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

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Abstract

Bitterling fishes and unionid mussels are involved in a two-sided coevolutionary association. On one side, bitterling exploit unionids by ovipositing in their gills. On the other side, unionids develop via a larval stage (glochidium) that attach to fish gills. Both interactions are parasitic and expected to have negative consequences for the host. Here we examined 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; the rose bitterling *Rhodeus ocellatus* and Chinese pond mussel *Sinanodonta woodiana*. As predicted, we observed an increase in routine MO_2 in mussels parasitised 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 glochidia infections on maximum oxygen uptake rate (MO_2max) but glochidia-infected bitterling showed consistently lower oxygen consumption rates during recovery from MO_2max. Our results suggest that acute costs of this mutually parasitic relationship may be at least partly mitigated by adaptations to limit infection 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 encystement 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 Palearctic (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 developing embryos display a range of specialised adaptations to ensure 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).

Whilst 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. As developing bitterling embryos can potentially disrupt the water flow of the gills and damage the

62 ciliated gill epithelium (Stadnichenko & Stadnichenko, 1980), the effectiveness of both functions
63 may be compromised by the presence of bitterling embryos in the gills (Mills *et al*., 2005).

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

74 In turn, unionid mussels have parasitic larvae (glochidia) that are obligatory parasites of
75 fish, including bitterling. Female mussels discharge ripe glochidia into the water column where
76 they attach to the fins and gills of fish and become encysted by host tissue until they metamorphose
77 into a juvenile mussel (Arey, 1932; Dudgeon & Morton, 1984; Douda *et al*., 2017a). Encystment
78 of glochidia by the host causes tissue swelling, and in the case of attachment to the gills can result
79 in fusion of gill filaments and lamella (Meyers *et al*., 1980; Howerth & Keller, 2006; Thomas *et*
80 *al*., 2014). The resulting reduction in surface area for gas exchange can lead to respiratory stress
81 and fish mortality at extreme glochidia loads (Howerth & Keller, 2006; Taeubert & Geist, 2013).
82 At ecologically relevant loads, glochidia infections can elicit a suite of physiological and
83 behavioural responses. These include increased ventilation rates (Crane *et al*., 2011; Thomas *et al*.,
84 2014), increased haematocrit (Meyers *et al*., 1980; Filipsson *et al*., 2017), immune response (Dodd
85 *et al*., 2006; Rogers-Lowery *et al*., 2007; Barnhart *et al*., 2008), impaired osmoregulation (Douda

86 *et al*., 2017b) and decreased feeding activity (Crane *et al*., 2011; Österling *et al*., 2014; Filipsson
87 *et al*., 2016). Consequently, glochidia infection may alter energy budgets in the host by increasing
88 maintenance metabolism (Filipsson *et al*., 2017) and swimming costs (Slavík *et al*., 2017), resulting
89 in decreased post-infection growth rates (Ooue *et al*., 2017).

90 The negative effects of glochidia on host energetics may not be limited to maintenance
91 metabolism but may also affect maximum oxygen uptake rate as a direct consequence of impaired
92 gill function. Glochidia, as well as any other gill parasites, may affect maximum metabolic rate,
93 with ecologically relevant consequences. Impaired gill function can have a direct impact on a host's
94 ability to escape predators, alter the outcome of agonistic encounters or affect prey capture success
95 (Clark *et al*., 2013; Norin & Clark, 2016). By reducing the aerobic scope for activity, it may affect
96 how well a fish copes with environmental perturbations, such as elevated temperature or hypoxia
97 (Claireaux & Lefrançois, 2007). In Atlantic salmon *Salmo salar*, amoebic gill disease caused a
98 substantial decrease in both maximum oxygen uptake and swimming speed (Hvas *et al*., 2017),
99 and a reduction in maximum swimming speed was observed in sea lice-infected rainbow trout
100 *Oncorhynchus mykiss* (Wagner & McKinley, 2004) and glochidia-infected brown trout *Salmo*
101 *trutta* (Taeubert & Geist, 2013). The ability to recover from peak exercise levels can also be
102 compromised in parasitized fish (Wagner *et al*., 2005), as demonstrated by consistently increased
103 ventilation rates during recovery in glochidia-infested brown trout (Thomas *et al*., 2014). Bitterling
104 have evolved behavioural adaptations to minimize the risk of glochidia infections (Rouchet *et al*.,
105 2017) and decrease glochidia load by inhibiting their attachment and shedding them prior to their
106 successful metamorphosis (Douda *et al*., 2017a; Modesto *et al*., 2017), further suggesting that
107 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 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 characterised (Kitamura, 2005, 2006a; Douda *et al*., 2017a; Rouchet *et al*., 2017). We conducted two experiments. 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. Next, we infected adult *R. ocellatus* with glochidia of *S. woodiana* and measured maximum oxygen uptake rates (MO_2max) after exercise and during recovery. We chose to focus on aspects of aerobic performance, anticipating stronger effects, than on maintenance metabolism. We predicted a decrease in MO_2max in infected fish compared to non-infected fish, but an increase in MO_2 during recovery as a result of impaired gill function.

MATERIALS 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, Chinese Academy of Science (IHB), Wuhan, where they were housed in two separate glass aquaria (1.0 x 0.75 x 0.5 m; 30 fish per tank) supplied with de-chlorinated 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 at least 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 infection 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 de-chlorinated 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) prior to dissection.

EXPERIMENTAL INFECTION OF *R. OCELLATUS*

Gravid *S. woodiana* females were used to infect *R. ocellatus* with glochidia. Ripe glochidia were collected by flushing the marsupium with water with 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 infecting bitterling (Douda *et al.*, 2017a). A suspension of glochidia was prepared containing 4165 ± 1916 (mean ± s.d.) viable glochidia L^{-1}. Bitterling (1.00 ± 0.28 g) were placed in the glochidia 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-infected) fish were exposed to the same protocol, except 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 in Steffensen, 1989). Respirometry was performed in an isolated, air-conditioned room (22.0 ± 0.1°C) with 24 hours dimmed lighting. Respirometers were placed in a 1.0 x 0.5 x 0.30 m holding tank. Twenty percent of the water in the holding tank was replaced daily with de-chlorinated 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–4mini (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 to 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 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. 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 infections; no experimental infections were conducted.

Experimentally infected (N=12, mean wet mass: 0.99 g ± 0.22 SD) and non-infected (N=12, mean wet mass: 1.02 g ± 0.33 SD) *R. ocellatus* were first subjected to a 3 min chasing protocol, followed by 30 s air exposure (Clark *et al*., 2013) to achieve maximum oxygen uptake (MO_2max), before being placed in the respirometers (90 mL cylindrical glass chambers, diameter 45 mm). To ensure 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. 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 hours, while recording MO_2 during recovery, after which the chasing protocol was repeated to obtain a second value of MO_2max. Bitterling RMR were not measured because a reliable RMR estimate requires at least 24 hours 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 hours (Nie *et al*., 2017).

Following 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 and 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 to existing sequences using a GenBank Blast search. Bitterling species (Acheilognathinae) are well covered on GenBank database (Chang *et al*., 2014; Kawamura *et al*., 2014), including all *Rhodeus* species in the study area. A total of 7 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

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. MO_2 measurements 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 where 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 version 3.3.3 (R Development Core Team, 2017).

The routine metabolic rate (RMR) of mussels was estimated as the lower 10th percentile of MO_2 measurements and expressed per g of soft tissue dry weight. 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 (log-transformed) as covariate. Because 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 (that 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 infected and non-infected mussels (rather than using the number of bitterling embryos as a continuous covariate) provided concordant results.

The effect of glochidia load on bitterling MO_2max was analysed using Linear Mixed Models (LMM) 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 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 ICC (intraclass correlation) function in the *psych* package (Revelle, 2017). The effect of glochidia infection 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: $t_{36} = 1.04$, $P = 0.315$, $R^2_{adj} = 0.001$; Fig. 1A). However, for the subset of mussels with early stage embryos (residing in the mussel's water tubes), there was a significant positive relationship between routine MO_2 and the number of early embryos in the gills (LM: $t_{11} = 7.72$, $P = 0.018$, $R^2_{adj} = 0.36$; Fig 1B). Overall, mean routine metabolic rate (RMR) of mussels was 0.25 mg O_2 g dm^{-1} h^{-1} ± 0.06 SD (mean dry mass: 10.12 g ± 4.42 SD).

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: $t_{18.2} = 1.08$, $P = 0.239$), nor when only including glochidia attached to the gills ($t_{18.3} = 1.67$, $P = 0.113$), despite a lower MO_2max in fish with the highest infection rate (Fig. 2). Between the first and second MO_2max measurement, infected fish had significantly lower MO_2 compared to non-infected fish (GLS, treatment effect: $\chi^2 = 5.71$, df =

1, $P = 0.017$), but with the same temporal decline in oxygen uptake after the chasing protocol (treatment by time interaction: $\chi^2 = 0.66$, df = 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 infected mussels. We demonstrated that infected mussels had higher routine oxygen uptake rates when hosting bitterling embryos at early developmental stages (that reside between gill lamellae and distract water circulation), but later stages (that occupy the suprabranchial cavity) had no significant impact on mussel respiration. In glochidia-infected bitterling we predicted a reduced ability to maximize oxygen uptake (MO_2max) at maximum 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, glochidia-infested bitterling had consistently lower oxygen uptake rates during recovery.

No 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

289 epithelial surfaces, thereby compromising gill function. This effect has previously been
290 demonstrated in two species of unionids harbouring embryos of European bitterling, with a
291 negative correlation between ventilation rate and number of bitterling embryos in the gills (Mills
292 *et al*., 2005). Although we found a positive correlation between the number of embryos and oxygen
293 consumption rate it does not necessarily imply that mussels had increased ventilation rates. In filter
294 feeding bivalves, the ventilatory flow is scaled to feeding requirements and greatly exceeds
295 requirements for gas exchange (Krogh, 1941). Oxygen uptake only becomes coupled to ventilation
296 at low flow rates where the diffusive resistance is increased by a thickening of the diffusive
297 boundary layer on the gill surface (Barker Jørgensen *et al.*, 1986). Furthermore, the ciliary pump
298 is highly energy efficient and the energetic cost of ventilation in mussels is low (Barker Jørgensen
299 *et al*., 1986). It is, therefore, possible to achieve increased oxygen uptake concurrent with decreased
300 ventilation, and an elevated MO_2 does not necessarily reflect increased energy expenditure from
301 increased ventilation.

302 It is unlikely that the positive correlation between mussel RMR and number of embryos is
303 due to respiration of the bitterling embryos. There are no known data on the respiration rate of
304 bitterling embryos, but MO_2-age relationships were measured at a similar temperature for embryos
305 of a cichlid fish, *Pseudocrenilabrus multicolor*, with embryos of a comparable size, with estimates
306 of $MO_2 = 0.05\ e^{0.66age}$ at the age of 0-5 days and $MO_2 = 0.62\ e^{0.19age}$ at the age of more than 5 days
307 (Mrowka & Schierwater, 1988). Early bitterling embryos at 5 days would thus have an estimated
308 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
309 measured oxygen consumption. The underlying physiological mechanisms behind an increased
310 routine MO_2 in mussels hosting bitterling embryos are unclear but may be associated with immune
311 responses, stress responses, or tissue repair (Robar *et al*., 2011). Regardless of mechanism, an

312 increased maintenance metabolism would limit the amount of energy that can be directed towards
313 growth and may explain the impaired growth observed in mussels hosting European bitterling
314 embryos (Reichard *et al*., 2006).

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

324 The implications of glochidia infections for fish respiration are not well understood, but in
325 at least two species of fish; rainbow darters *Etheostoma caeruleum* and brown trout *Salmo trutta*,
326 glochidia–infected fish showed increased ventilation frequencies compared to non-infected fish. In
327 rainbow darters, ventilation frequency remained elevated during routine activities throughout the
328 infestation period (Crane *et al*., 2011), while in brown trout glochidia–infected fish had consistently
329 elevated ventilation frequencies following intense exercise, taking longer to return to non-stressed
330 values (Thomas *et al*., 2014). Glochidia–infested brown trout also showed reduced swimming
331 performance with reduced critical swimming speeds (Taeubert & Geist, 2013), which all points to
332 impaired oxygen uptake capabilities in glochidia–infected fish. In contrast to these observations,
333 and our results for bitterling presented here, Filipsson *et al*. (2017) reported a small increase in
334 maximum metabolic rate in juvenile brown trout naturally infected with glochidia of the freshwater

pearl mussel *Margaritifera margaritifera* when compared to non-infected fish. Besides the possibility for species-specific differences in the effects of glochidia on fish hosts, the conflicting outcome may also be explained by temporal effects. While we studied the acute effects of glochidia attachment on MO_2max, Filipsson *et al*. (2017) observed higher MO_2max in naturally infested fish that may have hosted glochidia for several months, since *M. margaritifera* glochidia remain encysted in their hosts for approximately 10 months (Bauer & Vogel, 1987). Given that glochidia-infested brown trout also had elevated metabolic rates at rest, they may 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 maximum metabolic rate to maintain aerobic scope (Bonneaud *et al.,* 2016). Even when infected with the same parasite, fish may also show species-specific responses. Rainbow trout *Oncorhynchus mykiss* infected with the microsporidium gill parasite *Loma salmonae* increased both routine and maximum metabolic rate, whereas the brook trout *Salvelinus fontinalis* lowered routine metabolic rate with no change in maximum metabolic rate (Powell *et al*., 2005). Therefore, a response in MO_{2max} may be both specific to host species, to parasite identity and may vary over the course of infection.

Although we did not observe a significant effect on MO_2max in glochidia–infected bitterling, the direction of the trend was a reduced MO_{2max} in the most heavily infected 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 infected fish (average: 8.0, range: 0-20) per individual. This number does not reflect unsuccessful experimental infestation, as comparable infestation loads were observed in naturally infected fish from the same population (Douda *et al*., 2017a). Instead, the low glochidia attachment success on *R. ocellatus* gills may be a consequence of the close coevolutionary association between bitterling and mussel. All tested bitterling species, including

359 *R. ocellatus*, have lower numbers of glochidia initially attaching to them, and substantially lower
360 infection success than that seen in other freshwater fishes coexisting with *S. woodiana* (Douda *et*
361 *al*., 2017a). Thus, it appears that bitterling can limit the number of glochidia that attach to a level
362 at which the impact of infection on gill function is negligible.

363 Contrary to our predictions, glochidia–infected bitterling did not have elevated oxygen
364 uptake rates during recovery compared to non-infected fish but instead had consistently lower MO_2.
365 This finding might reflect a behavioural response to infection in bitterling through reducing their
366 spontaneous activity levels (i.e. turning inside the respirometer). Reducing activity levels may even
367 be a general and adaptive response to glochidia infections. For example, chub *Squalius cephalus*
368 infected with glochidia of *Anodonta anatina* were less active in open field tests and dispersed less
369 in a natural setting, especially during the early phases of infection (Horký *et al*., 2014). In brown
370 trout, glochidia load was negatively correlated with feeding activity (Österling *et al*., 2014), general
371 activity levels and aggression (Filipsson *et al*., 2016). Assuming that glochidia attachment acutely
372 impairs MO_2max and maximum swimming speed (Taubert and Geist, 2013), reducing activity
373 levels would be an adaptive response to limit the risk of predation since the chance of escaping a
374 predator would be reduced. Reduced activity may also be an adaptive response to avoid further
375 infections given that high glochidial loads may lead to mortality (Howerth and Keller, 2006;
376 Taubert and Geist, 2013). While we did not record activity levels in bitterling, we propose that the
377 observed decrease in MO_2 might have reflected an adaptive behavioural response to glochidia
378 infection as observed in other species. This prediction may be further tested by measuring activity
379 levels together with oxygen consumption rates pre- and post-infection.

380 In conclusion, we demonstrated that unionid mussels infected by early-stage bitterling
381 embryos showed an elevated routine metabolic rate, though this relationship might have been

382 driven by a selective preference by bitterling for spawning in mussels with high oxygen
383 consumption rates. *Rhodeus ocellatus* did not suffer a significant acute cost of *S. woodiana*
384 glochidia infestation, measured in terms of a reduced maximum oxygen uptake. However,
385 glochidia–infected fish had lowered oxygen uptake rates during the recovery phase between
386 MO_2max measurements, possibly due to a behavioural response. Our results suggest that bitterling
387 and mussels can minimize the acute costs in their bi-directional parasitic relationship. Given the
388 long-term coevolution between unionid mussels and bitterling in East Asia, mussels are capable of
389 ejecting bitterling embryos to a level that may curtail any negative consequences, while bitterling
390 appear capable of limiting their glochidia load to the level that mitigates significant negative
391 consequences.
392
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Figure legends

Fig. 1 Mass-specific routine metabolic rate (RMR) in *S. woodiana* hosting *R. ocellatus* embryos. The relationship between RMR and (A) total number of embryos (early and late stages) and (B) number of early stage embryos is shown. Five specimens with no embryos (not included in analysis) are shown in open (black) circles). The least squares regression lines are shown with 95% confidence intervals indicated by a shaded area.

Fig. 2 Effect of glochidia load on maximum oxygen consumption rates (MO_{2max}) of *R. ocellatus*. (A) total number of attached (fins + gills) glochidia, (B) glochidia attached to gills only. Repeated measurements are shown.

Fig. 3 Oxygen consumption rates in glochidia-infected *R. 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 infected (open circles) and 10 non-infected (filled circles) individuals. Error bars represents 95% confidence intervals.