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Energetic costs in the relationship between bitterling and mussels in East Asia

CAROLINE METHLING[1], KAREL DOUDA[2], HUANZHANG LIU[3], ROMAIN ROUCHET[1], VERONIKA BARTÁKOVÁ[1], DAN YU[3], CARL SMITH[1,4,5,6] and MARTIN REICHARD[1,*]

[1]*The Czech Academy of Sciences, Institute of Vertebrate Biology, Brno, Czech Republic*
[2]*Department of Zoology and Fisheries, Czech University of Life Sciences Prague, Prague, Czech Republic*
[3]*The Key Laboratory of Aquatic Biodiversity and Conservation, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China*
[4]*Department of Ecology and Vertebrate Zoology, University of Łódź, Łódź, Poland*
[5]*School of Biology, University of St Andrews, St Andrews, UK*
[6]*Bell Pettigrew Museum of Natural History, University of St Andrews, St Andrews, UK*

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Bitterling fishes and unionid mussels are involved in a two-sided co-evolutionary association. On the one side, bitterling exploit unionids by ovipositing in their gills. On the other side, unionids develop via a larval stage (glochidium) that attaches to fish gills. Both interactions are parasitic and expected to have negative consequences for the host. Here, we examine the effects of this association on the metabolic rates of mussel and fish hosts by measuring oxygen uptake rates (MO_2). Measurements were performed on two widespread and broadly coexisting species, namely the rose bitterling *Rhodeus ocellatus* and Chinese pond mussel *Sinanodonta woodiana*. As predicted, we observed an increase in routine MO_2 in mussels parasitized by bitterling, but only when hosting early stages of bitterling embryos that reside in the interlamellar space of the gills and obstruct water circulation. Hosting later-stage bitterling embryos (that reside in the suprabranchial cavity outside the host gills) was not associated with a higher routine MO_2. We did not observe an acute negative effect of glochidial infestations on maximal oxygen uptake rate (MO_2max), but glochidia-infested bitterling showed consistently lower oxygen consumption rates during recovery from MO_2max. Our results suggest that acute costs of this mutually parasitic relationship might be mitigated, at least in part, by adaptations to limit infestation rates.

ADDITIONAL KEYWORDS: Acheilognathinae – branchial parasites – evolutionary arms race – metabolic rate – Unionidae.

INTRODUCTION

Host–parasite interactions are evolutionarily dynamic relationships that often involve costly consequences of parasitism for hosts. Parasites decrease fitness when hosts divert resources into increased maintenance costs, for example by the host mounting an immune response or engaging in tissue repair (Robar *et al.*, 2011), leaving less energy available to direct towards other metabolic functions, such as growth and reproduction.

The relationship between bitterling fishes and unionid mussels (Mills & Reynolds, 2003; Reichard *et al.*, 2010; Rouchet *et al.*, 2017) is an outstanding example of a two-sided host–parasite association. In this relationship, bitterling parasitize mussels by ovipositing into their gills and, reciprocally, mussels parasitize bitterling (and other fishes) by encystment of their larvae (glochidia) on fish gills and fins (Smith *et al.*, 2004). Bitterling (Acheilognathinae) are small cyprinid fishes distributed widely in East Asia, with a single species complex in the West Palaearctic (Chang *et al.*, 2014). All bitterling exploit unionids as oviposition sites and for subsequent embryo development (Wiepkema, 1961; Reynolds *et al.*, 1997; Smith *et al.*, 2004). During the breeding period, female bitterling place their eggs in the gills of a mussel through the exhalant siphon of the host mussel. Bitterling eggs lodge in the interlamellar spaces and water tubes of the mussel, and the

*Corresponding author. E-mail: reichard@ivb.cz

developing embryos display a range of specialized adaptations to ensure that they remain in place (Kim & Park, 1985; Aldridge, 1999). In the later stages of their development, bitterling embryos move from the water tubes of the gills and reside in the suprabranchial cavity (Aldridge, 1999).

Although bitterling have evolved adaptations to maximize the survival of their offspring, host mussels have evolved counter-adaptations to minimize the costs associated with hosting bitterling eggs and embryos (Smith *et al.*, 2000; Mills & Reynolds, 2003, Mills *et al.*, 2005; Reichard et al., 2006, 2010). The gills of unionid mussels serve two vital functions: feeding and gas exchange. Given that developing bitterling embryos can potentially disrupt the water flow of the gills and damage the ciliated gill epithelium (Stadnichenko & Stadnichenko, 1980), the effectiveness of both functions may be compromised by the presence of bitterling embryos in the gills (Mills *et al.*, 2005).

Hosting the embryos of European bitterling *Rhodeus amarus* was shown significantly to impede the growth of the European mussel *Unio pictorum* (Reichard *et al.*, 2006). Although bitterling embryos may potentially compete directly with the host mussel for oxygen and nutrients (Spence & Smith, 2013), the underlying mechanisms behind reduced growth in parasitized mussels are yet to be identified. Elevated maintenance costs from tissue repair and immune responses are assumed to have a negative impact on the growth of host organisms (Robar *et al.*, 2011) and are represented by elevated metabolic rates at rest. To mitigate those costs, mussels have evolved mechanisms to reduce the number of bitterling embryos, primarily by expelling them from the gills using a powerful jet of water generated by rapid shell closure (Kitamura, 2005) and by diverting the ovipositor of a spawning female away from the gill chamber (Reichard *et al.*, 2010).

In turn, unionid mussels have parasitic larvae (glochidia) that are obligatory parasites of fish, including bitterling. Female mussels discharge ripe glochidia into the water column, where they attach to the fins and gills of fish and become encysted by host tissue until they metamorphose into juvenile mussels (Arey, 1932; Dudgeon & Morton, 1984; Douda *et al.*, 2017a). Encystment of glochidia by the host causes tissue swelling and, in the case of attachment to the gills, can result in fusion of gill filaments and lamellae (Meyers *et al.*, 1980; Howerth & Keller, 2006; Thomas *et al.*, 2014). The resulting reduction in surface area for gas exchange can lead to respiratory stress and fish mortality at extreme glochidial loads (Howerth & Keller, 2006; Taeubert & Geist, 2013). At ecologically relevant loads, glochidial infestations can elicit a suite of physiological and behavioural responses. These include increased ventilation rates (Crane *et al.*, 2011; Thomas *et al.*, 2014), increased haematocrit (Meyers *et al.*, 1980; Filipsson *et al.*, 2017), immune response (Dodd *et al.*, 2006; Rogers-Lowery *et al.*, 2007; Barnhart *et al.*, 2008), impaired osmoregulation (Douda *et al.*, 2017b) and decreased feeding activity (Crane *et al.*, 2011; Österling *et al.*, 2014; Filipsson *et al.*, 2016). Consequently, glochidial infestation may alter energy budgets in the host by increasing maintenance metabolism (Filipsson *et al.*, 2017) and swimming costs (Slavík *et al.*, 2017), resulting in decreased post-infestation growth rates (Ooue *et al.*, 2017).

The negative effects of glochidia on host energetics may not be limited to maintenance metabolism but may also affect maximal oxygen uptake rate (MO_2max) as a direct consequence of impaired gill function. Glochidia, like any other gill parasites, may affect maximal metabolic rate, with ecologically relevant consequences. Impaired gill function can have a direct impact on the ability of a host to escape predators, alter the outcome of agonistic encounters or affect prey capture success (Clark *et al.*, 2013; Norin & Clark, 2016). By reducing the aerobic scope for activity, it may affect how well a fish copes with environmental perturbations, such as elevated temperature or hypoxia (Claireaux & Lefrançois, 2007). In Atlantic salmon *Salmo salar*, amoebic gill disease caused a substantial decrease in both MO_2max and swimming speed (Hvas *et al.*, 2017), and a reduction in maximal swimming speed was observed in sea lice-infested rainbow trout *Oncorhynchus mykiss* (Wagner & McKinley, 2004) and glochidia-infested brown trout *Salmo trutta* (Taeubert & Geist, 2013). The ability to recover from peak exercise levels can also be compromised in parasitized fish (Wagner *et al.*, 2005), as demonstrated by consistently increased ventilation rates during recovery in glochidia-infested brown trout (Thomas *et al.*, 2014). Bitterling have evolved behavioural adaptations to minimize the risk of glochidial infestation (Rouchet *et al.*, 2017) and decrease glochidial load by inhibiting their attachment and shedding them before their successful metamorphosis (Douda *et al.*, 2017a; Modesto *et al.*, 2018), further suggesting that glochidia represent a significant burden for their fish hosts.

In the present study, we examined the proximate metabolic costs associated with hosting bitterling embryos by mussels and hosting glochidia by adult bitterling. We used two common and widespread species in East Asia, the Chinese rose bitterling *Rhodeus ocellatus* and the Chinese pond mussel *Sinanodonta woodiana*. Both species coexist across a large region of East Asia, with overlapping reproductive periods (Dudgeon & Morton, 1983; Zheng & Wei, 1999; Kitamura, 2006b), and their association has been relatively well characterized (Kitamura, 2005, 2006a; Douda *et al.*, 2017a; Rouchet *et al.*, 2017). We conducted two experiments.

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First, we used field-collected *S. woodiana* mussels from a site where they coexist with *R. ocellatus* and measured their routine oxygen consumption in relation to the load of bitterling embryos in their gills. We predicted that hosting bitterling embryos would negatively affect energy budgets of mussels via an increase in mussel routine metabolic rate (RMR). We further predicted a correlation between elevated RMR and the number of bitterling embryos hosted by a mussel, and that this correlation would be stronger for early embryos. Second, we infested adult *R. ocellatus* with glochidia of *S. woodiana* and measured MO_2max after exercise and during recovery. We chose to focus on aspects of aerobic performance, anticipating stronger effects, rather than on maintenance metabolism. We predicted a decrease in MO_2max in infested fish compared with non-infested fish, but an increase in MO_2 during recovery, as a result of impaired gill function.

MATERIAL AND METHODS

Animals and husbandry

Rhodeus ocellatus were collected during late April and early May 2014 from two sites, Lake Bao'an (30°17′25.4″N, 114°43′48.9″E) and Lake Niushan (30°20′25.0″N, 114°31′48.7″E), using baited fish traps. Fish were transported in cool, aerated water to the Institute of Hydrobiology (IHB), Chinese Academy of Science, Wuhan, where they were housed in two separate glass aquaria (1.0 m × 0.75 m × 0.5 m; 30 fish per tank) supplied with dechlorinated tap water and a 20 mm layer of washed sand. Fish were fed daily with frozen bloodworms and commercial flake food. Water temperature was maintained at 24 ± 1 °C using aquarium heaters, with 10% of the water in each aquarium replaced daily to maintain water quality. Light conditions followed a natural 13 h light–11 h dark photoperiod. Fish and mussels were maintained for ≥ 14 days in captive conditions before measurements of oxygen consumption.

Sinanodonta woodiana mussels were collected from earthen fishponds near the town of Jianli, Hubei Province, China (29°42′29.4″N, 112°58′37.5″E) on 17 and 27 April 2014 (at the peak of the bitterling spawning season). Gravid mussels for experimental infestation by glochidia were collected from Lake Niushan. All mussels were transported in cool, aerated water to the IHB, where they were housed in large plastic tubs (Jianli mussels, N = 43, 2000 L; Bao'an mussels N = 13, 1350 L) containing dechlorinated tap water to a depth of 0.3 m and continuously aerated. Water temperature was maintained at 24 °C by air conditioning. Approximately 5% of the water was replaced daily. Mussels were fed phytoplankton regularly.

Husbandry and experimentation adhered to the legal regulations on animal welfare in China and the Czech Republic. Experiments were concluded by dissection of experimental fish and mussels to estimate the level of their parasitism, and all were killed with an overdose of anaesthetic (MS-222) before dissection.

Experimental infestation of *R. ocellatus*

Gravid *S. woodiana* females were used to infest *R. ocellatus* with glochidia. Ripe glochidia were collected by flushing the marsupium with water using a syringe. The viability of glochidia was confirmed by observing their snapping action in a sodium chloride solution, and only glochidia from females with > 90% viability were used for infesting bitterling (Douda *et al.*, 2017a). A suspension of glochidia was prepared containing 4165 ± 1916 (mean ± SD) viable glochidia L^{-1}. Bitterling (1.00 ± 0.28 g) were placed in the glochidial suspension for 15 min and then transferred to an aerated 5 L bath for 2 h to rinse off non-attached glochidia before beginning the respirometry measurements. Control (non-infested) fish were exposed to the same protocol, except that these fish were placed in a glochidia-free suspension.

Respirometry

For both mussels and bitterling, metabolic rates were estimated indirectly by measuring oxygen consumption rates using computerized intermittent flow-through respirometry (general procedure reviewed by Steffensen, 1989). Respirometry was performed in an isolated, air-conditioned room (22.0 ± 0.1 °C) with 24 h dimmed lighting. Respirometers were placed in a 1.0 m × 0.5 m × 0.30 m holding tank. Twenty per cent of the water in the holding tank was replaced daily with dechlorinated water from an adjacent reservoir tank. Water temperature was maintained at 24.0 ± 0.1 °C, and a submersible pump was placed in the holding tank to ensure complete mixing of water. The respirometers were fitted with two sets of tubing: one recirculation loop and one for periodically flushing the respirometer with water from the outside holding tank. Each inlet/outlet was fitted with baffles to ensure proper mixing inside the respirometer. Chamber oxygen partial pressure (pO_2) was measured with an OXY-4 mini (PreSens, Germany) fibre-optic O_2 transmitter, placed in the recirculation loop and recorded by the AutoResp4 software (Loligo Systems, Denmark).

For *S. woodiana* (N = 38, shell size 101–167 mm), the mussels were removed from their holding tank in the morning, gently brushed to remove epibionts, and transferred to the respirometer holding tank. The following morning, mussels were placed individually

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in the submerged respirometers made from water- and air-tight plastic containers (450 or 1100 mL). Chambers were periodically flushed for 4 min, followed by a closed 2 min wait period to reach steady state, with a 10 min closed measuring period. Oxygen consumption was measured continuously for 12 h, after which mussels were dissected to quantify the number of bitterling embryos in their gills. The soft tissue of each mussel was dried for 48 h at 65 °C and weighed. The MO_2max of mussels was not estimated because there is no protocol to elicit MO_2max in mussels. All bitterling embryos in the mussel gills originated from natural infestations; no experimental infestations were conducted.

Experimentally infested (N = 12, mean ± SD wet mass: 0.99 ± 0.22 g) and non-infested (N = 12, mean ± SD wet mass: 1.02 ± 0.33 g) *R. ocellatus* were first subjected to a 3 min chasing protocol, followed by 30 s air exposure (Clark *et al.*, 2013) to achieve MO_2max, before being placed in the respirometers (90 mL cylindrical glass chambers, diameter 45 mm). To ensure that pO_2 never fell below 80% saturation during the closed phase, a cycle of 3 min flushing, 1 min wait and 2.5 min measurement period was used. The MO_2max was determined as the first measurement after placing the fish inside the chamber (immediately before the closed phase). Fish were then left undisturbed in the chambers over the subsequent 2.5 h, while recording MO_2 during recovery, after which the chasing protocol was repeated to obtain a second value of MO_2max. Bitterling RMR was not measured because a reliable RMR estimate requires ≥ 24 h of continuous MO_2 measurements, and estimates of bitterling RMR were not needed for our study aims. All respirometry was done on individuals that had fasted for 24 h (Nie *et al.*, 2017). After respirometry, bitterling were killed with an overdose of anaesthetic (MS-222), and the total number of glochidia attached to fins, gills and body was quantified under a stereomicroscope.

Bitterling embryo identification

Bitterling embryos were removed during mussel dissections, staged according to Nagata & Miyabe (1978) and Aldridge (1999) and morphologically determined to the genus level. Morphologically, all recovered embryos belonged the genus *Rhodeus* and apparently of the same species. Given that morphological identification of *Rhodeus* embryos is not possible (Liu *et al.*, 2004), the embryos were stored in 96% ethanol for identification by genotyping.

A sample of 15 embryos from 15 different host mussels was genotyped for a 797-bp-long fragment of mitochondrial gene for cytochrome *b* (*CYTB*). Genetic analysis followed the protocol of Bohlen *et al.* (2006). Haplotypes were compared with existing sequences using a GenBank Blast search. Bitterling species (Acheilognathinae) are well covered in the GenBank database (Chang *et al.*, 2014; Kawamura *et al.*, 2014), including all *Rhodeus* species in the study area. A total of seven different *CYTB* haplotypes (12 polymorphic sites) was detected, and all genotyped embryos were confirmed as *R. ocellatus*, with an estimated 99–100% similarity to archived sequences. All generated sequences have been uploaded to GenBank (accession numbers MG544112–MG544118).

Data analysis

The oxygen consumption rate (MO_2) was derived from the decrease in respirometry chamber oxygen partial pressure (pO_2) during the measuring period using the function: $MO_2 = V[d(pO_2)/dt]\alpha$, where V is the volume of the chamber and α is the specific oxygen solubility. Measurements of MO_2 where the regression coefficient (r) of the slope $d(pO_2)/dt$ was < 0.96 were excluded from the analysis (Chabot *et al.*, 2016). This included periods when mussels had closed shells and did not ventilate. All MO_2 measurements were corrected for microbial respiration recorded in empty chambers before and after each experiment. All statistical analyses were performed using R v.3.3.3 (R Development Core Team, 2017).

The routine metabolic rate (RMR) of mussels was estimated as the lower tenth percentile of MO_2 measurements and expressed per gram of soft tissue dry weight (dm). The effect of bitterling embryos on mussel respiration rate was analysed using a linear model (LM) with Gaussian distribution, with mass-specific MO_2 as the response variable and the number of bitterling embryos (logarithmically transformed) as covariate. Given that there were biological reasons to assume a stronger effect of early stage bitterling embryos (which reside in the mussel water tubes) than later stage embryos (which reside in the suprabranchial cavity) (Kim & Park, 1985; Aldridge, 1999), the same analysis was performed on a subset of mussels hosting early stage embryos. An alternative analysis that directly compared infested and non-infested mussels (rather than using the number of bitterling embryos as a continuous covariate) provided concordant results.

The effect of glochidial load on bitterling MO_2max was analysed using linear mixed models (LMMs) in the *lme4* package (Bates *et al.*, 2015), with mass-specific MO_2max as response variable and the number of glochidia as predictor. A separate analysis was performed with the total number of glochidia attached (sum of glochidia on body and gills) and glochidia attached to the gills only. Individual fish was included as a random term in the model to accommodate two

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separate MO_2max measurements for each fish. Sex and wet mass were initially included as covariates but had non-significant effects and were excluded from final models. The repeatability of MO_2max measurements was analysed using the intraclass correlation (ICC) function in the *psych* package (Revelle, 2017). The effect of glochidial infestation on bitterling respiration rate while recovering from exercise was analysed using a generalized least squares (GLS) model in the *nlme* package, with mass-specific MO_2 as the response variable. Treatment (presence or absence of glochidia), time (2.5–131 min after chasing) and their interaction were included as covariates. Time was included as a random intercept in the model to account for a temporal component from repeated measurements of the same individuals.

RESULTS

Mussel respiration

There was no significant effect of hosting bitterling embryos on mussel RMR when both early and late stage embryos were included (LM: $t_{36} = 1.04$, $P = 0.315$, adjusted $R^2 = 0.001$; Fig. 1A). However, for the subset of mussels with early stage embryos (residing in the water tubes of the mussels), there was a significant positive relationship between RMR and the number of early embryos in the gills (LM: $t_{11} = 7.72$, $P = 0.018$, adjusted $R^2 = 0.36$; Fig. 1B). Overall, the mean ± SD RMR of mussels was 0.25 ± 0.06 mg O_2 (g dm)$^{-1}$ h^{-1} (mean ± SD dry mass: 10.12 ± 4.42 g).

Bitterling respiration

The first and second MO_2max measurements were highly repeatable within individuals ($ICC_1 = 0.595$, $F_{18,19} = 3.94$, $P = 0.002$). There was no significant effect of glochidia on bitterling MO_2max when all attached glochidia were included (LMM: $t_{18.2} = 1.08$, $P = 0.239$) or when including only glochidia attached to the gills ($t_{18.3} = 1.67$, $P = 0.113$), despite a lower MO_2max in fish with the highest infestation rate (Fig. 2). Between the first and second MO_2max measurement, infested fish had significantly lower MO_2 compared with non-infested fish (GLS, treatment effect: $\chi^2 = 5.71$, d.f. = 1, $P = 0.017$), but with the same temporal decline in oxygen uptake after the chasing protocol (treatment by time interaction: $\chi^2 = 0.66$, d.f. = 1, $P = 0.418$; Fig. 3).

DISCUSSION

We examined the proximate energetic costs of reciprocal parasitism between bitterling fish and unionid mussels. We predicted that hosting bitterling embryos would be associated with an elevated routine oxygen uptake rate reflecting increased maintenance costs for infested mussels. We demonstrated that infested mussels had higher routine oxygen uptake rates when hosting bitterling embryos at early developmental stages (which reside between gill lamellae and distract water circulation), but later stages (which occupy the suprabranchial cavity) had no significant impact on mussel respiration. In glochidia-infested bitterling, we predicted a reduced ability to maximize oxygen uptake at maximal metabolic rate and a reduced ability to recover from intense exercise as a direct result of compromised gill function. We found no significant effect of glochidia on MO_2max and, contrary to our predictions,

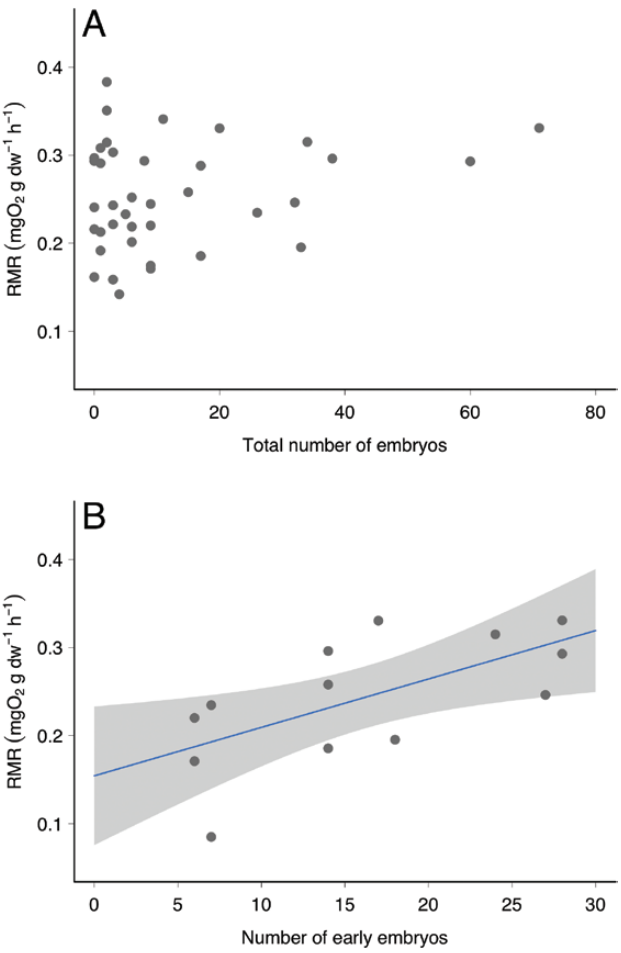

Figure 1. Mass-specific routine metabolic rate (RMR) in *Sinanodonta woodiana* hosting *Rhodeus ocellatus* embryos. A, relationship between RMR and the total number of embryos (early and late stages). B, relationship between RMR and the number of early stage embryos. The least squares regression lines are shown, with 95% confidence intervals indicated by the shaded area.

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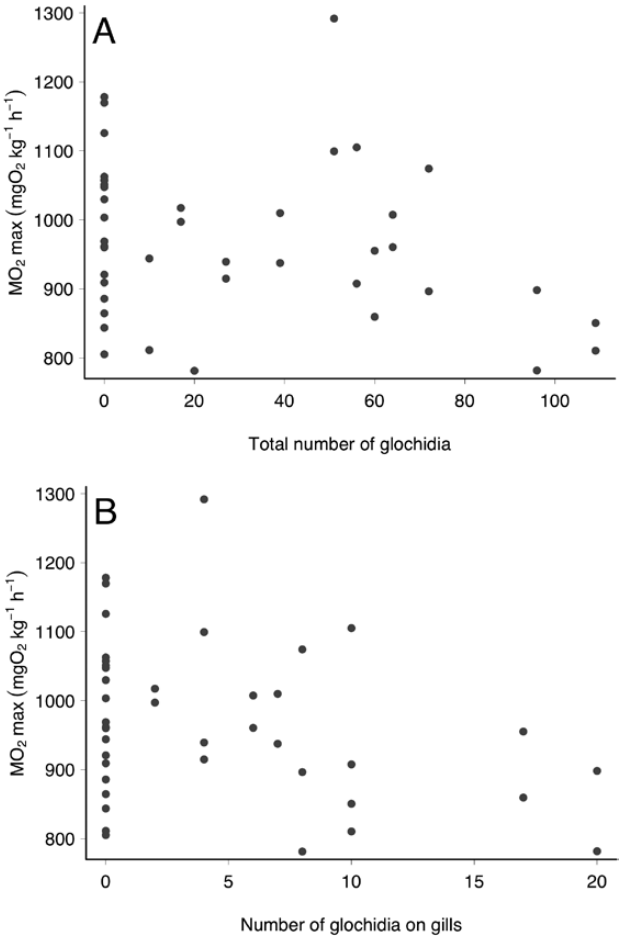

Figure 2. Effect of glochidial load on maximal oxygen consumption rates (MO_2max) of *Rhodeus ocellatus*. A, total number of attached (fins + gills) glochidia. B, glochidia attached to gills only. Repeated measurements are shown.

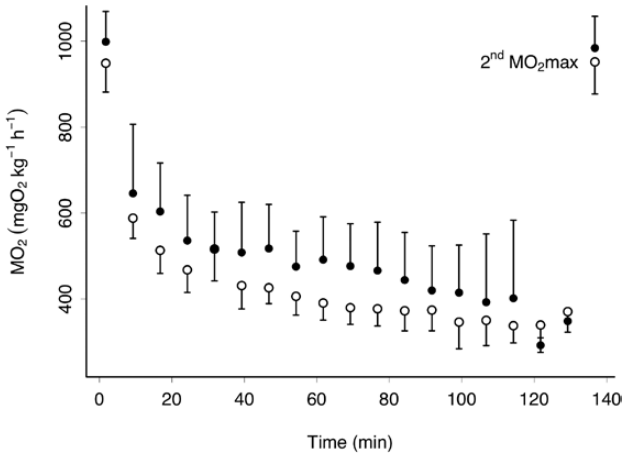

Figure 3. Oxygen consumption rates in glochidia-infested *Rhodeus ocellatus*. Fish were subjected to a chasing protocol to elicit MO_2max and left to recover before a second measurement of MO_2max. Points represent mean values of 12 infested (open circles) and ten non-infested (filled circles) individuals. Error bars represents 95% confidence intervals.

glochidia-infested bitterling had consistently lower oxygen uptake rates during recovery.

The lack of an increase in routine oxygen consumption rates in mussels hosting late stage bitterling embryos suggests that their presence does not impose a significant energetic cost at this developmental stage. This finding is consistent with the observation that late stage embryos are no longer closely associated with mussel gill epithelia and typically reside in the suprabranchial cavity (Aldridge, 1999). In contrast, harbouring early embryos was associated with increased oxygen uptake rates by mussels. At this stage, bitterling embryos are lodged in the interlamellar space of the gill, obstructing water flow through the gills and distorting the lateral cilia bands on the epithelial surfaces, thereby compromising gill function. This effect has been demonstrated previously in two species of unionids harbouring embryos of European bitterling, with a negative correlation between ventilation rate and the number of bitterling embryos in the gills (Mills *et al.*, 2005). Although we found a positive correlation between the number of embryos and oxygen consumption rate, it does not necessarily imply that mussels had increased ventilation rates. In filter-feeding bivalves, the ventilatory flow is scaled to feeding requirements and greatly exceeds requirements for gas exchange (Krogh, 1941). Oxygen uptake becomes coupled to ventilation only at low flow rates, where the diffusive resistance is increased by a thickening of the diffusive boundary layer on the gill surface (Barker Jørgensen *et al.*, 1986). Furthermore, the ciliary pump is highly energy efficient, and the energetic cost of ventilation in mussels is low (Barker Jørgensen *et al.*, 1986). It is, therefore, possible to achieve increased oxygen uptake concurrent with decreased ventilation, and an elevated MO_2 does not necessarily reflect increased energy expenditure from increased ventilation.

It is unlikely that the positive correlation between mussel RMR and the number of embryos is attributable to respiration of the bitterling embryos. There are no known data on the respiration rate of bitterling embryos, but MO_2–age relationships were measured at a similar temperature for embryos of a cichlid fish, *Pseudocrenilabrus multicolor*, with embryos of a comparable size, with estimates of $MO_2 = 0.05e^{0.66age}$ at the age of 0–5 days and $MO_2 = 0.62e^{0.19age}$ at the age of > 5 days (Mrowka & Schierwater, 1988). Early bitterling embryos at 5 days would thus have an estimated

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MO_2 of 1.36 μg O_2 h^{-1} and at day 21, 33.5 μg O_2 h^{-1}, accounting for a maximum of 0.075% of the measured oxygen consumption. The underlying physiological mechanisms behind an increased routine MO_2 in mussels hosting bitterling embryos are unclear but might be associated with immune responses, stress responses or tissue repair (Robar *et al.*, 2011). Regardless of mechanism, an increased maintenance metabolism would limit the amount of energy that can be directed towards growth and might explain the impaired growth observed in mussels hosting European bitterling embryos (Reichard *et al.*, 2006).

Given that our measurements involved naturally infested mussels, active choice of host mussels by bitterling offers an alternative explanation to the positive relationship between the number of early embryos and mussel oxygen uptake rate. Bitterling are capable of perceiving the quality of the mussel as an incubation site for their eggs. The cues used for choice of mussel are the absolute levels of dissolved oxygen in the exhalant siphon and the oxygen gradient between mussel inhalant and exhalant siphons (Smith *et al.*, 2001; Phillips *et al.*, 2017). Therefore, active choice of mussels with high oxygen consumption might explain the positive relationship between mussel oxygen consumption and the number of bitterling embryos. This possibility could be tested by measuring bitterling oviposition preference in mussels with known oxygen consumption levels.

The implications of glochidial infestations for fish respiration are not well understood, but in at least two species of fish, rainbow darters *Etheostoma caeruleum* and brown trout *S. trutta*, glochidia-infested fish showed increased ventilation frequencies compared with non-infested fish. In rainbow darters, ventilation frequency remained elevated during routine activities throughout the infestation period (Crane *et al.*, 2011), whereas in brown trout glochidia-infested fish had consistently elevated ventilation frequencies after intense exercise, taking longer to return to non-stressed values (Thomas *et al.*, 2014). Glochidia-infested brown trout also showed reduced swimming performance, with reduced critical swimming speeds (Taeubert & Geist, 2013), all of which points to impaired oxygen uptake capabilities in glochidia-infested fish.

In contrast to these observations and to our results for bitterling presented here, Filipsson *et al.* (2017) reported a small increase in maximal metabolic rate in juvenile brown trout naturally infested with glochidia of the freshwater pearl mussel *Margaritifera margaritifera* when compared with non-infested fish. Besides the possibility for species-specific differences in the effects of glochidia on fish hosts, the conflicting outcome might also be explained by temporal effects. We studied the acute effects of glochidial attachment on MO_2max, whereas Filipsson *et al.* (2017) observed higher MO_2max in naturally infested fish that may have hosted glochidia for several months, because *M. margaritifera* glochidia remain encysted in their hosts for ~10 months (Bauer & Vogel, 1987). Given that glochidia-infested brown trout also had elevated metabolic rates at rest, they might have made cardiorespiratory compensations to increase MO_2max to sustain aerobic scope for activity in the long term. Notably, immune-challenged mosquitofish *Gambusia holbrooki* (with elevated maintenance metabolism) were able to upregulate maximal metabolic rate to maintain aerobic scope (Bonneaud *et al.*, 2016). Even when infested with the same parasite, fish may also show species-specific responses. Rainbow trout *Oncorhynchus mykiss* infested with the microsporidium gill parasite *Loma salmonae* increased both routine and maximal metabolic rate, whereas the brook trout *Salvelinus fontinalis* lowered routine metabolic rate, with no change in maximal metabolic rate (Powell *et al.*, 2005). Therefore, a response in MO_2max may be both specific to host species and parasite identity and may vary over the course of infestation.

Although we did not observe a significant effect on MO_2max in glochidia-infested bitterling, the direction of the trend was a reduced MO_2max in the most heavily infested fish, as we predicted. We propose the modest effect to be a consequence of the low number of encysted glochidia on the gills of experimentally infested fish (average: 8.0; range: 0–20) per individual. This number does not reflect unsuccessful experimental infestation, because comparable infestation loads were observed in naturally infested fish from the same population (Douda *et al.*, 2017a). Instead, the low glochidial attachment success on *R. ocellatus* gills may be a consequence of the close co-evolutionary association between bitterling and mussel. All tested bitterling species, including *R. ocellatus*, have lower numbers of glochidia initially attaching to them and substantially lower infestation success than that seen in other freshwater fishes coexisting with *S. woodiana* (Douda *et al.*, 2017a). Thus, it appears that bitterling can limit the number of glochidia that attach to a level at which the impact of infestation on gill function is negligible.

Contrary to our predictions, glochidia-infested bitterling did not have elevated oxygen uptake rates during recovery compared with non-infested fish but instead had consistently lower MO_2. This finding might reflect a behavioural response to infestation in bitterling through a reduction in their spontaneous activity levels (i.e. turning inside the respirometer). Reducing activity levels might even be a general and adaptive response to glochidia infestations. For example, chub *Squalius*

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cephalus infested with glochidia of *Anodonta anatina* were less active in open field tests and dispersed less in a natural setting, especially during the early phases of infestation (Horký *et al.*, 2014). In brown trout, glochidial load was negatively correlated with feeding activity (Österling *et al.*, 2014), general activity levels and aggression (Filipsson *et al.*, 2016). Assuming that glochidia attachment acutely impairs MO_2max and maximal swimming speed (Taeubert & Geist, 2013), reducing activity levels would be an adaptive response to limit the risk of predation, because the chance of escaping a predator would be reduced. Reduced activity might also be an adaptive response to avoid further infestations given that high glochidial loads may lead to mortality (Howerth & Keller, 2006; Taeubert & Geist, 2013). Although we did not record activity levels in bitterling, we propose that the observed decrease in MO_2 might have reflected an adaptive behavioural response to glochidial infestation, as observed in other species. This prediction might be tested by measuring activity levels together with oxygen consumption rates pre- and post-infestation.

In conclusion, we demonstrated that unionid mussels infested by early-stage bitterling embryos showed an elevated RMR, although this relationship might have been driven by a selective preference by bitterling for spawning in mussels with high oxygen consumption rates. *Rhodeus ocellatus* did not suffer a significant acute cost of *S. woodiana* glochidial infestation, measured in terms of a reduced MO_2max. However, glochidia-infested fish had lowered oxygen uptake rates during the recovery phase between MO_2max measurements, possibly owing to a behavioural response. Our results suggest that bitterling and mussels can minimize the acute costs in their bidirectional parasitic relationship. Given the long-term co-evolution between unionid mussels and bitterling in East Asia, mussels are capable of ejecting bitterling embryos to a level that might curtail any negative consequences, whereas bitterling appear capable of limiting their glochidial load to the level that mitigates significant negative consequences.

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