A proximate cue for oviposition site choice in the bitterling (Rhodeus sericeus)

CARL SMITH,* KARINA RIPPON,* ALEX DOUGLAS* and PAVEL JURAJDA†
*School of Biological Sciences, Queen Mary, University of London, London, U.K.
†Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno, Czech Republic

SUMMARY
1. We investigated two possible proximate cues used for oviposition site choice by females of the bitterling (Rhodeus sericeus), a freshwater fish that spawns on the gills of live unionid mussels. The two cues were the flow velocity and/or oxygen content of water emerging from the exhalant siphon of a mussel.
2. Field observations showed that female bitterling always inspected the exhalant siphons of mussels before they spawned in them. Siphon inspection was not always a prelude to spawning and it may serve as a means of assessing mussel quality. Female skimming behaviour, swimming over a mussel without spawning, may also be used to assess mussel quality, although the mechanism for this is unclear.
3. Measurements of the flow velocity of water emerging from the exhalant siphons of four mussel species (Anodonta anatina, A. cygnea, Unio pictorum and U. tumidus) showed a significant difference among species, with U. tumidus having the highest mean flow velocity and U. pictorum the lowest.
4. Measurements of the change in oxygen concentration of water entering a mussel inhalant siphon and leaving its exhalant siphon in field and laboratory studies showed a significant difference among the four mussel species, with A. cygnea exhibiting a significantly higher change in oxygen concentration than the other species.
5. The presence of bitterling embryos in the gills of a mussel significantly increased its oxygen consumption whereas larval glochidia had no significant effect. We discuss oxygen availability as a possible proximate cue for oviposition site choice in bitterling.

Keywords: freshwater mussel, glochidia, oxygen availability, pseudobranch

Introduction
Oviposition site choice can have significant fitness consequences for offspring (Huey, 1991; Rosenzweig, 1991; Thomson & Pellmyr, 1991), and can shape life-history evolution (Resetarits, 1996). Sites for oviposition may vary in quality, or may already contain the eggs of other individuals, including other species. Individuals must also choose whether to deposit all their eggs in one site or among several sites. A variety of cues may be used to assess oviposition site quality.

Godfray (1994) reviewed oviposition choice in parasitoids, in which species, size, age and condition can determine host quality. A variety of visual, tactile or chemical cues may be used by parasitoids to assess the suitability of hosts for oviposition.

Here we investigate two possible cues for oviposition site choice by the bitterling, Rhodeus sericeus (Pallas), a freshwater fish that spawns on the gills of living unionid freshwater mussels. Female bitterling develop long ovipositors that they use to place their eggs between the gill lamellae of a mussel through the exhalant siphon (Duyvéné de Wit, 1955). Male bitterling defend territories around mussels to which they lead females to spawn. Males fertilize the eggs by releasing sperm over the inhalant siphon of the
mussel, so that water filtered by the mussel carries the sperm to the eggs (Breder & Rosen, 1966). Female bitterling can spawn in more than one mussel and lay 50–100 clutches of 1–6 eggs during a breeding season. Mussels may contain multiple clutches (Wiepkema, 1961). Thus, spawning by bitterling is analogous to superparasitism in parasitoids, whereby a clutch of eggs is deposited on a host that has already been parasitized by a member of the same species (Godfray, 1994). Embryonic development of the eggs is completed inside the mussel gill and lasts from 3 to 6 weeks (Aldridge, 1999). At least four mussel species, Anodonta anatina (L.), A. cygnea (L.), Unio pictorum (L.), and U. tumidus (Philipsson), are used by bitterling as spawning hosts (Aldridge, 1997). Smith et al. (2000a) used field and laboratory experiments to show that female bitterling spawn at a significantly higher rate in A. anatina, U. pictorum and U. tumidus than in A. cygnea. Female bitterling were further shown to spawn at a significantly lower rate in mussels already containing many bitterling embryos. The survival of embryonic bitterling in their host mussel was significantly negatively density-dependent for all mussel species. The strength of the relationship between mortality rate and embryo density differed significantly among the four mussel species, with the strength of density-dependent mortality higher for A. cygnea than the other three species tested (Smith et al., 2000a). Smith et al. (2000a) proposed that the flow rate and/or oxygen content of water emerging from the exhalant siphon of a mussel might serve as a proximate cue for oviposition site choice. We, therefore, assessed the relationship between the mussel inspection behaviour of female bitterling immediately prior to spawning and spawning frequency and also investigated flow velocity and oxygen content of water emerging from the exhalant siphon of a mussel as possible cues for assessing the suitability of a mussel for successful embryo development.

Methods

Bitterling behaviour

Field data on spawning behaviour were collected in May 1998 in Lake Dédova in the south-east of the Czech Republic, at the centre of the natural range of the bitterling in Europe (Kottelat, 1997). Tests were conducted in three arenas, spaced approximately 30 m apart, around the lake margin, 2–3 m from the shore and in water approximately 40–60 cm deep. Two snorkellers observed the spawning behaviour of male and female bitterling presented with eight freshwater mussels; two each of A. anatina, A. cygnea, U. pictorum, and U. tumidus, placed in flowerpots filled with sand, arranged in a circle in a predetermined random order. A snorkeller observed the mussels from a distance of 1.0–1.5 m. Pilot studies had shown that bitterling would continue territorial and breeding behaviour in the presence of a snorkeller. Following a spawning, the location of each of the mussels under test was rearranged according to the next random pattern. After a second spawning, all the mussels in the arena were replaced. Some results from this experiment have been published elsewhere (Smith et al., 2000a; Smith, Reynolds & Sutherland, 2000b); none are repeated here.

Female bitterling perform a head-down ‘siphon inspection’ behaviour prior to spawning (Wiepkema, 1961), in which they position themselves with their head over the mussel’s exhalant siphon, often with the body orientated vertically, for up to 3 s before either spawning or moving away. They also perform a ‘skimming’ behaviour that involves the female moving quickly forward over the exhalant siphon of a mussel but without inserting her ovipositor or releasing eggs. Spawning and skimming are not mutually exclusive; females may skim over a mussel and then subsequently spawn in it. We recorded the rate of siphon inspection and skimming behaviour for a succession of female bitterling that were led to mussels for spawning by territorial males. To avoid pseudoreplicating bitterling behaviour we recorded distinguishing features, such as lost scales or the presence of external parasites of fish at arenas. Individual females spent relatively little time at arenas and sometimes individual distinguishing features were not recorded. For 30 females for which we did record distinguishing features only two returned to spawn again in our test arenas during the experiment, and neither spawned with the same male or in the same mussels. Consequently, the behavioural results we obtained for all fish are probably to include little pseudoreplication.

Mussel flow velocity and oxygen consumption

The freshwater mussels used were collected by a diver from the River Cam and Burwell Lode, Cambridgeshire in July 1999. Four species of mussel...
were used: *A. anatina*, *A. cygnea*, *U. pictorum* and
*U. tumidus*. They were transported to Queen Mary,
University of London, aquarium in river water and
housed in an aerated 0.63 m³ glass aquarium contain-
ing a 100-mm deep layer of fine gravel. Within
2 weeks of collection mussels were labelled individu-
alily by removing a 10 mm patch of periostracum
just below the umbo using sandpaper and marked
with a waterproof pen. Mussels were fed daily
with approximately 6 L of algae, predominantly
*Chlamydomonas* sp. and *Chlorella* sp.

Water velocity at the exhalant current of each
mussel was estimated directly by measuring the
movements of fine suspended particles departing
the exhalant siphon viewed using a narrow beam of
light against a dark background. This technique is
commonly used for measuring the flow velocities of
fluids (Fage, 1933; Crapper, Bruce & Gouble, 2000).
Measurements were made in a darkened room. A
slide projector was used to direct a narrow light beam
into the end of a glass aquarium measuring 350
(length) × 70 (width) × 250 (depth) mm. A narrow
light beam was obtained by projecting light through a
black slide engraved with an approximately 1 mm
clear slit. The light beam had a width of approxi-
mately 15 mm as it entered the aquarium. The back
panel of the aquarium was lined with black plastic
with a 1-mm graduated rule across it to provide scale.
A shaded lamp was placed 400 mm away from the
back of the aquarium to illuminate the divisions on
the scale. The aquarium contained a 50-mm deep
layer of fine gravel so that the test mussels could bury
themselves in a natural position.

A stopwatch, accurate to 0.01 s, was clamped to the
front of the aquarium and illuminated with a small
spotlight. A video camera was placed approximately
80 mm from the front of the aquarium such that it
focused on the scale, stopwatch and the exhalant
siphon of a mussel placed in the aquarium. The camera
was linked to a video monitor and video recorder. A
mussel was placed gently into the gravel in the
aquarium and allowed at least 10 min to adjust to the
conditions in the aquarium and to adjust its position
before measurement began. After this time the water
was stirred to suspend fine sediments in the gravel. The
amount of sediment in the aquarium was low to enable
single particles to be readily seen. During experiments
mussels did not produce pseudo faeces in response to
the suspension of sediment. Consequently, we do not
believe our results are confounded by the gills of the
mussels becoming clogged with particles.

Once particles were seen to be emerging from a
mussel’s exhalant siphon in a steady stream the
stopwatch was started and the flow of particles
emerging from the exhalant siphon was recorded
using the video. At least 8 min of continuous filtering
was recorded for each mussel. After completion of
recording the mussel was removed from the tank and
its length was recorded. Water temperature in the
aquarium was recorded during trials.

In order to calculate water velocity at the exhalant
siphon of each mussel, the rate of movement of
particles emerging from the centre of the exhalant
siphon of each mussel was estimated from video
recordings. Once a steady current of particles was
seen to be emerging from the exhalant siphon in the
video recordings, an individual particle was identified
and the recording paused with the particle approxi-
mately 10 mm from its emergence point from the
exhalant siphon. The stopwatch display was read and
a marker was placed on the screen where the particle
was visible. The particle was then tracked, frame by
frame approximately 20 mm across the screen. The
stopwatch display was again read and a second
marker attached to the screen where the particle was
visible. The exact distance between these markers was
measured using callipers to the nearest 1 mm and the
time taken to travel that distance was calculated to
the nearest 0.01 s. The absolute distance travelled by
the particle was calculated using the 1 mm scale
visible in the recordings. Thus, the velocity of a
particle emerging from a mussel siphon was calcula-
ted in mm s⁻¹. The velocities of five particles leaving
each mussel were calculated. A pilot study showed
that five estimates for each mussel provided a repre-
sentative mean velocity. Because of parallax, the
distance between mussel and scale would result in
small overestimates of flow velocity. Thus the exha-
lant siphon of the mussel was 5–10 mm in front of the
scale so that an overestimate of between 1–3% could
be expected. This degree of error was considered
sufficiently small to be ignored. Measurements were
completed for 54 mussels.

The oxygen concentration of water entering the
inhalant siphon of a mussel and that leaving the
exhalant siphon was also measured. The difference in
oxygen concentration between inhalant and exhalant
siphons was assumed to be removed by the mussel.
Measurements were made in an aquarium measuring 450 (length) × 250 (width) × 250 (depth) mm with 50 mm depth of gravel substrate. The aquarium was aerated at each end to achieve an even distribution of oxygenated water. Each of the individually marked mussels used for flow velocity measurements was tested. To make measurements a mussel was first placed gently in the gravel in centre of the aquarium and allowed to adjust to its surroundings and to adjust its position for 5–10 min before readings were made. An oxygen probe was clamped over the inhalant siphon of the mussel so that it was as near as possible to the tip of the siphon but not making contact with it, to measure the oxygen concentration of water entering the mussel. The probe was attached to an oxygen meter, with a precision of 0.1 mg O2 L–1, that was calibrated daily. Once the mussel began filtering at a steady rate, readings were taken at 30 s intervals for at least 5 min. The same procedure was used to measure oxygen concentration at the exhalant siphon. The oxygen content of the water column in the experimental aquarium was allowed to stabilize before further measurements were made. Measurements were completed for 34 mussels.

Oxygen consumption of mussels and embryos

A field study was conducted to measure the oxygen consumption of mussels under natural conditions to validate laboratory measurements and to measure the oxygen consumption of mussels containing bitterling embryos. Field data were collected in May 2000 in the same lake in which behavioural observations were made. A total of 80 mussels were used in the experiment, 20 each of A. anatina, A. cygnea, U. pictorum and U. tumidus. Within species, mussels were assigned equally to one of two treatments, ‘with embryos’ and ‘without embryos’. In the ‘without embryos’ treatment, a 10-mm wide strip of transparent acetate sheet was fixed onto either side of the mussel shell, using polyacrylamide glue, to form a loop. The loop was positioned over the exhalant siphon and approximately 10 mm clear of the siphon, so that it prevented female bitterling from spawning but allowed water to be expelled normally. In the ‘with embryos’ treatment a loop of acetate was similarly fixed to the mussel then cut, to permit bitterling to spawn into the mussel. Pairs of mussels belonging to each treatment were placed in flowerpots filled with sand and placed around the margin of the lake for 3 weeks before oxygen measurements were made. The change in oxygen concentration between inhalant and exhalant siphon of each mussel was measured as in the laboratory trials. A diver positioned the oxygen probe over the mussel siphons and four readings were made at each siphon. The gills of all the mussels for which recordings were made were removed the same day and fixed in 4% formalin. Gills were later dissected under a low power microscope and bitterling embryos counted. The presence in the gills of developing larval glochidia was also noted. Water temperature during the taking of oxygen measurements was 24.7 °C.

We estimated the total oxygen demand of bitterling embryos only, incubated in mussel gills to assess their impact on the mussel oxygen budget. Data for oxygen consumption of bitterling embryos are not available. Consequently, estimates of the oxygen consumption of embryos were made using the oxygen consumption–age relationship of Mrowka & Schierwater (1988) for the cichlid Pseudocrenilabrus multicolour (Schoeller). Pseudocrenilabrus multicolour eggs are comparable in size to those of bitterling, 1.5 mm along their longest axis, and thus probably to have similar oxygen requirements. For P. multicolour embryos of age 1–5 days, oxygen consumption followed the exponential regression: oxygen consumption (µg O2 embryo–1 h–1) = 0.05 e0.66x, where x is the age in days. For offspring older than 5 days the regression took the form: oxygen consumption (µg O2 offsprin g–1 h–1) = 0.62 e0.19x. Mrowka & Schierwater (1988) measured the respiratory rate of P. multicolour embryos and larvae at 27 °C, 2.3 °C higher than in the present study. The effect of this marginal difference in temperature on the oxygen consumption rates of embryos is not known, but is probably slight (Blaxter, 1992) and was ignored. We investigated the effects of varying embryo number, embryo age, and mussel filtration rate on oxygen availability in mussels assuming an ambient oxygen level of 10 mg O2 L–1.

Results

Bitterling behaviour

Field observations showed that female bitterling only spawned in a mussel if they had first inspected its exhalant siphon. However, there was no significant difference in the inspection rate of mussels in which
they subsequently spawned and those in which they did not spawn (Mann–Whitney, \( W_{68,139} = 14127, P = 0.164 \)) (Fig. 1). Among the inspected mussels there was no significant association between spawning and skimming (\( \chi^2 \)-test, \( \chi^2 = 2.22, \text{d.f.} = 1, P = 0.136 \)) (Fig. 2). Further, there was a significant negative correlation between spawning rate and skimming rate (both variables log \( 10 + 1 \) transformation) in mussels in which females spawned or skimmed (Pearson’s correlation, \( r = -0.44, P < 0.001 \)).

**Mussel flow velocity and oxygen consumption**

An analysis of covariance (ANCOVA), using mussel length and water temperature as covariates, showed a significant difference in the velocity of water emerging from the exhalant siphons of the four mussel species tested (ANCOVA, \( F_{3,42} = 3.63, P = 0.023 \)). The highest flow velocity was shown by *U. tumidus* with a mean flow velocity of 43 mm s\(^{-1} \) (±3.1), the lowest by *U. pictorum* with a mean of 27 mm s\(^{-1} \) (±1.9) (Fig. 3). A significant difference in the change in oxygen concentration was also found between inhalant and exhalant siphons, with mussel length and water temperature as covariates (log \( 10 \) ANCOVA, \( F_{3,22} = 18.80, P < 0.001 \)), with *A. cygnea* showing the greatest change in oxygen concentration (Fig. 4).

**Mussel and embryo oxygen consumption**

The acetate loops fixed over the exhalant siphon of the ‘without embryos’ treatment sometimes became dislodged, or failed to or only partially prevented spawning because they were incorrectly placed. Consequently, to analyse these data we treated the number of embryos in a mussel as a continuous variable rather than as a treatment group. Thus the data were analysed using a two-way ANCOVA with

![Fig. 1](image1.png) The mean (±1 SE) inspection rate (h\(^{-1} \)) by female bitterling of the exhalant siphons of mussels into which they either subsequently spawned or did not spawn.

![Fig. 2](image2.png) The frequency of spawning and skimming by female bitterling in 210 independent trials in the field.

![Fig. 3](image3.png) The mean (±1 SE) flow velocity (mm s\(^{-1} \)) of water from the exhalant siphons of four species of mussel.

![Fig. 4](image4.png) Mean percentage reduction (±1 SE) in oxygen concentration of water filtered by four species of mussel.
mussel species as a fixed factor, presence of glochidia as a random factor and number of embryos in the gills of the mussel as covariate. There was a significant difference among mussels in the change in oxygen concentration between inhalant and exhalant siphons, with *A. cygnea* showing the greatest change (ANCOVA, \( F_{3,48} = 4.76, P = 0.006 \)). There was no significant difference between mussels with and without glochidia (ANCOVA, \( F_{1,48} = 3.88, P = 0.055 \)). The number of embryos in the gills of mussels was a highly significant covariate (ANCOVA, \( F_{1,48} = 14.05, P < 0.001 \)). Mussel length was not a significant covariate (ANCOVA, \( F_{1,48} = 0.15, P = 0.698 \)).

Estimates of bitterling embryo oxygen consumption varied from < 1% total available oxygen to > 90% (Figs 5 & 6). Assuming a constant mussel filtration rate of 2 L h\(^{-1}\), oxygen consumption was greatest with older embryos at high densities (Fig. 5). With 257 embryos in a mussel (the greatest number of embryos found in a single mussel, C. Smith, personal observations) of 25 days old, 92% of available oxygen entering a mussel might be used by embryos. Assuming an even age distribution of embryos, oxygen consumption was predicted to be greatest at low filtration rates and high embryo abundance (Fig. 6).

*Fig. 5* Estimated percentage of available oxygen used by bitterling embryos brooded on the gills of mussels of varying age (days) and abundance; closed square one embryo, open circle 10 embryos, closed triangle 50 embryos, open square 100 embryos, closed circle 200 embryos, open triangle 257 embryos. 257 embryos are the maximum numbers found in a mussel (C. Smith, personal observations). Estimates assume an ambient oxygen concentration of 10 mg O\(_2\) L\(^{-1}\) and a mussel filtration rate of 2 L min\(^{-1}\).

*Fig. 6* Estimated percentage of available oxygen used by bitterling embryos brooded on the gills of mussels varying in filtration rate (L min\(^{-1}\)) and abundance; closed square one embryo, open circle 10 embryos, closed triangle 50 embryos, open square 100 embryos, closed circle 200 embryos, open triangle 257 embryos. Estimates assume an ambient oxygen concentration of 10 mg O\(_2\) L\(^{-1}\) and an even age distribution of embryos.
Discussion

Female bitterling always inspected mussels before spawning, but inspection was not always a prelude to spawning. We detected no difference in the inspection rate of mussels in which a female subsequently spawned in and those that were not used. During siphon inspection the female may obtain information about mussel quality. Smith et al. (2000a) proposed two proximate cues that bitterling may use to choose a mussel for oviposition site choice. These were the flow rate and/or the oxygen content of water emerging from the exhalant siphon of a mussel. Of these two cues, the change in oxygen concentration between inhalant and exhalant siphons most closely matched bitterling spawning choices. Bitterling avoid spawning in A. cygnea (Smith et al., 2000a), and this species was shown to significantly reduce the oxygen content of its inhalant current in comparison with the other three mussel species in both field and laboratory studies. Bitterling also avoid mussels containing high numbers of embryos (Smith et al., 2000a), and embryo number significantly covaried with oxygen removal in mussels. Thus, we provide circumstantial evidence for oxygen being the cue that is used by female bitterling for oviposition site choice. Both mussel species and fullness with embryos are significant determinants of the oxygen content of the exhalant current of a mussel, and the spawning frequency of female bitterling is negatively associated with mussels with low oxygen levels in their exhalant currents (i.e. in A. cygnea and mussels with a high embryo density).

Bitterling may be sensitive to dissolved oxygen concentration. Alternatively, or in addition, they may be sensitive to the products of respiration, such as CO₂, or changes in water chemistry resulting from respiration, such as decreased pH, which correlate with oxygen availability in mussels. In either case, siphon inspection may provide information on oxygen conditions in the mussel that a female is investigating.

While terrestrial vertebrates generally possess chemoreceptors for breathing that are sensitive to CO₂ and pH, aquatic organisms typically possess oxygen receptors (Milsom, 1998). In teleost fishes, sites for oxygen chemoreception have not been determined (Butler & Metcalfe, 1983), although the pseudobranch has increasingly been implicated in serving this function (Laurent & Dunel-Erb, 1984; Bridges et al., 1998; Hedrick & Jones, 1999). In teleosts the pseudo-branch is located in the cranial part of the subpercular cavity and is exposed to water that enters the opercular cavity through the mouth of the fish. Mussel inspection behavior in the bitterling would suffuse the pseudobranch with water emerging from the mussel, and oxygen concentration in the exhalant flow of the mussel might be detected by the pseudo-branch. Territorial male bitterling also inspect the siphons of mussels in their territory and may also use information from mussel inspection in decisions about which mussel to lead females to that enter their territory to spawn.

Bitterling embryos are dependent on their host to provide a supply of oxygenated water. Embryos possess particular adaptations for low oxygen conditions, including an extensive fin fold circulation, the ethanol pathway for glycolysis and the absence of a chorion; features not shown by embryonic stages of other cyprinids (Suzuki & Hibiya, 1984; Dmitrijeva et al., 1985; van Waarde, van den Thillart & Verhagen, 1993; Aldridge, 1999). Aldridge (1997) showed that the survival and development of bitterling embryos is compromised under low oxygen conditions. Thus, oxygen may be limiting in mussels and embryos may compete with conspecifics for oxygen on the mussel gill. Under these conditions females could maximize embryo survival and rate of development by spawning on mussels with a high oxygen content. As a cue for spawning host choice, the oxygen content of the exhalant current and/or the products of respiration may provide a reliable indication of oxygen levels in a mussel.

Our estimates of oxygen consumption (Figs 5 & 6) show that at high embryo densities, among older embryos, and in mussels that filter low water volumes, competition for oxygen may increase. However, these estimates are based on the embryonic oxygen consumption of an unrelated fish species, and measurements of bitterling embryonic oxygen consumption rates will be needed to clarify these predictions. Competition among embryos for oxygen may lead to mortalities at high embryo densities, and could account for the significant density-dependent mortality among embryos shown by Smith et al. (2000a) and the significantly higher density-dependent mortality in A. cygnea. Embryos may also compete with their mussel host for oxygen and compromise mussel growth, survival or glochidial development.
The more efficient removal of oxygen by *A. cygnea* than the three other species tested may be associated with adaptations of this species to life in anoxic conditions (Zs.-Nagy, Holwerda & Zandee, 1982). A consequence of the greater efficiency of oxygen removal by *A. cygnea* is that it is less heavily used as a spawning host by bitterling.

Larval glochidia had no significant effect on the change in oxygen concentration between inhalant and exhalant siphon. In unionids, the glochidia are brooded in the interlamellar spaces of the outer gill demibranchs and are nourished with leucocytes from parental blood (Ellis, 1978; Dillon, 2000). Tankersley & Dimock (1993) found a significant reduction in the oxygen consumption of mussels brooding glochidia of the unionid bivalve *Pyganodon cataracta*. They attributed this result to a loss in oxygen extraction efficiency in gravid mussels through distortion of the gills. Our results showed a reverse, although non-significant trend. Thus, gravid mussels may face two effects; increased oxygen demand by glochidia, counterbalanced by reduced gill efficiency.

We found that skimming behaviour was significantly negatively correlated with spawning rate. After mussel siphon inspection females may use skimming as a further test of mussel quality, in a similar way to parasitoids that insert their ovipositors into hosts but without releasing eggs (Godfray, 1994). Skimming behaviour may provide extra information to a female about mussel quality, although a mechanism is not apparent.

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