



Benefits and costs to mussels from ejecting bitterling embryos: a test of the evolutionary equilibrium hypothesis

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(Received 1 July 2004; initial acceptance 19 August 2004;
final acceptance 12 September 2004; published online 7 April 2005; MS. number: 8184)

Two major hypotheses of host–parasite interactions have been proposed to explain cases where hosts do not defend themselves against parasites. The evolutionary lag hypothesis suggests that there has been insufficient time for a host response to evolve, whereas the evolutionary equilibrium hypothesis proposes that host defence does not evolve because it carries costs that outweigh the benefits. We tested potential benefits and costs of host defence in an unusual interaction, between a freshwater fish, the European bitterling, *Rhodeus sericeus*, and live unionid mussels that are used as hosts for the fish's eggs. We found a significant reduction in ventilation rate of mussels that were incubating bitterling embryos, which became more severe with an increasing number of embryos. We tested the hypothesis that the risk of ejecting a mussel's own larvae while ejecting bitterling embryos has constrained the evolution of a host response. This predicts that brooding female mussels would retain more bitterling larvae than males or nonbrooding females. This prediction was not supported: brooding female mussels contained as many larvae per accessible gill as, and fewer in total than, males or nonbrooding females. In summary, based on the costs and benefits of ejection that we measured, we found no evidence in support of the evolutionary equilibrium hypothesis. However, other differences between mussel species, such as gill structure as measured in this study, ventilation rates and differences in the distance eggs are lodged into the gills, may contribute to differences in egg ejection rates.

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Symbioses encompass a range of interspecific relationships, from parasitism, such as infectious diseases (Allison 1982), to mutualisms, such as those found between ants and *Acacia* (Janzen 1966). The outcome of such interactions can be explained as the result of reciprocal evolutionary changes between the two or more noninterbreeding populations (Thompson 1994). Theoretical advances in our understanding of coevolution have come from a range of host–parasite interactions (Anderson & May 1982; Thompson & Burdon 1992; Takasu et al. 1993; Takasu 1998), but notably from behavioural studies of avian brood parasitism (Rothstein 1990; Davies 2000). The first step in a coevolutionary relationship is often the evolution of a host defence against a parasite, resulting from a fitness cost of the parasite

to the host. In turn, a counteradaptation against the host defence may be selected for in the parasite, to which the host reciprocates, resulting in a coevolutionary arms race.

One of the remaining conundrums in the study of coevolution is why some hosts accept parasites or parasitic eggs that incur fitness costs. Two general hypotheses have been proposed which may help to explain the lack of a host response: the evolutionary lag and evolutionary equilibrium hypotheses. The evolutionary lag hypothesis suggests the absence of a host defence is merely a result of insufficient evolutionary time for either the mutation to arise or for the defence behaviour to spread through the host population (Rothstein 1975; Dawkins & Krebs 1979; Davies & Brooke 1989a; Soler & Møller 1990). In contrast, the evolutionary equilibrium hypothesis suggests that, although there has been time for the evolution of defence behaviour in hosts, the costs of such a defence outweigh the benefits, thereby preventing the evolution of defensive behaviour (Zahavi 1979; Rohwer & Spaw 1988; Davies & Brooke 1989b; Lotem et al. 1992; Marchetti 1992).

Variation in host responses to a potential parasite has been recognized in an unusual interaction involving

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a freshwater fish, the European bitterling, *Rhodeus sericeus*, and its mussel hosts (Unionidae) (Mills & Reynolds 2003a). The European bitterling is a member of the subfamily Acheilognathinae (Family Cyprinidae) and has an obligate spawning relationship with living mussels. It co-occurs with four principal mussel species over much of its European range: *Unio pictorum*, *U. tumidus*, *Anodonta anatina* and *A. cygnea* (Reynolds et al. 1997; Smith et al. 2000a; Mills & Reynolds 2002a). During the reproductive season, male bitterling aggressively defend an area containing one or more mussels and attract females with courtship displays (Candolin & Reynolds 2001, 2002a, b; Mills & Reynolds 2003b). Female bitterling initially inspect the males, but their final spawning decisions are based on the quality of the mussels in the male's territory (Candolin & Reynolds 2001). They prefer mussels that do not already contain bitterling embryos (Smith et al. 2000b; Candolin & Reynolds 2001) and that pump a larger volume of water per unit time (Mills & Reynolds 2002b). Females insert their long ovipositors through the exhalant siphon and lodge their eggs in the gills of the mussels (Wiepkema 1961; Heschl 1989). They lay two to six eggs in each mussel (Mills & Reynolds 2002a), which are fertilized when the male's sperm is drawn into the mussel with the inhalant respiratory current (Duyvene de Wit 1966). There is no evidence to suggest that bitterling sperm provide mussels with any growth benefits (Mills & Reynolds 2003a). The eggs develop within the mussel for 2–4 weeks, after which the young bitterling leave via the mussel's exhalant siphon (Reynolds et al. 1997; Aldridge 1999a). Bitterling survival can be reduced when high phosphate or low oxygen and algal concentrations in the surrounding waters cause premature embryo ejection (Reynolds & Guillaume 1998; Mills & Reynolds 2004).

The four mussel species used by bitterling for spawning in the U.K. differ from one another in their rates of egg ejection (Mills & Reynolds 2002a). Two species, *U. pictorum* and *U. tumidus*, show particularly low levels of egg ejection, which suggests that only weak levels of defence have evolved in these mussel species. The bitterling was introduced to the U.K. around 100 years ago, so the evolutionary lag hypothesis may explain the lack of a host response to bitterling eggs in the U.K. However, we consider this unlikely, as similarly low rates of egg ejection were found in the same two species from the Czech Republic, where the mussel hosts have an ancient history of sympatry with bitterling (Smith et al. 2000b; Mills & Reynolds 2002a).

The evolutionary equilibrium hypothesis, which predicts that the costs of a host defence against a parasite outweigh the benefits of the defence, has not been tested in the bitterling–mussel interaction. If the evolutionary equilibrium hypothesis is to be applied to the bitterling–mussel relationship, the mussels must (1) incur a fitness cost by housing bitterling eggs, and (2) incur a potentially greater cost by expelling bitterling eggs. There is reason to suspect that bitterling embryos may affect mussels negatively. The embryos measure 2.6×1.7 mm (Aldridge 1999a) and over 100 eggs are typically found in the most popular host species (Mills & Reynolds 2003a).

To test the evolutionary equilibrium hypothesis, we investigated the costs to mussels from housing bitterling

eggs, by manipulating the presence of eggs and comparing differences between species of mussels in ventilation rates. The differences we found led us to consider why some mussel species do not eject bitterling eggs routinely. Gravid female mussels are known to abort their larvae (glochidia) prematurely under conditions of low oxygen (Tankersley & Dimock 1993), and we have often observed glochidial ejection by mussels sharing their aquaria with bitterling, especially when bitterling spawn. To test whether the differences in host defence were due to differential costs of ejection through loss of a mussel's own larvae, we examined the numbers of embryos carried in relation to the mussel's sex and reproductive state and predicted that brooding female mussels would retain more bitterling larvae than males or nonbrooding female mussels. Finally, we tested for differences between species in gill structure that may affect mussel ejection rates.

METHODS

Potential Benefits of a Host Response

We first tested the hypothesis that bitterling embryos may impair ventilation rates of mussels. Ventilation rates of *U. pictorum* and *A. anatina* were compared before bitterling spawning, after spawning while carrying bitterling embryos, and after bitterling larval emergence.

We collected by hand 40 individuals of each of the two mussel species *U. pictorum* and *A. anatina* from Reach Lode, a tributary of the River Cam, Cambridgeshire, U.K., at the point of confluence with Wicken Lode, N.G.R.: TL 545 696 during March and April 2000. The mussels were maintained in artificial pond water in outdoor plastic pools (140 × 90 cm and 30 cm high), covered in a 10-cm layer of washed sand, with 20-cm depth of water and were fed daily 3 litres of a live algal suspension derived from an outdoor pool that had been seeded with *Chlorella vulgaris*.

On 16 May 2000, 80 mussels were placed in plastic troughs (75 × 22 cm and 17 cm high) lined with 10 cm layer of washed sand and covered with plastic netting (1-cm² holes at 0.5-cm intervals) so that they were inaccessible to bitterling, but their ventilation rate was not affected, and placed in Reach Lode to acclimatize. After 1 week, we measured the ventilation rate of all 80 mussels at the site with a small thermistor probe following the methods described in detail by Mills & Reynolds (2002b).

After acclimatization, five mussels of each species were transferred into each of eight plastic troughs of the same size as used during the acclimatization. Four of the troughs were re-covered with plastic netting and the other four troughs were left uncovered to allow bitterling to spawn. These manipulations resulted in two treatments: mussels inaccessible or accessible to bitterling. We returned the troughs to Reach Lode for 4 weeks until 20 June, after which we remeasured the ventilation rates of the mussels from both treatments at the site. We dissected the mussels in two troughs from each treatment and counted the eggs present in the gills. The remaining two troughs from each treatment were re-covered with plastic netting and returned to the study site to allow for larval emergence.

On 7 July, when the majority of bitterling larvae would have left the mussels, we removed the mussels from the site and remeasured their ventilation rates. Each mussel was kept in a separate plastic trough at the University of East Anglia and monitored twice daily for any remaining larval emergence. These remaining mussels were returned to the study site at the end of the experiment.

Potential Costs of a Host Response

Twenty *U. pictorum*, *U. tumidus*, *A. anatina* and *A. cygnea* were collected by hand from Reach Lode, on 3 May 2004, and all external shell parameters were measured. We dissected the mussels and counted the advanced larvae (pigmented eyes, a nearly fully absorbed yolk sac and at least 7.5 mm in length). These larvae would have been approximately 21 days old and would have left the mussels within approximately 7 days (Aldridge 1999a). We examined mantle samples of each mussel under an Olympus BX50 compound microscope and used the presence of sperm or glochidia to determine a mussel's sex. We determined the reproductive state of female mussels from the absence or presence of glochidia in the outer gills (nonbrooding and brooding females, respectively). The outer gills are capped off during glochidial brooding and thus bitterling embryos can be laid only into the two inner gills of brooding females (Mills & Reynolds 2003a). To account for any confounding effect of gill accessibility, we calculated the number of larvae per accessible gill in each mussel.

Differences in Gill Structure

At the time of dissection we removed the inner gill from each mussel and observed it under a Wild Heerbrugg M8 dissecting microscope, using $\times 20$ magnification. The widths of at least four water tubes from the inner gill were measured as the distance between two lamellae (± 0.01 mm) of 20 individuals and compared among the four mussel species.

Data Analysis

We carried out ANCOVAs to examine the number of larvae carried in wild mussels, using mussel species and sex/reproductive state as fixed factors and mussel length and water tube width as covariates. Post hoc tests were carried out on mussel species and sex/reproductive state using the contrasts procedure in SPSS 11.0 for Mac OS X (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Potential Benefits of a Host Response

The ventilation rates of *U. pictorum* and *A. anatina* were reduced by the presence of eggs and embryos in their gills (Fig. 1a, b). After 4 weeks, the ventilation rates of mussels that were accessible to bitterling were significantly lower

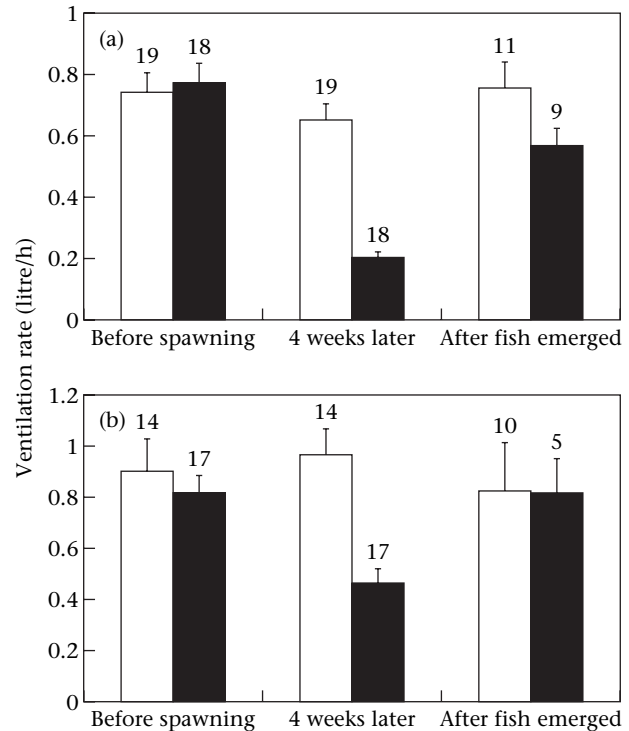


Figure 1. The mean \pm SE ventilation rates of mussels before bitterling spawned at the start of the experiment, after spawning 4 weeks later and after the majority of bitterling had emerged 2.5 weeks later in (a) *U. pictorum* and (b) *A. anatina*. Numbers above bars are sample sizes. \square : Mussels not accessible to bitterling; \blacksquare : mussels accessible to bitterling.

than their rates at the beginning (paired *t* test: *U. pictorum*: $t_{17} = 8.15$, $P < 0.001$; *A. anatina*: $t_{16} = 3.15$, $P < 0.006$). Mussels, of both species, that were inaccessible to bitterling showed no change in ventilation rate (*U. pictorum*: $t_{18} = 1.1$, $P = 0.29$; *A. anatina*: $t_{13} = 0.23$, $P = 0.82$).

After a further 4 weeks, with the bitterling larvae having left the accessible mussels, the ventilation rates increased and returned to (*A. anatina*) or towards (*U. pictorum*) their original values at the start of the experiment (*U. pictorum*: $t_8 = 1.52$, $P = 0.17$; *A. anatina*: $t_4 = 0.14$, $P = 0.89$; Fig. 1a, b). The ventilation rates of mussels that were not accessible to bitterling again showed no change (*U. pictorum*: $t_{10} = 0.74$, $P = 0.48$; *A. anatina*: $t_9 = 1.07$, $P = 0.31$).

The ventilation rates of *U. pictorum* decreased with the number of bitterling embryos and larvae in their gills (linear regression: *U. pictorum*: $R^2 = 0.60$, $F_{1,13} = 19.4$, $P = 0.001$; *A. anatina*: $R^2 = 0.08$, $F_{1,9} = 0.81$, $P = 0.391$; Fig. 2). The reduction in ventilation rate as a percentage of the original ventilation rate in *U. pictorum* also became more severe with increasing number of bitterling embryos and larvae in the gills (linear regression line: *U. pictorum*: $R^2 = 0.60$, $F_{1,13} = 13.33$, $P = 0.005$; *A. anatina*: $R^2 = 0.17$, $F_{1,9} = 1.58$, $P = 0.245$).

Potential Costs of a Host Response

There was a significant difference in the total number of advanced larvae in wild mussels between species and

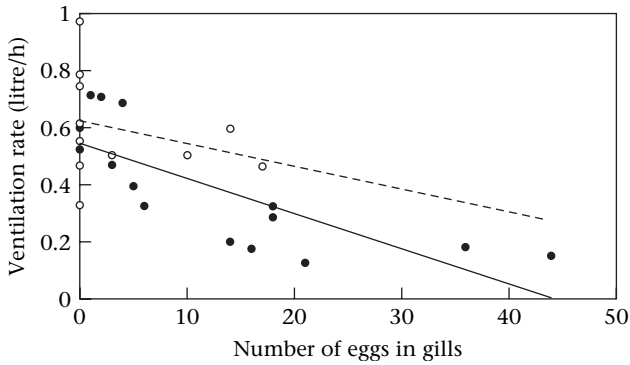


Figure 2. Relations between the number of eggs present in the gills of a mussel and the mussel's ventilation rate; ●: *U. pictorum*; $Y = 0.545 - 0.012X$ (—); ○: *A. anatina*; $Y = 0.625 - 0.008X$ (---).

mussels of different sex and reproductive state (univariate model, species: $F_{3,77} = 4.03$, $P = 0.011$; sex/reproductive state: $F_{2,78} = 5.62$, $P = 0.005$; Fig. 3a). As predicted from our previous study (Mills & Reynolds 2002a), fewer larvae were found in the gills of *A. cygnea* than in the three other species (post hoc: $P = 0.004$), and fewer larvae were found in the gills of *A. anatina* than in *U. pictorum* and *U. tumidus* (post hoc: $P = 0.019$). These differences reflect both bitterling preferences and mussel ejection rates (Mills & Reynolds 2002a). More larvae were found in the gills of male mussels than in brooding female mussels (post hoc:

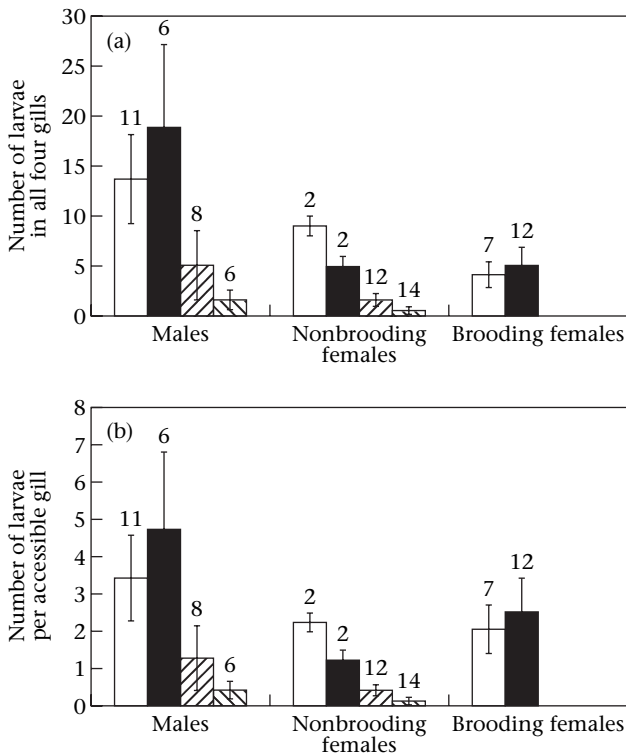


Figure 3. The mean + SE number of bitterling larvae carried in wild mussels (a) in all four gills and (b) per accessible gill (mean number of larvae divided by number of accessible gills) in relation to mussel sex and reproductive state. □: *U. pictorum*; ■: *U. tumidus*; ▨: *A. anatina*; ▩: *A. cygnea*. Numbers above bars are sample sizes.

$P < 0.001$). However, there was no difference in the number of larvae found in nonbrooding females compared to males (post hoc: $P = 0.152$) or compared to brooding females (post hoc: $P = 0.086$). There was no significant interaction between mussel species and sex/reproductive state ($F_{4,76} = 0.59$, $P = 0.67$), nor was there an effect of the covariates, mussel length or water tube width, on the number of larvae carried (length: $F_{1,79} = 0.96$, $P = 0.33$; water tube width: $F_{1,79} = 0.7$, $P = 0.41$).

As with the total number of larvae carried, we found significant differences in the number of larvae per accessible gill between mussel species ($F_{3,77} = 3.1$, $P = 0.032$; Fig. 3b). Similarly, fewer larvae per gill were found in *A. cygnea* than in all three other mussel species (post hoc: $P = 0.012$) and fewer larvae per gill in *A. anatina* than in *U. pictorum* and *U. tumidus* (post hoc: $P = 0.035$). However, there was no longer an effect of sex/reproductive state on the number of larvae per gill ($F_{2,78} = 1.98$, $P = 0.145$), and still no interaction between species and sex/reproductive state ($F_{2,78} = 0.42$, $P = 0.791$). Neither of the covariates, mussel length or water tube width affected the number of bitterling larvae per gill (length: $F_{1,79} = 1.27$, $P = 0.264$; water tube width: $F_{1,79} = 0.173$, $P = 0.679$).

Differences in Gill Structure

Species, sex and reproductive state of mussels had significant effects on the width of water tubes from the inner gills (species: $F_{3,77} = 5.58$, $P = 0.002$; sex/reproductive state: $F_{2,78} = 8.6$, $P < 0.001$; Fig. 4). The species that ejects bitterling eggs most consistently, *A. cygnea*, had significantly wider water tubes than all three other mussel species (post hoc: $P = 0.001$). Males had wider water tubes than nonbrooding females (post hoc: $P = 0.007$). However, there was no effect of mussel length on water tube width ($F_{1,79} = 0.01$, $P = 0.936$).

DISCUSSION

This study shows that European bitterling embryos significantly reduce their mussel hosts' ventilation rates (Figs 1 and 2), thereby providing the strongest demonstration

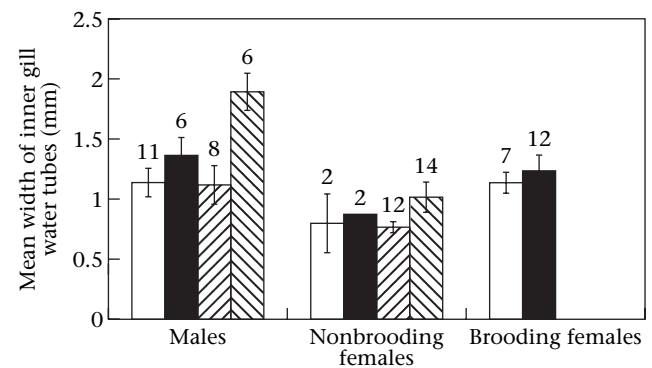


Figure 4. The mean + SE width of water tubes from the inner gills in relation to the sex and reproductive state of the four mussel species: □: *U. pictorum*; ■: *U. tumidus*; ▨: *A. anatina*; ▩: *A. cygnea*. Numbers above bars are sample sizes.

available to date of a cost to mussels from housing bitterling embryos. In addition, differences in oxygen concentration between inhalant and exhalant siphons are greatest in mussels that contain embryos (Smith et al. 2001). Bitterling embryos may lower ventilation rates by obstructing water flow through the gills and by distorting the gills and the lateral cilia on their surface. These effects may have more severe consequences for filter feeding than for oxygen uptake, because bivalve gills function more importantly in feeding than in oxygen exchange (Tankersley 1996). Baker & Hornbach (1997) found that starvation, caused by reduced food intake and (or) increased metabolic costs, is the major mechanism that reduces fitness and eventually causes mortality of unionid mussels infected with zebra mussels, *Dreissena polymorpha*. Furthermore, lowering of the feeding rate together with a constant or slowly decreasing metabolic demand leads to a drastic reduction of somatic growth followed by loss of mass and death in the marine mussel *Mytilus edulis* (Sukhotin et al. 2003). We therefore suggest that bitterling should be regarded as parasites of mussels, whose impairment of ventilation rates throughout the 4-month bitterling spawning season is likely to reduce food intake rate and may translate into long-term effects on mussel growth, reproduction or survival. This suggests that there should be selection on mussels to defend themselves against bitterling.

Under the evolutionary equilibrium hypothesis, a constraint on evolving a host response could be the risk of ejecting glochidia at the same time, analogous to costs of birds damaging their own eggs when rejecting avian brood parasites (Davies 2000). One prediction of this hypothesis is that female mussels brooding glochidia might be constrained from ejecting bitterling embryos, and would therefore contain more advanced bitterling larvae than males and nonbrooding females. This prediction is more relevant to *Unio* species because the bitterling spawning period coincides more with their glochidial brooding period than with that of *Anodonta* species (Aldridge 1999b). As no differences have been found in the number of eggs laid in mussels of different sex and reproductive state (Mills & Reynolds 2003a), the numbers of larvae found in wild mussels should reflect mussel ejection rates. Contrary to the prediction that brooding female mussels would retain more bitterling embryos, we found significantly more bitterling larvae in males (Fig. 3a). This difference disappeared when we accounted for differences in the number of gills accessible to bitterling (Fig. 3b), but this result still did not support the prediction. We conclude that differences between mussels in costs of premature ejection of their own glochidia when ejecting bitterling embryos are unlikely to explain differences in ejection behaviour between mussels.

As the lack of ejection in *Unio* species, which receive most eggs and have the lowest rates of ejection, was not resolved by glochidial brooding, we looked for differences between mussel species in gill structure. Each mussel gill is compartmentalized dorsoventrally into water tubes and it is in these compartments that the bitterling develop (Mills & Reynolds 2003a). Bitterling embryos are a unique wedge

shape, which may make the mussel less able to dislodge them from their gills (Mills & Reynolds 2003a). Our results show that the widest water tubes are found in *A. cygnea*, the mussel species with the highest rate of egg ejection (Mills & Reynolds 2002a). Although the difference in water tube width may explain differences in ejection behaviour between *A. cygnea* and the other three species, water tube width does not explain differences in ejection either between *A. anatina* and *Unio* species or between the two *Unio* species. Therefore, other physiological traits may also be involved. In this study, we did not find a significant effect of water tube width on either the total number of larvae carried or the number of larvae carried per gill. To test definitively whether water tube width determines egg ejection rate, egg ejection should be compared in relation to tube width among mussel species into which a controlled number of eggs have been laid.

Although this study has revealed significant costs in terms of reduced ventilation rates to mussels from housing bitterling embryos, not all mussel species eject bitterling. The one aspect we considered as a potential cost of bitterling ejection, the loss of mussel glochidia, does not explain the low ejection rates in *Unio* species. Therefore, based on the costs of ejection in relation to brooding glochidia and the benefits of ejection in terms of reduced ventilation rates, we are unable to use the evolutionary equilibrium hypothesis to explain the differences in egg ejection between mussel species. Other costs and benefits of ejection, for example energetic costs and fitness benefits, respectively, may differ between mussel species, so the evolutionary equilibrium hypothesis still cannot be ruled out. For example, large marine mussels are more susceptible to low oxygen than smaller mussels, owing to their higher metabolic rates (Sukhotin et al. 2003). If a similar susceptibility exists in freshwater mussels, then conditions of reduced oxygen and food caused by bitterling parasitism would be more costly for the larger unionid *A. cygnea* and may explain their higher rates of embryo ejection. In terms of the second hypothesis, we have shown (Mills & Reynolds 2002a) that the differential ejection rates between species are unlikely to be caused by evolutionary lag; however, a more definitive test of this hypothesis would involve a comparison of mussel responses between areas that differ in their history of association with bitterling.

There is a growing body of evidence to suggest that physiological differences between mussel species may be the prime determinants of the ejection response. These include the following three findings: (1) the water tubes of *Unio* species and *A. anatina* are narrower than those of *A. cygnea*, which may allow bitterling eggs to be wedged more tightly into their gills (this study); (2) mussel ventilation rate, which is likely to be one of the methods used to eject bitterling embryos, increases from *U. pictorum* through *U. tumidus* and *A. anatina* to *A. cygnea* (Mills & Reynolds 2002b); and (3) the distance from the exhalant siphon that bitterling are lodged in the gills is greater in *Unio* than *Anodonta* species, which may make them harder to eject (Mills & Reynolds 2002a). These findings may, in combination, explain the rank order of egg ejection from *U. pictorum* through *U. tumidus* and *A. anatina* to *A. cygnea*.

Acknowledgments

We thank Brian Reeve and Roger Humphrey for their electronics expertise, time and help in making the thermistor flowmeter, Chris Wiskin and Thierry Fredou for their invaluable help with fieldwork and data collection, Richard Tankersley for insightful discussions, Paul Cooke, Geoff Eagles, Martin Perrow and Mark Tomlinson for technical assistance and Smith Bros Garage (Norwich) for parts. We also thank the John and Pamela Salter Charitable Trust for help with funding. This research was supported by a studentship from the Natural Environment Research Council to S.C.M.

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