_2 measurements, mussels were placed in outdoor tanks with a group of reproductively active bitterling for 10 days. All mussels were positioned in sand-filled plastic containers. Control mussels were placed in nylon mesh bags to prevent bitterling oviposition while treatment mussels were fully exposed to bitterling oviposition. Post-treatment measurements were performed after 10 days using the same procedure as in the pre-treatment measurements. After the MO_2 post-treatment measurement, mussels were dissected to quantify the number of bitterling embryos in their gills. Soft tissue was dried for 48 h at 65 °C and weighed. Control mussels were also checked for the presence of bitterling embryos, but none were found.

Neither shell length (LM: $t_{45}=0.42$, $P=0.680$) nor dry mass (LM: $t_{45}=1.54$, $P=0.132$) differed between control and treatment mussels. Mean shell length was 80.3 mm (95% CI 77.7–82.8 mm) and 81.0 mm (95% CI 78.6–83.3 mm), for control and infected mussels, respectively. Dry mass was 1.09 g (95% CI 0.9–1.28 g) and 1.31 g (95% CI 1.08–1.54 g) for control and infected mussels, respectively.

Experiment 2: glochidia infection of bitterling

Forty-eight bitterling were semi-randomly (size matched and with equal sex ratio) assigned to either control ($N=18$; wet mass: 1.7 g ± 0.6 standard deviation, SD) or treatment group ($N=30$; wet mass: 1.7 g ± 0.6 g). The treatment group was intentionally larger to accommodate the possibility that some bitterling would be resistant to glochidia infection, lowering the final effective sample size in the treatment group. After all MO_2 pre-treatment measurements were made, bitterling were infected with glochidia in three batches of 16 fish (six control, ten treatment). The batches were infected 4 days apart, so that post-treatment measurements were performed on day 1–4 post-infection and 16 days after pre-treatment measurements. The infection protocol followed Douda et al. (2017a). In brief, a suspension of glochidia was prepared by flushing the ripe marsupium of six gravid *S. woodiana* sampled in the River Morava with water from a syringe and diluting to a density of 4283 ± 2356 (mean ± SD) viable glochidia L^{-1}. Bitterling were placed in the circulating glochidia suspension (1 L) in individual tanks for 15 min and then returned to their tanks, except those to be measured on the first day of infection. Those fish were transferred to an aerated 2-L bath for 2 h to rinse off non-attached glochidia before MO_2 measurements were taken. Control fish were exposed to the same protocol, but with exposure to a glochidia-free suspension. After post-infection measurements,

fish were killed with an overdose of anaesthetic (clove oil) and the number of attached glochidia was quantified. No glochidia were found on control fish. Husbandry and experimentation adhered to legislation in the Czech Republic on animal welfare and the experimental protocol was approved (IVB, MO17/2).

Data analysis

Oxygen consumption rate (MO_2) was derived from the decrease in chamber oxygen partial pressure (pO_2) during the measuring period according to: $MO_2 = V(d(pO_2)/dt)\ \alpha$, where V is the volume of the chamber (minus volume of mussel/fish) and α is the specific oxygen solubility. MO_2 measurements for which the regression coefficient (R^2) of the slope $d(pO_2)/dt$ was < 0.96 were excluded from the analysis (Chabot et al. 2016). All MO_2 measurements were corrected for microbial respiration recorded in empty chambers before and after each experiment. The SMR of mussels and fish was estimated as the lower 20th percentile of MO_2 measurements. The maximum uptake rate (fish) was estimated as the first MO_2 after the chasing protocol.

The effect of hosting bitterling embryos on mussel respiration rate was analysed using linear models (LM) with the absolute difference in SMR (mg O_2 h^{-1}) between pre- and post-treatment estimate (ΔSMR) as the response variable and infection status (treatment, control) as a factorial predictor. To quantify the effect of bitterling embryo load on mussel respiration, we additionally used post-treatment SMR estimate (mg O_2 g^{-1} h^{-1}) as a response variable and the number of bitterling embryos (log-transformed) as a covariate. Possible effects of temperature on respiration rate were included in models as the difference between the outdoor tank temperature (daily average before measurement) and temperature in the respirometry setup. Dry mass was included as a covariate in regression models.

The effect of glochidia infection on bitterling SMR and MO_2max was analysed using linear models (LM) with the absolute difference in SMR and MO_2max between pre- and post-treatment measurement as the response variable (ΔSMR and ΔMO_2max) and infection status (treatment, control) as factorial predictor. To quantify the effect of glochidia load on bitterling respiration, we additionally tested post-treatment SMR and MO_2max estimates as response variables and the number of attached glochidia (log-transformed) as a covariate. The regression models were first tested using the total number of attached glochidia (given their predicted effect on host energetics), and then using the number of glochidia attached to gills (given their direct effect in oxygen uptake). The temperature difference between pre-treatment measurement housing and respirometry setup, the difference in wet mass between pre- and post-treatment measurements, and days post-infection were included in models. Models were initially fitted with all variables and interaction terms and model selection was employed by stepwise removal of terms based on the Akaike Information Criterion (AIC). Assumptions regarding homoscedasticity and normality of residuals were examined by visual inspection of residual plots and Q–Q plots of final models. All analyses were performed using R version 3.3.3 (R Development Core Team 2017).

Results

Experiment 1: mussel SMR increased with bitterling load

In general, mussels had lower oxygen consumption at the post-treatment measurement with an average decrease by 28.7% (95% CI 20.8–35.9%) for control and 5.7% (95% CI 0.55–10.8%) for infected mussels. The decrease in SMR (ΔSMR) between pre- and post-treatment estimates was significantly lower in bitterling-infected mussels (LM: $t_{41} = 6.32$, $P < 0.0001$, $R^2_{adj} = 0.48$; Fig. 1a). The average increase in temperature between outdoor holding tank and respirometry setup was 4.2 °C (95% CI 3.9–4.5 °C) and 5.4 °C (95% CI 4.4–5.9 °C), for pre- and post-treatment measurement, respectively, and did not significantly contribute to the variation in ΔSMR ($t_{42} = 1.99$, $P = 0.053$ and $t_{41} = 0.18$, $P = 0.861$ for pre- and post-treatment measurements, respectively).

After 10 days of exposure to bitterling oviposition, mussel hosted an average of 30 (range 3–65) embryos lodged in their gills. There was a significant positive correlation between the number of embryos (log-transformed) and mussel SMR while hosting embryos (LM: $t_{15} = 2.90$, $P = 0.011$; Fig. 1b). There was no correlation between number of embryos and pre-measurement SMR (LM: $t_{18} = 1.17$, $P = 0.257$). The temperature difference between outdoor tanks and experimental setup had a significant positive effect on post-treatment SMR estimates (LM: $t_{15} = 4.16$, $P = 0.001$) and there was a significant negative correlation between SMR (mgO_2 g^{-1} h^{-1}) and dry mass (LM: $t_{15} = 14.03$, $P < 0.001$).

Experiment 2a: glochidia infection increased host SMR only at day 4 post-infection

Fish SMR decreased between pre- and post-treatment measurements, with a 6.8% (95% CI 1.9–15.6%) and 7.5% (95% CI 1.4–13.7%) decrease in control and glochidia-infected fish, respectively.

There was a significant effect of glochidia infection on ΔSMR, but this depended on time post-infection, with an interaction between treatment group (control/infected)

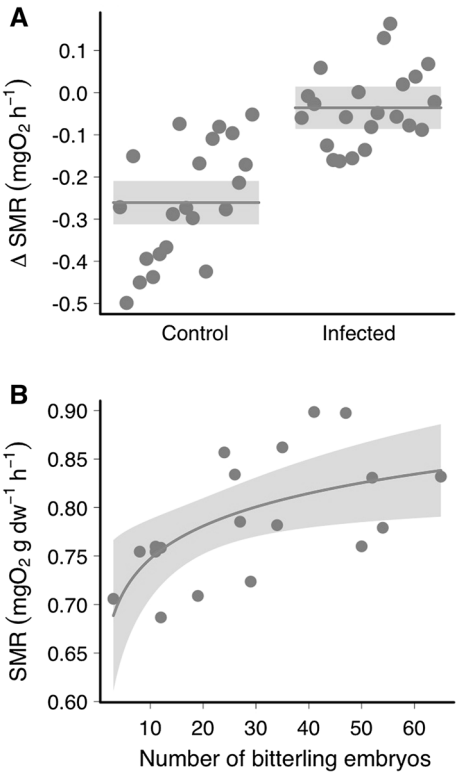

Fig. 1 Effect of hosting *Rhodeus amarus* embryos on the standard metabolic rate (SMR) of *Anodonta anatina*. **a** Differential change in the SMR in control and bitterling-infected mussels ($N=43$). **b** Association between the number of bitterling embryos and mussel SMR ($N=19$ infected mussels). Least squares regression lines are shown with 95% confidence intervals indicated by shaded areas. Note that regression line was fitted for log-transformed values of the number of bitterling embryos

and days post-infection (day 1–day 4) (LM: $t_{37}=2.78$, $P=0.009$; Fig. 2). On day 1 post-infection, ΔSMR was higher in control fish compared to infected fish. On day 2 and 3 post-infection, the difference in ΔSMR between control and infected fish diminished. On day 4 post-infection, ΔSMR was higher in infected fish than in control fish (Fig. 2). The ΔSMR was also affected by the temperature difference between holding tank and respirometer setup at the pre-treatment measurement (LM: $t_{37}=-4.51$, $P<0.001$).

Infected fish had on average 19 (range 0–77) glochidia attached in total, with an average of two (range 0–8) glochidia attached to their gill filaments. There was no correlation between the number of glochidia and post-treatment SMR estimates when all attached glochidia were included (LM: $t_{21}=0.09$, $P=0.931$; Fig. 3a). However, the number of glochidia attached to the gills (log-transformed) positively correlated with post-treatment SMR (LM: $t_{14}=2.97$, $P=0.011$; Fig. 3b).

Experiment 2b: glochidia load on host gills was associated with a higher MO_2max

Maximum oxygen uptake MO_2max was likewise generally lower in post-treatment measurements, with a decrease of 17.8% (95% CI 10.0–25.5%) and 14.8% (95% CI 9.4–20.2%) for control and treatment fish, respectively. There was no significant difference in ΔMO_2max between control and treatment fish (LM: $t_{39}=1.29$, $P=0.205$; Fig. 4a), but MO_2max was positively correlated with the number of glochidia attached to the gills (LM; $t_{14}=2.20$, $P=0.048$; Fig. 4b). On average, fish gained body mass between the measurements, with no significant difference between control and treatment fish (LM: $t_{46}=-1.18$, $P=0.241$). Control fish gained 8.7% (95% CI 1.6–15.7%) and treatment fish gained an average of 4.0% (95% CI −0.7 to 8.7%) of their body mass. The difference in body mass between measurements had a predictable allometric scaling effect on ΔMO_2max (LM: $t_{39}=-3.16$, $P=0.004$) and post-treatment MO_2max estimates (LM; $t_{12}=-3.81$, $P=0.003$); a mass gain resulted in a lower mass-specific MO_2max.

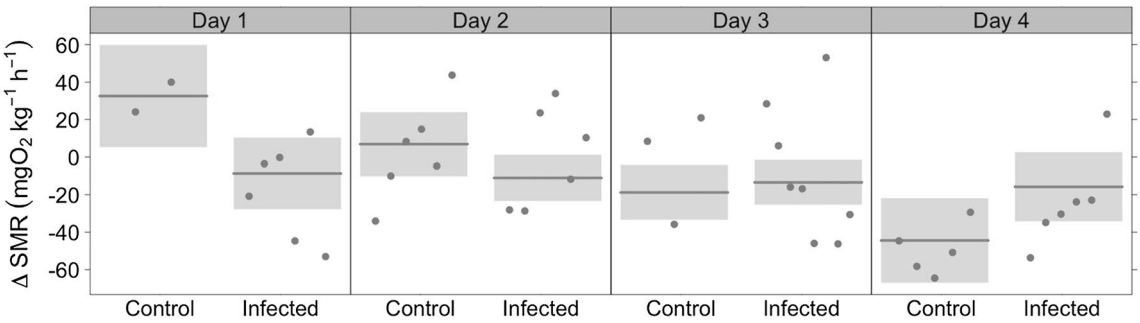

Fig. 2 Effect of *Sinanodonta woodiana* glochidia infection on the standard metabolic rate (SMR) of *Rhodeus amarus* over 4 days post-infection ($N=42$), expressed as differential change in SMR between control and experimentally infected fish. Predicted means are shown with 95% confidence intervals indicated by shaded areas

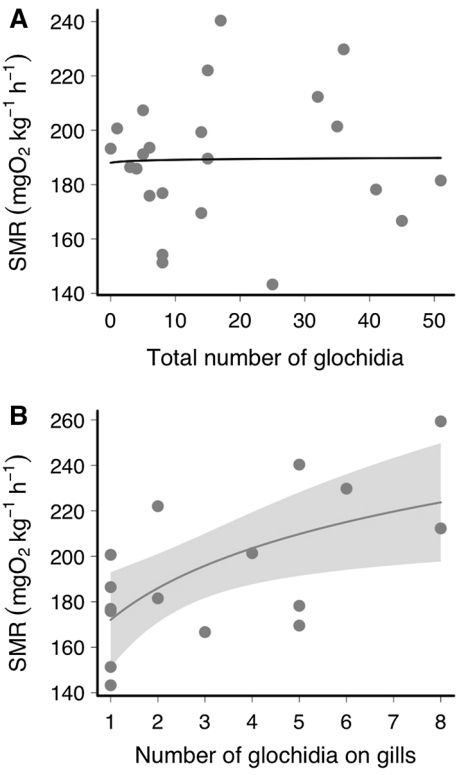

Fig. 3 Standard metabolic rate (SMR) of *Rhodeus amarus* infected with *Sinanodonta woodiana* glochidia. **a** Relationship between SMR and total number of attached glochidia (on fins, body and gills combined) ($N=23$ infected fish). **b** Relationship between SMR and number of glochidia attached to the gills ($N=19$ infected fish). Least squares regression lines are shown with 95% confidence intervals indicated by shaded areas. Note that regression line was fitted for log-transformed values of the number of bitterling embryos

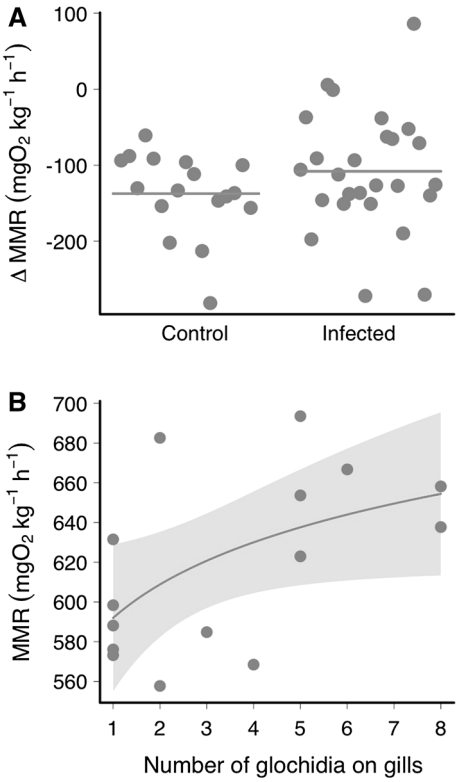

Fig. 4 Effect of a *Sinanodonta woodiana* glochidia infection on maximum oxygen uptake rate (MO_2max) in *Rhodeus amarus*. **a** Mean differential change in MO_2max in control and glochidia-infected fish ($N=43$). **b** Relationship between number of glochidia attached to gills and MO_2max ($N=15$ infected fish). Least squares regression line is shown with 95% confidence intervals indicated by shaded areas

Discussion

We demonstrated that both partners in the bitterling–mussel relationship suffered an energetic cost associated with hosting the parasitic stages of their symbiont. Hosting bitterling embryos was associated with a relative increase in the maintenance metabolism of host mussels compared to control, non-infected mussels. Similarly, glochidia infection elevated SMR in bitterling hosts, though the effect was time-dependent (apparent only after 4 days of infection) and strongest when glochidia were attached to the gills. We did not find any negative effect of glochidia infection on MO_2max, presumably due to low glochidia prevalence on the gills (only 12% of attached glochidia infected host gills). Instead, the MO_2max was positively correlated to glochidia load.

Costs to host mussels

We confirmed our prediction that hosting bitterling embryos is energetically costly and measurable as an increase in host SMR. While we found an overall decrease in SMR between the first and second measurements across all mussels, this decrease was much lower in infected than in control mussels. The general decrease in SMR between measurements may have been related to the thermal conditions (Chen et al. 2001) or changes in phytoplankton concentration and composition throughout the experimental period. Although phytoplankton was seemingly plentiful in all tanks, there may have been differences in species composition and cell concentration between holding and experimental tanks. Mussels adjust feeding rates to algal concentrations (Jørgensen 1996) and particle size (Kiørboe and Møhlenberg 1981). Since we anticipated such variation when choosing natural conditions for infections over a strictly standardized environment, our paired before–after design accommodated any potential biases.

We further confirmed that SMR positively correlates with the number of bitterling embryos residing in mussel gills. This finding corroborated an earlier observation of this relationship in naturally infected *S. woodiana* hosting embryos of the East Asian bitterling, *R. ocellatus* (Methling et al. 2018). In that study, the functional relationship

between MO_2 and the number of bitterling embryos was confounded, with a possibility that parental bitterling selected mussels with a high MO_2 for oviposition. Bitterling choose host mussels on the basis of oxygen availability for their embryos (Smith et al. 2001; Phillips et al. 2017). We ruled out that active oviposition choice of host mussels drove the pattern in our current dataset, as there was no relationship between pre-treatment SMR and the number of bitterling embryos (that varied from 3 to 65 embryos per mussel). Finally, the respiration of the bitterling embryos themselves cannot directly account for increased oxygen consumption. MO_2–age relationships of 5-day-old embryos of a cichlid fish (*Pseudocrenilabrus multicolor*) with a comparable size and measured at a similar temperature (Mrowka and Schierwater 1988) suggest that bitterling embryos have accounted for a maximum of 0.1% of the measured MO_2.

The proximate causes of the increased SMR in mussels hosting bitterling embryos are unclear. The mussel gill is a relatively large organ and is densely covered with cilia. Since the beating frequency of cilia is directly proportional to ATP expenditure (Clemmesen and Jørgensen 1987), the increase in SMR would be most directly explained by increased beating frequency of cilia. However, this contradicts a previous study reporting a negative correlation between *A. anatina* ventilation rate and number of *R. amarus* embryos (Mills et al. 2005). Such a discrepancy could be explained if the presence of bitterling embryos in the gills makes ciliary pumping less efficient, perhaps if distortion of ciliary bands alters fluid dynamics (Riisgård et al. 2015). A study measuring both ventilation and respiration of unionid mussel is needed to clarify this issue.

The present study mechanistically links an earlier finding that the growth of mussels hosting bitterling embryos is impaired over a period of at least 4–19 weeks (Reichard et al. 2006). Elevated maintenance metabolism means that less energy can be allocated to other fitness-related organismal functions such as growth and reproduction.

Costs to host fish

Glochidia infections were predicted to increase SMR in bitterling hosts and this effect was only manifested 4 days post-infection. The time lag in a measurable metabolic response to glochidia infection may have several explanations. First, glochidia attachment most likely elicited a stress response, with a release of cortisol. In other fishes, plasma cortisol was positively correlated with glochidia load (Douda et al. 2016) and elevated cortisol exerts several secondary effects, such as a disruption of the hydromineral balance and disturbances in intermediary metabolism (Douda et al. 2017b). Restoring these balances is energetically costly and tertiary effects of increased energy expenditure and suppressed growth are, hence, observed after a short time lag following the initial stressor (Wendelaar Bonga 1997). The fact that an increase in SMR was only observed 4 days post-infection in *R. amarus* may, therefore, reflect the typical dynamic of an organismal stress response.

Second, initial non-specific defence mechanisms such as encystment, lysozyme activity and migration of leucocytes may not be associated with significant energetic costs, whereas specific immune defences (antibody production and cell proliferation) is a delayed process associated with energetic costs (Ackerman et al. 2000). Acquired immunity against glochidia has been reported in other fish species (Dodd et al. 2006; Rogers-Lowery et al. 2007) and repeatedly infected fish shed more glochidia (Treasurer et al. 2006; Donrovich et al. 2017). Activation of the specific immune-system could, therefore, offer another explanation for the lag in a measurable response of infected bitterling. Given the effective resistance of bitterling to primary glochidia attachment (Dudgeon and Morton 1984; Douda et al. 2017a; Huber and Geist 2019), bitterling may have evolved acquired immunity against glochidia though it remains to be directly tested.

The implications of glochidia infections on host energetics have not been thoroughly documented. Similar to our data on bitterling, brown trout *Salmo trutta* with very high glochidia infection had increased SMR (Filipsson et al. 2017) and common carp *Cyprinus carpio* infected with *S. woodiana* glochidia possessed increased energy expenditure during routine activity (Slavík et al. 2017). Increased energy expenditure at rest may result in trade-offs with less energy being available for other activities, such as foraging, growth and reproduction. Indeed, decreased foraging activity (Österling et al. 2014) and associated decreases in body mass (Crane et al. 2011), condition (Douda et al. 2017b) and growth rate (Ooue et al. 2017) in glochidia-infected fish are commonly reported. While the positive association between the number of glochidia on gills and host SMR corroborates that hosting glochidia is energetically costly, that correlation ceased when glochidia attached to the fins were included. Overall, these observations suggest that while the energetic costs of glochidia attachment to the fins are relatively minor, glochidia attachment to the gills can be particularly costly to bitterling.

Contrary to our prediction, we did not detect reduction in the maximum oxygen uptake (MO_2max) in infected bitterling. When attached to gills glochidia can cause reductions in surface area for gas exchange (Meyers et al. 1980; Howerth and Keller 2006) but, unexpectedly, we found a modest positive (rather than negative) correlation between MO_2max and the number of glochidia attached to gills. The lack of reduction in MO_2max in infected fish could be explained by the low number of glochidia attached to the gills in this study; i.e. approximately two glochidia per g fish. This level of infection is a considerably lower than an average of 30–75

glochidia on similarly sized rainbow darters *Etheostoma caeruleum* that demonstrated increased ventilation rates (Crane et al. 2011), and a mean of 37 glochidia per juvenile brown trout that took longer to reach basal ventilation rates after peak exercise (Thomas et al. 2014). In adult brown trout, a reduction in critical swimming speed (indicating loss of aerobic performance) was only observed at infection loads of > 900 glochidia per g fish (Taeubert and Geist 2013). The low number of glochidia attached to gills in the present study was unlikely to have caused any significant damage to the gill filaments, or bitterling were fully able to compensate for that damage, perhaps by an increase in haematocrit. Increased haematocrit is a typical stress response and has been observed in glochidia-infested fish (Meyers et al. 1980; Filipsson et al. 2017; but see Thomas et al. 2014) and glochidia-infected brown trout had both elevated haematocrit and higher MO_2max compared to non-infected fish (Filipsson et al. 2017). This suggests that an increased blood oxygen carrying capacity may even overcompensate for any loss of uptake capacity by the gills. Therefore, elevated haematocrit could explain why we observed that MO_2max was positively associated with glochidia load.

Conclusions

In conclusion, we demonstrated that infection by bitterling embryos was associated with elevated SMR in host mussels and, reciprocally, glochidia infection was associated with an elevated SMR in bitterling fish. The cost to the bitterling was: (1) only detected 4 days post-infection, (2) strongest when glochidia were attached to the gills, and (3) did not affect MO_2max. This outcome suggests the role of stress response, including increased oxygen carrying capacity and increased energy expenditure to restore homeostasis. These responses mitigate the costs of infection rather than challenge parasite fitness and are therefore all components of a tolerance of parasite infection. At the same time, there are behavioural responses and physiological signatures of parasite resistance to bitterling and glochidia infections. Mussels can expel bitterling eggs and embryos (Mills and Reynolds 2002; Kitamura 2005; Reichard et al. 2007) and bitterling largely avoid glochidia infections (Dudgeon and Morton 1984; Douda et al. 2017a; Huber and Geist 2019). Therefore, the coevolutionary dynamics between bitterling and mussels appears to utilize both aspects of coping with a parasite; tolerance and resistance (Robar et al. 2011). It is possible that the relative importance of tolerance and resistance varies across the coevolutionary relationship between bitterling and mussels, with some species resisting parasitism of their symbionts while others pay costs and tolerate their partner (Knutie et al. 2016). Recent human-assisted translocations may challenge local species with new symbionts and offer particularly suitable settings to study the energetics of coevolution. To that end, the bitterling–mussel coevolutionary relationship offers a tractable system for further titrating the relative importance and effectiveness of tolerance and resistance.

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Author contribution statement CM, KD and MR conceived the study. CM performed the experimental work with the help of KD, CM analysed data and drafted the paper. All authors contributed to interpretation and writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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