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REVIEW

The reproductive ecology of the European bitterling (*Rhodeus sericeus*)

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Abstract

The European bitterling *Rhodeus sericeus* Pallas is a freshwater fish belonging to the Acheilognathinae, a subfamily that has an unusual spawning symbiosis with freshwater mussels. Female bitterling possess long ovipositors that they use to place their eggs onto the gills of a mussel through the mussels' exhalant siphon. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel. The embryos develop inside the mussel for approximately a month, eventually leaving the mussel as actively swimming larvae. Because they use a discrete spawning site that can be readily manipulated they have recently been used in studies to understand oviposition choice with respect to mate and spawning site quality, host–parasite coevolution, alternative male mating tactics, and linking behavioural decisions with population dynamics. The current state of knowledge of bitterling reproductive ecology is reviewed and synthesized, and future directions for research on this species are proposed.

Key words: Acheilognathinae, intersexual conflict, mate choice, sperm competition, symbiosis, *Rhodeus sericeus*

INTRODUCTION

The European bitterling *Rhodeus sericeus* Pallas (Acheilognathinae, Cyprinidae) is a freshwater fish with an unusual spawning symbiosis with freshwater mussels. During the spawning season, males develop bright nuptial coloration and defend territories around mussels. Female bitterlings develop long ovipositors that they use to place their eggs onto the gills of a mussel through the mussel's exhalant siphon. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Developing embryos reside inside the mussel for *c.* 1 month during which time they develop into actively swimming larvae. Bitterlings display remarkable morphological, physiological and behavioural adaptations for using mussels as spawning sites and they represent a valuable model in behavioural, population and evolutionary ecology. The value of the bitterling arises from it having a spawning site that can be easily manipulated and assessed for quality. They can also be observed under natural conditions and they adapt readily to laboratory conditions.

Scientific interest in the bitterling over the past 50 years has been sporadic. Wiepkema (1961) provided a detailed description of their reproductive behaviours. Ovarian development in European and the related rose bitterling *Rhodeus ocellatus* (Kner) has received attention because of the former use of bitterlings as test animals to assay mammalian reproductive hormones (e.g. Duyvené de Wit, 1940; Asahina, Iwashita *et al.*, 1980; Asahina, Hanyu *et al.*, 1981; Asahina, Nishina *et al.*, 1981; Honda, 1982). Basic population parameters for European bitterling have been collected in several ecological studies, notably by Holčík (e.g. Holčík, 1958, 1959, 1960). Recently there has been renewed scientific interest in the bitterling. They have been used in studies on host specialization (Aldridge, 1997; Reynolds *et al.*, 1997; Smith, Rippon *et al.*, 2001; Mills & Reynolds, 2002), mate and oviposition choice (Smith, Reynolds, Sutherland *et al.*, 2000; Candolin & Reynolds, 2001; Smith, Douglas *et al.*, 2002), alternative mating tactics (Kanoh, 1996, 2000; Candolin & Reynolds, 2002*a*,*b*; Smith, Douglas *et al.*, 2002; Smith, Reichard & Jurajda, 2003), and in linking behavioural decisions with population dynamics (Smith, Reynolds & Sutherland, 2000; Douglas, 2003). Our aim in this review is to synthesize the disparate literature on the European bitterling (hereafter referred to simply as bitterling unless otherwise qualified) to provide a summary of what is known about the reproductive ecology of the species and to identify gaps in our knowledge for future research.

Although using a living invertebrate as a spawning substrate is unusual, it is not unique to bitterling, and at

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least four other groups of fishes have comparable, though less well understood, modes of reproduction. Liparid snailfish in the genus *Careproctus* possess an anteriorly positioned ovipositor that enables them to deposit their eggs in the branchial chambers of lithodid crabs (Melville-Smith & Louw, 1987). The benefits to snailfish seem to be that their embryos and larvae obtain constant aeration and protection from predators. The presence of liparid embryos seems to damage the crab's gills, which are compressed resulting in necrosis of gill tissue (Somerton & Donaldson, 1998). The tubesnout *Aulichthys japonicus* (Brevoort) (Aulorhynchidae) spawns its eggs in the peribranchial cavity of the ascidian *Halocynthia roretzi* (Drasche) (Uchida, 1934). A species of *Barbus* in Lake Tanganyika is also reported to spawn on the gills of a bivalve mollusc, probably *Pseudospatha tanganyicensis* (Smith) (Vanderplank, 1941). Finally, cyprinids of the genus *Sarcocheilichthys* spawn in freshwater mussels (Breder & Rosen, 1966).

Previous reviews of bitterling biology include a summary by Breder & Rosen (1966) in their monograph on fish reproductive modes. This review predates all recent research on bitterling reproductive ecology. In 1995 and 1999, Holčík reviewed the biology of bitterling, although a detailed treatment of the behavioural aspects of bitterling biology was not provided.

Scope of the review

Our review begins with a brief summary of the current taxonomic status of the bitterling, its distribution and a description of its external appearance and the anatomy that is relevant to its reproductive ecology. The mussels that act as reproductive hosts, which species are used by bitterlings, and the nature of the symbiotic relationship between mussel and bitterlings are then considered. The reproductive ecology of females and males is then examined. First, the factors affecting egg production and the mechanism of spawning are briefly reviewed. Female oviposition and mate choice is examined and their consequences for individual fitness and population dynamics discussed. In reviewing reproduction in the male, territoriality and spawning behaviour, male oviposition choice and alternative mating tactics and sperm competition are addressed. The section ends by considering intersexual conflict in oviposition choice in bitterlings. Embryo development is then reviewed and the possibility of sibling rivalry among bitterling embryos addressed. Finally, areas for future research with bitterlings that have been highlighted in this review are discussed.

Taxonomy and distribution

European bitterlings are small cyprinid fish belonging to the subfamily Acheilognathinae, a group including 40 (Arai, 1988) or 60 (Nelson, 1994) species. All these fishes, except *Rhodeus sericeus*, are restricted to Asia and share the same characteristic reproductive style (Nelson, 1994), though the phylogenetic relationships of this group are still unclear (Okazaki *et al.*, 2001). The classification of the European bitterling has been problematic, because of its discontinuous distribution across its range. In the west of its distribution, it is found in Europe and Asia Minor. In the east, bitterling are reported from the River Amur system, Sakhalin Island and rivers emptying into Peter the Great Bay and Sea of Japan (Holčík, 1999). Western populations are sometimes considered a separate species, *R. amarus* Bloch, distinct from the eastern *R. sericeus*. The western species was later reduced to a subspecies of the eastern, with the designation *R. sericeus amarus* (Bloch) (Svetovidov & Eremeev, 1935). However, Holčík & Jedlička (1994) demonstrated that the characters used to separate the eastern and western species/subspecies were size and temperature dependent and could not be reliably used to separate the two, reverting to the designation *R. sericeus* for both the eastern and western populations. In a recent review of the taxonomy of European freshwater fish, Kottelat (1997) re-classified the western bitterling as *R. amarus*, but without clear justification. For the purpose of this review, Holčík & Jedlička's (1994) designation of the European bitterling as *R. sericeus* is adopted. Bogutskaya & Komlev (2001) recently described a new species of bitterling, *R. colchicus*, from west Transcaucasia, though the precise taxonomic status of this fish, and its relationship with *R. sericeus*, is not yet clear.

The habitat of the bitterling is intimately tied to the distribution of freshwater unionid mussels. Typical habitats are sluggish river backwaters, oxbows, lakes, ponds and irrigation canals (Holčík, 1995, 1999). However, bitterling are also found in faster flowing rivers (Przybylski & Ziêba, 2000; Reichard, Jurajda, Šimková *et al.*, 2002), and larval and early juvenile bitterling can constitute the major component of fishes drifting in rivers in the Danube basin (Reichard, Jurajda & Ondračková, 2002).

Morphology and anatomy

Bitterling are relatively small fish, rarely exceeding 70 mm standard length (from the tip of the snout to the origin of the caudal fin). They are relatively deep-bodied, with a body depth 29–45% of their standard length (Holčík, 1995; Reichard, 1998). At the start of the spawning season, the female develops an ovipositor, a long, tube-shaped structure inserted into a conical, muscular organ in the abdomen wall. The conical organ is usually retracted, but is extended during spawning. At its base, the ovipositor opens into the oviduct and bladder. The length of the ovipositor is highly variable, and at its longest shortly before or after spawning when it may exceed the body length of the female. Outside the spawning season females can be readily distinguished from males by a much reduced, but obvious, ovipositor.

During the spawning season, bitterlings display a marked sexual dimorphism in their coloration. Sexually mature males develop a pink flush on their ventral and lateral sides, becoming almost red near the pectoral fins. The lateral stripe is vivid green, while the dorsal sides become dark violet. The dorsal fin is almost black, with a red triangle at its tip, and the anal fin is red with a dark border. Small, white, conical epithelial tubercles, the so-called pearl organs, typical of sexually mature cyprinids, develop around the nostrils and above the eyes (Witkowski & Rogowska, 1991). The dorsal section of the iris is red in sexually mature males and remains pigmented throughout life after sexual maturity. In contrast to the bright colours of the male, the colour of the females remains unchanged during the spawning season: grey-green dorsally, silvery laterally and yellowish ventrally, and without red pigment around the iris.

THE MUSSEL HOST

Mussel species used for oviposition

Bitterlings spawn primarily on the gills of living freshwater mussels of the family Unionidae, but they have also been found using the Margaritiferidae (Zhul'kov & Nikiforov, 1988; D. G. Smith & Hartel, 1999). Within the unionids, bitterlings use several species across its range. In the west of its distribution, bitterling use *Anodonta anatina* (L.), *A. cygnea* (L.), *Unio pictorum* (L.), *U. tumidus* Philipsson (Wiepkema, 1961; Balon, 1962; Aldridge, 1997; Smith, Reynolds, Sutherland *et al.*, 2000), and *Pseudanodonta complanata* (Rossmässler) (C. Smith, pers. obs.). Stadnichenko & Stadnichenko (1980) report bitterling embryos in *U. rostratus* Haas. Balon (1962) found developing embryos in *U. pictorum* at three sites in the River Danube system, but failed to find them in *A. cygnea* or *Dreissena polymorpha* (Pallas). Kryzhanovskii (1949) and Kryzhanovskii *et al.* (1951) report bitterling embryos in *Unio* sp. Wiepkema (1961) conducted experiments using *Unio* sp. only, though he reports that bitterlings showed an interest in *Anodonta* sp. In the East of its distribution, Holčík (1999) reports bitterlings using *Anodonta* sp., *Unio* sp. and *Cristaria plicata* Leitch. Fish of the genus *Rhodeus* are also reported to have been found in the margaritiferid mussels *Margaritifera sachalinensis* Shadin (Zhul'kov & Nikiforov, 1988), and *Dahurinaia dahurica* (Middendorff) (D. G. Smith & Hartel, 1999). The morphology of the branchial lamella of margaritiferid mussels lack continuous vertical septa, and the group are considered primitive in the naiad mussel group (Dillon, 2000). The implications for the development of bitterling embryos in the different gill environments of unionid and margaritiferid mussels are not clear, though D. G. Smith & Hartel's (1999) finding suggests a lack of specificity in the range of mussel hosts that can be used by bitterlings (see Female oviposition choice).

Where bitterlings have been naturalized, they use native unionid mussel species. In North America bitterling embryos have been found in *Anodonta cataracta* Say and *Elliptio complanata* (Lightfoot) (Bade, 1926; Breder, 1933). Under laboratory conditions, bitterlings spawned readily in two South African mussel species, *Aspatharia wahlbergi* Krauss and *U. caffer* Krauss, with healthy larvae emerging from these mussels (Holčík & Duyvené de Wit, 1962).

Breder (1933) believed that bitterlings were capable of using any available species of freshwater unionid mussel for spawning. Though bitterlings will readily use mussel species with which they have no experience or evolutionary history, there is evidence to show that they are choosy about which mussels they will use (see Female/male oviposition choice).

Relationship between mussel and bitterling

Bitterlings and mussels are symbionts in that there exists a long-term intimate relationship between the two (Paracer & Ahmadjian, 2000). However, the form of the symbiosis, whether mutualistic, commensal or parasitic, is not evident. The nature of the relationship depends on the effect of bitterlings on mussels and the effect of mussels on bitterlings, and thus is ultimately dependent on the costs and benefits to each.

Unionid mussels have larvae known as glochidia that are obligate ectoparasites on fish (Dillon, 2000). Glochidia are brooded in the interlamellar spaces of the outer demibranch of the mussel gill and are released into the water column. They are composed of a tiny (100–450 μm) hinged valve that snaps shut on contact with a fish, typically attaching to the gills (*Unio* spp.) and fins (*Anodonta* and *Pseudanodonta* spp.). Once attached, the glochidia are encysted by host tissue, remaining attached for up to several months and obtaining nourishment from their host. Attachment to fishes is obligatory for glochidial development in most species and is also thought to serve in dispersal. High levels of infection by glochidia may be lethal to fish (Myers & Millemann, 1977). The relationship between bitterling and mussel has popularly been considered mutualistic on the premise that bitterlings use mussels as spawning sites while the mussels benefit by using bitterlings to disperse their glochidia (e.g. Wheeler, 1978). However, evidence for a mutualistic symbiosis is weak.

In a laboratory study, Holčík & Lohniský (1958) noted that glochidia readily attached to *Poecilia reticulata* Peters, *Xiphophorus helleri* L. and *Tanichthys albonubes* Lin, but not to bitterlings. Similarly, a laboratory study by Aldridge (1997) measured the intensity of infection and the duration of attachment by glochidia to bitterlings compared with *Rutilus rutilus* (L.), *Scardinius erythropthalmus* (L.), *Perca fluviatilis* L. and *Gasterosteus aculeatus* L. Glochidia of *Anodonta anatina*, but not *A. cygnea*, attached to bitterlings, but were all lost after 5 days. This contrasted with much higher intensities of infection by glochidia of both *A. anatina* and *A. cygnea* on the other fish species in the trial (650 *A. anatina* glochidia on a *P. fluviatilis*) and for periods of > 50 days.

Blažek (2000) conducted an extensive field study on the prevalence of glochidia on fishes in each month for 2 years in 16 small lakes in the Czech Republic. He screened a total of 657 *Rhodeus sericeus*, 763 *Rutilus rutilus*, and 692 *Perca fluviatilis* of which seven, 91 and 271 were infected with glochidia, respectively. The maximum intensity of infection of bitterlings by glochidia was two *Unio* sp. glochidia on one fish. In contrast, in *R. rutilus* the maximum intensity of infection was 142 *Unio* sp., and for *P. fluviatilis* it was 1244 *Anodonta* sp. Similar studies of parasite prevalence in fishes by Bychovskij (1962) and Ergens *et al.* (1975) have shown that bitterlings infected with glochidia are rare. Fukuhara *et al.* (1986) found that the intensity and prevalence of infection of *Rhodeus ocellatus* by *Anodonta woodiana* (Lea) glochidia was consistently lower than for other fish species that they surveyed over a 12-month period in a Japanese pond. Thus, the evidence suggests that bitterlings either avoid glochidial infection or lose glochidia shortly after their attachment. There is evidence that fish can develop an immunological response to glochidia that protects them against infection (Bauer, 2001; Jansen, Bauer & Zahner-Meike, 2001). Although there is no direct evidence for such immunological protection for bitterlings, their close association with mussels would make such a response adaptive, and it is a possible explanation for the low prevalence and intensity of infection of bitterlings by glochidia. The marked failure of bitterlings to host glochidia points away from a mutualistic symbiosis with mussels.

Given that there are so few studies on the effect of bitterling embryos on mussels, it is difficult to separate a commensal symbiosis, in which bitterlings benefit and mussels suffer no reduction in fitness, from a parasitic one, in which bitterlings gain and mussels lose. However, two studies provide circumstantial evidence for a cost to the mussel, suggesting a parasitic, rather than commensal, relationship. Stadnichenko & Stadnichenko (1980) conducted a histological and histochemical analysis of the effect of bitterling embryos on *Unio rostratus*. Bitterling embryos seemed to inhibit free water circulation through the gills of a mussel, which could have a negative impact on normal filtration by the gills, and therefore on the feeding and respiration of the mussel. Bitterling embryos, which were wedged in the gill interlamellar spaces (see Embryonic development), compressed and sometimes damaged the ciliated epithelium in the gills, again with a possible detrimental effect on gill function. Glycogen and RNA levels in the gill epithelium in the proximity of embryos were lower than at sites without embryos, a possible consequence of damage by the embryo. Stadnichenko & Stadnichenko (1980) concluded that bitterlings have a measurable adverse impact on mussels. A second study that hints at a cost for mussels is that of Smith, Rippon *et al.* (2001), who measured oxygen consumption of mussels under field and laboratory conditions. Bitterling embryos in mussels significantly increased the oxygen consumption of a mussel, suggesting that mussel and embryos may be in competition for oxygen.

The nature of the bitterling–mussel symbiosis remains equivocal, though we have found no evidence to support a mutualistic relationship. While the lack of clear evidence of a fitness cost to mussels suggests a commensal relationship, this reflects a lack of appropriate studies. There is some evidence that the bitterlings may parasitize mussels. Further research is needed to clarify the nature of the symbiosis, and particularly to investigate the extent to which mussels suffer gill damage from bitterling embryos and compete with them for oxygen.

THE REPRODUCTIVE ECOLOGY OF THE FEMALE

Ovarian development and oviposition

Bitterlings spawn between April and August in the west of their distribution, with a peak in spawning occurring in May (Papadopol, 1960; Holčík, 1995, 1999; Douglas, 2003). Spawning occurs over a similar period in the Amur system, in the east of their distribution (Zhul'kov & Nikiforov, 1988). The environmental cues that trigger gonad maturation are rising temperature and increasing photoperiod in *Rhodeus ocellatus* (Nishi & Takano, 1979; Asahina & Hanyu, 1983), and probably also in *R. sericeus*. They are batch spawners with determinate fecundity; breeding season fecundity is fixed at the onset of the spawning season, with egg number in the ovary declining over the spawning season (Smith, Reynolds & Sutherland, 2000). Sexual maturity is reached by both sexes in 1 year, when the fish are *c.* 30–35 mm standard length (Reichard & Jurajda, 1999). Holčík (1958) reports an exceptional case of bitterling achieving sexual maturity in < 1 year, spawning at a standard length of 25 mm.

In a study of the population dynamics of bitterlings in a series of oxbow lakes in the Czech Republic, the spawning population was predominantly (94%) composed of 1-year-old fish, with small numbers of 2-year-olds (Smith, Reynolds & Sutherland, 2000). The sex ratio in the same populations was always 1:1 for both age-classes (Smith, Reynolds & Sutherland, 2000). Breeding season fecundity is size dependent and reported to vary from 40 eggs for a naturalized population in eastern England (Aldridge, 1997) to 480 eggs (Zhul'kov & Nikiforov, 1988) for a population in the River Tym' on Sakhalin Island. Smith, Reynolds & Sutherland (2000) dissected the ovaries of 77 females before spawning and made direct counts of yolked oocytes. The relationship between female size and fecundity took the form: $F = 0.07.L^{2.165}$, where $F =$ fecundity and $L =$ fish standard length in mm. A typical breeding season fecundity is probably between 80 and 250 eggs (Abdurakhmanov, 1962; Schaumburg, 1989; Holčík, 1995, 1999; Aldridge, 1997; Smith, Reynolds & Sutherland, 2000). Ovipositor length varies cyclically throughout the spawning season, the ovipositor shortening during inter-spawning intervals, then lengthening as spawning approaches. Bouts of spawning last 1–3 days, with the interval between bouts usually 5–7 days (C. Smith, pers. obs.), though this period may be variable depending on feeding conditions and temperature. Once

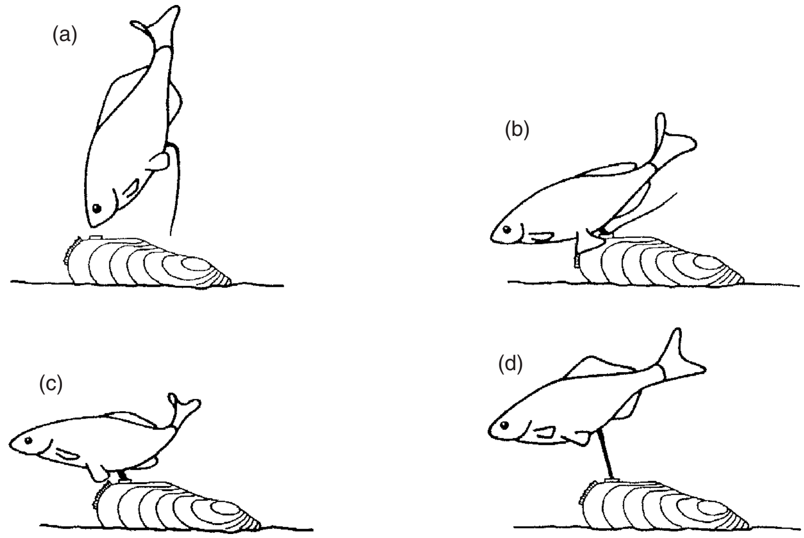

Fig. 1. Spawning of female bitterling *Rhodeus sericeus* in the exhalant siphon of a freshwater mussel: (a) head-down or mussel inspection posture; (b) insertion of the conical organ; (c) insertion of the ovipositor; (d) removal of the ovipositor. Modified from Keenleyside (1979).

a batch of eggs has ovulated, females are capable of spawning on up to at least five occasions on the same day (C. Smith, pers. obs.).

Duyvené de Wit (1955) and Wiepkema (1961) describe the sequence of events involved in oviposition. The role of urine in oviposition was proposed by Bretschneider & Duyvené de Wit (1947) and confirmed by Matsubara (1994) in *Rhodeus ocellatus*. In females that are ready to spawn, the ovipositor is extended and appears flaccid. To oviposit, the female orientates herself at an angle of $\sim 75^\circ$ from horizontal so that the tip of her snout is close (1–5 mm) to the exhalant siphon of a mussel, the *siphon inspection* or *head-down posture* (Fig. 1a). The muscular conical organ at the base of the ovipositor (see Morphology and anatomy) is extended and a batch of 1–6 ovulated eggs is positioned at the basal opening of the ovipositor, behind which urine collects. After holding position for several seconds, the female quickly sweeps forward and down and pushes the conical organ into the exhalant siphon of the mussel (Fig. 1b). The conical organ contracts and the urine, under pressure, forces the eggs quickly through the entire length of the ovipositor. As the eggs pass down it, the ovipositor stiffens and unfurls into the exhalant siphon and deep into the gill cavity of the mussel (Fig. 1c). The eggs are ejected and the female rises quickly, withdrawing the flaccid ovipositor (Fig. 1d). Oviposition is rapid and completed in < 1 s. The eggs are relatively large at 2.4–3.1 mm along their longest axis, pale yellow and appear yolky. After spawning they have a pronounced oval shape and are dense, sinking quickly in freshwater.

As a prelude to spawning, females with extended ovipositors sometimes engage in a behaviour in which they perform a spawning action, but without inserting their ovipositor into the mussel. This behaviour, which typically entails the female making contact with the mussel siphon with the base of their ovipositor, was termed *touching* by Wiepkema (1961) and *skimming* by Smith, Rippon *et al.* (2001). The function of this behaviour, if any, is not evident. It may simply be an attempt at spawning in which the female misses the exhalant siphon with the conical organ. Some studies have used this behaviour, rather than spawning itself, as an index of mussel choice by bitterlings (e.g. Heschl, 1989; Candolin & Reynolds, 2001). However, Smith, Rippon *et al.* (2001) found a significant negative correlation between skimming over a mussel by a female and the probability of the female subsequently spawning in the mussel, proposing that the behaviour serves in assessment of mussel quality. Skimming behaviour is frequently performed in aquaria and under natural conditions by females with partially retracted ovipositors that are not ready to spawn (Wiepkema, 1961; C. Smith, pers. obs.). No studies have yet addressed the question of whether the ovipositor has tactile or chemosensory receptors for sampling the internal environment of a mussel.

Holčík (1959) reported that bitterling do not necessarily require mussels for reproduction under natural conditions. This finding was based on a field study of a population of bitterlings in an oxbow lake in the Elbe Basin of the Czech Republic. In our experience, female bitterlings do not release eggs in the absence of mussels. Wiepkema (1961) reports bitterlings releasing eggs in the absence of a male, and on one occasion in the absence of mussels. Further, Duyvené de Wit (1955) induced females to release eggs by manipulating their behaviour using dummy mussels (see Female oviposition choice). However, description of some crucial details of Holčík's (1959) study are lacking. Consequently, we believe that unless other studies can demonstrate the same finding, Holčík's (1959) proposal that bitterlings are able to reproduce successfully without mussels under natural conditions should be disregarded.

Female oviposition choice

Oviposition begins with a female bitterlings recognizing a mussel as a potential spawning site. Duyvené de Wit (1955) stimulated bitterlings to spawn by setting up a glass tube through which a current of water was passed between the valves of an empty mussel shell. The same result has been reported by Boeseman *et al.* (1938), though Bresse (1954) was able to elicit a greater response, particularly from females with less spawning experience, if the water current contained the smell of mussels. A systematic study of the cues used by female and male bitterlings to recognize mussels as spawning sites was conducted by Heschl (1989). He showed that there is a hierarchy of responses to cues, and these were additive. The weakest response, measured as siphon inspection and skimming rate, was to visual cues of mussel siphons, the greatest response was to the smell or taste of a living mussel, and an intermediate response was to a current of water. Other factors that affected the response of bitterlings were the direction of flow and the shape of the flow field. Vertical flow with an oval flow field elicited the strongest response. Mussel shape was important only in that an object with a horizontal edge was favoured.

After recognizing a mussel as a potential spawning site, the female may exert some choice over whether or not to spawn in the mussel. Wiepkema (1961) believed that female bitterlings showed no choice for the mussel species they used for spawning. However, later studies provide evidence that occupancy of mussels by bitterling embryos varies with mussel species. After dissecting the gills of mussels, Balon (1962) concluded that bitterlings show a preference for *Unio* spp. over *Anodonta* spp. Reynolds *et al.* (1997) found that in mussels collected from a canal in eastern England, *U. pictorum* released significantly more larval bitterling than *A. cygnea*. Aldridge (1997) dissected the gills of 161 mussels from the same population and found most developing bitterling embryos in *U. pictorum* and none in *A. cygnea*. Thus, it seems that there are differences in the occupancy and release of bitterling embryos and larvae from mussels.

There are several possible explanations for this apparent differential use of mussels by bitterlings. First, bitterlings may not discriminate among mussels, but rather mussels may have evolved defences against bitterling embryos, with the result that mussels determine the number of embryos in their gills. Bitterling embryos are sometimes ejected from mussels (Balon, 1962; Kondo, Matsumura *et al.*, 1987; Aldridge, 1997; C. Smith, pers. obs.), and ejections can vary under certain environmental conditions (Reynolds & Guillaume, 1998). We term this the *ejection hypothesis*. Alternatively, choice may be a consequence of variation in interspecific host accessibility. Aldridge (1997) proposed that some mussels may be inaccessible for bitterlings to spawn in because of the orientation of the mussels in the substrate, closure of siphons when not feeding and respiring, or when disturbed. We term this the *accessibility hypothesis*. Choice of mussels may reflect the coevolutionary history of mussel and bitterlings. Reynolds *et al.* (1997) discuss an hypothesis that host specialization by bitterlings may have arisen from an evolutionary arms race with mussels. For mussel species with a long evolutionary association and/or sympatry with bitterlings, a stronger selection on bitterlings for host adaptation would be expected than for mussel species with low sympatric overlap and a short evolutionary association. Thus, the bitterling and mussel host symbiosis may mirror that of the cuckoo (*Cuculus canorus* L.) and its avian hosts. We term this the *coevolutionary history hypothesis*. This hypothesis infers reciprocal evolutionary responses by mussels and bitterling. Finally, choice may be related to some aspect of mussel quality as a host for bitterling embryos, which females can discriminate (Smith, Reynolds & Sutherland, 2000). Mussel quality may vary temporally, as well as with host species or physiological condition. We term this the *host quality hypothesis*.

A study by Smith, Reynolds, Sutherland *et al.* (2000) shows that the final hypothesis most adequately explains our current understanding of differential mussel use by bitterling. The study included field and laboratory experiments and investigated oviposition choice by female bitterling. As part of the study, four species of mussel (*Anodonta anatina*, *A. cygnea*, *Unio pictorum* and *U. tumidus*), each at two levels of fullness with embryos (high and low) were presented to bitterling in a shallow lake and observed by divers. The mussels in which females spawned were recorded, along with other variables, such as the orientation of mussels and the time they spent with their siphons open. The results showed that female bitterling avoided spawning in mussels that already contained high numbers of embryos and avoided one species, *A. cygnea*. A laboratory study further showed that these choices were adaptive; embryo mortality in mussels was strongly density dependent and the strength of density dependence was significantly higher in *A. cygnea*. The results of the experiment do not support the accessibility hypothesis. Mussel siphon angle and opening time did not vary with mussel species or fullness but there was still a clear choice of mussels by females. The ejection hypothesis can also be questioned based on a comparison of spawning choices and bitterling releases from mussels by Smith, Reynolds & Sutherland (2000). They placed 885 mussels of four species from nine populations in fine mesh bags over 24 h and counted the number of bitterling larvae released. They showed that *A. anatina* and *Unio pictorum* released most bitterling larvae and *A. cygnea* the fewest. *Unio tumidus* was intermediate between these species. Thus, releases of embryos from mussels matched the spawning choices of females, and losses of embryos from mussels need not be invoked to explain variation in occupancy of mussels by bitterling embryos.

In accordance with the coevolutionary hypothesis, bitterlings spawn readily and the embryos develop successfully in mussel species with which they have no sympatry or evolutionary history (e.g. Dence, 1925; Bade, 1926; Breder, 1933; Holčík & Duyvené de Wit, 1962; Schmidt, Samaritan & Pappantoniou, 1981). The study by Reynolds *et al.* (1997), however, provides evidence against the coevolutionary hypothesis. The study showed differences in mussel use by bitterlings from a population naturalized in eastern England, at the earliest in the mid-1970s (Aldridge, 1997). This population of bitterlings uses native mussels that have no association with bitterlings, and the mussels have had no opportunity to evolve responses to bitterlings. Females from this population, however, make almost identical oviposition choices to those using mussels with a long association with bitterlings, whereas the coevolutionary hypothesis would predict that all bitterling-naïve mussels would be used equally. Another failure of the coevolutionary hypothesis as an explanation for mussel choice by bitterlings is that it does not account for the strong effect of choice with respect to fullness with embryos.

Smith, Rippon *et al.* (2001) propose a possible mechanism for density-dependent mortality of embryos in mussels and the cue used by female bitterlings in host choice. They showed that in both field and laboratory studies, the change in oxygen concentration of water entering a mussel's inhalant siphon and leaving its exhalant siphon was significantly different among four mussel species, with *A. cygnea* exhibiting a significantly lower oxygen concentration in its exhalant siphon (6.8 mg l^{-1}, SE = 0.17) than three other species; *A. anatina* 7.8 mg l^{-1} (SE = 0.24), *U. pictorum* 7.6 mg l^{-1} (SE = 0.17), *U. tumidus* 7.5 mg l^{-1} (SE = 0.10). The presence of bitterling embryos on the gills of a mussel significantly increased the change in oxygen between inhalant and exhalant siphons, as did developing glochidia. They proposed that the oxygen content of water leaving the exhalant siphon is the proximate cue for oviposition choice by bitterlings, and that density-dependent mortality of bitterling embryos in mussels arises through competition for oxygen (see Sibling rivalry). Smith, Rippon *et al.* (2001) also measured the flow rate of water from the exhalant siphons from mussels, but found no correlation with female mussel choice.

Mills & Reynolds (2002) detected a positive correlation between mussel flow rate and female choice of mussels within species. However, they also found that mussel flow rates were highest for *A. cygnea*, the species of mussel actively avoided by bitterlings for spawning, bringing into question flow rate as a proximate cue for mussel choice. Unfortunately Mills & Reynolds (2002) did not measure the oxygen consumption of mussels in their study, and were unable to independently confirm Smith, Rippon *et al.*'s (2001) findings. However, Mills & Reynolds (2002) concurred with the earlier conclusion of Smith, Rippon *et al.* (2001), that oxygen conditions inside a mussel provide the most probable basis for female mussel choice.

The presence of developing glochidia in a mussel gill may affect bitterling spawning in several ways. The glochidia, which are brooded in the two outer gill demibranchs, consume oxygen and may adversely affect normal gill function, thereby reducing the quality of a mussel as a bitterling spawning site (Tankersley & Dimock, 1993; Smith, Rippon *et al.*, 2001). Also, the glochidia fill the outer gills of the mussel to the extent that they become distended, perhaps preventing the gill from receiving bitterling eggs, and it is rare to see bitterling embryos in the outer gills of mussels loaded with glochidia (C. Smith, pers. obs.). It is also possible that the likelihood of sperm reaching eggs through a mass of glochidia is reduced. The mussels commonly used by bitterlings for spawning are gravid with glochidia during different periods, at least in the west of their distribution. *Anodonta* spp. are gravid for most of the year, except May, June and July, and thus are free of glochidia during the bitterling spawning season (Schaumberg, 1989; Pekkarinen, 1993; Piechocki & Dyduch-Falniowska, 1993; Aldridge, 1999). In contrast, *Unio* spp. brood their glochidia from April to August, throughout the bitterling spawning season. Consequently, male *Unio* spp., which do not brood glochidia, should be the most suitable hosts for bitterlings, whereas both sexes and hermaphrodites of *Anodonta* spp. are predicted to be equally suitable. These predictions are supported by our own observations (C. Smith, pers. obs.).

The findings of Smith, Reynolds, Sutherland *et al.* (2000), Smith, Rippon *et al.* (2001) and Mills & Reynolds (2002) contradict the idea that host choice is tailored to particular mussel species, at least in *R. sericeus*. Bitterlings seem to display a broad host specificity for all unionid and margaritiferid mussels, irrespective of evolutionary associations at the species level. Host preferences within unionid and margaritiferid mussels seem to be related to host quality, measured in relation to oxygen conditions inside the mussel, again irrespective of evolutionary associations. This explanation accounts for variation in the choice of mussels with respect to species and fullness with bitterling embryos, the presence of developing glochidia in the mussel, and for the fact that bitterlings use mussels irrespective of their evolutionary history with the mussel population or species. Whether females base choice on a threshold oxygen concentration or in relation to the quality of recently encountered mussels is unclear, but could be readily investigated. It would also be valuable to test the host specificity and preferences of Asian bitterling species to determine the hypothesis of host choice to which they best conform.

Mussel size has not been shown to be a significant determinate of oviposition choice in any study. Among species, *A. cygnea* is the largest mussel used by bitterlings, with lengths of > 200 mm recorded (Kerney, 1999; C. Smith, pers. obs.), and this species is usually avoided. Within species, we have never detected an effect of mussel size on bitterling choice. Young, Clayton & Bernard (1990) conducted a study on risk-sensitive foraging in bitterlings and breeding site choice in which they varied breeding site quality by using mussels of different sizes, proposing that larger mussels represent better quality sites. They present no evidence, however, to support their proposal that larger mussels represent better quality spawning sites. Košík (2001) detected a weak negative correlation between mussel size and the number of embryos in *A. cygnea*. The lack of a strong effect of mussel size on choice and density-dependent mortality (Smith, Reynolds, Sutherland *et al.*, 2000) may arise because the positioning of eggs on the gills of a mussel is dependent on the length of the female's ovipositor. In both large and small mussels the embryos tend to be aggregated in the region of the gill nearest the exhalant siphon, within the reach of the ovipositor, and density-dependent effects may be localized within the gill. The largest mussel seen used by a bitterling was a 215-mm *A. cygnea*, and the smallest a 26-mm *A. anatina* (C. Smith, pers. obs).

Oviposition choice by female bitterlings may have consequences for bitterling population dynamics. Smith, Reynolds & Sutherland (2000) conducted a behavioural and demographic study of bitterlings and used a population model incorporating game theory decisions and measurements of demographic parameters to provide predictions of population size among 13 populations. The model predicted that female oviposition decisions, while maximizing individual fitness, caused a significant reduction in population size compared with randomly distributed spawnings. This effect arose because a random distribution of eggs among mussels would lead to enhanced survival of embryos in those mussels receiving few spawnings, ultimately resulting in better recruitment and a larger population size. This result accords with the paradigm that behaviours of individuals are selected to maximize individual lifetime reproductive success, not the long-term interests of the population, and illustrates the link, and potential importance, of behaviour for population studies.

No studies have properly addressed clutch size in bitterlings. Females may exert some control over the number of eggs they release at spawning, which may vary with mussel or mate quality, though this has yet to be tested.

Female mate choice

Sexual selection may operate by either direct selection on preference genes or indirect genetic benefits. In many species both direct and indirect selection may operate, and untangling the effects of each is problematic. Bitterlings are ideal for identifying the relative importance of direct and indirect mate choice benefits, because male quality (an indirect benefit) can be manipulated independently of oviposition site quality (a direct benefit). Male quality is thought to vary with male size and with extent and intensity of coloration (Schaumburg, 1989; Smith, Douglas *et al.*, 2002). Oviposition site quality varies with the number of embryos already on the gills of mussels, the presence of glochidia, and with mussel species (Smith, Reynolds & Sutherland, 2000; Smith, Rippon *et al.*, 2001).

Two studies have addressed the relative importance of direct and indirect mate choice benefits using bitterlings. In an aquarium study, Candolin & Reynolds (2001) showed that some male traits (intensity of red coloration of the eye and anal fin) correlated with the probability that a female would follow a territorial male to a mussel. Male quality did not, however, affect the decision to spawn with the male; rather, this was dependent on mussel quality measured as mussel fullness with embryos. In a field study by Smith, Douglas *et al.* (2002), females chose mussels for spawning on the basis of the number of embryos already present in the mussel and the body size and/or extent of red coloration of the eyes of the male guarding the mussel (male size and colour were correlated). There was a significant interaction between these direct and indirect benefits of oviposition choice. However, there was no effect of the number of mussels in the territory of a male on female mate choice. Vigour of male courtship was not different between high and low quality males, though low quality males suffered more interruptions to courtship by rivals. Thus, female bitterlings seem to spawn at disproportionately high rates with large and colourful males with high quality mussels, suggesting both direct and indirect benefits are important. Mussel quality ultimately determines oviposition choice, though inspection of mussels only occurs if the male guarding the mussel has intense and extensive coloration or is able to court the female without interruption from other males. In an aquarium study of a population of bitterlings recently naturalized in eastern England, Candolin & Reynolds (2002*a*) showed that the time until a female attempted to spawn decreased with male density. They proposed from these results that territorial males may 'tolerate' sneaking males, and may thereby obtain access to more females, a situation similar to that reported for the Azorean rock-pool blenny *Parablennius sanguinolentus parvicornis* (Oliveira *et al.*, 2002). Tolerance of a smaller, associated satellite male in Azorean rock-pool blennies increases the reproductive fitness of a territory holder, because the associated satellite male also helps to defend the territory from intruders (Oliveira *et al.*, 2002). However, we have never observed tolerance of rival males under natural conditions in 9 years of field studies on bitterlings (e.g. Schaumberg, 1989; Kanoh, 1996; Smith, Reynolds & Sutherland, 2000; Smith, Reynolds, Sutherland *et al.* 2000; Smith, Rippon *et al.*, 2001; Smith, Douglas *et al.*, 2002; Smith, Reichard *et al.*, 2003), and this observation may be an artefact of an aquarium study. Bitterling territories typically encompass 4–10 m^2, becoming unstable as territory size decreases to < 1 m^2 (Schaumberg, 1989). In the study by Candolin & Reynolds (2002*a*), territoriality was investigated in aquaria considerably smaller than the minimum territory size of bitterlings. Alternatively, the observed tolerance of rivals may have been a peculiarity of the recently naturalized population that they studied.

THE REPRODUCTIVE ECOLOGY OF THE MALE

Territoriality and spawning

During the spawning season, males guard territories around freshwater mussels. Males often defend only a single mussel, but will also guard several in their territory. Males defend a territory of between 4 and 10 m^2 (Schaumberg, 1989). Surprisingly few studies have been conducted on territoriality in bitterling, even though territory quality can be so readily manipulated in this species. In *R. ocellatus*, size seems to be the main determinant of the ability to hold a territory (Kanoh,

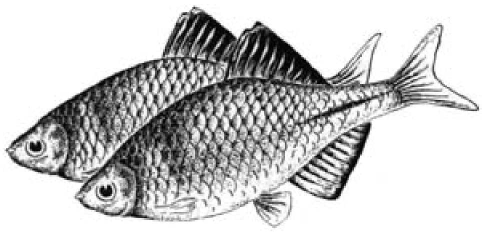

Fig. 2. Male bitterling *Rhodeus sericeus* engaging in parallel swimming. Modified from Wiepkema (1961).

2000), and this is also probably true for European bitterlings (see Alternative mating tactics and sperm competition). The agonistic and territorial behaviour shown by males is described by Duyvené de Wit (1955) and Wiepkema (1961), to which we add our own comments based on 9 years of field and laboratory observations. At the start of the spawning season, males begin to take an interest in mussels, inspecting the siphons of mussels, and they begin to show aggressive behaviour to other fish. Agonistic behaviour towards other males includes *finspreading*, in which the dorsal and anal fins are extended. Two males displaying to each other in this way may engage in *parallel swimming*, which involves the fish swimming alongside each other with their fins spread (Fig. 2). Males may attempt to push or strike an opponent using their body or caudal fin in so-called *jerking* behaviour. A male defending a mussel may also swim at other fish and strike them with its head in a behaviour termed *headbutting* (Fig. 3). Males may headbutt another fish by striking them on the flanks, in which case scales may be dislodged from the opponent. Alternatively, two males may engage in this behaviour head to head. While performing these behaviours, the colours of the male often intensify noticeably. Agonistic behaviour is directed at territorial and non-territorial males and non-spawning females. Aggression may also be directed at other species. We have often seen aggression between male bitterling and embryo-guarding male sunbleak *Leucaspius delineatus* (Heckel).

If a female with an extended ovipositor enters his territory, a male bitterling engages her in courtship behaviour. He will swim quickly towards the female and *quiver*, undulating his body at a high frequency and low amplitude. During quivering the male may turn side-on to the female, exposing his lateral side. The male then swims towards a mussel in his territory while continuing to quiver, termed *leading* behaviour (Fig. 4). If the female follows, the male will lead her directly to the mussel. If the female stops, the male will swim back to her and may quiver and attempt to lead her again. After successfully leading a female to a mussel, the male performs a behaviour comparable with skimming in females (see Ovarian development and spawning), in which he adopts a siphon inspection posture (Fig. 5a) before sweeping forward and down quickly over the inhalant siphon of the mussel, but without touching it (Fig. 5b). While moving forward the male releases sperm, occasionally visible as a greyish cloud. When this pre-oviposition ejaculation by the male is complete, the male positions himself close to the mussel and continues to quiver. If another fish approaches while a female is inspecting the siphon of a mussel, the male will attack the intruder aggressively, headbutting and charging the fish repeatedly.

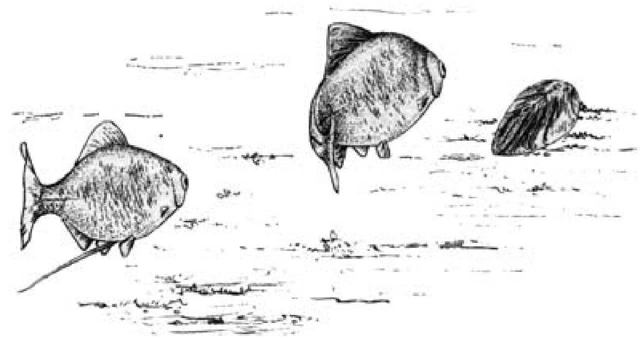

Fig. 4. Male bitterling *Rhodeus sericeus* leading a female to a mussel. Modified from Wiepkema (1961).

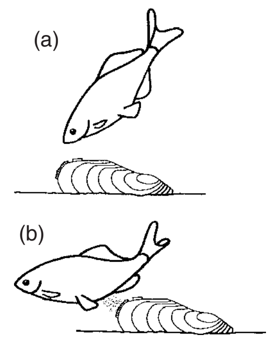

Fig. 5. Spawning of male bitterling *Rhodeus sericeus* in the inhalant siphon of a freshwater mussel: (a) head-down or mussel inspection posture; (b) sperm release. Modified from Keenleyside (1979).

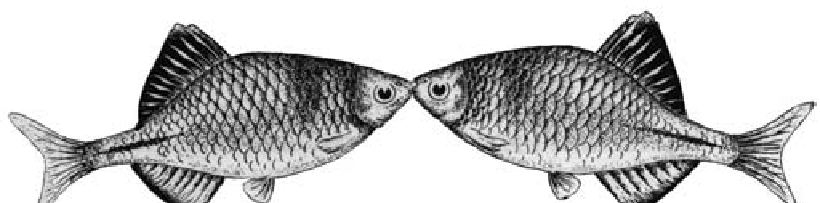

Fig. 3. Male bitterling *Rhodeus sericeus* engaging in headbutting behaviour. Modified from Wiepkema (1961).

If, after examining the exhalant siphon of a mussel, the female spawns, the male will immediately ejaculate into the inhalant siphon, sometimes repeatedly and particularly if another male also attempts to release sperm (see Alternative mating tactics and sperm competition). Spawning is completed when the male chases the female away from the mussel. After the female has released her eggs, the behaviour of the male changes abruptly. He becomes overtly aggressive and will not tolerate the presence of other fish near the mussel for about 30 s. Other males seem to recognize that the eggs have been released (Smith, Reichard *et al.*, 2003), and often chose this moment to try and 'sneak' fertilizations. Spawning by females is clearly distinguishable to human observers and likely to be equally clear to male bitterlings. Alternatively or additionally, males may perceive the odour or taste of the eggs, and/or the urine that propels them through the ovipositor.

Occasionally, as the female withdraws her ovipositor after spawning, eggs are ejected from the exhalant siphon of the mussel because they have not lodged in the gill properly. Both male and female will quickly eat eggs ejected by the mussel.

Male oviposition choice

Males are able to make oviposition choices, and may exert some control over female oviposition choice, by leading females to particular mussels. The extent to which males are able to discriminate among mussels has never been specifically investigated. However, Smith, Douglas *et al.* (2002) found that the leading behaviour of males largely corresponded with female choice of mussels for spawning. Males tended to avoid leading females to *A. cygnea* and to mussels with high numbers of embryos in them (Smith, Douglas *et al.*, 2002; Smith, Reichard *et al.*, 2003). Other factors, particularly the presence of other males, may affect male oviposition choice and this may result in male and female choice not always corresponding (see Intersexual conflict).

Alternative mating tactics and sperm competition

Reproductive behaviour can vary within members of the same sex and a variety of alternative mating strategies/tactics have been described (Birkhead & Møller, 1998). Alternative mating strategies/tactics have been observed in a range of species, though they seem to be particularly common in fishes (Gross, 1984). Gross (1996) identified two main forms of alternative mating strategies/tactic. The first is an *alternative strategy*, in which two or more strategies can be observed, but the individuals displaying them obtain the same fitness benefits through frequency-dependent selection. With an alternative strategy, male body form may vary markedly, associated with genetic polymorphism. The second is the *conditional strategy*, which is associated with genetic monomorphism, but in which male tactics are status or condition dependent (for example on the basis of size). The tactics may have unequal fitness benefits, though the tactic chosen by an individual provides it with the highest fitness benefit given its status. Gross & Repka (1998) have questioned the distinction between the different classes of alternative reproductive strategies/tactics, instead designating the different mating strategies/tactics seen among males all as different conditional tactics within the same reproductive strategy.

Alternative mating tactics have been considered as reproductive parasitism (Taborsky, 1994), and are often associated with sperm competition (Birkhead & Møller, 1998). Sperm competition is a term used to describe competition between the sperm of two or more males for the fertilization of an egg (Parker, 1970).

The 'typical' male reproductive tactic in bitterlings is to guard a territory containing mussels and to attract females to spawn in them (see The reproductive ecology of the male). However, a variety of alternative mating tactics that may be associated with sperm competition are found in bitterlings and are described by Kanoh (1996, 2000) for *Rhodeus ocellatus*. The same male mating tactics have been observed in *R. sericeus* (Smith, Douglas *et al.*, 2002; Smith, Reichard *et al.*, 2003). One tactic is to 'sneak' fertilizations. Sneaking involves males fertilizing the eggs of females that have been courted by rival males, thereby avoiding any costs of courtship. Sneaking by bitterlings can be accomplished by releasing sperm into the inhalant siphon of a mussel in which a female has recently spawned or will shortly spawn. Both territorial and non-territorial males intrude into the territories of males that have successfully courted a female and attempt to release sperm into the territory holders' mussel. Sperm competition between sneaking male bitterlings and the resident territorial male occurs within the gill chamber of the mussel. Territorial and non-territorial male bitterlings also perform pre-oviposition ejaculation into mussels, in which they ejaculate sperm into the inhalant siphon of a mussel before a female bitterlings releases her eggs, sometimes before a female has approached the mussel. Pre-oviposition ejaculation may enable males to obtain precedence for their sperm in the event that a female does spawn, though the period for which a sperm remains capable of fertilizing an egg is not known. Kanoh (1996) showed that *R. ocellatus* sperm stripped from a male remained capable of fertilizing an egg for at least 3 min in a Petri dish. Group sneaking also occurs in bitterlings, when up to 60 non-territorial males invade a territory and release sperm into a mussel. Male bitterlings in these sneaking groups engage in sperm competition not only with the resident territorial male, but also with all other males in the group that release sperm. Thus, there are at least four mating tactics shown by bitterlings: 'typical' pair spawning, sneaking by solitary territorial males, sneaking by solitary non-territorial males, and group sneaking. Each of these tactics may involve pre- or post-oviposition ejaculation or both.

Sneaking can be common under natural conditions. Smith, Douglas *et al.* (2002) saw 10 of 11 territorial male bitterlings suffer sneaking by other males in 47 of 69 separate spawnings. Kanoh (2000) witnessed 147 sneaking events from a total of 229 spawnings, and M. Reichard *et al.* (pers. comm.) used genetic markers to show that in 43 of 52 spawnings, a male other than the territory holder successfully fertilized some eggs. In response to sneaking, territorial male bitterlings display two adaptations for sperm competition with sneakers. Males may avoid leading females to mussels into which sneakers or other territorial males have already released sperm, an example of sperm competition avoidance. Males may also increase their rate of sperm release into mussels in which sneaking males have released sperm, termed 'sperm loading' (Smith, Douglas *et al.*, 2002). Smith, Reichard *et al.* (2003) discovered that male bitterlings did not avoid mussels, or increase their ejaculation rate, if frozen-thawed bitterling sperm was released into a mussel, suggesting that males cannot detect a rival's sperm. However, the presence of another male in proximity to a mussel elicited an aggressive response from a territorial male and caused males to avoid leading females to the mussel. In response to group spawning, male bitterlings did not attempt sperm loading, and males sometimes abandon defence of their territory for a short period (Smith, Douglas *et al.*, 2002). This response of the territorial male fits with Parker *et al.*'s (1996) model which predicts that males should increase ejaculate expenditure in the face of increasing sperm competition from another male, but reduce sperm expenditure as the number of rivals increases (Smith, Douglas *et al.*, 2002). Candolin & Reynolds (2002*b*) also showed that male bitterlings adjust their ejaculation rates, but not ejaculate volume, in relation to the density of competing males as predicted by Parker *et al.* (1996) before a female spawns. After a female had spawned they observed that males increased their ejaculation rate and aggression, irrespective of the number of competing males. The same pattern was shown in a field study by Smith, Reichard *et al.* (2003), who fitted estimates of ejaculate expenditure to Parker *et al.*'s (1996) model, finding a significant correlation of observed data to the predictions of the model. Smith, Reichard *et al.* (2003) further tested whether territorial male bitterlings responded to the number of competing males or the total frequency of rival ejaculations, showing that the number of competing males, not ejaculations, was the principle cue used by males in assessing sperm competition intensity.

The success of different sneaking tactics, measured as the number of offspring fathered, seems to differ. Kanoh (2000) used allozyme analysis to show that the territorial tactic was the most successful, sneaking by solitary non-territorial males the next most successful, and group sneaking the least successful tactic. From Kanoh's (2000) study it is not clear what the success is of combining sneaking and territory holding, or of pre- *vs* post-oviposition ejaculation. However, we have recently developed DNA microsatellites for bitterlings (Dawson *et al.*, 2003) that will enable us to identify paternity with a high degree of certainty. Ongoing research will investigate the relative fitness benefits of different mating tactics and the counter-tactics shown by male bitterlings for sperm competition.

The quality as well as the quantity of sperm may be important in sperm competition in bitterlings. The sperm of the Chinese bitterling *R. sinensis* seems to show some unusual features that may be related to the unusual breeding system of bitterlings (Guan & Afzelius, 1991). Further research on the morphology and motility of bitterling sperm among and within species would prove valuable in understanding adaptations for sperm competition and fertilization within the mussel gill chamber.

In our own and Kanoh's research on bitterling alternative mating tactics, mussels are always limiting and not all males are able to defend a mussel. Under conditions where mussel numbers exceed sexually mature males, it might be expected that each male would defend their own territory. However, we have observed sneaking tactics in natural populations where the ratio of bitterling to mussels was 58 mussels:1 male bitterling. For small or drab males, sneaking tactics may offer the only means of reproducing (see Female mate choice).

Intersexual conflict

An intersexual conflict arises when males and females differ in how they can best maximize their reproductive success (Partridge & Hurst, 1998). For example, mate guarding can reduce the proportion of ova that are fertilized (Warner *et al.*, 1995), or prevent females from obtaining the benefits of genetic variability within a clutch (Andersson, 1994). Sexual conflicts can also arise when females are forced or coerced into undergoing multiple matings, which can compromise female survival (Parker, 1970; Chapman *et al.*, 1998), increase energy expenditure (Daley, 1978; Scheider & Lubin, 1998), increase the risk of contracting diseases (Hunter *et al.*, 1993), or of injury (Clutton-Brock & Parker, 1995). Sexual conflict commonly arises through adaptations by males to sperm competition (Stockley, 1997), with females evolving a variety of counter-measures for combating the mating costs imposed by males (Eberhard, 1996).

In bitterlings, female oviposition choice seems to be adaptive, through maximizing embryo survival (see Female oviposition choice). Mate quality can influence female oviposition choice (see Female mate choice), and females may attempt to maximize fertilization success during mating. While male oviposition choice largely mirrors that of females when leading females to mussels, territorial males avoid those mussels into which rival males have recently released sperm (Smith, Douglas *et al.*, 2002). Thus, male oviposition choice may not always maximize embryo survival, but may maximize male fitness by ensuring paternity. Because male and female oviposition choice may not always correspond, there is a possibility of an intersexual conflict in oviposition choice in bitterlings. Further research is needed to clarify this potential sexual conflict in the choice of oviposition site.

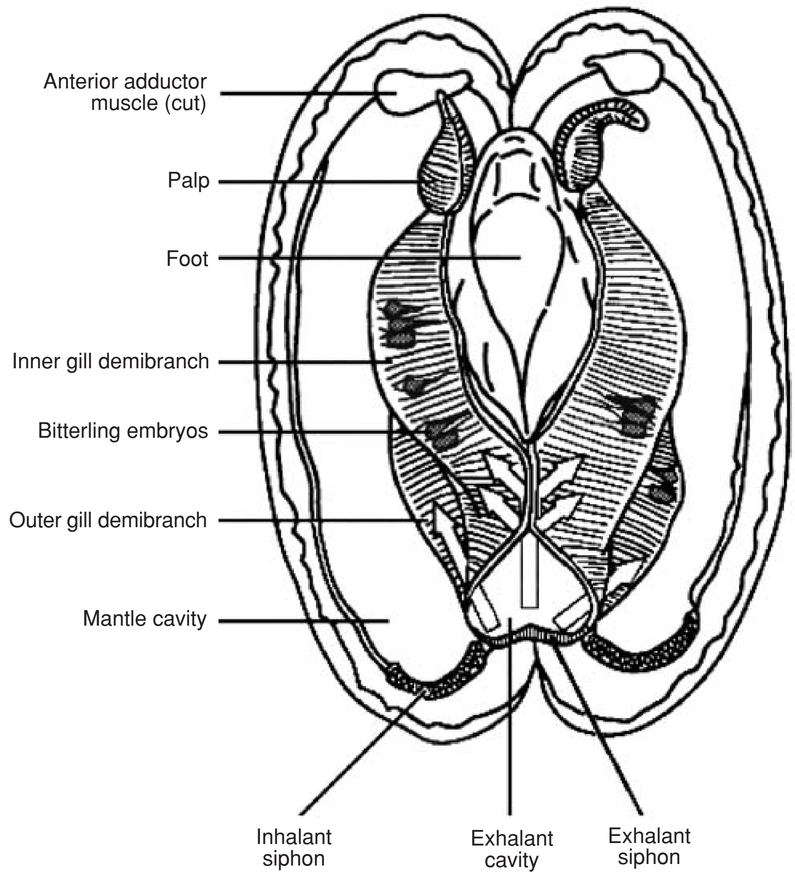

Fig. 6. The location of bitterling *Rhodeus sericeus* embryos on the gills of a freshwater mussel. Arrows indicate the route taken by the ovipositor into the gill. Modified from Aldridge (1999).

THE OFFSPRING

Embryonic development

There are several unique features of bitterling embryos that enable them to develop on the gills of mussels. Superficially, embryo development in *R. sericeus* resembles that of other bitterling species (Suzuki & Hibiya, 1984; Kim & Park, 1985; Aldridge, 1999).

During spawning, the female bitterlings deposits her eggs in the inner and outer gill demibranchs of the mussel, the eggs lodging in the interlamellar spaces (Fig. 6). The large size of bitterling eggs ensures that they fit snugly into the interlamellar space. There is no evidence that the eggs attach to the gills. A single egg may reside in the interlamellar space, though we have seen up to five in the same space. Ninety-nine per cent of the embryos develop with their heads down and their tails towards the opening of the demibranch (Aldridge, 1999).

The maximum number of embryos reported from a mussel is variable. Aldridge (1999) found a maximum of 63 bitterlings in a *Unio tumidus* from Little Mere, U.K. Balon (1962) reports a maximum of 19 embryos in a *U. pictorum* from a canal in the River Danube floodplain in Slovakia, while Zhul'kov & Nikiforov (1988) found up to 15 embryos in *Margarinata sachalinensis* in the River Tym'. D. G. Smith & Hartel (1999) report up to 10 embryos in the margaritiferid *Dahurinaia dahurica* in the River Belaya in the Amur basin. We have found a maximum of 257 bitterling embryos in an *A. anatina* from an oxbow lake in the Czech Republic. For the other mussel species investigated, a maximum of 147 embryos was found in *A. cygnea*, 149 in *U. pictorum* and 157 in *U. tumidus* (C. Smith & P. Jurajda, pers. obs.). The minimum number was a single embryo for all four species. Among Asian bitterling, Kondo, Yamashita & Kano (1984) give a maximum of 94 embryos of *Tanakia lanceolata* in an unidentified mussel in Gion Creek, Japan.

Complete development within the mussel, from spawning to the emergence of larvae, lasts *c.* 1 month. Aldridge (1999) found the mean development time from spawning to exogenous feeding (when larvae emerge) was 28 days. Holčík (1999) gives a range of 20–30 days. We have recorded development times of between 26 days (in a *Unio tumidus*) and 45 days (in a *U. pictorum*), with a mean of 34 days (SE = 5.2 days). We have found no evidence of differences in the development time of bitterlings incubated in *A. anatina*, *A. cygnea*, *U. pictorum*, and *U. tumidus*. Reynolds *et al.* (1997) also found no difference among the same four mussel species in the developmental rate of embryos. The main abiotic factors

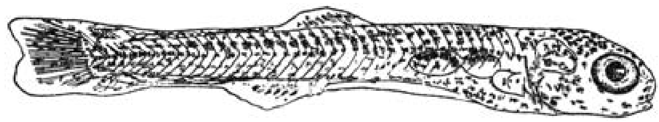

Fig. 7. Bitterling *Rhodeus sericeus* larvae at emergence from the mussel. Modified from Kryzhanovskii (1949).

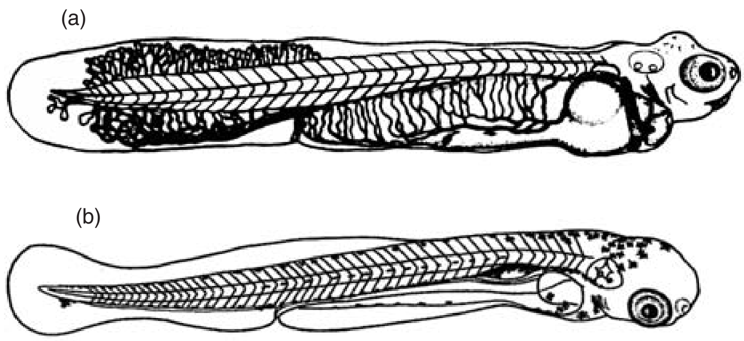

Fig. 8. Embryonic vascularization in two species of freshwater fish at the same developmental stage: (a) bitterling *Rhodeus sericeus* at *c*. 8 mm; (b) roach *Rutilus rutilus* at *c*. 7.2 mm. Modified from Kryzhanovskii (1949).

that determine the development rate of fish embryos are oxygen availability and temperature (Kamler, 1992), and this seems to be true for bitterlings (Aldridge, 1997).

Bitterlings emerge from the mussel once they have absorbed their supply of yolk and are about to begin exogenous feeding. No studies have investigated whether the embryos feed within the mussel. Aldridge (1999) gives the total length of the larvae at emergence as 10.5 mm. We have recorded the total length at emergence as 8.4 mm (SD = 0.22) (for ethanol preserved specimens), and Holčík (1999) gives a size of 9–10 mm. Reynolds *et al.* (1997) note that the size of larvae emerging from *A. anatina*, *A. cygnea*, *U. pictorum*, and *U. tumidus* does not differ significantly, but without giving a mean size. At emergence from the mussel, the larvae have well-developed pectoral fins and a swim bladder and are active swimmers, though the ventral fin-fold is not fully differentiated (Fig. 7). Shortly before emergence, the larvae are found occupying the exhalant cavity of the mussel gill. Kondo, Matsumura *et al.* (1987) report that *R. ocellatus* emerged from mussels exclusively between sunset and sunrise. Our data also suggest that emergence of *R. sericeus* larvae is more common at night (C. Smith, pers. obs.).

The dominant feature of the early life of bitterlings is their requirement for oxygen. Restricted oxygen availability retards the development of fish embryos and can cause death (for review see Kamler, 1992). Bitterlings display several unique physiological and morphological features of their embryonic development that seem to be adaptations to low oxygen conditions (Dmitrijeva *et al.*, 1985; Holčík, 1999). Bitterlings may face unusually low oxygen conditions on the gills of a mussel for several reasons. First, they are exposed to water that is likely to be depleted of oxygen because it has passed over the respiratory surfaces of the mussel gill (Aldridge, 1999). Mussels also sometimes close their valves when stressed or disturbed, thereby severely restricting the amount of oxygen passing into the gills (Aldridge, 1999). Bitterling eggs are also relatively large, possibly to enable them to wedge in the gills, and consequently have a high *per capita* oxygen requirement (Aldridge, 1999). Finally, bitterling eggs can be found at high densities in mussels and may compete with each other for oxygen (Smith, Reynolds, Sutherland *et al.*, 2000; Smith, Rippon *et al.*, 2001).

The first adaptation shown by bitterlings to low oxygen conditions is the short time to hatching. Bitterlings hatch after *c*. 36 h at a total length of *c*. 3.3 mm. This interval is considerably shorter than for other temperate cyprinids (Aldridge, 1999) and faster than zebrafish *Danio rerio* (Hamilton-Buchanan), tropical cyprinids with a rapid embryonic development (Kimmel *et al.*, 1995). The loss of the egg capsule (chorion) at hatching removes an important barrier to oxygen diffusion (Kamler, 1992). Experimental removal of the chorion increases the oxygen consumption of fish embryos and accelerates development rate (Hayes, Wilmot & Livingstone, 1951). Bitterling embryos are also capable of using the ethanol pathway for glycolysis (Waarde *et al.*, 1993), which enables them to tolerate low oxygen conditions. Perhaps the most striking adaptation of bitterlings for hypoxia is the extent of development of the cutaneous embryonic respiratory system, with a highly vascularized yolk sac and dorsal and anal fin-fold. Figure 8 shows a bitterling embryo along with a roach *Rutilus rutilus* Rafinesque embryo of the same developmental stage for comparison (roach are a typical temperate cyprinid, sympatric with bitterlings over much of their range). This extensive vascular system of bitterling is retained by the embryo until its emergence from the mussel (Dmitrijeva *et al.*, 1985), and enables the embryo to efficiently extract oxygen over almost its

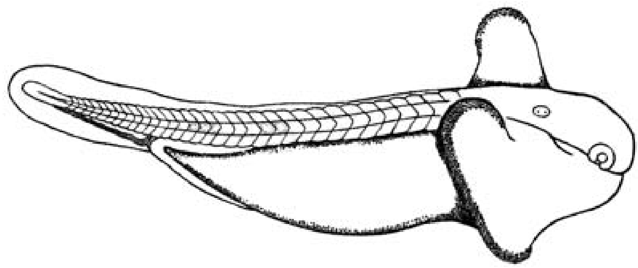

Fig. 9. Bitterling *Rhodeus sericeus* larvae during development in the mussel gill chamber, showing wing-like yolk projections. Length *c.* 5 mm. Modified from Kryzhanovskii (1949).

entire body surface. These adaptations are shown by other bitterling species (Suzuki & Hibiya, 1984). Smith, Rippon *et al.* (2001) provide evidence that female bitterlings use the oxygen content of the water flowing from the exhalant siphon as the proximate cue for determining the quality of a mussel for oviposition.

Other unique adaptations of bitterlings seem to help the embryo hold itself in position on the mussel gill, despite exposure to the exhalant flow from the mussel gills. The eggs of bitterlings are large (2.4–3.1 mm along their longest axis) and ellipsoidal. At spawning, this size and shape of the egg provides a secure fit between the mussel's gill lamellae. After hatching the embryo rapidly develops dorsal yolk projections, a pair of protuberances that serve to keep the embryo wedged between the gill lamellae (Bade, 1926) (Fig. 9). Approximately 4 days after hatching, the embryo begins to move. The actively swimming embryo is positively rheotaxic and negatively phototaxic, two adaptations that also ensure it retains its position in the gill (Dmitrijeva *et al.*, 1985). Fukuhara, Nagata & Maekawa (1982) describe the presence of small scaly tubercles on the yolk sac of six species of Asian bitterlings of the genus *Rhodeus*. These tubercles may also assist the embryo to lodge in the gills of its mussel host. We have attempted to dislodge embryos from the gills of mussels using fish anaesthetics, but this has always failed. The ineffectiveness of anaesthetics suggests that bitterlings passively maintain themselves in mussels, relying on the embryo lodging securely between the gill lamellae.

Embryos are sometimes lost from the gills of mussels (Kondo, Matsumura *et al.*, 1987; C. Smith, pers. obs.). Reynolds & Guillaume (1998) found that losses of bitterling embryos from mussels increased if the mussel was exposed to high levels of phosphate, and they speculated that mussels had control over the embryos in their gills and were able to expel them. As they acknowledge, however, their experimental design did not account for the direct effects of phosphate on the bitterling embryos, and there is no substantial evidence to support the idea that mussels can actively expel bitterlings. Losses of embryos from mussels are more likely to be because they are inadvertently dislodged from the gills by movements of the mussel, or by the death of embryos from suffocation at high densities. Our own research has shown that if mussels are disturbed or handled, the muscular contraction of the mussel as it closes its valves can dislodge bitterling embryos, particularly if the mussel contains a high density of embryos. This observation suggests that mussels may not detect the presence of embryos and any ejections are accidental. Reynolds & Guillaume (1998) conclude that mussels may not always eject bitterlings because there may be some benefit to hosting them. They do not, however, say what that benefit would be, and so far no evidence for a mutualistic bitterlings–mussel symbiosis has been found (see Relationship between mussel and bitterling).

Sibling rivalry

Competition for oxygen among bitterling embryos in the gills of a mussel is the most likely cause of density-dependent mortality of embryos (Smith, Reynolds, Sutherland *et al.*, 2000; Smith, Rippon *et al.*, 2001), and represents an intriguing form of sibling rivalry (Mock & Parker, 1997). Bitterling embryos in a mussel can be all full-siblings, a mixture of full and half-siblings and/or a mixture of full and half-siblings and unrelated individuals. Competition for oxygen can have sub-lethal effects, particularly a reduced rate of embryonic development, as well as lethal effects through suffocation. Thus, there is a possibility that developing embryos may modulate their oxygen consumption according to the relatedness of the embryos around them. Mock & Parker (1997) review the theoretical framework for exploring sibling rivalry. The sib-competition scramble model of Godfray & Parker (1992) can be used to predict the 'evolutionary stable strategy' for competition over oxygen in relation to relatedness among bitterling embryos in a mussel. Empirical tests of hypotheses for oxygen consumption and development rate could be performed using a micro-respirometer and an artificial mussel for rearing embryos.

FUTURE RESEARCH

Our review has highlighted several areas that we believe to be priorities for future research with this species. (1) The nature of the bitterlings–mussel relationship remains equivocal, particularly the impact of bitterling embryos on mussels. The challenge remains to show whether the symbiosis is that of host and parasite or of commensals.

(2) Because the quality of males and oviposition sites can easily be manipulated, intersexual conflict over oviposition choice can be readily investigated in bitterlings. In particular, it would be valuable to identify the extent to which males can influence female choice of mussel, and the possible direct and indirect benefits to a female of allowing a male to influence her oviposition decisions.

(3) Genetic studies will shed light on the success of the different alternative mating tactics shown by male bitterlings, as well as the relationships among embryos in mussels, and will contribute to our understanding of bitterling population dynamics.

(4) A particularly exciting field of study is the dynamics of fertilization within the gills of a mussels, the adaptations of bitterling sperm for fertilizing eggs inside the mussel gill, and the optimum timing of ejaculation of males for fertilizing an egg in relation to mussel characteristics and sperm competition. A combination of empirical and theoretical approaches to this question would be especially fruitful.

(5) There are *c*. 44 species of bitterlings worldwide, many with sympatric distributions. Interspecific comparisons, for example, in oviposition choice will be extremely valuable in testing current hypotheses in bitterling reproductive ecology. Investigation of interspecific competition among adults for mussels and between embryos within mussels would be particularly interesting.

CONCLUSIONS

(1) Bitterlings display remarkable morphological, physiological and behavioural adaptations for using mussels as spawning sites and they represent a valuable model in behavioural, population and evolutionary ecology. They have provided insights into oviposition choice, sexual selection, mate choice, sexual conflict, alternative male mating tactics and linking behavioural decisions with population dynamics. The value of the bitterling arises from it having a spawning site that can be easily manipulated and assessed for quality. They can also be observed under natural conditions and they adapt readily to laboratory conditions.

(2) The nature of the symbiotic relationship between mussels and bitterling is ambiguous. There is no evidence that the symbiosis is mutualistic. The absence of a fitness cost to mussels suggests that the relationship may be commensal, though this may only reflect a lack of appropriate studies. There is some evidence that bitterlings parasitize mussels, and further research is needed to clarify the nature of the symbiosis, concentrating on the extent to which bitterling embryos damage the gills of mussels and compete with mussels for oxygen.

(3) Female bitterlings are choosy about which mussel they use for spawning. Choice has been shown to vary with mussel species, fullness of a mussel with bitterling embryos, and the presence of glochidia. Female oviposition choices appear adaptive, with females choosing mussels that maximize the survival of their offspring. Mortality of bitterling embryos on the gills of mussels is strongly density dependent, with the strength of density dependence varying among mussel species. Density-dependent mortality of embryos is probably related to the oxygen conditions in a mussel, with embryo deaths being caused by suffocation. Female bitterlings seem to use the oxygen content of the exhalant flow from a mussel in oviposition choice, thus female choice is related to host quality.

(4) Male bitterlings show at least four different mating tactics, territoriality and 'typical' pair spawning, sneaking by solitary territorial males, sneaking by solitary non-territorial males, and group sneaking. Each of these tactics may involve males depositing sperm into the inhalant siphon of the mussel before or after the female spawns. All these tactics are widespread and common under natural conditions, and it is likely that strong sperm competition occurs among males in the gills of mussels. Males display adaptations to sperm competition, including sperm loading and leading females away from mussels in which rivals have released sperm. Molecular genetic studies are needed to clarify the fitness benefits of alternative mating tactics and male adaptations for sperm competition.

(5) While female oviposition choices maximize offspring survival, male choices seem to maximize paternity, and there may be an intersexual conflict over oviposition choice. The extent to which male bitterlings can influence female choice of mussel is unclear, though there seem to be both costs and benefits to a female of allowing a male to influence her oviposition decisions.

(6) Bitterling embryos possess numerous adaptations for development on the gills of mussels, and particularly for the low oxygen conditions in a mussel. These adaptations include early hatching, the anaerobic ethanol pathway for glycolysis, and an unusually well-developed embryonic respiratory system. Embryos probably compete with each other and possibly with their host mussel for oxygen.

(7) While the recent scientific interest in the reproductive ecology of bitterlings has yielded valuable insights into a variety of behavioural and evolutionary questions, bitterlings remain a relatively poorly studied group of fishes. Our review highlights weaknesses in our understanding of bitterling biology and will encourage further research on these remarkable animals.

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