Sperm competition and male mating tactics in the bitterling fishes

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Abstract

Sperm competition can play a significant role in the evolution of animal mating systems and is a widespread phenomenon occurring when the ejaculates of one male coincide or overlap with the ejaculates of another. The relative reproductive success of individual males within a population is determined to a large extent by sperm competition and adaptations to maximise a males success when faced with sperm competition are common.

Bitterling are a group of freshwater fishes that lay their eggs on the gills of living freshwater mussels, using the mussel as a protective environment for embryo development and utilising the mussels own respiration to ensure fertilisation. This unusual spawning mechanism, using a spawning site that can be easily manipulated, makes bitterling ideal for investigating sperm competition and mating system evolution.

Here, using a range of bitterling species, a series of aquarium experiments were conducted, as well as morphological and histological studies of the sperm and testes. It was shown that males were highly sensitive to sperm competition, ejaculating at a higher frequency and subsequently becoming more sperm depleted where sperm competition was high. There were few differences between mating tactics except in relative testis size, where larger males had proportionally larger reproductive apparatus, but ejaculates were of a similar size. The timing of ejaculates was found to be crucial, with a peak in sperm concentration within the mussel mantle cavity 30 seconds after ejaculation. The spatial clustering of fertilisation opportunities and OSR were found to affect ejaculate frequency, ejaculate distribution among mussels, the dominance of guarder males and subsequently the opportunity of subordinate males to sneak fertilisations. Significant differences in the spermatogenic strategy and the structure of the reproductive apparatus among species were identified, as well as significant differences between species in the morphology of spermatozoa.

Darwin's Fish

Darwin had a fish named Very Brighte
'Twas marked with spots of orange amongst the white
Such a fine example of selective breed'n
'Twould be a shame to see him eat'n.

Charles D., taught this very special fish
To take a walk on a contrivance; called a leash.
On days when weather was good and fair
Brighte was released from his leather snare.

Under trees so green and supple, the two
Frolicked, as only friends could do.
One day they took a different path
As they were deep in thought, discussing math.

They approached a glen so inviting
The fish dictating, Darwin writing.
There in the cooling shade,
A brook's babbling sound was made.

'Memberances of times in distant past
Thru his brain the pictures flashed.
Caused him (the fish, not Darwin)
To leap right in.

'Twas here, the poor fish did discover
A truth known to father, mother, sister, brother.
Pollywogs, as well, have found it's true,
One cannot go home to waters, blue.

As he sank into the deep,
Poor Darwin's fish began to weep.
Sink or Swim, he did remember.
But forgot all else, that day in September.

Perhaps he forgot how to swim and drowned.
Yet his body was never found.
But Darwin was convinced that it was evolution,
That brough Brighte's life to its conclusion.

Sidi J. Mahtrow

Contents

Chapter 1: Introduction

Sperm Competition and Sexual Selection

Selection acting through competition for mates (intra-sexual) or through mate choice (inter-sexual) is referred to as sexual selection (Darwin 1859, 1871; Møller 1994, 1998; Birkhead and Møller 1998). Sexual selection arises when variance in the number of males that successfully mate is unequal (Andersson 1994; Møller and Ninni 1998) and is related to male phenotypic characters (Andersson 1994). Where individuals must compete for mating opportunities with rivals, or attract the attention of mates, there is often strong selection for morphological adaptations that provide a competitive advantage over rivals; either through enhanced fighting ability (Weckerly 2001; Kokko and Brooks 2003) or an increased attractiveness to mates, though the two may be linked (Møller and Ninni 1998).

While intra-sexual competition is most obvious in the form of pre-copulation male aggression, intra-sexual selection may also occur post-copulation; females, even of monogamously paired species, will often mate with more than one male (Marshall et al. 2007; Leibgold et al. 2006). The resulting overlap of ejaculates results in sperm competition, an area of intra-sexual competition that, since Parker's (1970) early work on the mating system of the yellow dungfly, has been studied in almost every taxa (Marconato et al. 1995; Gage and Barnard 1996; Birkhead and Møller 1998; Stockley 2002). Sperm competition is now recognised as an important mechanism of sexual selection that has shaped the evolution of plant and animal mating systems, influencing male reproductive anatomy, physiology, and behaviour (Smith 1984; Birkhead and Møller 1998; Andersson 1994; Eberhard 1996; Delph and Havens 1998; Parker 1998, 1990; Simmons 2001).

Sperm competition theory predicts that males should adjust their reproductive expenditure according to the risk (the probability of competing with another males ejaculate) (Parker et al. 1997), and intensity (the actual number of competing ejaculates) (Parker et al. 1996) of sperm competition. The way in which sperm competition operates at the gametic level remains relatively poorly understood because of the difficulty of investigation and the confounding responses of each sex; males are predominantly adapted to avoid sperm competition or maximise their individual fertilisation success, while females often show the opposite response, especially where females are sperm limited (Parker et al. 1997, 1999). However, it has been demonstrated that in vertebrates at least, sperm competition functions as a lottery, where the more sperm a male releases at a mating the greater his chances of siring offspring (Parker 1997; Birkhead and Møller 1998; Preston et al. 2001).

In species where the risk of sperm competition is high males often show adaptations to sperm competition, including adaptations of testis size and structure (Byrne et al. 2002), ejaculate size (Briskie 1993; Møller 1988; Briskie 1993; Gage and Barnard 1996; Stockley et al. 1996, 1997; Birkhead and Møller 1998; Sicotte 2002), and sperm morphology (Gage 1994; Stockley et al. 1997; Snook 2005), or through increased fighting ability, and other behavioural adaptations for sperm competition avoidance (Anderson 1994; Polak 1994; Alcock 1996; Sokolovska et al. 2000; Jones and Hutchings 2001; Preston et al. 2001; Carlini et al. 2002; Smith et al. 2002). Sperm competition theory predicts that among species there will be a positive correlation between ejaculate expenditure and the risk of sperm competition (Parker 1982). Within species, once the number of competing males exceeds two there is predicted to be a negative correlation between sperm competition intensity (the extent to which ejaculates overlap at a mating), and ejaculate expenditure (Parker et al.

1996), though few studies have demonstrated this effect (Pilastro et al. 2002; Smith et al. 2003).

Ejaculates are physiologically expensive to produce (Van Voorhies 1992; Olsson et al. 1997; Wedell et al. 2002), and males are predicted to adjust ejaculate expenditure in response to sperm competition intensity. Thus, if the intensity of sperm competition is high (i.e. more than two rivals males participating at a spawning) the optimal tactic for a male may be to reduce ejaculate expenditure, since the probability of fertilising eggs diminishes with the number of competing males that attempt to fertilise the same ova (Parker et al. 1996). In contrast when there is a single rival, males should invest more in ejaculates (Ball and Parker 1998). If a male faces no competition at a mating, he is predicted to release only enough sperm to achieve fertilisation. Research has shown males from multiple taxa to be highly sensitive to sperm competition, tailoring ejaculate expenditure to local conditions and thereby, maximising their long-term reproductive success (Gage 1991; Pilastro et al. 2002; Pizzari et al. 2003; Harris and Moore 2005a; Del Barco-Trillo and Ferkin 2006) by out-competing rivals and minimising sperm depletion (Rondeau and Sainte-Marie 2001; Wedell et al. 2002).

When males increase sperm expenditure in response to sperm competition (Gage 1991), gain access to multiple matings through dominance or female mate choice (Preston et al. 2001; Carlini et al. 2002), or have a finite quantity of sperm (Damiens and Boivin 2005), sperm depletion as well as sperm competition will play a role in determining a males success (Hoelzel et al. 1999; Preston et al. 2001; Wedell et al. 2002). Sperm depletion occurs when a male cannot produce enough sperm to maintain ejaculate size over successive ejaculates, and is thought to be common across multiple taxa (Birkhead and Møller 1998; Birkhead and Fletcher 1995; Wedell

et al. 2002). Where sperm depletion occurs females risk reduced fitness through their ova not being fertilised (Brockman et al. 1994). As a result, where females risk becoming sperm limited they are often highly promiscuous, to ensure sufficient sperm reserves are available to fertilise all their ova.

The study of sperm competition at the gametic level is difficult, often intrusive and risks influencing natural behaviour (Parker et al. 1997, 1999). Fish with large eggs and external fertilisation (in most cases) offer a solution, and are ideal for testing predictions of sperm competition theory. Fishes show a remarkable diversity of mating systems (Taborsky 1994) and among the greatest range of sperm competition intensities of any animal group (Birkhead and Møller 1998). They exhibit examples of internal fertilisation (Evans and Magurran 2001; Kvarnemo and Simmons 2004; Naud and Havenhand 2006), and external fertilizers (Robertson 1996; Kiflawi et al. 1998; Taborsky 1998), including more unusual externally-spawning species that use other living organisms to provide their young with parental care and protection (Breder and Rosen 1966; Smith and Louw 1987; Smith et al. 2004).

Of all externally fertilising fish species, bitterling offer an unusually amenable system for use in sperm competition research. They are a group of approximately 40 (Arai 1988) species of freshwater fishes belonging to the Acheilognathinae, a subfamily of the Cyprinidae largely confined to Asia, but with a single species in Europe, that has an unusual spawning symbiosis with freshwater Unionid mussels; laying their eggs in mussel gill cavities (Zhul'kov and Nikiforov 1988; Smith and Hartel 1999; Smith et al. 2004). Bitterling are small, rarely exceeding 70 mm from the tip of the snout to the origin of the caudal fin (Standard Length) (Smith et al.

2004). They are relatively deep bodied, with a body depth 29–45% of their standard length (Holčík 1995, 1999; Reichard 1998; Smith et al. 2004).

While previously thought of as a mutualism, recent research suggests that the bitterling-mussel relationship should, at least in the case of the European bitterling *Rhodeus amarus*, be considered as parasitism. Somerton and Donaldson (1998) showed that the presence of embryos can damage mussel gill tissue. Recently Reichard et al. (2006) showed that bitterling are parasites of freshwater mussels, significantly affecting their growth and overall fitness.

In Asia, the centre of distribution for bitterling fishes, the relationship between bitterling and mussels may be more mutualistic (Reichard et al. 2006). Asian species of bitterling normally have both a higher load of mussel larvae (glochidia) on them (Kadlec et al. 2003; Smith pers. com.) and considerably more eggs are ejected from mussels after spawning (Reichard et al. 2005). Differences between *R. amarus* and the Asian species of bitterling in glochidia load and egg rejection rate may represent an evolutionary lag where fish and mussels in Asia have co-evolved to parasitize each other, while European mussels have not yet evolved responses to bitterling. Asian mussel species appear to have developed glochidia larvae which can successfully attach to bitterling, while bitterling eggs and larvae have become morphologically adapted to maintain their position within the mussel gill cavity and avoid ejection. In contrast, *R. amarus* invaded Europe relatively recently from Asia (Reichard et al. 2005; Van Damme et al. 2007) and subsequently there appears to be an evolutionary lag between parasite and host, where bitterling are able to take advantage of mussels with minimal risk of infection from glochidia (Kadlec et al. 2003; Mills and Reynolds 2003; Reichard et al. 2005).

The Biology of Bitterling Fishes

The biology of the European bitterling, *R. amarus*, is reviewed by Smith et al. (2004) and appears to broadly resemble that of all other species so far studied. At the start of the spawning season, male bitterling develop bright nuptial coloration, start inspecting the siphons of mussels, and begin to aggressively defend territories around mussels (Smith et al. 2004). During the spawning season female bitterling develop long ovipositors which they use to place their eggs onto the gills of a mussel through the mussel's exhalant siphon (Smith et al. 2004). The ovipositor is a long, tube-shaped structure opening within the abdomen into the oviduct and bladder. The ovipositor is highly variable in length, at its shortest outside the breeding season, just protruding from the abdomen, and at its longest at spawning, when it may exceed the body length of the female. During the reproductive period, each female goes through several cycles; typically a single day spawning, followed by a resting period of between 7 and 10 days. Males, which may play both the role of a guarder or a sneaker with no clear morphological differences, (Candolin and Reynolds 2002a, 2002b; Smith et al. 2002, 2004; Reichard et al. 2005) 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. Ejaculations are sometimes visible as a greyish cloud. Ejaculations are frequent both pre and post-oviposition (Reichard et al. 2004) with an average of 15 ± 1.3 ejaculations per mating (Reichard et al. 2004b). Importantly, in European bitterling under natural conditions, presumably through sperm precedence and because of the time it takes sperm to reach the eggs, pre-oviposition ejaculations are more successful in fertilizing spawned eggs than ejaculates released at spawning (Reichard et al. 2004a). Once fertilized, embryos develop inside the mussel for

approximately a month, before leaving the mussel as actively swimming larvae (Aldridge 1999).

Mating Behaviour

A variety of alternative mating tactics, (which are thought to be similar across all bitterling species) have been described for *R. ocellatus* (Kanoh 1996, 2000) and *R. amarus* (Smith et al. 2002; Smith et al. 2003). Typically the mating behaviour of male bitterling follows the sneak-guard model of Parker (1990), where some males adopt a guarder tactic (guarding a territory containing mussels and courting females) and others mate as sneakers (joining a spawning pair with no investment in territorial defence or courtship, and investing primarily in sperm competition (Taborsky 1994; Smith et al. 2003, 2004; Reichard et al. 2005). Sneaking is a common and successful alternative mating tactic; Kanoh (2000) recorded 147 sneaker male ejaculations in just 229 spawnings, while Reichard et al. (2004) found that sneaker males had achieved paternity in 43 of 52 separate recorded spawning events in an artificial pond.

Mating tactics in bitterling are opportunistic rather than fixed with an individual switching mating tactic to take advantage of local conditions. Mating tactic appears to be determined by, male size, operational sex ratio, male density and the spatial clustering of mussels (Kanoh 2000; Smith et al. 2004). According to the hypothesis of Gross and Repka (1998) the different mating tactics seen among bitterling males should be seen as different conditional tactics within the same reproductive strategy as males can play either tactic at the same spawning site, and the success of each tactic does not have a fixed success rate.

Guarder males are often found defending a single mussel, but if capable may guard several within a territory of approximately 1 m^2, though territory sizes are

usually considerably smaller than this maximum. The main determinant of the ability of a male to hold a territory is thought to be size (*R. ocellatus*, Kanoh 2000; *R. amarus*, Smith et al. 2004). Working with *R. amarus* Reichard et al. (2005) found that male size was the main variable underpinning male dominance and, therefore, that larger body size was favoured in both intersexual and intrasexual selection. Reichard et al. (2005) also found that while females were choosy over who fathered their offspring, in reality they had limited influence over the paternity of their offspring as females were often controlled by dominant males, who limited the mating opportunity of rivals. Female mate choice also operates in bitterling (Smith et al. 2002, 2004; Reichard et al. 2005) with evidence for a female preference for sneaker males, perhaps in response to the effect of sperm depletion on ejaculate size in dominant males (Wedell at al. 2002; Reichard et al. 2005; Smith and Reichard 2005).

Bitterling also participate in group spawning, often when fish are at high densities, and up to 60 non-territorial males may invade a territory to ejaculate over a mussel (Smith et al. 2004). Where group spawning occurs, male bitterling face sperm competition from not only the guarding male, but from all other males in the spawning group (Smith et al. 2002). In response to group spawning, Smith et al. (2002) showed that guarder males avoided repeatedly ejaculating over mussels (hereafter referred to as 'sperm loading'), and in some cases abandoned defence of their territory for a short period.

Thus, there are at least four mating tactics shown by bitterling: 'typical' pair spawning, sneaking by guarder males, sneaking by non-territorial males, and group spawning. The success (number of offspring fathered) of each different sneaking tactic is varied, with guarder males the most successful, followed by sneaking by solitary non-territorial males, and group spawning the least successful (Kanoh 2000).

While only a few males hold a territory at any one time, mating tactic is thought to change over time with young males initially acting mainly as sneakers, before adopting a predominantly territorial role as they grow.

Sperm Competition Risk

While all bitterling appear to share similar mating tactics (Kondo et al. 1984; Nagata 1984; Smith et al. 2000, 2004; Kitamura 2006), they differ in spawning season length and clutch size. For example, *R. amarus* has a substantially shorter spawning season (*R. amarus*; 6 weeks (Douglas 2003; Reichard et al. 2004) than either *R. sinensis* (3-4 months) or *R. ocellatus* (5-6 months) (Kondo et al. 1984; Nagata 1984)). Despite differences in seasonal length the overall seasonal fecundity is approximately the same among species, (Smith et al. 2000; Kitamura 2005; Reichard unpublished data for *R. sinensis*) though importantly, there are differences in clutch size and value. While small clutches of eggs are produced several times a day by female *R. amarus* (typically 3-4 eggs) and *R. ocellatus* (typically 4-5 eggs), female *R. sinensis* lay large clutches with a mean (SE) of 16 (2.6) eggs per spawning act, possibly only once or twice during a given period of receptivity (Smith et al. 2004; Reichard et al. 2007).

Females and their ova are a resource that can become clustered both in space and time. In bitterling the spatial distribution of mussels, the length of spawning season and female clutch size determines the intensity of spatial and temporal clustering, local operational sex ratio, and therefore also the level of sperm competition (Shuster and Wade 2003). While bitterling species show similar reproductive strategies (Kondo et al. 1984; Nagata 1984; Smith et al. 2000, 2004; Kitamura 2006), spawning season length and female clutch size both vary between species. Therefore the temporal and spatial clustering of fertilisation opportunities

differs, suggesting that in some species, for example where clutch sizes are large but infrequent, the competition at spawnings will be much greater than in a species where spawnings are more frequent and clutch sizes are small. Therefore, it is assumed that while bitterling species sharing a similar seasonal fecundity, natural sperm competition risk should vary among bitterling species, according to clutch size and spawning season length.

Pre and Post-Oviposition Ejaculates

Guarder and sneaker male bitterling of all species will often perform pre- and post-oviposition ejaculations into mussels. In pre-oviposition ejaculations a male will ejaculate over the inhalant siphon of a mussel before a female bitterling has released her eggs and often before a female has even approached the mussel (Reichard et al. 2004).

Pre-oviposition ejaculation may provide an opportunity for males to obtain sperm precedence in the event that spawning takes place (Reichard et al. 2004a). The period for which a sperm remains capable of fertilizing an egg (after ejaculation) is not known, though 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, and Reichard et al. (2005), using parentage analysis, showed that the sperm of a male released 14 minutes before spawning was successful in fertilising an egg. Sperm motility, as with other teleost fish decreases rapidly after ejaculation into freshwater. However bitterling sperm may be similar to that of the three-spined stickleback, (*Gasterosteus aculeatus*), where due to osmotic shock sperm become immotile shortly after ejaculation, but can be reanimated by the females ovarian fluids (Le Comber et al. 2004).

In female bitterling, before oviposition the muscular conical organ at the base of the ovipositor is extended and a batch of 1–6 ovulated eggs is positioned at the basal opening of the ovipositor, behind which urine collects. As the female swoops forward to spawn, the conical organ contracts and the urine forces the eggs quickly through the entire length of the ovipositor, unfurling and stiffening into the interlamellar gill spaces within the mussel gill (Matsubara 1994). The urine of female bitterling is high in amino acids which appear to influence male behaviour, triggering pecking in low light (where males peck the female around the anal fin and ovipositor) and ejaculation under natural lighting (Kawabata et al. 1992; Kawabata 1993). Amino acids or other chemicals within female urine may also stimulate sperm or reverse the affects of osmotic shock. If this is the case it may explain the finding by Reichard et al. (2005) that sperm released 14 minutes prior to spawning were successful in fertilising ova.

Ejaculate Frequency

Candolin and Reynolds (2002b) and Smith et al. (2003) showed that guarder male responses to sperm competition risk fitted with Parker et al.'s (1997) prediction that males adjust ejaculation rates in response to sperm competition intensity. While participating in pre and post-oviposition ejaculations males also increase the frequency of sperm releases into mussels where sneaker males have ejaculated, Smith et al. (2002) termed this 'sperm loading'. This behaviour in bitterling fits with the model proposed by Parker (1998) for externally fertilising fishes, where males top up or maintain sperm concentrations by ejaculating over the same spot at regular intervals.

When experimenting with frozen-thawed bitterling sperm, Smith et al. (2003) found that male bitterling could not detect the presence of sperm within the mussel. Instead male responses to sperm competition were triggered by the presence of other males in proximity to mussels which often resulted in an aggressive response from guarder males and caused them to avoid leading females to the mussel. Therefore, it can be assumed that in bitterling male responses to sperm competition are based on visual rather than olfactory cues and secondly that rather than responding to ejaculate number, male bitterling respond to the number of males present at a mussel (Smith et al. 2003), though both are a reflection of the level of sperm competition intensity.

Sperm Morphology

As demonstrated in other species, sperm quality as well as the numerical size of ejaculates may be important in sperm competition between male bitterling (Stockley et al. 1997). The bitterling group is ideal for comparative studies on the affect of sperm competition on sperm morphology and quality, as the mating system among species is similar, while the risk of sperm competition differs significantly. There is little work on the effect of sperm competition on either sperm quality or sperm morphology in bitterling. However, the sperm of the Chinese species *R. sinensis* and *R. ocellatus* have been shown to possess features on the sperm head (Guan and Afzelius 1991; Ohta 1991) similar to that observed by Kessel et al. (1983) in the zebra fish (*Danio rerio*). Ohta (1991) showed that sperm possessed intramembranous particles on the sperm head which they suggested may function in locating the egg and specifically the micropile.

Research on *R. ocellatus* and *R. sinensis* suggests that bitterling spermatozoa have a primitive morphology similar to that of most other externally fertilising teleost

fishes, consisting of a sperm head, a midpiece and a flagellum. In both *R. ocellatus* and *R. sinensis* a head and neck region can be distinguished, each with a spherical shape. Spermatozoa have a large mitochondrion section and centrioles in the middle piece. As in some blenniid species (Lahnsteiner and Patzner 1990a), the flagellum arises in the nuclear notch at the lateral side of the spermatozoon, at the border between the head and neck region. The midpiece was shown to encircle the root of the flagellum (Ohta 1991). The proximal portion of the flagellum is surrounded by a sleeve of plasma membrane (arising from a flattened section of the midpiece) which appears separated from the head piece, except at the centriolar fossa.

Aims

While the mode of spawning in bitterling makes them an interesting, unusual and extremely useful model species, research into sperm competition in bitterling is limited mainly to behaviour and remains relatively superficial when compared with other model species (Parker 1970; Stockley et al. 1997; Birkhead and Møller 1998). A unique aspect of bitterling biology is their use of freshwater mussels as a spawning site. However, little is known about the processes which occur within the mussel, resulting in fertilisation, except the work of Ohta (1991) on the sperm egg fusion and the function of intramembranous particles on the sperm head. Therefore, the aims of this study were to build on previous behavioural and morphological work utilising a wider range of species than any previous studies to gain a fuller understanding of sperm production, sperm demand, sperm competition, sperm allocation and sperm morphology across bitterling species.

During research *Rhodeus amarus*, *R. ocellatus*, *R. sinensis*, *Tanakia himantegus*, *T. limbata*, *Acheilognathus barbatulus*, and *A. tabira*, were all used for

observations on the external morphology of spermatozoa. We also used *D. rerio* as a control species for morphological observations of spermatozoa. However, for all but one study (Chapter 6: Sperm Morphology) only three species of bitterling; the European bitterling, *R. amarus*, the rose bitterling, *R. ocellatus* and the Chinese bitterling, *R. sinensis* were used. The three species were selected for their adaptability to laboratory conditions, their obvious and frequent spawnings and the associated spawning behaviour.

Thesis Structure

- **Chapter 2: Guarder Male Response to Sperm Competition and, Ejaculate Characteristics in the European Bitterling.**

  In this chapter I discuss (i) the behavioural responses of males to sperm competition and (ii) examine the quality of guarder and sneaker male ejaculates.

  Part i) Sperm competition theory predicts that among species ejaculate expenditure during mating is positively correlated with the risk of sperm competition, whereas within species, where more than two males compete ejaculate expenditure is predicted to correlate negatively with sperm competition intensity; males are expected to reduce ejaculate expenditure where several rivals are present but increase there investment where rival numbers are lower than two (Parker et al. 1996).

  While in bitterling, spawning can take place as a pair, with just a male and female present, more frequently a female will mate in the presence of at least two males (Kanoh 1996, 2000; Smith et al. 2002; Smith et al. 2003) and therefore guarding males must adapt to both conditions of low and high sperm

competition intensity. Using only *R. amarus* I aimed to identify the response of guarder male bitterling to sperm competition. Where sperm competition intensity is great, do males respond by escalating aggression, increasing the frequency of ejaculates, or both, and at what point do males stop escalating competition?

Part ii) Where sperm competition occurs, sperm quality (swimming speed, swimming direction, spermatozoa longevity), and not only the numerical size or frequency of ejaculates, is important to male fertilisation success (Stockley et al. 1997). Sperm size can influence sperm competition as longer sperm generate greater flagella forces (Katz et al. 1989) and, therefore, swim faster (Gomendio and Roldan 1991). However, with a fixed resource budget the production of larger spermatozoa may result in a forced reduction in the number of spermatozoa a male can produce and either fewer, or numerically smaller ejaculates (Gomendio and Roldan 1991; Parker 1982; Stockley et al. 1997; Burness et al. 2004). In contrast, smaller sperm may allow the production of more gametes and, therefore, afford greater success where sperm compete numerically (Parker 1982, 1997, 1999).

Sperm quality has been shown to vary between males and mating strategies (Neff et al. 2003) and while in bitterling both guarder and sneaker males release sperm both pre and post-oviposition, sneakers release pre-oviposition ejaculates significantly more often than guarders and may have different requirements in terms of sperm quality and especially longevity. While mating tactics in bitterling appear to change over the ontogenetic gradient it is possible that at different stages of their life cycle males vary their investment

into spermatozoa production to increase their reproductive fitness. I aimed to identify any differences between the sperm of guarder and sneaker males.

- **Chapter 3: Male Sperm Depletion and Female Sperm Limitation in the European Bitterling.**

  Here I investigated both the effect to which sperm depletion affects ejaculate size, and the effect of sperm competition on the severity of sperm depletion (i), and I also observed the effect of the risk of sperm limitation on female behaviour (ii).

  Part i) Sperm depletion, or prudent control of ejaculate size by males, can have consequences for both male and female reproductive success, where females can become sperm limited, and in males depleted individuals release progressively smaller ejaculates and often suffer the consequent reduction in reproductive success (Pitnick 1993; Royer and McNeil 1993; Stockley et al. 1997; Rondeau and Sainte-Marie 2001; Preston et al. 2001; Shapiro et al. 1994; Rasotto and Shapiro 1998). Where a male is successful in attracting mates he may mate extremely frequently and may not be able to produce enough sperm to maintain ejaculate size. If dominant males become sperm depleted, while sperm competition is widespread, smaller males, which often mate at a lower frequency, are likely to be less depleted and therefore more successful in covert contests through sperm competition. In this situation while the number of copulations a male achieves may remain consistent or even increase, the proportion of offspring he will sire, if competing with un-depleted males, will decrease. Male bitterling (especially *R. amarus*) mate frequently during the spawning season and will release multiple ejaculates at each spawning. Therefore, I aimed to assess whether sperm depletion was a

limiting factor in male reproduction. I observed ejaculate size over individual spawnings and over the course of a day, monitoring sperm concentrations under both low and high sperm competition intensity.

Part ii) Sperm limitation in females can lead to inter-female competition for access to males (Saether et al. 2001) and the evolution of male mate choice (Preston et al. 2001) as where females are sperm limited they may have insufficient sperm to fertilise their ova. Often females trade off the cost of infertility, against "good" paternal genes (Wedell et al. 2002) as where a male is extremely popular, females which chose to mate with that male risk reduced fecundity, as unless he has infinite sperm reserves she will only receive a proportion of his total sperm reserve, and a smaller proportion the more popular he is (Jones 2001; Saether et al. 2001). There is ample evidence that females solicit matings from males, and mate with multiple partners to increase their access to sperm and thereby circumvent the risk of sperm depletion (Wedell et al. 2002; Gray 1997). In *R. amarus*, Smith and Reichard (2005) demonstrated that females achieved higher fertilisation rates when mating with multiple rather than solitary males and secondly that females perform conspicuous behaviours associated with spawning more frequently close to sneakers, and spawned more eggs close to high-quality sneakers. Therefore, I aimed to observe any sexual conflict and identify any adaptations which enable males to avoid sperm depletion and maximise reproductive success, or which in females, maximises the chances of the female receiving sufficient sperm to fertilise all her eggs.

- **Chapter 4: Spatial Clustering of Spawning Sites and Operational Sex Ratio in Three Species of Bitterling.**

Here I investigated the effect of the spatial clustering of resources and OSR on male reproductive success and mating tactics. The spatial and temporal clustering of resources can influence the level of competition between rivals (Shuster and Wade 2003). Female bitterling and their ova can be considered as a resource critical for spawning and therefore the clustering of females in space and time can influence the local operational sex ratio and the level of sperm competition. I wished to measure the fertilisation success of males within different mating tactics and observe the behaviour of males under different intensities of spatial and temporal clustering, to identify if the success of different mating tactics varied with female clustering.

- **Chapter 5: Spatial Clustering of Spawning Sites; as a Mesocosm Study With Rose Bitterling.**

  I investigated the effect of the spatial clustering of mussels over a large scale on male reproductive success and mating tactics. I aimed to measure the success of different mating tactics and observe the behaviour of males under different intensities of spatial clustering, to identify if the success of different mating tactics varied with the spatial clustering of mussels on a large scale.

- **Chapter 6: Sperm Morphology of Seven Species of Bitterling.**

  The morphology of spermatozoa among fishes is varied, (Robinson and Prince 2003), with spermatozoa morphology reflecting numerous different selective pressures that range from spawning environment to sperm competition (Billard 1986; Lahnsteiner and Patzner 1990; Jamieson 1991; Stockley et al. 1996, 1997). Sperm function and morphology can be greatly affected by the morphology and biochemistry of the female reproductive tract (Dybas and Dybas 1981; Briskie and Montgomerie 1992), though in bitterling the

importance of the female reproductive tract is replaced by mussel gill morphology and physiology. Building on work by Ohta (1991) I aimed to understand the external morphology of bitterling spermatozoa and to identify if spermatozoa show any adaptation to spawning environment or sperm competition risk.

- **Chapter 7: Testis Structure and Spermatogenesis in three species of Bitterling.**

  In species where sperm competition risk is high males may not only have a high gonadosomatic index (Harcourt et al. 1981; Gage 1994; Hosken and Ward 2001), but may also control ejaculate size (Shapiro et al. 1994; Rasotto and Shapiro 1998). Other adaptive features include the sperm ducts, which can produce mucus that acts to parcel and protect the ejaculate, or continue the development of prematurely released spermatids (Lahnsteiner and Patzner 1990b; Rasotto and Mazzoldi 2002). The frequent ejaculations and a highly specialised spawning environment seen in bitterling may require specific spermatogenic adaptations that could include mucus production or rapid spermatogenesis. Therefore, I aimed to identify the structure of the testes and the nature of spermatogenesis in bitterling, and related my findings to spawning site or differences in sperm competition risk among species.

Chapter 2. Guarder male response to sperm competition and ejaculate characteristics in the European bitterling (*Rhodeus amarus*)

Abstract

Sperm competition is a widespread phenomenon that occurs when ejaculates of one male coincide or overlap with the ejaculates of another. Sperm competition risk is the probability that ejaculates will overlap during a mating, while sperm competition intensity is the extent to which ejaculates overlap when sperm competition occurs. The risk of sperm competition is expected to vary with the spatial and temporal distribution of fertilisations. When sperm compete to reach an egg, the morphology of spermatozoa, not just sperm numbers are of importance in sperm competition. Morphological adaptations such as larger mitochondria, which may confer greater sperm longevity, or a longer flagellum, which may increase sperm swimming speed, can make some spermatozoa more effective in sperm competition than others.

Bitterling are fishes that lay their eggs on the gills of living freshwater mussels, which makes them ideal for investigating sperm quality and the effects of sperm competition intensity on male mating behaviour. In aquarium experiments the intensity of sperm competition was manipulated to investigate the response of guarder males in terms of ejaculate frequency, and separately tested both sneaker and guarder male sperm quality and ejaculate size. It was shown that *Rhodeus amarus* (a species with a high temporal clustering of fertilisations) was sensitive to the intensity of sperm competition and modulated ejaculation rate in response to the intensity of sperm competition. It was also demonstrated that the most effective time to ejaculate was 30 seconds before female oviposition and that, as anticipated due to the opportunistic

mating tactics seen in bitterling, sperm quality and ejaculate size does not vary between male mating tactics.

INTRODUCTION

In species in which males guard females, or resources critical for female reproduction (such as sites for oviposition), a small number of males are sometimes able to monopolise matings. For example, male southern elephant seals (*Mirounga leonina*) guard harems of females, with the largest and most aggressive males fathering a disproportionate number of offspring (Hoelzel et al. 1999; Carlini et al. 2002). A consequence is high variance in the reproductive success of males (Jones et al. 2001). The principal factors that drive variance in male reproductive success are the spatial and temporal distribution of fertilisations (Emlen and Oring, 1979; reviewed by Shuster and Wade, 2003) and male density (reviewed by Kokko and Rankin, 2006). When fertilisations are spatially clustered, some males are able to monopolise matings and variance in reproductive success among males is predicted to be high. Conversely, when fertilisations are spatially dispersed variance among males tends to be low. Temporal clustering of fertilisations produces the opposite effect; if most potential mates are receptive simultaneously the opportunity for a small number of males to monopolise matings is low. Consequently, temporal clustering of fertilisations tends to result in low variance in reproductive success among males. Alternatively, if matings are distributed over a longer period, a few dominant males may monopolise matings, leading to an increased variance in male reproductive success (Shuster and Wade, 2003).

Because the temporal and spatial distribution of fertilisations are major determinants of variance in male reproductive success, these processes also drive the evolution of alternative male mating tactics (Møller, 1998), which are associated with sperm competition (Parker, 1998). A common form of alternative mating tactic is

sneaking, where males attempt to mate with a female courted by another male and thereby avoid the cost of courtship. In mating systems where sneaking is common, the risk of sperm competition will be high; the ejaculates of two or more males have a high probability of overlap during a mating. Thus a high risk of sperm competition results in the evolution of sexually selected male adaptations, including adaptations in testis and ejaculate size, as well as sperm quality (Briskie, 1993), sperm morphology (Snook, 2005), and behavioural adaptations for sperm competition avoidance (Smith et al. 2002). The relationship between male adaptations and risk of sperm competition across taxa is reviewed by Birkhead and Møller (1998).

Alternative mating tactics are common among teleost fishes (Petersen and Warner, 1998). For example, in sockeye salmon (*Oncorhynchus nerka*) males are either large territory holders or precocially mature 'jacks'. Jacks mate solely by sneaking and show behavioural and morphological adaptations for this mating strategy (Foote et al. 1997). In the bluegill sunfish (*Lepomis macrochirus*) males are characterised by three alternative mating tactics. Parental males dig nests, court females and provide care to the eggs and embryos, 'sneakers' dart in and out of nests, ejaculating between spawning pairs, while 'satellites' mimic females. Sneakers in this species are believed to produce relatively larger ejaculates than parental males, though parental male sperm has greater longevity and, independently of sperm number, appears more successful at fertilisation (Neff et al. 2003). In addition, in many fishes males act as sneakers opportunistically, for example in threespine sticklebacks (*Gasterosteus aculeatus*) (Wootton, 1976), rainbow darters (*Etheostoma caeruleum*) (Fuller, 1998) and European bitterling (*Rhodeus amarus*) (Smith et al. 2004).

Sperm competition theory predicts that for a given species, ejaculate expenditure will correlate positively with the risk of sperm competition (Parker, 1982). However, sperm competition intensity (the number of competing ejaculates at a given mating), is predicted to correlate negatively with ejaculate expenditure (Parker et al. 1996). When there are no competitors at a mating, a male is predicted to release only enough sperm to achieve fertilisation. With a single rival, males are predicted to increase ejaculate expenditure, but as the number of rival males increases above two the ejaculate expenditure of individuals is predicted to decline as the probability of fertilisation success is reduced (Ball and Parker, 1998).

While sperm competition may influence male reproductive behaviour, it may also have a selective influence on sperm morphology and sperm quality (Stockley et al. 1997). For fishes there are two models, predicting the effect of sperm competition on sperm allocation and sperm morphology; the instantaneous fertilization model where internal fertilization occurs at one particular instant usually some time after mating, and the continuous fertilization model for most externally fertilizing fish species, where fertilization occurs in a continuous fashion immediately after mating (Parker 1993; Balshine 2001). In the continuous fertilization model, sperm size is predicted to increase with sperm competition intensity (Ball and Parker 1996) as males release sperm simultaneously initiating a race for the available ova. Therefore, as sperm competition intensity increases, males should increase both sperm number and sperm swimming speed to maximize the number of collisions between sperm and eggs.

Sperm morphology is inherently linked to sperm quality; where sperm competition selects for increased sperm size, competition selects for increased sperm quality specific to the type of competition (Byrne et al. 2003). For example, where

swimming speed is an important aspect of sperm quality, tail length is expected to be selected to increase (Parker 1993; Ball and Parker 1997, 1998; Stockley et al. 1997). Similarly, where longevity is an important factor in fertilisation success (most commonly in internally fertilising species) the mitochondrial section of spermatozoa may be enlarged to provide extra energy supplies (Stockley et al. 1997). Adaptations in spermatozoa morphology usually increase the cost of individual spermatozoa production, and either increase the overall cost of spermatogenesis or, if sperm are produced with a limited energy budget, reduce the numerical size of ejaculates (Stockley et al. 1997).

The cost of morphological adaptation in spermatozoa is not limited purely to the energetic cost of spermatogenesis. For example adaptations to increase the ability of sperm to affix themselves to the egg or increase the likelihood of sperm and egg meeting, such as swimming in a spiralling motion may reduce sperm speed and in turn significantly reduce the actual distance travelled by sperm.

Here I use the European bitterling fish, *Rhodeus amarus*, to investigate the effect of sperm competition intensity on male mating tactics, ejaculate expenditure and sperm quality between male mating tactics. Bitterling fishes use living freshwater mussels as spawning sites. During the spawning season, males aggressively defend territories around mussels (Smith et al. 2003, 2004; Reichard et al. 2005). Females develop long ovipositors and deposit their eggs on the gills of a mussel, through the exhalent siphon. Males fertilise the eggs by releasing sperm over the inhalant siphon. The reproductive biology of *R. amarus* is reviewed by Smith et al. (2004). Because bitterling use a discrete spawning site that can easily be manipulated and assessed for quality they offer a valuable model for understanding male and female mating decisions and behavioural and morphological adaptations to the risk and intensity of

sperm competition. Bitterling adapt well to aquarium conditions and produce large quantities of sperm that can be readily stripped for analysis.

In this study I measured the responses of guarding males to variation in the intensity of sperm competition and compared guarder and sneaker male sperm quality and ejaculate size. Guarder male responses were measured in terms of ejaculate expenditure (rate of ejaculation and size of ejaculates) and sperm quality (sperm swimming speed and motility), which was measured using the Hobson sperm tracker, with analysis conducted using CASA (Computer Assisted Sperm Analysis). It was predicted that males would reduce ejaculate expenditure in the presence of several rivals as predicted by the model of Parker et al. (1996). It was also predicted that ejaculate size would vary as a function of male size and mating tactic. Sneaker males, which tend to be smaller than guarders, face a higher risk of sperm competition than guarder males and were predicted to produce larger ejaculates of a higher sperm quality. I also aimed to estimate sperm concentrations within the mantle cavity of mussels to infer optimal tactics for males faced with sperm competition.

METHODS

R. amarus used in the study were collected from the River Kyjovka in the southeast of the Czech Republic. Fish were transported in river water to the Institute of Vertebrate Biology in Brno and stored in a garden pond. *Unio tumidus* mussels were collected at the start of the bitterling spawning season by hand from an oxbow lake close to the R. Kyjovka, transported to Brno and stored in a second pond. *U. tumidus* are widespread and common in central Europe and commonly used by *R. amarus* for spawning (Smith et al. 2000b). Experimental work with *R. amarus* was conducted

during May 2005 in the aquarium facility at the Institute of Vertebrate Biology in aquaria measuring 60 cm (length) x 40 cm (width) x 40 cm (depth). During experiments, fish were exposed to a natural light: dark cycle.

Intensity of Sperm Competition and Ejaculation Frequency

I investigated the effect of the intensity of sperm competition on guarder male behaviour by manipulating the number of rivals to which guarder males were exposed. For *R. amarus*, a single *U. tumidus* (mean length 103.1, SD 2.41 mm), was placed in a small sand-filled flower pot in the centre of an experimental aquarium. A square-sided glass jar measuring 12 cm (width) x 12 cm (depth) x 22 cm (height) was placed 24 cm from the mussel. A single male and a female with an extended ovipositor were placed in the aquarium. The fish were allowed to settle for at least one hour before observations began. During this time the mussel was covered with a perforated plastic cup to prevent spawning, but allow the fish to see and smell it. Males were sequentially exposed to two treatments; sneakers or no sneakers, with the order of treatment selected randomly. In the sneaker treatment two rival males were placed in the glass jar; in the no sneaker treatment the jar was empty. Behavioural observations during trials, where the guarder was seen to display to and attack the confined male, confirmed that guarding males still recognised the captive males as potential sneakers. Trials were also used to confirm the optimum position of the glass jar, so that guarding males courted females rather than focusing all their attention on driving off the captive sneakers.

After one hour the mussel was uncovered and the number of ejaculations by the experimental male recorded by an observer for 20 min. After 20 min the mussel was covered and rival males either added or removed from the jar. Fish were allowed

to settle for a minimum of 30 min before a second 20-min observation took place. After observations were complete all fish were removed, their Standard Length (length from the tip of the snout to the base of the tail) measured to the nearest 1 mm and none were used in the experiment again. During trials males placed in the glass jar did not show a response to guarding males, but displayed low level courtship behaviour to females by extending their fins. Sneakers swam naturally and did not show signs of distress during the short period they were confined. After the experiment was completed all fish were released back into the River Kyjovka. A total of 11 paired replicates were completed.

Estimating Ejaculate Size

An experiment was conducted to estimate the change in sperm density over time within the mantle cavity of mussels, following ejaculation over the inhalant siphon by male bitterling. This study was conducted to enable estimates of ejaculate size to be made.

Using a sharp file a 2 mm long, 1 mm wide hole was carefully made in the shell of a *U. tumidus* mussel. A BD Venflon winged cannula was inserted into the hole and the needle removed from the cannula sheath. The tip of the cannula was gently positioned so that it rested inside the mantle cavity 5 mm from the inhalant siphon. The cannula wings were glued to the mussel shell to hold it in position. Approximately 1 m of 5 mm diameter plastic tubing was fixed to the cannula and a syringe fitted to the other end (see Appendix 2a). No detrimental effect of fitting cannulas to mussels was observed; mussels continued to filter normally and mussel survival was 100%. Cannulas were fitted to 16 (mean length 103.1, SD 2.41 mm) *U. tumidus* mussels which were then randomly selected from a storage tank for each

experiment. No mussels containing glochidia larvae in their outer gills were used, as glochidia may affect mussel filtration (Tankersly and Dimock, 1993). After completion of experiments the cannulas were removed.

To make estimates of sperm density a single male *R. amarus* was housed in an experimental aquarium measuring 60 cm (length) x 40 cm (width) x 40 cm (depth) with a *U. tumidus* to elicit territorial behaviour. A female with an extended ovipositor was gently released in the experimental aquarium and a randomly chosen mussel with a cannula fitted was used to replace the original mussel. Males were allowed to ejaculate once over the inhalant siphon of the experimental mussel before a water sample was collected from the mantle cavity using the cannula after a pre-determined time interval. Intervals were: 0, 10, 30, 60, 120 or 240 seconds following ejaculation by the male. Water samples were collected by drawing 0.3 ml from within the mussel mantle cavity using the syringe. The plastic tubing was detached at the junction with the cannula and the 0.3 ml sample was released into a 1.0 ml Eppendorf. The sample was pipetted onto a haemocytometer and a count made of the number of spermatozoa in the water sample. For each sample, three counts were made, from which the mean density of spermatozoa in the mantle cavity was estimated. Estimates of ejaculate size were made for 10 males with a single mussel each. A record was made of fish standard length and mussel length. Fish and mussels were used only once during the experiment. Females did not spawn in mussels during trials; all ejaculations were pre-oviposition.

Intensity of Sperm Competition and Ejaculate Size

I investigated ejaculate size under two different intensities of sperm competition, controlled by varying the number of rival males to which a guarding male was

exposed. Experiments with *R. amarus* were conducted during May 2005. A total of 14 *U. tumidus* (mean length 100.2, SD 2.2 mm) were fitted with cannulas in the way described above. Male *R. amarus* used in the experiment (mean SL 41.07, SD 3.96) were captured by a diver from the captive River Kyjovka population held at the Institute of Vertebrate Biology in Brno.

The experimental protocol followed that in section (a), except that males were presented with mussels fitted with cannulas and, directly after the first ejaculation the male was disturbed by the observer to prevent further ejaculations. A water sample was taken after 30 seconds and the density of sperm estimated from the water sample; from part (b) it had been established that spermatozoa density reached a peak in the mussel mantle cavity after this period. To avoid the possibility of males becoming sperm limited a minimum of 30 minutes was left between the first and second sperm competition treatment.

After finishing a replicate all fish (SL and weight) and mussels (maximum posterior – anterior shell length) were measured.

Computer-Assisted Analysis of Sperm Quality

A total of 24 males were examined, 12 guarder's and 12 sneakers, the tactics of which were determined during behavioural experiments. Fish were humanely killed and testes were immediately extracted and one testis added to 20 μl of water and disrupted to release sperm into the water; the remaining testis was left within the body cavity. 1 μl of the sperm/water mixture was immediately (within 15 s) added to a 12-well multitest slide (ICN, Basingstoke, UK) for tracking. All samples were analysed at room temperature, and the microscope stage allowed to cool for 10 minutes between each pair of samples. Sperm were video-taped for 4 minutes using

an Olympus BH-2 microscope with a ×40 objective (negative-high phase contrast) linked to a Sony Hyper HAD black and white video camera with a charge-coupled diode iris via an MTV-3 adaptor.

After completion of videoing the procedure was repeated using the second testis. Video tapes were analysed using CASA (version 7V2B) in conjunction with the Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, Derbyshire, UK). CASA settings were optimised for bitterling sperm, and sperm from each sample were tracked for 22, 15 second intervals. The following sperm motility variables were examined: VCL: curvilinear velocity (μms−1), the sum of incremental distances moved in each frame divided by total track time; VSL: straight line velocity (μms−1), the straight line distance between the start and end points of the track, divided by track time; BCF: beat cross frequency (Hz), the frequency with which the actual path crosses the smoothed path; ALH: amplitude of lateral head displacement (μm), the average deviation in the smoothed path, derived from the difference in linearity between the smoothed and sampled paths; LIN: linearity (%), the straight line distance between the start and end points of the track, divided by the sum of the incremental distances along the actual path. Using the CASA data sperm longevity was also calculated by simply recording the times that sperm ceased to be moving and then comparing data between mating tactics.

Data Analyses

All data were tested for normality using a Kolmogorov-Smirnov test. Ejaculations are presented as a rate per 10 min. While sperm densities are presented per ml^3. A paired *t*-test was used to test the effect of treatment on ejaculation rate and a 1-way ANOVA with a Tukey's HSD *post hoc* test to test the effect of time on sperm densities within

the mussel mantle cavity after ejaculation. Paired *t*-tests were used to compare ejaculate size between treatments. Two sample *t*-tests were used to compare sperm quality parameters between mating tactics. Statistical analyses were conducted using MINITAB 14.

RESULTS

Ejaculation Frequency and Ejaculate Size

Male *R. amarus* showed a significantly reduced ejaculation frequency in the presence of sneakers (paired *t*-test, $\log_{10}$ transformed data, $t_{10} = 2.24$, $P = 0.049$; Fig 1).

There was a significant effect of time after ejaculation on the density of *R. amarus* sperm in the mantle cavity of mussels (1-way ANOVA, $F_{5,54} = 25.21$, $P < 0.001$). A Tukey's HSD *post hoc* test showed that the density of sperm increased quickly following ejaculation, with a significant peak after 30 seconds, and a slow but statistically significant decline between 60 and 240 seconds (Fig. 2). I detected no significant effect of sperm competition intensity on ejaculate size in *R. amarus* (paired *t*-test, $t_{10} = 1.52$, $P = 0.145$).

There was no significant correlation between male body length and ejaculate size in *R. amarus* (Pearson's correlation, $r_{25} = 0.140$, $P = 0.496$).

Sperm Quality

No significant difference between guarding males and sneaker males was seen in VCL (two sample *t*-test, $t_{10} = 1.03$, $P = 0.318$), VSL ($t_{10} = 1.31$, $P = 0.211$), BCF ($t_{10} = 0.96$, $P = 0.346$), ALH ($t_{10} = 0.26$, $P = 0.802$), LIN ($t_{10} = 1.27$, $P = 0.224$) or

activity (t_{10} = 0.94, P = 0.370) and no significant difference in sperm longevity between mating tactics (t_{10} = 0.08, P = 0.934).

DISCUSSION

I experimentally manipulated the intensity of sperm competition to investigate its effect on male ejaculate expenditure, measured as ejaculate frequency and ejaculate size, in the European bitterling. I also measured the change in sperm concentration in the mussel mantle cavity and compared the sperm quality characteristics of guarder and sneaker males.

I showed that the rate of ejaculation decreased significantly with an increased intensity of sperm competition in *R. amarus*, but there was no significant effect of exposure to sneakers on ejaculate size. I observed the pattern of sperm concentrations within the mussel mantle cavity after ejaculation and established the peak of sperm concentration in the gill cavity to be at approximately 30 s after ejaculation. I also established that there was no difference in ejaculate size between males adopting guarder and sneaker roles. There were no significant differences between guarder and sneaker males in any parameter related to sperm quality.

In *R. amarus* ejaculation rate decreased in response to an increase in the intensity of sperm competition. Sperm competition risk is relatively high in *R. amarus*, a result of high temporal clustering of females over a short spawning season (Smith et al. 2003). Consequently, male *R. amarus* may be sensitive to the intensity of sperm competition; males may modulate ejaculate expenditure in accordance with the predictions of theoretical models (Parker et al. 1996). Guarder males are predicted to reduce their investment on ejaculate expenditure as the guarantee of paternity

diminishes. The benefit (paternity gain) per unit of energy expended on ejaculates will decrease as rival male number increases, assuming the number of competing ejaculates increases alongside rival male number (Parker, 1998). Alternatively, *R. amarus* may reduce ejaculation rates when rivals are present as males may use ejaculatory behaviour as a visual cue that a spawning has occurred (Smith et al. 2003); males under threat of sperm competition may constrain ejaculation rates if ejaculation is used as a visual cue by other males to attempt to sneak fertilisations (Le Comber et al. 2003; Spence and Smith 2005).

I found no evidence that male *R. amarus* vary ejaculate size in response to the intensity of sperm competition. Candolin and Reynolds (2002) in their study of ejaculate frequency also attempted to quantify ejaculate size by measuring the size of sperm clouds over a mussel when male bitterling ejaculated. Using video footage, they measured the size of ejaculates of different males under low and high sperm competition risk. Here I used a more accurate technique that measured sperm density inside the mantle cavity of the mussel, close to where fertilisation of ova takes place. The current results confirm Candolin and Reynolds (2002) speculative conclusion that ejaculate size of *R. amarus* males does not vary as a function of sperm competition intensity.

Results for *R. amarus* showed that once males ejaculated over the inhalant siphon of a mussel, sperm density in the mantle cavity peaked after 30 s (Fig. 2). This time lag has implications for both sneaker and guarder mating tactics, since it implies that sperm released immediately before oviposition may have precedence in fertilising eggs. Pre-oviposition ejaculation is common in *R. amarus* (Candolin and Reynolds, 2002; Smith et al. 2002, 2003; Reichard et al. 2004a) and there is evidence

that pre-oviposition ejaculates do have a higher probability of fertilising ova than post-oviposition ejaculates (Reichard et al. 2004b).

Our results also demonstrated that sperm remains within the mussel mantle cavity for an extended period (>40 sperm mm^{-3} after 240 s) following ejaculation (Fig. 2). This result is supported by a paternity study by Reichard et al. (2004a) who showed that an ejaculation by a male can fertilise eggs at least 14 minutes after ejaculation, considerably longer than has formerly been thought (Kanoh, 1996). These results show that males need not be present at a spawning to achieve fertilisations, though the probability of fertilisation success may be directly related to the number of sperm in the mussel gill cavity at oviposition (Reichard et al. 2004b). In addition, I did not test the viability or motility of sperm remaining in the mussel gill cavity, and these will play a vital role in determining fertilisation success.

As shown earlier in this study, and also by Reichard et al. (2004) who showed that sperm released 14 minutes before spawning were successful in fertilising ova, sperm remain within the mussel for prolonged periods. Sperm also have to move significant distances from the initial point of ejaculation to the ova. Therefore, with both swimming speed and longevity important factors in determining fertilisation success, I anticipated finding differences in sperm quality between sneaker and guarder males. Sneakers often release pre-oviposition ejaculates and, therefore, I would expect them to have sperm with an increased overall longevity. In contrast, guarding males normally release ejaculates at spawning, or just after and their sperm might have an enhanced swimming speed so that they would rapidly reach the ova. However, I found no significant difference between the spermatozoa of males displaying either tactic, based on any measure of sperm swimming speed, or sperm longevity. The lack of any differences between guarder and sneaker males probably

reflects the fact that mating tactics are opportunistic in European bitterling and usually linked to changes over the ontogenetic gradient, rather than specific male morphs (Foote et al. 1997; Neff et al. 2003).

Our data showing that there was no difference in ejaculate size between large and small males suggests that smaller male *R. amarus* produced relatively (when bodyweight is considered), but not absolutely, larger ejaculates than larger males. This hypothesis is consistent with sperm competition theory, which predicts that smaller males should invest more in sperm production as they face a higher risk of sperm competition than larger males (Parker, 1990). Larger males, which may be able to attract females through courtship or to exclude other males through aggression, may face a lower risk of sperm competition and may be selected to invest resources into growth or ornamentation (Taborsky, 1994). The testes of small males might be either disproportionately large, as in *S. salar* (Gage et al. 1995) and *L. macrochirus* (Gross, 1982) in which sneaker males have a relative testis weight nearly twice that of dominant/territorial males. Alternatively, as data on bitterling testis morphology suggests (chapter 5), there may be morphological adaptations within the testes that allow males to control ejaculate size. In the bluehead wrasse (*Thalassoma bifasciatum*) males regulate the amount of sperm released between spawnings and there is a difference in the sperm allocations of territorial males and smaller, group-spawning males. In this species the posterior section of the sperm duct is divided, by connective tissue, into numerous small chambers. Ejaculation is controlled by muscle attached to the anal fin that encircles the chambers. Contraction of this muscle during ejaculation closes some of the chambers and may permit control of ejaculate size (Shapiro et al. 1994; Rasotto and Shapiro, 1998).

In conclusion, I found that there was no difference in the size or quality of ejaculates produced by sneaker or territorial males, and that while ejaculate size was unaffected by sperm competition intensity, ejaculation rates in *R. amarus* decreased significantly with an increased intensity of sperm competition. Following ejaculation, the density of sperm in the mantle cavity of a mussel increases rapidly, peaking after 30 s, and then slowly declining.

Figures

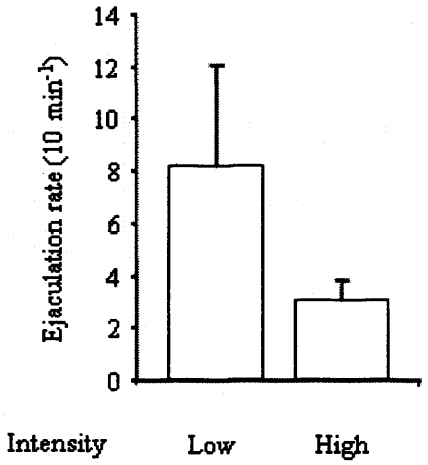

Figure 1. Mean rate of ejaculation (+1 SE) of *R. amarus* (RA) exposed to two levels of sperm competition intensity (Low – 0 rivals; High – 2 rivals).

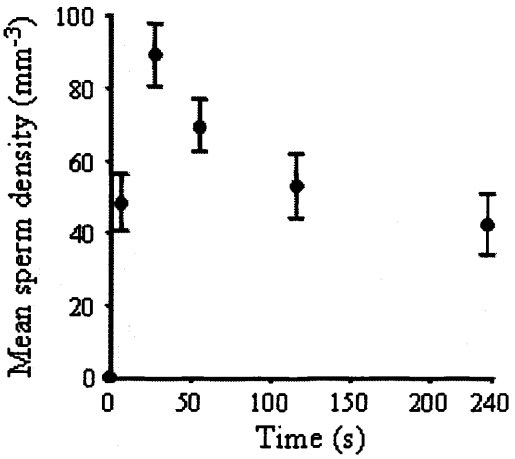

Figure 2. Mean density in mm^{-3} (±1 SE) of *R. amarus* spermatozoa in the mantle cavity of a freshwater mussel as a function of time after ejaculation.

Chapter 3: Male sperm depletion and female sperm limitation in the European bitterling (*Rhodeus amarus*).

Abstract

Sperm depletion occurs when males are unable to maintain ejaculate size over successive ejaculations, while female sperm limitation occurs when females receive insufficient spermatozoa to fertilize all their eggs. Sperm depletion in males and sperm limitation in females appears to be widespread across multiple taxa. I used the European bitterling (*Rhodeus amarus*), a fish that lays its eggs in the gills of freshwater mussels, to experimentally investigate sperm depletion over different temporal scales at different levels of sperm competition intensity. I also investigated deceptive behavioural signals that may reduce the risk of sperm depletion and sperm limitation by males and females respectively. I showed that the spermatozoa density of the ejaculates of guarding males declined over five consecutive ejaculations, but was significantly higher if the male was exposed to a rival, suggesting males were sensitive to sperm competition intensity and modulated ejaculate size in accordance with theoretical models. However, following successive matings over the course of a day guarder males exposed to rivals became significantly more sperm depleted than males exposed to low sperm competition intensity. During spawning males performed "false" ejaculations; ejaculations containing unusually low numbers of spermatozoa. The frequency of false ejaculations was significantly lower when guarder males were exposed to rivals. I also measured a significant effect of the number of rival males on female "skimming", a behaviour that may increase the probability of sperm competition thereby enhancing female fertility. I discuss the results with reference to

a possible intersexual conflict over the size and distribution of ejaculates in the context of male sperm depletion and female sperm limitation.

INTRODUCTION

In species in which females mate promiscuously the ejaculates of males may compete simultaneously to fertilize the same set of ova, a process termed sperm competition. Sperm competition is now recognized as an important mechanism of sexual selection that has shaped the evolution of animal mating systems (Andersson 1994; Eberhard 1996; Parker 1990, 1998; Simmons 2001). Where the probability of female promiscuity is high (a high risk of sperm competition) males often show adaptations to sperm competition, including adaptations of testis size and structure (Byrne et al. 2002), ejaculate size (Briskie 1993), sperm morphology (Snook 2005) and behavioural adaptations for sperm competition avoidance (Smith et al. 2002). Because ejaculates can be physiologically expensive to produce (Van Voorhies 1992; Olsson et al. 1997; Wedell et al. 2002), males are predicted to modulate ejaculate expenditure in relation to sperm competition. Thus, males should increase their ejaculation expenditure when competing with a single rival, but reduce expenditure if the intensity of sperm competition (the extent of overlap of competing male ejaculates) is high, since the probability of fertilizing eggs diminishes with the number of competing males that attempt to fertilize the same set of ova (Parker et al. 1996). Males from a range of taxa appear highly sensitive to sperm competition and tailor ejaculate expenditure to local conditions and thereby, presumably, maximize their long-term reproductive success (Gage 1991; Pilastro et al. 2002; Pizzari et al. 2003; Harris and Moore 2005; delBarco-Trillo and Ferkin 2006).

When males increase sperm expenditure in response to sperm competition (Gage 1991), gain access to multiple matings through dominance or female mate choice (Preston et al. 2001; Carlini et al. 2002), or have a finite quantity of sperm

(Damiens and Boivin 2006), sperm depletion as well as sperm competition may play a substantial role in determining paternity success (Hoelzel et al. 1999; Preston et al. 2001; Wedell et al. 2002). Sperm depletion occurs when a male cannot produce enough sperm to maintain ejaculate size in successive ejaculates, and is thought to be common in many taxa (Birkhead and Møller 1998; Birkhead and Fletcher 1995; Wedell et al. 2002).

Sperm depletion, or prudent control of ejaculate size by males, can have consequences for female reproductive success, possibly resulting in females becoming sperm limited; i.e. females may obtain insufficient sperm to fertilize all their ova (Pitnick 1993; Royer and McNeil 1993; Stockley 1997; Rondeau and Sainte-Marie 2001). In the bluehead wrasse (*Thalassoma bifasciatum*), an externally fertilizing fish, females prefer dominant males as mates but suffer reduced fertility when mating with these males as they produce smaller ejaculates than subordinate males (Shapiro et al. 1994; Rasotto and Shapiro 1998). The same can occur in lekking species, with females risking reduced fecundity when mating with dominant males (Jones 2001; Saether et al. 2001); females appear to trade off the cost of infertility, against "good" paternal genes (Wedell et al. 2002).

Sperm limitation can lead to inter-female competition for access to males (Saether et al. 2001) and the evolution of male mate choice (Preston et al. 2005). There is also ample evidence that females solicit matings from males, and mate with multiple partners, to increase their access to sperm (Wedell et al. 2002). For example, female red-winged blackbirds (*Agelaius phoeniceus*) seek extra-pair copulations to improve their reproductive success, which may thereby circumvent sperm depletion in their mates (Gray 1997). In the European bitterling (*Rhodeus amarus*), a fish that lays its eggs in the gills of freshwater mussels, Smith and Reichard (2005)

demonstrated that females achieved higher fertilization rates when mating with multiple rather than solitary males.

I used the European bitterling to investigate the effect of sperm competition intensity on male sperm depletion over successive ejaculations during a single spawning event, and successive spawning events over a day of matings. I also investigated the behavioural responses of males and females to the risk of male sperm depletion. During the spawning season male bitterling either aggressively defend territories around mussels or perform sneaking behaviour, though males can play both roles and there are no morphological differences between territory holders and sneakers (Candolin and Reynolds 2002a, 2002b; Smith et al. 2002, 2004; Reichard et al. 2005). Females develop long ovipositors, which they use to place their eggs into the gills of a mussel. 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. Both pre and post-oviposition ejaculations are performed by male bitterling; pre-oviposition ejaculations appear to have a higher probability of fertilization success through sperm precedence (Reichard et al. 2004a). The reproductive biology of *R. amarus* is reviewed by Smith et al. (2004). European bitterling offer a valuable model for understanding mating decisions and assessing sperm competition and sperm depletion; the mean ± s.e. rate of ejaculation by a guarder male is high, at 15 ± 1.3 ejaculations per mating (Reichard et al. 2004b), they use a discrete spawning site that can be readily manipulated, and they perform unambiguous behaviours associated with spawning (Smith et al. 2004).

The response of guarder male European bitterling to different levels of sperm competition is consistent with the strategy predicted by models of sperm competition intensity (Parker et al. 1996); guarder males maximize ejaculation rate when

competing with a single rival, but reduce ejaculation rate with increasing numbers of rivals (Smith et al. 2003). Accordingly, I predicted that with a single rival present guarder males faced a higher risk of sperm depletion than males without competition from rivals.

During spawning female bitterling perform a conspicuous behaviour (termed "skimming") which entails the female making contact with the mussel siphon with the base of her ovipositor, but without inserting her ovipositor into the mussel gill or releasing eggs. Skimming behaviour is distinct from "missed" ovipositions, in which the female attempts to spawn but misses the mussel siphon and deposits her eggs on the substrate (Smith et al. 2004). Skimming appears to mimic spawning behaviour and is thought to perform the function of signaling the readiness of a female to spawn (Smith and Reichard 2005). Skimming may also serve as a cue for males to ejaculate over a mussel (Smith et al. 2004). Female bitterling are less likely to experience sperm limitation when several males participate in a mating (Smith and Reichard 2005). Therefore, I further predicted that females would increase the rate of skimming behaviour, in response to the number of males present at a spawning.

METHODS

Study Organisms

R. amarus used in experiments were caught using a small Seine net from an oxbow lake adjacent to the River Vistula near the village of Soczewka (52°32 N; 19°34 E), Central Poland. A total of 280 *R. amarus* were transported in river water to the University of Łódź and stored in indoor aquaria. *Unio tumidus* mussels were collected for experiments by hand before the start of the bitterling spawning season from the

Sulejów reservoir, Poland (51°24 N; 19°52 E), transported to the University of Łódź and stored in an outdoor pond. *U. tumidus* are widespread and common in Central Europe and are readily used by *R. amarus* for oviposition (Smith et al. 2000, 2001, 2004).

Experimental work with *R. amarus* was conducted during May 2006 in the aquarium suite of the University of Łódź. Experimental aquaria measured 60 (length) x 40 (width) x 40 cm (depth). All fish used in the study were exposed to a natural light: dark cycle and fed frozen chironomid larvae and dried fish flake food.

At the end of each experiment fish standard length (length from the tip of the snout to the base of the tail) and mussel total length (maximum shell length from anterior to posterior) were measured to the nearest 1 mm. All fish and mussels were returned to the locations from which they were collected and were used only once in experiments.

Estimating Ejaculate Size

To estimate the density of spermatozoa in ejaculates, a BD Venflon winged cannula (Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was attached to the outer surface of 14 *U. tumidus* mussels. The cannula wings were glued to the mussel shell, so that the sheath of the cannula lay along the shell, with the tip resting immediately adjacent to the inhalant siphon. A 1 m long piece of 5 mm diameter plastic tubing was fixed to each cannula and a syringe fitted to the open end. Immediately following ejaculation by a male bitterling, a sample of 0.3 ml of water was collected at the inhalant siphon of the mussel by drawing water into the plastic tubing using the syringe. The plastic tubing was detached at the junction with the cannula and the water sample was released into a 1.0 ml Eppendorf. The sample was gently mixed

then pipetted onto a haemocytometer (Neubauer improved, VWR International) and a count made of the number of spermatozoa in the sample. Trials with cannulas in different positions, using dyes to represent an ejaculate, confirmed that sampling a volume of 0.3 ml of water from just in front (within 5mm) of the inhalant siphon of the mussel accurately collected the entire ejaculate released over the mussel inhalant siphon; collecting a larger volume did not yield a higher estimate of sperm concentration. For each sample, three counts were made, from which the mean density of spermatozoa in the ejaculate was estimated and expressed as spermatozoa mm^{-3}. I observed no detrimental effects of attaching cannulas to mussels; mussels continued to filter normally and mussel survival was 100%. Mussels containing glochidial larvae in their outer gills were excluded from the study, since glochidia may affect the rate of mussel filtration (Tankersley and Dimock 1993).

Sperm Depletion over the Course of a Mating

This experiment was conducted to estimate whether the size of ejaculates by a male *R. amarus* declined over a series of consecutive ejaculations, under two treatments of sperm competition intensity.

Six cannulas, three on each side, were attached to a *U. tumidus* mussel in the way described, with the tips of each cannula resting adjacent to the mussel's inhalant siphon (see Appendix 2b). A single male and a female bitterling that was ready to spawn (with an extended ovipositor) were released into an experimental aquarium with an experimental mussel (covered with a plant pot). Two randomly assigned treatments were used; low sperm competition intensity, where the female and guarding male were alone, or high sperm competition intensity, where a single smaller rival male was also added. Rival males were haphazardly selected from a pool

of small males and were used only once. Once fish had settled (guarder male courting female and displaying to rival male, female responding to courtship; typically between 30 and 60 min), the mussel was uncovered and observations started. Water samples were collected immediately after each of the first five ejaculations by the guarding male, from randomly selected cannulas and the time interval between ejaculates recorded. Collection of ejaculates did not unduly disturb spawning fish, with the interval between consecutive ejaculations by guarding males as short as 8 s.

A total of 13 replicates of each treatment were completed. Rival males were rarely able to ejaculate over the mussel, though if they did the replicate was discarded so that estimates of ejaculate size were for guarder males only. A different mussel was used for each paired replicate. Data were analyzed using a randomized-complete-block paired ANOVA (Sokal and Rohlf 1995).

Sperm Depletion over the Course of a Day

A second experiment was conducted to investigate changes in male ejaculate size in successive matings, over the course of a day of matings.

A single winged cannula was attached to a *U. tumidus* mussel in the way described, placed in an experimental aquarium and covered with a flower pot. A second, exposed mussel with no cannula attached was placed together with a male bitterling and two females with extended ovipositors, into the same experimental aquarium. Two treatments were used; guarder male alone without a rival male, and guarder male with a rival male present. The guarder and rival male were allowed to court the females and ejaculate over the uncannulated mussel throughout the day. Two females were used to ensure that spawnings occurred over the entire day. At three periods during the day the cannulated mussel was uncovered and the

uncannulated mussel covered. The density of spermatozoa in the ejaculates of guarding males was estimated by collecting a water sample in the way described previously. After removing a water sample the cannulated test mussel was again covered and the original uncannulated mussel uncovered so that fish could continue to spawn.

Fish were randomly assigned to treatments, placed in aquaria, and presented with an uncannulated mussel at 0700 each morning that trials were conducted. The first water sample was collected in the morning (0900-1000), a second at midday (1200-1300) and the final sample in the afternoon (1500-1700). A total of 12 replicates of each treatment were completed. Data were analyzed using a balanced two-way ANOVA.

Association Between Skimming Behaviour and Intensity of Sperm Competition

This experiment was conducted to measure the effect of sperm competition intensity on the rate of female skimming behaviour.

A single female, with an extended ovipositor, and a randomly selected guarder male were released into an aquarium with a mussel as a spawning site and allowed to settle for 1 hour. Three treatments were used; the guarder male and female were either alone, with a single rival, or with three rival males. Rival males were haphazardly selected from a pool of small males and were used only once. Treatments were imposed in a pre-determined random order. Once males started courting females, and females inspected mussels, the frequency of female skimming behaviour was recorded for 8 minutes. A total of 14 replicates of each treatment (0, 1 or 3 rivals present) were completed.

Data Analyses

All data were tested for normality using a Kolmogorov-Smirnov test and for homoscedasticity using a Bartlett test. Data that deviated from normality were ranked and the rank transformation statistic calculated (Kepner and Robinson 1988). A One-way ANOVA was used to compare the sperm concentration within the mussel after consecutive ejaculations under low and high sperm competition intensity. I also used a One-way ANOVA with a Tukey's HSD *post hoc* test to compare the rate of female skimming among treatments. A paired *t*-test was used to test for a difference in the time intervals between ejaculations among treatments. A Pearson's correlation was used to test for a link between both male size and spermatozoa concentration, and rival male size and sperm concentration after ejaculation. I used a balanced ANOVA to compare sperm concentrations over the course of a day and a paired *t*-test to compare the number of false ejaculations by guarder males between treatments.

RESULTS

Sperm Depletion Over The Course Of A Mating

There was a significant difference in the density of spermatozoa in ejaculates at the inhalant siphon of experimental mussels over five consecutive ejaculations (randomized-complete-block paired ANOVA, ranked data: $F_{4,108} = 10.27$; $P < 0.001$); with the first ejaculation always larger than the last (Fig. 1). There was also a significant difference between low and high sperm competition intensity treatments ($F_{1,108} = 11.01$; $P = 0.001$; Fig. 1), mean guarder male spermatozoa density was greater when a rival was present than when males were alone with a female. There was no significant interaction between treatment and ejaculation order ($F_{4,108} = 0.52$;

P = 0.719); the effect of sperm competition intensity was comparable among ejaculations.

There was no significant difference in the mean interval among ejaculations between treatments (paired t-test; $t_{10} = 0.59$; $P = 0.569$; mean ± s.e. = 34 ± 4.6 s), nor was there a significant correlation between guarder male length and spermatozoa density in ejaculates (Pearson's correlation; $r_{14} = 0.239$, $P = 0.296$), or rival male length and spermatozoa density of guarder male ejaculates (Pearson's correlation; $r_{14} = 0.366$, $P = 0.219$).

Sperm Depletion Over The Course Of A Day

There was a significant difference in the density of spermatozoa in the ejaculates of guarder males among the three time periods (balanced ANOVA, ranked data; $F_{2,66} = 14.24$, $P < 0.001$). Mean spermatozoa density was higher in the morning (mean ± s.e. = 293.95 ± 37.12 spermatozoa mm^{-3}) than in the afternoon (mean ± s.e. = 133.61 ± 11.65 spermatozoa mm^{-3}). There was no significant difference between treatments ($F_{1,66} = 1.78$, $P = 0.187$; Fig. 2), though there was a significant interaction between treatment and time of day ($F_{2,66} = 5.49$, $P = 0.006$); the change in density of spermatozoa was significantly greater when guarder males were exposed to a rival (Fig. 2).

The Effect Of Sperm Competition On The Frequency Of "False" Ejaculations

During experiments on sperm depletion over a mating and over a day it was observed that, although males performed ejaculatory behaviour, the spermatozoa density of ejaculates was sometimes either zero or unusually low (< 60 spermatozoa mm^{-3}). These ejaculations were termed "false" ejaculations since they were substantially

lower than the average spermatozoa density detected after "normal" male ejaculations (mean ± s.e. = 200 ± 8.88 spermatozoa mm^{-3}), resulting in a bimodal distribution of spermatozoa densities in ejaculates (Fig. 3). For the first experiment, which tested sperm depletion over the course of a mating, I compared the frequency of false ejaculations between the high and low sperm competition intensity treatments. I detected a significant difference between treatments (paired *t*-test, $\log_{10}$ transformation; $t_{13} = 3.84$, $P = 0.002$; Fig. 4), with false ejaculations significantly less frequent when a rival was present than when a guarder male was alone with a female. Note that "false" ejaculations were not excluded *a posteriori* from data analyses to investigate sperm depletion over a mating and over a day.

Association Between Skimming Behaviour And Intensity Of Sperm Competition

There was a significant difference between the rate of female skimming behaviour among sperm competition intensity treatments (one-way ANOVA, $\log_{10}$ transformation; $F_{2,50} = 3.66$, $P = 0.033$; Fig. 5). A Tukey's HSD *post hoc* test showed that females skimmed at a significantly higher rate when three rivals were present compared to the treatment where no rivals were present. There were no differences between the remaining treatments.

DISCUSSION

When males mate with a succession of females they run the risk of sperm depletion. A consequence for females of male sperm depletion is that they may receive insufficient sperm during mating to fertilize all their eggs (Wedell et al. 2002). In this study I investigated the consequences for male European bitterling, of successive

ejaculations during a spawning, and successive spawnings over the course of a day, at two levels of sperm competition intensity, measured in terms of the density of spermatozoa in ejaculates. In addition, I measured the effects of sperm competition intensity on female "skimming" behaviour, which is believed to serve as a signal to males that a female is about to spawn (Smith and Reichard 2005). Finally, I also identified "false" ejaculatory behaviour by males; males performed ejaculatory movements, but either failed to release spermatozoa or released unusually low numbers, and I investigated the effect of sperm competition intensity on the frequency of this behaviour.

I showed that over five consecutive ejaculations the density of spermatozoa in ejaculates declined significantly. I also detected a significant effect of sperm competition intensity; spermatozoa densities were significantly higher overall when males were exposed to a potential rival. In a second experiment I detected a decline in spermatozoa density over the course of a day of continuous spawning. In this case I detected a significant interaction between temporal and sperm competition intensity effects; although males exposed to rivals initially produced the highest density of spermatozoa in ejaculates, over a day males experienced a significantly greater decline in spermatozoa densities than males that were not exposed to rivals.

Two processes could account for these results. The first is that male European bitterling appear able to modulate the size of ejaculates when confronted by a rival male; at least in the short term. Previous studies have demonstrated an increase in ejaculation rate in the face of increased sperm competition intensity (Candolin and Reynolds 2002a; Smith et al. 2002, 2003). Candolin and Reynolds (2002a) also attempted to measure the size of ejaculates of European bitterling but failed to detect an effect of sperm competition intensity. They used stills from video recordings of

male ejaculations and estimated the size of sperm clouds. However, this method may not have been sufficiently precise to detect an effect, and is not as accurate as direct sperm counts (Candolin and Reynolds 2002a).

The mechanism by which male bitterling could exert control over ejaculate size may be similar to that in the bluehead wrasse, *T. bifasciatum*. In this species the posterior section of the sperm duct is divided by connective tissue into numerous small chambers and ejaculate size is under muscular control (Shapiro et al. 1994; Rasotto and Shapiro 1998). On the basis of pilot studies analogous structures appear to be present in the testes of European bitterling (Pateman-Jones, unpublished data), and ongoing research will address this question (chapter 7).

The results of our second experiment suggest that males may be unable to sustain elevated spermatozoa densities in ejaculates over the course of a day and risk sperm depletion; this appears more likely when in competition with a rival. This result has consequences for the mating tactics of guarder males and females. A guarder male bitterling under natural conditions faces wide variation in the number of rivals at a mating; from solitary mating with a female, to competition with a single rival, to occasions when groups of 60 or more males may attempt to participate in a spawning (Smith et al. 2000, 2001, 2002, 2003, 2004). If guarder males tailor ejaculate size to the circumstances of each spawning in which they participate they may be able to achieve a higher reproductive success than a fixed ejaculate size. On occasion, for example in the absence of rivals, this may entail a male conserving sperm at a cost of failing to fertilize an entire clutch of eggs (Smith and Reichard 2005). For females, attempts by guarding males to conserve sperm that result in some of their eggs going unfertilized may prove a substantial cost. Consequently, the interests of guarder males and females may not coincide; with the optimal tactic for

males being one of prudent sperm allocation among females, while that for females is for more lavish sperm expenditure, by multiple males, that ensures fertilization.

An outcome of this potential conflict in bitterling appears to be the evolution of "deceptive" signaling by both sexes (Semple and McComb 1996; Searcy and Nowicki 2005). Female bitterling engage in what I have termed "skimming" behaviour, which closely mimics spawning (Smith et al. 2001, 2004; Smith and Reichard 2005). This behaviour appears to attract the attention of males (Smith and Reichard, unpublished data) and elicits ejaculation (Smith et al. 2004). The adaptive value of this behaviour appears to arise through ensuring males load a mussel gill with sperm before the eggs are released and, thereby, maximizing their chance of fertilization. In the present study I demonstrated a significant effect of the number of participants at a mating and the frequency of skimming by females. Females also engage in skimming at a significantly higher rate when spawning with a guarding male over mussels situated close to a rival (Smith and Reichard 2005). Guarding males similarly engage in what I have termed "false" ejaculations; the performance of ejaculatory behaviour, but with the release of very low numbers of spermatozoa or none at all. Performing "false" ejaculations may serve to elicit spawning by females, but has an adaptive value in conserving male sperm reserves. However, this behaviour loses its adaptive value in the face of sperm competition, and I detected a significantly lower frequency of false ejaculations by guarder males when a male rival was present at a spawning.

A further consideration arising from our results is in the context of mating tactics adopted by sneaker males to guarder male sperm depletion. Sneakers themselves face the risk of sperm depletion. However, sneakers show greater flexibility in the spatial distribution of their ejaculates (Zięba, unpublished data) and

may also vary them temporally. For example, our results suggest that sneaking may be more successful for males that attempt to parasitize the matings of guarding males in the late afternoon than in the morning. Future work will explore sneaker tactics in the context of sperm depletion and the rate of sperm replenishment.

The density of spermatozoa in the ejaculates of guarder male *R. amarus* declined significantly over consecutive ejaculations, but experiments showed that males were sensitive to sperm competition intensity and released ejaculates with a higher density of spermatozoa when competing with a rival. Over a day of matings guarder males also showed a temporal decline in the density of spermatozoa in ejaculates, but particularly so when competing with a rival, suggesting sperm depletion. It was also demonstrated that males may perform deceptive behaviour by performing "false" ejaculations, a tactic that may enable males to conserve sperm. Finally, I found a significant effect of the number of rival males on female "skimming" behaviour, which may increase the probability of sperm competition and thereby female fertility.

Figures

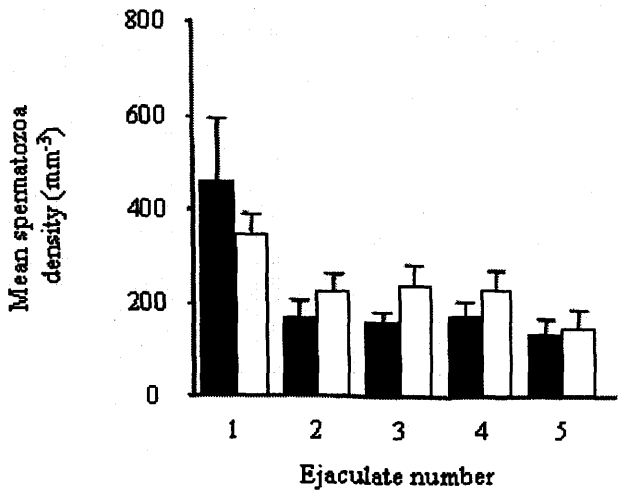

Figure 1. Mean (+standard error; SE) density of spermatozoa in five consecutive ejaculates of territorial male *R. amarus* at high (white bars) and low (black bars) levels of sperm competition intensity.

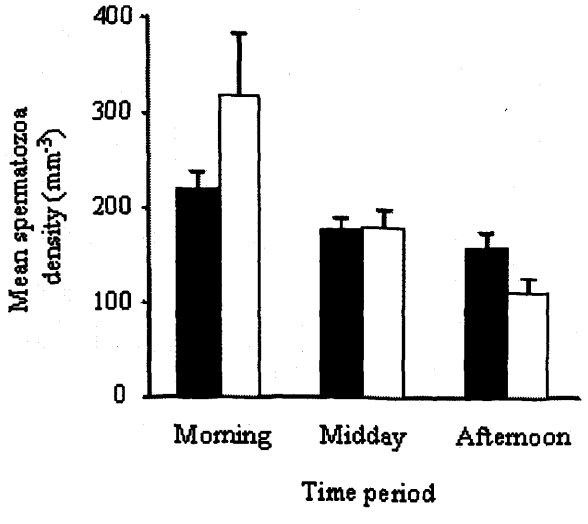

Figure 2. Mean (+standard error; SE) density of spermatozoa in three ejaculates collected in the morning (0900-1000), at midday (1200-1300), and afternoon (1500-1700) of territorial male *R. amarus* at high (white bars) and low (black bars) levels of sperm competition intensity.

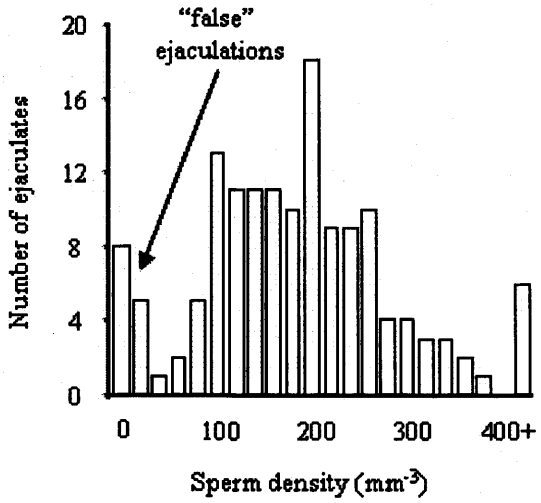

Figure 3. Frequency distribution of spermatozoa density of ejaculates of territorial male *R. amarus* for pooled data from test of sperm depletion over a spawning. Ejaculations <60 spermatozoa mm^{-3} are labeled as "false" ejaculations.

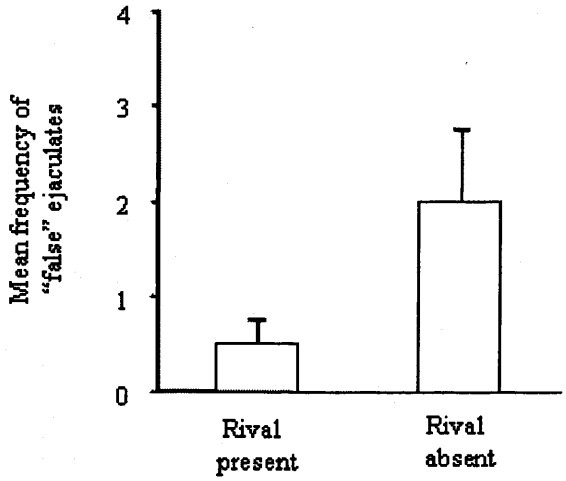

Figure 4. Mean (+standard error; SE) frequency of "false" ejaculations by territorial male *R. amarus* at two levels of sperm competition intensity.

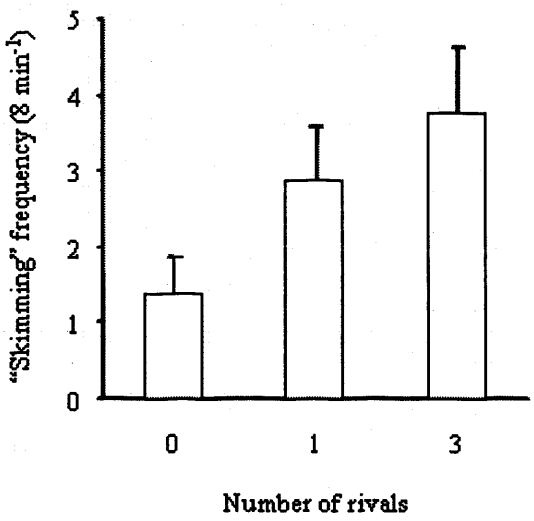

Figure 5. Mean (+standard error; SE) female *R. amarus* "skimming" frequency at three levels of sperm competition intensity.

Chapter 4. Spatial clustering of spawning sites and Operational Sex Ratio in three species of bitterling; *Rhodeus amarus*, *R. sinensis* and *R. ocellatus*

Abstract

In species where males defend patches of resources critical for female reproduction there is often high variance in male reproductive success. The relative reproductive success of individual males within a population is thought to be determined to a large extent by the operational sex ratio and the spatial clustering of fertilisations. Here, I investigated the effect of the spatial clustering of fertilisation opportunities and the operational sex ratio (OSR) on male aggression, and the frequency and distribution of ejaculates in three closely related species of bitterling; *Rhodeus amarus*, *R. ocellatus* and *R. sinensis*. Bitterling are freshwater fishes that lay their eggs on the gills of living freshwater mussels, making them ideal for investigating the effect of the spatial clustering of fertilisation opportunities on male behaviour. In aquarium experiments I manipulated the spatial clustering of spawning opportunities and OSR by varying the spatial clustering of spawning sites, and manipulating the number of females ready to spawn. Our data showed that the spatial clustering of fertilisation opportunities and OSR both affect male ejaculation rate and ejaculate distribution among mussels, the dominance of guarder males and subsequently the opportunity of subordinate males to sneak fertilisations.

INTRODUCTION

Variance in male reproductive success is often high in species with resource-defence polygyny, where males defend patches of resources critical for female reproduction (Emlen and Oring 1977; Alcock 1996; Simmons et al. 1999). The relative reproductive success of individual males in a population is thought to be determined to a large extent by the operational sex ratio (OSR) and the spatial and temporal clustering of fertilisations (Emlen and Oring 1979; Shuster and Wade 2003). When fertilisations are spatially clustered around resources essential for female reproduction, some males are able to monopolise matings and variance in reproductive success among males is predicted to be high (Alcock 1996; Simmons et al. 1999; Groddeck et al. 2004). Conversely, when fertilisations are spatially dispersed variance among males tends to be low (Brownwell and Ralls 1986; Møller 1988; Dahl 1993; Stockley and Purvis 1993; Brown and Weatherhead 1999).

Similarly the population OSR can affect individual reproductive success (Emlen and Oring 1977). The OSR is the ratio of males to females that are ready to mate, and is recognized as a central concept in understanding variation in mating competition (Emlen 1976; Emlen and Oring 1977; Clutton-Brock and Parker 1992; Kvarnemo and Ahnesjö 2002; Reichard et al. 2004). The OSR can affect male reproductive success and males are sensitive to changes in the OSR (Kvarnemo and Ahnesjö 2002). When the OSR is male biased subordinate males may be out-competed for mating opportunities by more dominant males (Alberts et al. 2003; Shuster and Wade 2003) increasing variance in reproductive success among males. A male-biased OSR often leads to an increase in interference competition among males for matings, which can affect mating tactics, reproductive success and mate choice

(Emlen 1976; Enders 1993; Jirotkul 1999; Kvarnemo and Ahnesjö 2002). For example, there may be a change in mating behaviour; typically where pair spawning and territoriality break down to be replaced by group spawning (Fuller 1999; Kanoh 2000). In contrast, if the OSR is at unity (females are all receptive simultaneously), or female biased, the opportunity for monopolising mating opportunities by a small number of males will be limited and the variance in reproductive success among males will be low (Moyer et al. 1983; Taborsky 1994; Halliday 1998; Vieites et al. 2004).

High variance in male reproductive success drives the evolution of alternative male mating strategies and tactics (Gross 1991; Taborsky 1994, 1998), including sperm competition (Møller 1998; Parker 1998; Petersen and Warner 1998; Smith et al. 2002). Alternative mating tactics are common among teleost fishes (Petersen and Warner 1998). The most common form of alternative mating behaviour is represented by the sneak-guard model of Parker (1990). Here, some males adopt a guarder tactic (guarding a resource critical to reproduction, courting females, and in some species performing paternal care), while others mate as sneakers (joining a spawning pair with no investment into territorial defence or courtship, and investing primarily in sperm competition) (Foote et al. 1997; Neff et al. 2003). Where sneaking is common, guarder males often face a high risk of sperm competition; ejaculates have a high probability of overlapping with those of rivals (Parker 1998). High sperm competition risk drives male adaptations that may affect sperm behaviour and morphology (Gage 1994; Snook 2005), testis and ejaculate size (Møller 1988; Briskie 1993; Sicotte 2002), and behavioural adaptations to avoid sperm competition (Smith et al. 2002).

Here I used bitterling fishes to investigate the effect of the spatial clustering of fertilisation opportunities and OSR on male mating tactics. Bitterling fishes exhibit

resource-defence polygyny; they use only living freshwater mussels as spawning sites. During the spawning season, males either act as guarders, aggressively defending territories around mussels and courting females, or principally adopting sneaky mating tactics (Smith et al. 2003, 2004; Reichard et al. 2005). Males can adopt both tactics, but are highly consistent in their roles in a given context. At high male densities territoriality ceases and all males participate in group spawning (Kanoh 2000; Mills and Reynolds 2003; Reichard et al. 2004a,b). At ovulation female bitterling develop long obvious ovipositors, which they use to place their eggs through the mussel's exhalant siphon into its gill chamber. To spawn a female quickly inserts her ovipositor into the gill cavity of the mussel and deposits a clutch of eggs, the size of clutch varying among species (Smith et al. 2004). Males fertilise the eggs by releasing sperm into the inhalant siphon of the mussel. Sperm is released in discrete ejaculations, when a male sweeps quickly and obviously over the inhalant siphon of a mussel. Ejaculations are frequent, both before and after eggs are laid (Reichard et al. 2004a), and pre-oviposition ejaculations are frequently successful in fertilizing subsequently spawned eggs (Reichard et al. 2004a). Because bitterling use a discrete spawning site that can easily be manipulated, and because females develop long, obvious ovipositors when they are about to spawn, they represent a valuable model for investigating the effects of the spatial distribution of fertilisation opportunities and OSR on male behaviour.

I used three closely related species of bitterling (Okazaki et al. 2001); the European bitterling, *Rhodeus amarus*, the Chinese rose bitterling, *R. ocellatus*, and the Chinese bitterling, *R. sinensis*, to investigate the effects of the spatial clustering of fertilisation opportunities and OSR on male mating behaviour in relation to the natural spawning conditions in each species.

The reproductive biology of *R. amarus*, which is similar to all other bitterling fishes so far investigated, is reviewed by Smith et al. (2004). The three study species show similar reproductive strategies (Kondo et al. 1984; Nagata 1984; Smith et al. 2000, 2004; Kitamura 2006), though the European bitterling has a substantially shorter spawning season (6 weeks) than the two Chinese species (3-6 months) (Kondo et al. 1984; Nagata 1984; Reichard et al. 2007). Nevertheless, the overall seasonal fecundity of all three study species is similar at 200-300 eggs, depending on female size (Smith et al. 2000; Kitamura 2005; Reichard et al. 2007). However, there are differences in clutch sizes among species. Mean (± SE) clutch size per spawning in *R. amarus* is typically 2.9 (±0.2) (Smith et al. 2000), in *R. ocellatus* 4.4 (±1.3) (Reichard et al. 2007) and in *R. sinensis* 15.7 (±2.6) (Reichard et al. 2007). During the reproductive period, females go through several spawning cycles, in each bout laying 30-50 eggs. Typically a spawning bout lasts a single day, followed by a resting period of between 7 and 10 days (though the interval between spawning bouts may be longer at the end of the spawning season). Therefore, there are natural differences among the bitterling species tested in the level of male bias of the OSR during the spawning season together with the predicted effects of a biased operational sex ratio on monopolization by guarder males. It is reasonable to assume that the natural differences observed between the three study species in OSR and spawning season length mean that males of one species are likely to be more behaviourally adapted to certain conditions of spatial clustering or changes in OSR than another species. Consequently, I anticipated that the three bitterling species would respond differently to experimental manipulation of the spatial clustering of fertilization opportunities and the OSR.

I experimentally manipulated the spatial clustering of fertilisation opportunities and the OSR in each bitterling species, and measured the effects on guarder and sneaker male mating tactics. Spatial clustering of mating opportunities was manipulated by changing the distribution of spawning sites (freshwater mussels). OSR was manipulated by altering the number of females with extended ovipositors, so that the OSR was either male biased or at unity. Guarder and sneaker male responses were measured in terms of aggressive behaviour of guarders towards sneakers, guarder and sneaker ejaculation rates, and the distribution of ejaculates by both groups of males, among mussels.

I predicted that guarder male aggression and ejaculate frequency would be highest when the spatial clustering of fertilisation opportunities was low (mussels were dispersed) and OSR was 1. Under these conditions guarder males have least control over females and resources, and were predicted to escalate competition with other males through increased aggression and ejaculation rates. I also expected to observe significant differences between species in response to the spatial clustering of mussels and clustering of females, with some species being better adapted to clustering than others. I predicted that guarder *R. amarus*, which due to a short reproductive season with numerous spawning bouts has a high risk of sperm competition and a need to produce numerous small ejaculates, would invest most energy into sperm competition, spreading many ejaculates over the entire group of mussels. In contrast, while the frequency of spawnings in *R. sinensis* is considerably lower than *R. amarus*, due to the value of each female clutch the risk of sperm competition at spawning events is considerably higher in *R. sinensis* than either *R. amarus* or *R. ocellatus* which has both infrequent spawnings and small clutch sizes.

Therefore I anticipated that *R. sinensis* would invest most heavily into sperm competition avoidance through aggressive behaviour.

METHODS

Study Animals

Experimental *R. amarus* used in the study were collected using a Seine net from an oxbow lake adjacent to the River Vistula, near the village of Soczewka (52° 32' N; 19° 34' E), central Poland. A total of 280 *R. amarus* were transported in river water to the University of Łodz and stored in indoor aquaria. *Unio tumidus* mussels were collected by hand at the start of the bitterling spawning season, from Sulejow reservoir, Poland (51° 24' N; 19° 52' E), transported to the University of Łodz and stored in a second outdoor pond. *U. tumidus* are widespread and common in Central Europe and are readily used by *R. amarus* for spawning (Smith et al. 2000b, 2004). Experimental work with *R. amarus* was conducted over a six-day period in May 2006, in the aquarium facilities at the University of Łodz.

Experimental *R. ocellatus* were the first captive generation of 80 wild fish imported from China. *U. pictorum* mussels used in experiments with *R. ocellatus* were collected from the River Cam in Cambridgeshire, UK, in March 2005. Mussels were transported to the University of Leicester and stored in a large water tank on the roof of the Department of Biology and fed with phytoplankton. *U. pictorum* has a wide distribution; it is found in Europe and East Asia (Nagel 2000; Graf 2002) and its range overlaps with *R. ocellatus* in China. *R. ocellatus* readily use *U. pictorum* for spawning and their embryos develop successfully in the gill chambers of this mussel

(Reichard et al. 2007). Experimental work with *R. ocellatus* was conducted over a 12-day period (August 2005) at the aquarium facility of the University of Leicester.

R. sinensis used in experiments were collected from Lake Bao'an in the River Yangtze basin Central China (30° 50' N; 114° 16' E) and stored in the aquarium facilities at the Institute of Hydrobiology, of the Chinese Academy of Sciences, Wuhan. *Unio douglasiae* mussels were used in experiments with *R. sinensis* and were collected from Lake Poyang, China. This mussel is widespread in China and regularly used by *R. sinensis* for spawning (Reichard et al. 2007b). Mussels were transported to the Institute of Hydrobiology, stored in water tanks and fed with phytoplankton. Experimental work with *R. sinensis* was conducted over a six-day period (April 2006) in the aquarium facilities of the Institute of Hydrobiology.

Different mussel species do not elicit different male courtship or ejaculatory behaviour (Smith et al. 2000, 2001, 2004) and the filtration rates of *U. tumidus*, *U. pictorum* and *U. douglasiae* are comparable (Smith et al. 2001; Mills and Reynolds 2002; Reichard et al. 2007b). All experimental work (in China, Poland and the UK) was conducted in aquaria of identical size; 60 cm (length) x 40 cm (width) x 40 cm (depth), with fish exposed to a 16: 8 light: dark cycle at 23 °C. Prior to and during experiments all fish used in the study were fed frozen chironomid larvae and commercial dried flake food.

Experimental Protocol

Fish were exposed to two levels of spatial clustering of fertilisation opportunities (high and low), and two levels of OSR (4 and 1). The spatial clustering of fertilisation opportunities was manipulated by arranging the distribution of four mussels so that they were placed at the corners of a 5 x 5 cm square (high spatial clustering) or

dispersed, each at the corner of a 30 x 30 cm square (low spatial clustering). To manipulate OSR I exposed males to either a single female with an extended ovipositor (OSR = 4), or four females with extended ovipositors simultaneously (OSR = 1). This gave four experimental combinations; high spatial clustering with OSR at unity, high spatial clustering and a male-biased OSR, low spatial clustering with OSR at unity, and low spatial clustering with a male-biased OSR. Experimental protocols were identical for *R. amarus*, *R. ocellatus* and *R. sinensis*. A total of twelve replicates of each of the four combinations were completed for each of the three bitterling species; a total of 144 observation periods. While the conditions of clustering of females and mussels is considerably more regimented than those seen in the wild, in my experience the clustering 4 mussels in such an area or the number of receptive females as used in this experiment is not unusual in the wild.

Experimental mussels; *U. tumidus* for *R. amarus* (mean ± SD length, 83 ± 8.7 mm), *U. pictorum* for *R. ocellatus* (86 ± 5.2 mm), and *U. douglasiae* for *R. sinensis* (78 ± 6.1 mm), were selected haphazardly from storage tanks, placed in small sand-filled pots and covered with perforated clear plastic cups that enabled fish to see and smell the mussels but prevented them from spawning. Four males were captured from a stock aquarium and gently released into the experimental aquarium to be tested under all treatments. The spatial distribution of mussels was arranged according to the designated treatment; either low or high clustering. Depending on the OSR treatment, either a single, or a group of four females with extended ovipositors were captured from a separate holding aquarium and gently released into the experimental aquarium. Single and groups of females were selected haphazardly, but once placed within a group the same fish were used for all subsequent observation periods within each replicate.

In test groups a male was allowed to establish dominance over rivals and display territorial defence of the mussels, this usually occurred within 1-2 hours. Experiments were not started until a single male had become dominant and male dominance was determined by behavioural observations of male aggression. The remaining three subordinate males took on the role of sneakers. Once dominance was established, the mussels were uncovered and behavioural recording started. After eight minutes mussels were re-covered and the next treatment imposed, such that each group of males experienced all four treatments (in a predetermined random order). A minimum of 30 min. was allowed between observation periods to minimise the effects of disturbance on behaviour. The behaviours recorded were the frequency and location of guarder and sneaker male ejaculations, and rate of guarder male aggression. If a spawning occurred during the observation period, providing it did not occur within the first four minutes, the exact timing was noted and a rate of the different behaviours calculated from the pre-spawning observation period. However if spawning occurred within the first four minutes the female was replaced and the replicate was restarted after 30 minutes.

After all four observations were completed, the fish were removed and their Standard Length (SL; length from the tip of the snout to the origin of the tail fin) was measured to the nearest 1 mm. No male or mussel was used in more than a single replicate. Females may have been reused if they returned to spawning condition during the experiment, but not with the same males or mussels. After completion of the experiment fish were stored in a separate stock tank and wild fish were subsequently released at the location of their collection.

Data Analyses

All data were tested for normality using a Kolmogorov-Smirnov test and equality of variance using Bartlett's test. A two-way ANOVA was used to test for the effect of spatial clustering and OSR on aggression (the rate of aggression by guarder males directed at sneakers), guarder ejaculation rate (directly correlated with sneaker ejaculation rate), and variance in the distribution of ejaculates among mussels. A Bartlett's test was used to test for a significant difference in the variance of the distribution of ejaculates among mussels. A one-way ANOVA was used to test for differences in the frequency of ejaculations or aggression among species.

RESULTS

Guarder Male Aggression Rate

Spatial clustering and OSR treatments had no significant effect on the rate of guarder male aggression in *R. amarus* or *R. ocellatus* (two-way ANOVA; Table 1, Fig 1*a* & *b*). In *R. sinensis*, aggression rate was significantly higher with low mussel spatial clustering and low OSR, with no significant interaction between factors (Table 1, Fig 1*c*).

Guarder Ejaculation Rate and Distribution of Ejaculates Among Mussels

There was no significant effect of mussel spatial clustering or OSR on the ejaculation rate of guarder male *R. amarus* (two-way ANOVA, Table 1). In addition, the variance in the distribution of ejaculates among mussels did not vary among treatment combinations (Bartlett's test, $\log_{10}$ transformation, $p = 0.293$; Fig 2); the distribution of guarder male ejaculates was similar among all treatments.

The ejaculation rate of guarder male *R. ocellatus* was significantly higher when OSR was at unity, though there was no significant effect of spatial clustering and no interaction (two-way ANOVA, Table 1). In addition, variance in the distribution of ejaculates among mussels differed significantly among treatments (Bartlett's test, $\log_{10}$ transformation, $p = 0.001$, Fig 3); variance in the distribution of guarder male ejaculates was lowest when the spatial clustering of fertilisation opportunities was high and the OSR male biased; i.e. when guarders were best able to monopolise spawning sites and females.

In *R. sinensis,* there was no significant difference in guarder male ejaculation rate between treatment groups (two-way ANOVA, Table 1), though there was a significant difference in the variance of the distribution of ejaculates among mussels among treatment groups (Bartlett's test, $\log_{10}$ transformation, $p = 0.001$; Fig 4); like *R. ocellatus*, the variance in the distribution of ejaculates among mussels was lowest with a male-biased OSR and high spatial clustering of fertilisation opportunities.

The ejaculation rate of guarder males was significantly different among species (one-way ANOVA, $F_{2,141} = 30.21$, $p < 0.001$). A Tukey's HSD *post hoc* test showed that guarder male *R. amarus* ejaculated at a significantly higher rate than *R. ocellatus* and *R. sinensis*, though there was no significant difference between the latter two species.

Sneaker Ejaculation Rate and Distribution of Ejaculates Among Mussels

Variance in the distribution of sneaker male ejaculates among mussels was significantly different among treatment combinations (Bartlett's test, $p < 0.001$; Fig 2). Variance was lowest when the OSR was at unity and the spatial clustering of fertilisation opportunities low. This result suggests sneakers were most successful in

distributing ejaculates among mussels when conditions meant that the guarder male was least able to monopolise spawning sites and females.

Variance in the distribution of ejaculates among mussels differed significantly between treatment combinations (Bartlett's test, $\log_{10}$ transformation, $p = 0.023$; Fig 3); variance was lowest when the spatial clustering of mussels was low and the OSR male biased.

Variance in the distribution of ejaculates among mussels by sneaker male *R. sinensis* differed significantly among treatment combinations (Bartlett's test, $\log_{10}$ transformation, $p < 0.001$; Fig 4). Variance in the distribution of ejaculates was lowest when spatial clustering of mussels was high and OSR male-biased.

I detected a significant difference in the ejaculation rate of sneaker males among the three study species (one-way ANOVA, $F_{2,141} = 16.00$, $p < 0.001$). A Tukey's HSD *post hoc* test showed that the ejaculation rate of sneakers was significantly higher in *R. amarus* compared to *R. sinensis* and *R. ocellatus*, though there was no significant difference between *R. sinensis* and *R. ocellatus*.

DISCUSSION

I experimentally manipulated the OSR and spatial clustering of fertilisation opportunities, to investigate their effect on the reproductive tactics of guarder and sneaker males in three closely related species of bitterling.

I tested the prediction that with a low spatial clustering of mussels and even OSR, guarder male aggression and ejaculate frequency would be higher than when the spatial clustering of fertilisations was high and the OSR male-biased. I also predicted that male *R. amarus* would invest more energy in sperm competition, with a

higher ejaculation rate than either *R. ocellatus* or *R. sinensis* since the short spawning season and frequent spawning of *R. amarus* means that males are adapted to a high frequency of ejaculation where sperm competition is common. Guarder male *R. sinensis*, which due to large females clutch sizes are likely to face a high risk of sperm competition where spawnings occur than either *R. amarus* or *R. ocellatus*, were predicted to invest most energy into aggression, as a sperm competition avoidance strategy where dominant males try to monopolise infrequent, high value spawnings.

Guarder male *R. amarus* did not vary rates of aggression or ejaculation in response to treatments, and distributed ejaculations evenly among mussels within replicates. There was no significant difference in the distribution of ejaculates among mussels between treatment combinations, though the distribution of ejaculates among individual mussels by sneaker males did vary significantly with treatment; sneaker males distributed ejaculates evenly among all four mussels while spatial clustering was low and the OSR was male biased, but appeared constrained in their distribution of ejaculations under conditions of high spatial clustering.

Guarder male *R. ocellatus* varied ejaculation rate between treatments, showing the highest rate of ejaculation when OSR was at unity. Throughout the study guarder and sneaker males limited the distribution of their ejaculates to a restricted number of mussels. While theory suggests reducing sperm investment (Parker 1998), as male-male competition increases *R. ocellatus* maintain a consistent ejaculation rate, which suggests that males may be poorly adapted to face a high intensity of sperm competition.

I observed no significant difference in the ejaculation rate of guarder male *R. sinensis* among treatment combinations. Guarder male *R. sinensis* were significantly more aggressive when the OSR was 1 and spatial clustering low. Both guarder and

sneaker male *R. sinensis* varied the distribution of their ejaculates among mussels between low and high spatial clustering of mussels; guarder males distributed ejaculates evenly among all test mussels when spatial clustering was high, but limited the number of mussels they ejaculated over (the reverse of sneaker males) when spatial clustering was low. Where there were multiple fertilisation opportunities (OSR = 1) and widely distributed spawning sites (low spatial clustering of mussels), guarder male *R. sinensis* increased the proportion of their ejaculates they released into some mussels while reducing the proportion at others. In contrast, the ejaculates of guarder males were more evenly distributed among mussels when the OSR was male biased (4:1) and more easily monopolised.

The spatial clustering of mussels also influenced subordinate males; when mussel spatial clustering was low subordinates had a greater opportunity to inspect and ejaculate into mussels than when mussels were clustered and more readily defended by guarders. As sites of fertilisation become more clustered the clustering of males around the spawning sites and, therefore, the level of male-male interference competition is expected to increase, as there are few fertilisation opportunities outside the clustered group (Emlen and Oring 1977; Kanoh 2000, Shuster and Wade 2003). The same pattern is observed in southern elephant seals (*Mirounga leonina*), where females converge on small islands in the southern ocean to breed, and there is intense competition between mature males, which attempt to monopolise matings by guarding harems of females, though in this species the way in which males distribute ejaculates among females is not known (Hoelzel et al. 1999; Simmons et al. 1999; Carlini et al. 2002).

Where the OSR is at unity, subordinate males have a greater opportunity of participating in mating, either by switching tactic to act as a guarder, or as a parasite

of other males, (Emlen and Oring 1977, Kvarnemo and Ahnesjö 1996, 2002). In contrast, competition among males will be elevated when the OSR is male biased (Emlen and Oring 1977; Clutton-Brock and Parker 1992). Work by Enders (1993) on spider mites (*Tetranychus urticae*) showed that aggression was elevated under a male-biased OSR and that large males had a greater reproductive success than small males. The present study suggests a similar response. Here, as in other species (Emlen and Oring 1977, Kvarnemo and Ahnesjö 1996, 2002), when the OSR was low, subordinate males had more opportunities to court females and ejaculate into mussels because the guarder male was often preoccupied with courting females.

Despite a male-biased OSR and elevated male-male interference competition guarder males were able to maintain dominance over at least one mussel under each different combination of spatial clustering and OSR. However, unlike subordinate males, when spatial clustering of mussels and OSR were reduced guarder males limited the number of mussels they ejaculated over. Previous work with bitterling by Nagata (1985), Kanoh (2000), Mills and Reynolds (2003) and Reichard et al. (2004) showed that male bitterling adjust their mating behaviour in relation to OSR. They observed a decrease in aggressive and courtship behaviour and a change in mating tactics from pair to group spawning as the OSR became more male biased and density increases (Kanoh 2000; Mills and Reynolds 2003; Reichard et al. 2004). Our study used fewer fish than previous experiments and guarder males did not switch tactic from pair to group spawning.

Each species in the present study responded differently to changes in the spatial clustering of fertilisation opportunities and the OSR. Variation in responses may be explained by the natural length of spawning season in each species (*R. amarus*; 6 weeks (Smith et al. 2004), *R. sinensis* and *R. ocellatus*; 5-6 months (Kondo

et al. 1984; Nagata 1984)). In each species, spawning season length and the intervals between spawning bouts determines the natural male bias in the OSR and, therefore, the risk of sperm competition, which is predicted to increase as the OSR becomes more male biased. Thus, the spawning season length in each species of bitterling may determine the extent to which fish are adapted to cope with female temporal clustering and male interference competition.

The level of competition for fertilisations may be controlled not only by the OSR and the spatial clustering of mussels, but also by the value of each spawning. It has been shown that clutch sizes of *R. sinensis* are significantly larger than those of either *R. amarus* or *R. ocellatus* (Reichard et al. 2007). Therefore, due to the higher value of each egg clutch in *R. sinensis*, which results in a series of highly temporally clustered fertilisation opportunities over a relatively long spawning season, I would suggest that the risk of sperm competition at each spawning should be considerably higher than *R. ocellatus* and even *R. amarus*, where spawnings are temporally clustered (relatively evenly) over a short spawning season but considerably less spatially clustered, as despite the low frequency of spawnings in *R. sinensis*, when spawnings occur fertilisation opportunities (eggs) are heavily clustered in both space and time (Shuster and Wade 2003). Consequently, due to spatial and temporal clustering of fertilisations, it may be that male *R. sinensis* are adapted to a higher risk of sperm competition than either *R. amarus* or *R. ocellatus* and respond through intense aggression towards rivals.

In conclusion, this study demonstrates that as the spatial clustering of fertilisation opportunities decreases and the OSR is at unity, competition from subordinate males similarly increases. A consequence is that guarder males appear to concentrate their ejaculates on a smaller group of mussels that they are able to

maintain control over, rather than attempting to distribute their ejaculates among all potential spawning sites.

Figures

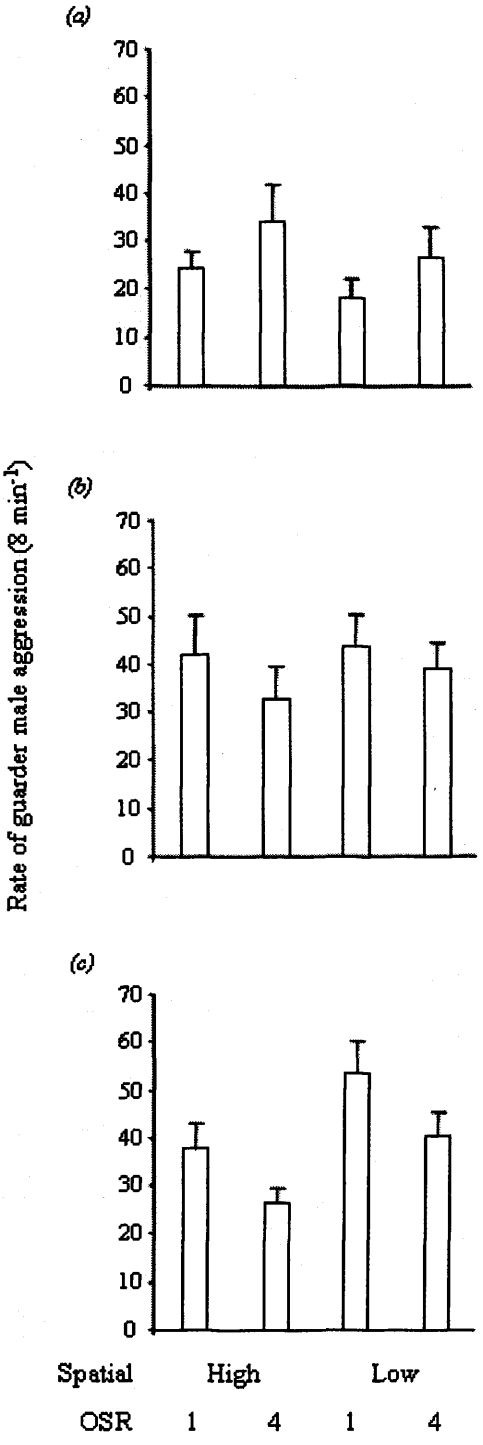

Figure 1. The rate of guarder male aggression towards rivals among spatial clustering and OSR treatment combinations. (*a*) *R. amarus*, (*b*) *R. ocellatus*, and (*c*) *R. sinensis*. Error bars are one standard error.

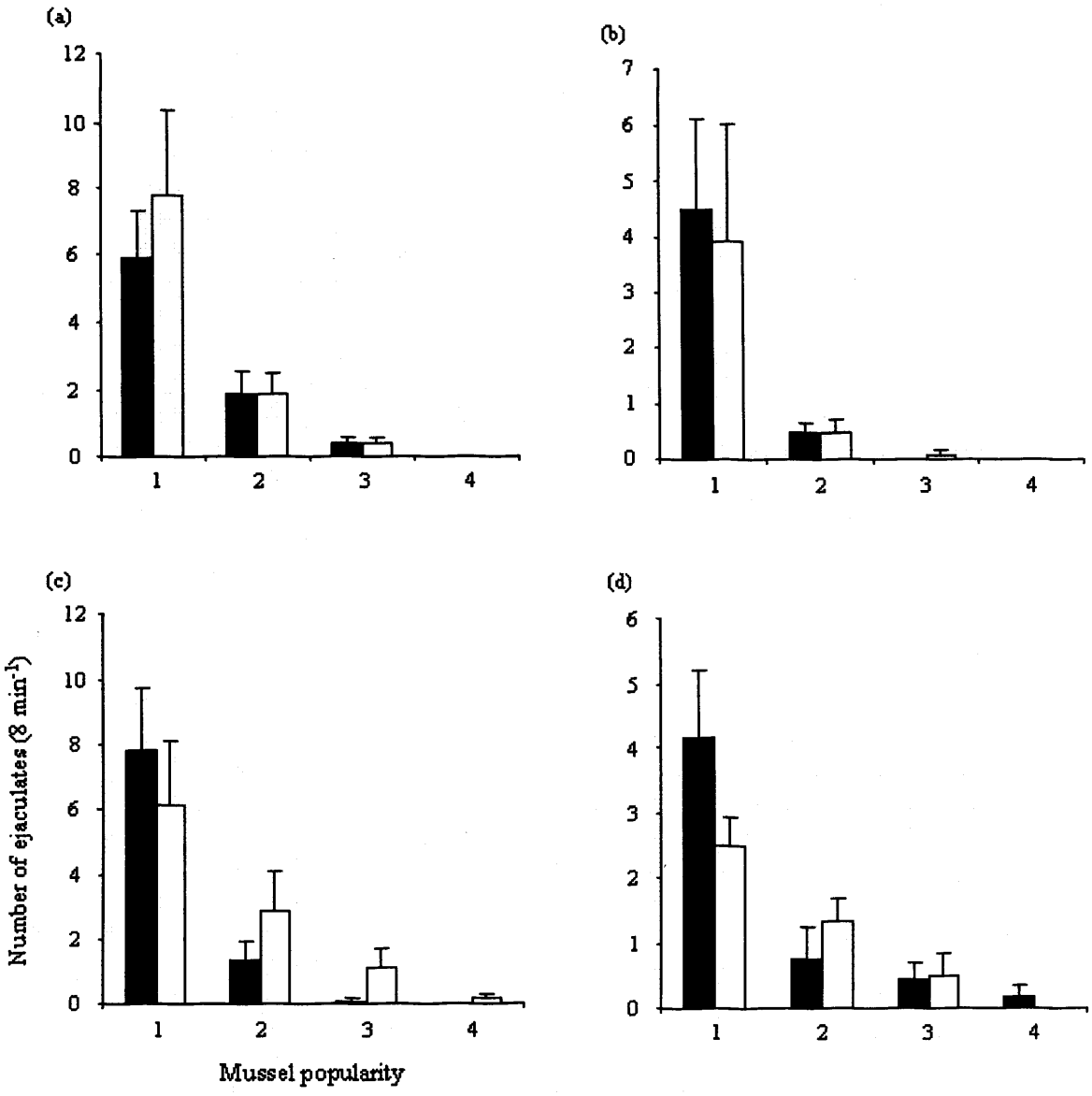

Figure 2. Mean ejaculates among mussels at two levels of spatial clustering of spawning sites and OSR in *R. amarus* demonstrating the change in the distribution of ejaculates as the clustering of mussels and females changes. X axis: numbers correspond to mussel popularity, in descending order. Y axis: number of ejaculates per mussel. (a) High spatial clustering of mussels and the Operational Sex Ratio (OSR) 1 (SH & OSR 1), (b) High spatial clustering of mussels and OSR 4 (SH & OSR 4), (c) Low spatial clustering of mussels and OSR 4 (SL & OSR 4), (d) Low spatial clustering of mussels and OSR 1 (SL & OSR 1). Black Bars represent sneakers, while white bars represent territorial males. Error bars are one standard error.

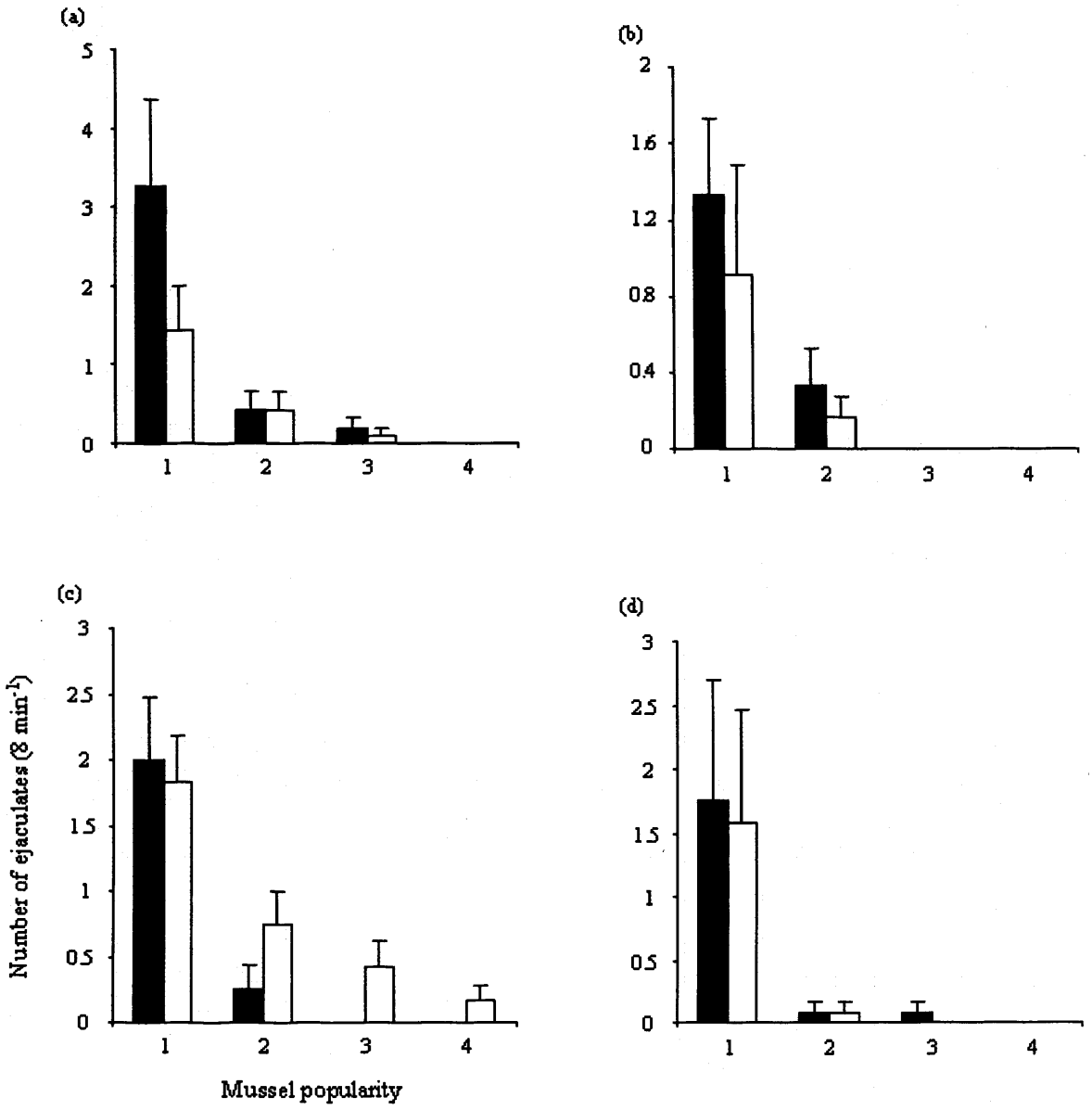

Figure 3. Mean ejaculates among mussels at two levels of spatial clustering of spawning sites and OSR in *R. ocellatus* demonstrating the change in the distribution of ejaculates as the clustering of mussels and females changes. X axis: numbers correspond to mussel popularity, in descending order. Y axis: number of ejaculates per mussel. (a) SH & OSR 1, (b) SH & OSR 4, (c) SL & OSR 4, (d) SL & OSR 1. Black Bars represent sneakers, while white bars represent territorial males. Error bars are one standard error.

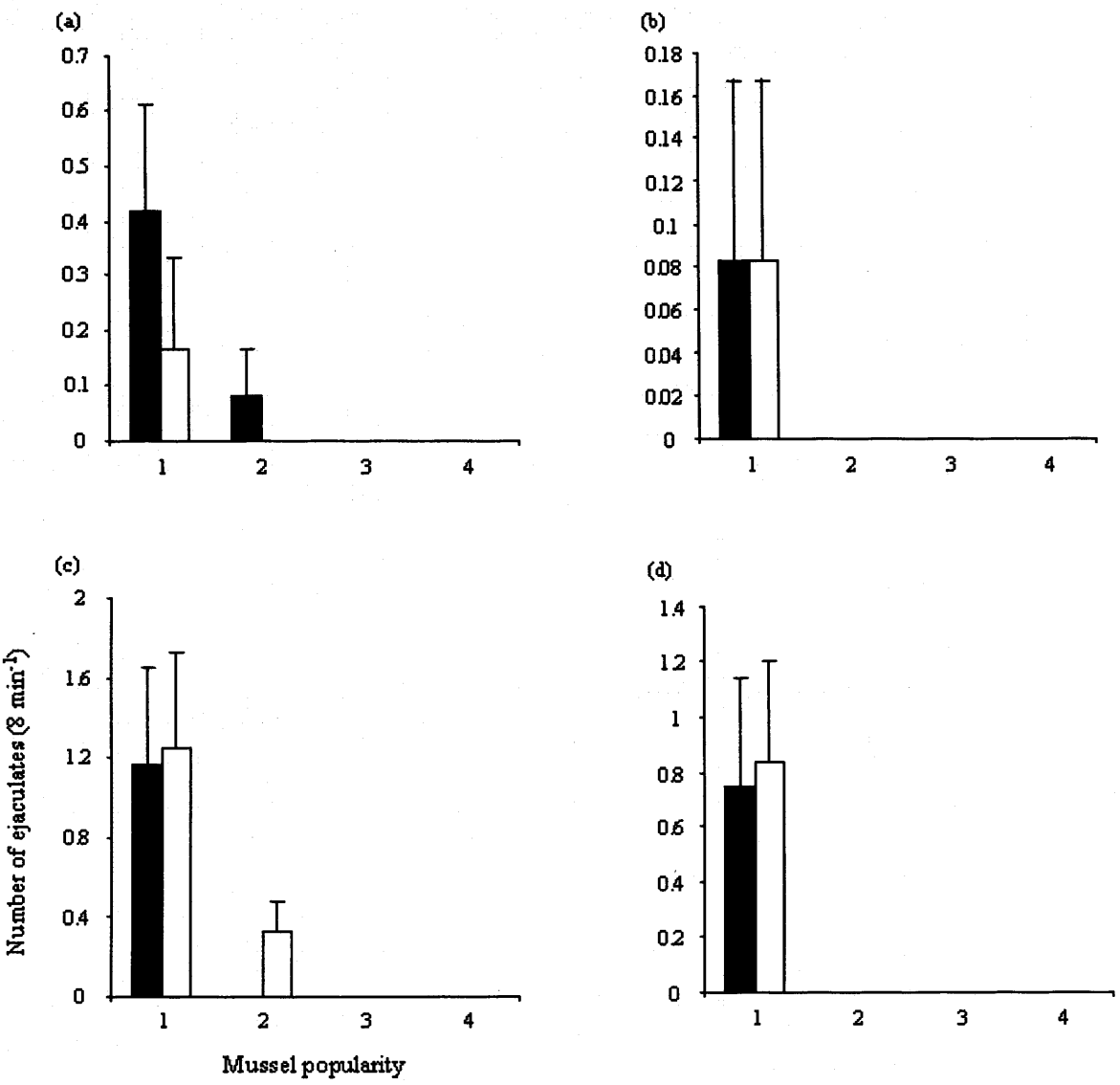

Figure 4. Mean ejaculates among mussels at two levels of spatial clustering of spawning sites and OSR in *R. sinensis* demonstrating the change in the distribution of ejaculates as the clustering of mussels and females changes. X axis: numbers correspond to mussel popularity, in descending order. Y axis: number of ejaculates per mussel. (a) SH & OSR 1, (b) SH & OSR 4, (c) SL & OSR 4, (d) SL & OSR 1. Black Bars represent sneakers, while white bars represent territorial males. Error bars are one standard error.

Table 1. Summary of results

	R. amarus		*R. ocellatus*			*R. sinensis*		
(a) Aggression	F	*p*	F	*p*		F	*p*	
Spatial	1.48	0.230	0.30	0.586		8.37	0.006	L>H
OSR	2.45	0.125	1.09	0.303		5.70	0.021	1>4
Interaction	0.01	0.920	0.12	0.733		0.03	0.871	
(b) Guarder ejaculation								
Spatial	1.39	0.244	2.56	0.117		2.58	0.115	
OSR	2.05	0.159	6.63	0.013	1>4	1.56	0.218	
Interaction	0.23	0.635	0.00	0.945		0.03	0.859	

Chapter 5. Spatial clustering of spawning sites; a mesocosm study with rose bitterling (*Rhodeus ocellatus*)

Abstract

Variance in male reproductive success is often high in species with resource-defence polygyny, where males defend patches of resources critical for female reproduction. The ability of males to monopolise fertilisation opportunities, and therefore the level of male-male competition and subsequent fertilisation success among males, is expected to vary with the spatial distribution of fertilisations. For example, where fertilisations are spatially clustered, variance in fertilisation success among males is expected to be high as a small number of males can often monopolise a disproportionately high number of all fertilisation opportunities. Here I test the effect of the spatial clustering of fertilisations on the mating behaviour of the rose bitterling (*Rhodeus ocellatus*). Bitterling are fishes that lay their eggs on the gills of living freshwater mussels, an easily manipulated spawning substrate, making them ideal for testing the effects of variation in spatial clustering on male mating tactics. In an aquarium experiment I varied the spatial clustering of fertilisations by manipulating the positions of 16 mussels on a large grid. The spatial clustering of mussels was found to control the ratio of guarder to sneakers males; as the clustering of mussels became more intense fewer males were able to hold territories and therefore the number of males acting as sneakers increased. I discuss our results with reference to opportunistic switching and the fertilisation success of males unsuited to the tactic they are forced to adopt.

INTRODUCTION

The number guarding males, the level of competition for fertilisations, and therefore the variance in success of males within a population is determined to a large extent by the Operational Sex Ratio (OSR) and the spatial and temporal clustering of fertilisations (Emlen and Oring 1979; Shuster and Wade 2003). Females have a lower reproductive potential than males due to the cost of egg production but rarely have to compete for access to males, or forgo fertilisation opportunities (Bateman 1948). In contrast because males are limited mainly by the number of mates with which they can mate, there is often direct competition between males for mating opportunities (Brockmann et al. 1994; Gross 1991; Preston et al. 2001; Carlini et al. 2002) and subsequently competition on a gametic level; i.e. sperm competition, where the ejaculates of rival males overlap (Parker 1990; Andersson 1994; Eberhard 1996; Parker 1998; Simmons 2001; Wedell et al. 2002; Wada et al. 2005).

Where fertilisations are spatially clustered a relatively small number of dominant males may monopolise a disproportionately large number (Alcock 1996; Hoelzel 1999; Simmons et al. 1999). In contrast, where fertilisations are more spatially dispersed dominant males are less able to control females or resources critical for reproduction and subordinate males may have more opportunity to participate in mating (Emlen and Oring 1977; Stockley and Purvis 1993; Brown and Weatherhead 1999; Shuster and Wade 2003).

Under conditions that give rise to high variance in male reproductive success alternative male mating tactics can evolve (Taborsky 2001). Alternative male mating tactics are particularly common among fishes (Petersen and Warner 1998), and examples of conditional switching of mating tactics are well known (Constanz 1975;

Taborsky 1985; Beauchamp 1990; Taborsky 1994, 2001; Ryan et al. 1990; Rasotto and Mazzoldi 2002).

Aside from size differences, dominant males are often morphologically distinct from subordinate males (Taborsky 1994). Morphological differences are often associated with physiological costs, which do not affect individuals that have not invested into morphological change (Frischknecht 1993; Taborsky 1994). The cost of morphological change is overcome by increased reproductive fitness which may be attributed to adaptations in among others, sperm behaviour and morphology (Gage 1994; Snook 2005), testis and ejaculate size (Møller 1988; Briskie 1993; Sicotte 2002), behavioural adaptations for sperm competition avoidance (Smith et al. 2002) and increased fighting ability (Anderson 1994; Polak 1994; Alcock 1996; Sokolovska et al. 2000; Jones and Hutchings 2001; Preston et al 2001; Carlini et al. 2002).

However, when fertilisations are spatially clustered, the number of territories may be diminished and therefore the least successful of the dominant, normally territorial males may be unable to hold a territory. Where large males are displaced by others competing for a limited resource, and are forced to act as sneakers (parasitizing pair spawnings or participating in group spawning) they are expected to have a low reproductive success, relative to their investment, as they are less suited to sneaker mating tactics (Anderson 1994; Taborsky 1994). The reverse may also occur where territories or females become so abundant that smaller subordinate males are more reproductively successful when they mate as a territorial rather than a sneaker male (Taborsky 1994, 2001; Clutton-Brock et al. 1997; Rasotto and Mazzoldi 2002). Therefore, the overall success of individual males within mating tactics is heavily dependent on the ratio of males, in each tactic, involved in mating and the spatial

clustering of resources critical for spawning; if there are fewer territories than territorial males, all but a few of the most dominant males will be displaced (Taborsky 1994).

Subordinate males face a high intensity of sperm competition as they almost always mate in the presence of rivals. Therefore, subordinates may require greater sperm numbers than predominantly pair spawning territorial males. It has been shown that subordinate males often produce more sperm, though not necessarily of a higher quality, than territorial males (Ryan et al. 1990; Rasotto and Mazzoldi 2002). While small males have no need to invest energy into territorial defence, large males must trade off the need to produce and conserve enough sperm to maintain reserves (to avoid sperm depletion), with allocating enough energy to maintain a territory (Fuller 1998; Rondeau and Sainte-Marie 2001; Wedell et al. 2002).

Here I use the rose bitterling, *Rhodeus ocellatus*, to investigate the effect of the spatial clustering of fertilisations on male mating tactics and ejaculate distribution among mussels. For a full description of the reproductive biology of the European bitterling *R. amarus* which is similar among all studied species see Smith et al. (2004). Bitterling fishes exhibit resource-defence polygyny: they use only living freshwater Unionid mussels as spawning sites. During the spawning season, males aggressively guard territories around mussels (Smith et al. 2003, 2004; Reichard et al. 2005). Females develop long ovipositors, which they use to place their eggs into the mussel gill chamber. Males fertilise the eggs by sweeping over the inhalant siphon of the mussel and ejaculating, an act which is sometimes visible as a greyish cloud. Because bitterling use a discrete spawning site that can easily be manipulated, and because females produce long, obvious ovipositors when they are ready to spawn,

they represent a valuable model for investigating the effects of the spatial distribution of fertilisations on male mating tactics and ejaculatory behaviour.

Here experimental manipulation of the spatial clustering of fertilisations was accomplished by varying the distribution of mussels within a large experimental aquarium. The temporal clustering of fertilisations (i.e. female number) was not manipulated, though the number of females with extended ovipositors in each replicate was recorded. Male responses were measured in terms of aggressive behaviour towards rivals, and ejaculation rate.

I predicted that the number of territories would vary between treatments, with the greatest number where mussels were evenly distributed. Where mussels were spatially clustered I predicted that only a few of the most dominant males would be capable of guarding a territory of clustered mussels. More males were expected to be aggressive when mussels were dispersed, as guarding behaviour would be more widespread. I also predicted that guarding males would ejaculate into a small group of mussels, while because sneakers are likely to only ever sire a small proportion of any clutch (Kanoh 2000), they were expected to invest into a larger more spatially dispersed group of mussels where participation in multiple spawning events may increase their overall fertilisation success.

METHODS

Experimental *R. ocellatus* were the first captive generation offspring of 80 wild fish imported from China. *Unio pictorum* mussels used in experiments with *R. ocellatus* were collected from the River Cam in Cambridgeshire, UK where they are abundant and easily collected. Mussels were transported to the University of Leicester and

stored in a large water tank on the roof of the Department of Biology and fed with phytoplankton. *U. pictorum* has a wide distribution; it is found in Europe (Nagel 2000) and East Asia (Graf 2002) and overlaps in its distribution with *R. ocellatus* in China. *R. ocellatus* readily use *U. pictorum* for spawning and their embryos develop successfully in the gill chambers of this mussel (Casalini 2007). Experimental work with *R. ocellatus* was conducted over a 12-day period during April 2006, at the aquarium facility of the University of Leicester.

Experimental Protocol

The experiment was conducted in a large rectangular aquarium measuring 170 x 140 x 120 cm in size (2858 litres), with a natural light: dark cycle at 23 °C. Forty-eight males and thirty-two females were stocked in the aquarium so that there were three males to every two females. I chose this ratio of males to females to ensure that females were a limited resource which males would have to compete for, and so that spawnings were not so frequent that they were difficult to monitor with just two observers. All fish were sexually mature. Prior to, and during, experiments all fish used in the study were fed frozen bloodworm and commercial dried flake food.

A 4 x 4 grid of 16 numbered points (1-16), each 40 cm from adjacent intersections was marked on the base of the tank using painted pebbles. I manipulated the spatial clustering of fertilisations by arranging the distribution of 16 selected mussels on the grid in three distinct levels of spatial clustering; with 1 mussel placed in all 16 positions, 2 mussels in 8 randomly selected positions, and 4 mussels at 4 randomly selected positions.

To restrict mussel movement and ease removal and relocation within the aquarium, each mussel was positioned in a manoeuvrable gravel-filled flowerpot.

Mussels were covered with perforated clear plastic cups that enabled fish to see and smell mussels but prevented them from spawning and then uncovered before observing fish behaviour. Mussels containing glochidia larvae in their outer gills were excluded from the study, as glochidia may affect mussel filtration (Tankersly and Dimock 1993) and therefore its quality as a spawning site.

The order that fish were exposed to spatial clustering intensities was randomly selected before the experiment began. At the end of each day, mussels were re-arranged in preparation for the following day's treatment. Where spawning had occurred mussels were replaced, as the presence of eggs within the mussel gill may effect mussel filtration and therefore, as with glochidia larvae, its quality as a spawning site (Smith et al. 2001). Fish were left to settle overnight, before beginning the next treatment, the following day.

Before each observation all mussels were uncovered and fish were fed. Once fish began to behave normally and inspect mussels a single male was randomly selected, (by waiting for a male to swim behind a square of acetate positioned on the exterior of the tank), and identified to ensure that he had not previously been observed. Fish were identified by estimating their standard length (SL) and recording any features specific to that fish, for example, fin shape, colouration and body shape. Trials were conducted to ensure that my estimates of length were both consistent and accurate, and also that I could readily identify fish already recorded. The experiment was stopped after 9 days (3 replicates of the 3 treatments) as it was felt that continuation would risk a reduction in the accuracy of my identification in comparison with the beginning of the experiment. In total 36 males were observed; 4 in each of the 9 days. The number of guarding males and the number of females with extended ovipositors was recorded in each observation.

Each day the behaviour of 4 males was recorded, each for a 20-minute period. During observations two observers jointly recorded behavioural data one dictating fish behaviour to the other, who recorded behaviour on a palm-top computer. Behaviours recorded were; male-male aggression (measured as aggression towards other males, minus aggression received from others), ejaculations and spawning events. The mussels at which ejaculation or spawning occurred were also recorded.

At the end of the experiment fish were released into a separate aquarium within the facilities of the University of Leicester. All mussels used in the experiment were stored on the roof of the University of Leicester Biology department until embryos had been released.

Data Analyses

All data were tested for normality using a Kolmogorov-Smirnov test. I used a Pearson's correlation to compare male size and the rate of male aggression and then male size and ejaculation rate. A one-way ANOVA was used to test for differences in ejaculation and aggression rate between spatial clustering treatments in each mating tactic. I also used a one-way ANOVA to compare the number of spawnings and the number of territories between the different levels of spatial clustering as well as sneaker placement of ejaculates, and time intervals between ejaculations among different mussels. A two-sample *t*-test was used to compare both the number of mussels that guarder males visited, but also the time intervals between ejaculations at specific mussels between mating tactics and treatments.

RESULTS

I detected a significant difference between the rate of aggression between small and large males (Pearson's correlation, log transformed data, $r_{1,36} = 0.517$, $p < 0.001$; Fig 1). Smaller males were significantly more likely to suffer aggression from rivals than larger males. Similarly large males are significantly more likely to be aggressive than small males (Pearson's correlation, log transformed data, $r_{1,36} = 0.415$, $p = 0.012$; Fig 1). However I detected no significant difference in guarder male aggression between the different levels of spatial clustering (one-way ANOVA, $F_{2,11} = 0.04$, $p = 0.960$),

There was a significant difference between the ejaculation rate of large and small males (Pearson's correlation, log transformed data, $r_{1,36} = 0.505$, $p = 0.017$) but no difference between the ejaculation rates of either tactic between different levels of clustering (sneaker; one-way ANOVA, $F_{2,23} = 2.36$, $p = 0.119$; guarder, $F_{2,11} = 0.94$, $p = 0.424$).

I found no significant difference in the number of spawnings between different levels of clustering, however, there was a difference in the total number of territories under each level of clustering, with the highest number of territories found when mussels were evenly spread, one on each grid intersection (one-way ANOVA, $F_{2,11} = 15.85$, $p < 0.001$; Fig 2).

Sneaker males released multiple ejaculates (mean ± s.e. = 6.032 ± 0.906) over as many as 5 mussels (mean ± s.e. = 2.06 ± 0.28; Fig 3, 4). However, of the mussels sneaker males visited, each male invested a significantly higher number of ejaculates into a specific mussel (one-way ANOVA, $F_{3,30} = 3.02$, $p = 0.047$; Fig 4). Guarder males released multiple ejaculates over a maximum of 2 mussels (mean ± s.e. = 1.28 ± 0.18) and there was no significant difference in the number of ejaculations between

mussels (two-sample t-test, $t_{11} = 2.15$ $p = 0.055$; Fig 4). Despite a difference in ejaculate distribution and frequency between tactics I detected no significant difference between the overall number of mussels that an individual male of either tactic ejaculated over (two-sample t-test, $t_{33} = 1.34$ $p = 0.190$) (mean ± s.e. = 1.81 ± 0.10).

I also found no significant difference in the time interval separating ejaculates between either, the first two mussels that a male visited (sneaker, two-sample t-test, $t_{11} = 2.02$, $p = 0.068$; guarder, two-sample t-test, $t_1 = 0.09$ $p = 0.941$; Fig 5), mating tactics (two-sample t-test, $t_{10} = 1.40$ $p = 0.193$; Fig 5), or treatments (sneaker, two-sample t-test, $t_6 = 1.92$ $p = 0.103$; guarder, two-sample t-test, $t_4 = 1.48$, $p = 0.214$).

DISCUSSION

The number of territories, the level of competition for fertilisation opportunities, and therefore the relative success of different male mating tactics, is controlled to a large extent by the spatial and temporal clustering of fertilisations (Emlen and Oring 1979; Shuster and Wade 2003; Kokko and Rankin 2006). In this study I investigated the effect of manipulating the spatial clustering of mussels, a resource critical for bitterling spawning, on male mating tactics.

I tested the prediction that the total number of territories was controlled by the intensity of spatial clustering; the greatest number expected to be where mussels were evenly dispersed, and the lowest number where mussels were clustered. Therefore the number of guarder males and subsequently the total level of aggression (from dominant to subordinate) was expected to be greatest when mussels were more dispersed. However, among individual guarding males I expected aggression to be

highest when mussels were clustered, as here competition from rivals for territories is greatest. I also anticipated that guarder males would ejaculate into a small group of mussels mainly within their territory, where due to limited rival access, they are likely to fertilise a large proportion of any spawned eggs (Kanoh 2000). While sneakers, which due to intense sperm competition are expected to only father a small proportion of any clutch (Kanoh 2000), are likely to ejaculate over a larger, more spatially dispersed group of mussels.

I showed that the clustering of mussels had little effect on either the frequency of ejaculates, aggression, or spawning, though it was significantly correlated with the number of territories held by dominant males. The average number of males guarding a single or a group of mussels was highest where mussels were evenly spread over the 16 grid intersections (mean ± s.e. = 9.25 ± 0.73) and lowest where mussels were clustered into 4 groups of 4 (mean ± s.e. = 4.75 ± 0.21). In addition I found a significant difference between sneaker and guarder males, in the distribution of ejaculates among mussels (Fig 2). Guarders released multiple ejaculates over a maximum of two mussels, while sneakers regularly ejaculated multiple times over as many as five mussels. I found that sneaker males ejaculated significantly more often over a single mussel than any others among the group they visited, while guarding males did not show a preference between mussels they visited (Fig 2).

Our data supports the hypotheses of Emlen and Oring (1979) and Shuster and Wade (2003) which suggest that the ratio of guarder-sneaker males is influenced by the spatial clustering of resources (Brownwell and Ralls 1986; Møller 1988; Dahl 1993; Stockley and Purvis 1993; Alcock 1996; Brown and Weatherhead 1999; Groddeck et al. 2004). When mussels were more spatially clustered, only the most dominant males were capable of holding a territory. Conversely, when fertilisations

were less spatially clustered, dominant males were unable to control more than a couple of mussels and therefore many small territories developed, allowing less dominant males to adopt the guarding role.

There was no variation in the rate of guarder male aggression or ejaculations during the experiment. However, the ratio of guarder-sneaker males varied with the spatial clustering of mussels; as spatial clustering became more intense, the number of individual territories decreased, while the number of males previously acting as guarders remained the same. Guarding males which were unable to maintain a territory under more intense spatial clustering were forced to switch mating tactic to sneaking and participation in group spawning. The success of males forced to switch tactic was not tested in this study, though as in other species, there may be a significant cost to males that invest into aggression, growth or colouration, but do not win a territory (Taborsky 1994). However, mating tactics are opportunistic in bitterling and therefore the costs to males of switching tactic are unlikely to be large as males are capable of performing either tactic. While according to Kanoh (2000) sneakers which become guarders will be considerably more successful, guarding males are expected to be less successful if they lose their territory and have to sneak fertilisations as unlike guarders, they will almost always mate in the presence of another male.

In addition, our data shows striking differences between mating tactics in the distribution of ejaculates among mussels; guarder males released multiple ejaculates over a small group of mussels within or adjacent to their territory (though, they were recorded releasing single ejaculates at other mussels), while sneaker males repeatedly visited multiple mussels to ejaculate at regular intervals (Fig 2). The difference in ejaculate distribution between mating tactics accurately highlights the differences in

access to mussels and fertilisations and the energetic limitations of both guarder and sneaker males. Dominant guarding males were expected to have access to multiple opportunities, but appeared to restrict the number of mussels they ejaculated over. The cost of leaving a territory undefended from sneaker males, whose competing ejaculates may significantly reduce guarder male paternity (Jennions et al. 1992; Brockmann et al. 1994; Kanoh 2000), may be higher than the relative benefit of attempting to sneak fertilisations at mussels outside their territory. Additionally, during both the short and longer term, the large number of mating opportunities that guarding males gain means that often dominant males may be affected by sperm depletion (Hoelzel et al. 1999; Preston et al. 2001; Wedell et al. 2002; Pateman-Jones et al. in review). Therefore, guarders may focus on reducing sperm competition, and therefore necessary sperm expenditure, through aggression, rather than competing openly in sperm competition at mussels outside their territories where sperm competition can escalate. Investing into a small group of mussels, where rival access is reduced and consequently where there is a chance of achieving high or total clutch paternity, with a reduced risk of sperm depletion, may be the most effective strategy for guarder males.

Visiting a small group of mussels may allow guarding males to better anticipate female spawning and therefore to accurately time the release of pre-oviposition ejaculates, which Reichard et al. (2004), together with our unpublished data on sperm concentrations within the mussel mantle cavity (chapter 2), suggests may be of importance in sperm competition. Parker (1998) suggested that males of externally fertilising species should regularly visit and ejaculate over spawning sites (termed 'topping up') to maintain sufficient sperm concentration to compete effectively with a rival ejaculate, should a spawning occur at that site. Our data

showed that males ejaculated at regular intervals in both tactics and therefore I suggest that further work with the bitterling system may be fruitful in testing Parkers (1998) hypotheses.

While guarding males appeared to limit the number of mussels they regularly visited, sneakers did not. Due to competition from larger males and female choice, sneakers are less likely to effectively court females, to maintain territories, or access guarded mussels to release ejaculates at spawning events, and hence face considerably lower fertilisation success than guarders at individual spawning events (Kanoh 2000). Therefore, sneaker males may achieve a higher fertilisation success by visiting multiple mussels, where guarding males may sometimes be absent or occupied (courting females or performing territorial defence), rather than competing with dominant males for access to specific mussels where their percentage of overall clutch paternity is likely to be low (Kanoh 2000). In our experiment smaller males rarely invested in courtship and appeared to have little influence over the position of spawning events (though sneaker presence may have some effect on female choice (Pateman-Jones et al. unpublished data)). Therefore, spreading ejaculates over multiple mussels may be the most effective strategy for sneaker males; minimising the cost of incoming aggression, time spent waiting for pair spawning, and maximising overall fertilisation success.

While releasing multiple ejaculates over more mussels than guarders, sneaker males were also shown to ejaculate at a significantly higher frequency. A higher frequency of ejaculations in sneaker males may reflect a lower threat of sperm depletion than that faced by larger guarding males or simply that sneakers face a higher risk of sperm competition (Parker 1998). Our unpublished data supports the later hypothesis as small males have a lower gonadosomatic ratio than large males but

produce ejaculates of a similar size (Gross 1982; Gage et al. 1995; Wedell et al. 2002; Pateman-Jones et al. in review).

In conclusion, the spatial clustering of mussels has a significant impact on the number of territories, the ratio of guarder to sneaker males, and therefore the reproductive success of males which change tactic. Significant differences in the spatial placement of ejaculates among mussels were observed between mating tactics. It appears that guarders limit their area of multiple ejaculations to a maximum of 2 mussels while sneakers regularly released multiple ejaculates over 4 or more mussels. The differences I observed may be indicative of the opportunities and limitations of each reproductive tactic; guarder males limit the number of mussels they visit due to the costs of sperm depletion and territory maintenance, while sneaker males consistently face sperm competition and consequently have to sneak ejaculations over multiple mussels to increase their relative paternity.

Figures

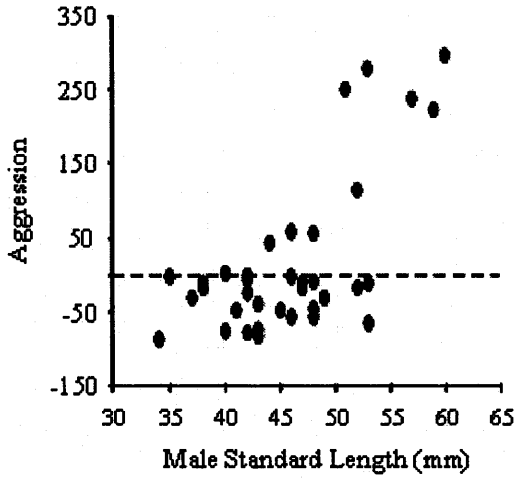

Figure 1. Level of aggression given by and received by males of different length in our study. X axis; male Standard Length (mm). Y axis; shows the number of attacks made by a male minus the number he received. Each marker point represents an individual male.

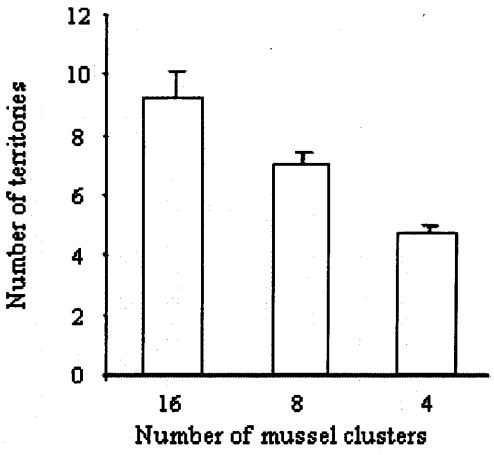

Figure 2. Mean number of territory holders in each of the three levels of mussel spatial clustering, where there was either 16, 8 or 4 clustered mussel groups. Error bars are one standard error.

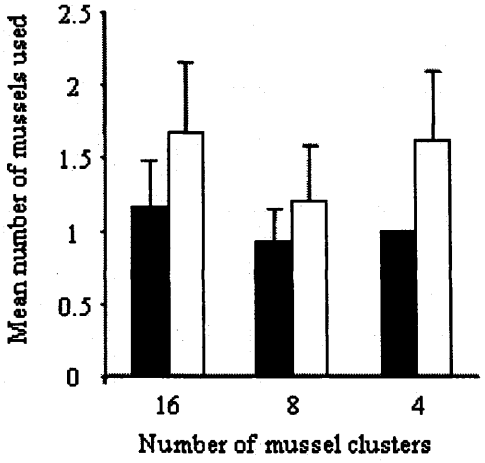

Figure 3. Mean number of mussels that sneaker and territorial males ejaculated over in each of the three different mussel spatial clustering levels (either 16, 8, or 4 groups of mussels). Error bars are one standard error.

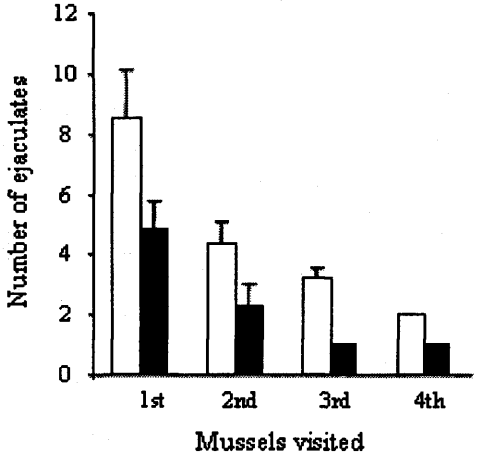

Figure 4. The number of ejaculates by males of each tactic into mussels and the number of mussels into which they released multiple ejaculates (territorials, up to 2; sneakers as many as 5). Black bars represent territorial males, while white bars represent sneakers. Error bars are one standard error.

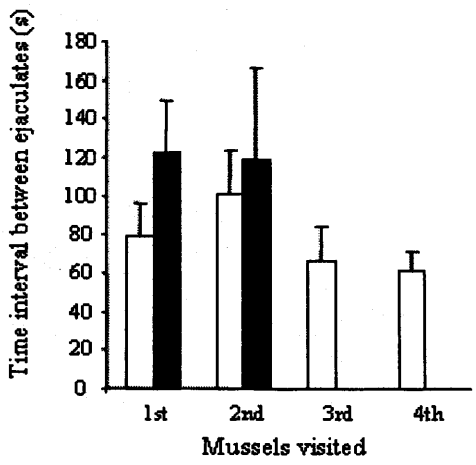

Figure 5. The average time intervals between ejaculates over a mussel buy sneaker and territorial males. The 4 mussels sneaker males visited most were included, while territorial males only released multiple ejaculates over a maximum of 2 mussels and therefore only 2 mussels were included. Black bars represent territorial males, while white bars represent sneakers. Error bars are one standard error.

Chapter 6. Sperm morphology in seven species of bitterling

Abstract

Sperm competition most commonly operates as a fair lottery. However, when sperm compete to fertilise an egg, the morphology of spermatozoa, not just sperm numbers plays a role in sperm competition. Morphological adaptations such as larger mitochondria, which may confer greater sperm longevity, or a longer flagellum, which may increase sperm swimming speed, can make some spermatozoa more effective in sperm competition than others. The continuous fertilization model, assumes that externally fertilising males will experience a higher level of sperm competition than in internally fertilising species as males simultaneously ejaculate and initiate a race for available eggs. As sperm competition intensity increases males should increase both sperm number and sperm swimming speed to maximize the likelihood of spermatozoa and eggs meeting. Longevity, which often correlates with mitochondrial size or number, is not important when there is intense sperm competition because most eggs will be fertilized soon after ejaculation.

In this study the sperm from 7 species of bitterling, fishes which lay their eggs on the gills of freshwater mussels, and which experience sperm competition, were compared.

Sperm morphology differed significantly among bitterling species and differences in sperm length and width were identified between bitterling and zebra fish (*Danio rerio*). I discuss our results in relation to sperm competition risk and spermatozoa adaptation to spawning environment.

INTRODUCTION

Sperm competition occurs when the ejaculates of two or more males overlap during a spawning (Parker 1970). Sperm competition greatly influences the success of mating males and as such is recognised as a force in the evolution of reproductive anatomy and behaviour (Smith 1984). Across a range of taxa research has shown a positive correlation between testis structure and ejaculates size and the risk of sperm competition (Harcourt et al. 1981; Stockley and Purvis 1993; Gage 1994; Stockley et al. 1997; Hosken and Ward 2001; Snook 2005). Sperm competition has been widely studied in fishes (Stockley et al. 1997) and adaptations to sperm competition are known to include features of sperm behaviour and morphology (Gage 1994; Johnson and Briskie 1999; Anderson and Dixon 2002; Byrne et al. 2003; Snook 2005), testis and ejaculate size (Briskie 1993; Sicotte 2002), and behavioural adaptations to avoid sperm competition by controlling females and dominating other males (Smith et al. 2002).

Sperm competition most commonly operates as a fair lottery with those males that release the most sperm being the most successful (Gomendio et al. 1998; Preston et al. 2001). While the relative number of sperm per ejaculate is of importance in a lottery-type situation, when ejaculates are matched numerically, sperm quality may also influence male reproductive success providing a competitive advantage over rival ejaculates (Stockley et al. 1997; Birkhead and Møller 1998; Balshine et al. 2001). Sperm quality can be defined as the fertilisation efficiency of a males ejaculate against the ejaculate of a rival, after removing sperm numbers as a factor in competition (Birkhead and Møller 1998; Snook 2005). Sperm traits such as size (LaMunyon and Ward 1998), longevity (Gage et al. 2004), viability (Hunter and

Birkhead 2002) and swimming speed (Gage et al. 2004) can all influence fertilisation efficiency.

Among fish the morphology of spermatozoa, like testis structure and size, is varied (Robinson and Prince 2003), though relationships between spermatozoa morphology and sperm competition are now more clear (Billard 1986; Lahnsteiner and Patzner 1990; Jamieson 1991; Stockley et al. 1996, 1997). Within the constraints of a fixed energy budget the production of small spermatozoa will give a numerically large ejaculate, while larger sperm with bigger flagellum or larger mitochondria may reduce the numerical size of the ejaculate, but could increase its competitive performance through an increase in sperm swimming speed or longevity (Parker 1982; Gomendio and Roldan 1991; Stockley et al. 1997; Burness et al. 2004; Snook 2005).

The selective pressures behind differences in spermatozoa morphology are less well understood, with a great range in sperm lengths within species (Snook 2005), and by 10 orders of magnitude among taxa (Chao et al. 1975; Pitnick et al. 1995). Two models relating to the relationship between sperm size and sperm competition have been proposed: for internal fertilisers, the instantaneous fertilization model where internal fertilization occurs at one particular instant, usually some time after mating, and the continuous fertilization model for most externally fertilizing fish species, where fertilization occurs in a continuous fashion immediately after mating (Parker 1993; Balshine 2001). In the continuous fertilization model, sperm size is predicted to increase with sperm competition intensity (Ball and Parker 1996). The model assumes that externally fertilizing species are likely to experience a relatively higher degree of sperm competition than internal fertilisers because males release sperm simultaneously, and sperm race for the available ova. Therefore, males should

increase both sperm number and sperm swimming speed to maximize the frequency that spermatozoa and eggs meet. Longevity is not important when there is intense sperm competition because most of the eggs will be fertilized shortly after ejaculation (Parker 1998).

Unlike other cyprinid fishes, bitterling have an unusual and specialised mode of reproduction utilising freshwater mussels as spawning sites (Smith et al. 2004). Reproduction is external but fertilisation occurs within the gill chamber of a freshwater mussel; the female inserts her eggs through the mussel's exhalent siphon and the male releases sperm over the inhalant siphon using the mussel's own filtration system to transport sperm to the eggs (Smith et al. 2004). The reproductive ecology of the European bitterling, which is similar to all other bitterling so far studied, is reviewed by Smith et al. (2004).

The external structure of spermatozoa in two bitterling species (*Rhodeus ocellatus* and *Acheilognathus lanceolatus* (Ohta 1991; Ohta and Matsuda 1995)) have already been described, with particular focus on intramembranous particles found on the sperm head which appear to aid in locating the egg or micropore. However, neither study related spermatozoa morphology to the bitterling mating system or inter/intra specific variation in sperm competition risk.

The mating system of all bitterling species is similar, however recent work has shown differences among species in the development of testis and sperm duct which may be related to sperm competition risk and the length of time intervals between spawnings (Chapter 7). In *R. ocellatus* and *R. sinensis* the development of the sperm duct and testes is not unusual and spermatogenesis occurs solely within the lumen wall. While both species have the same type of spermatogenesis each species faces a considerably different risk of sperm competition and accordingly there are differences

in the GSI; *R. sinensis*, which due to the intense spatial and temporal clustering of fertilisation opportunities (caused by large clutch sizes), has a high risk of sperm competition and larger reproductive apparatus than *R. ocellatus* which has a long spawning season and low clustering of fertilisations.

In contrast to *R. sinensis* and *R. ocellatus*, in *R. amarus*, which like *R. sinensis*, experiences a high risk of sperm competition, spermatogenesis is semicystic and there is a highly developed sperm duct, where spermatids continue developing after early release from cysts within the testis lumen. The differences between *R. amarus* and *R. sinensis* may be attributed to the length of the intervals between spawnings when sperm reserves can recover. Unlike *R. sinensis*, *R. amarus* has frequent periods of intense sperm demand (spawnings) and therefore relatively little time to recover sperm reserves. Having semicystic spermatogenesis together with a large sperm duct may be an adaptation to allow the rapid production of spermatozoa with relatively little investment into expensive testicular tissue.

Sperm morphology may also vary among species in relation to sperm competition risk. The similarity in spawning mechanism and the differences in sperm competition risk among bitterling fishes make bitterling ideal for testing the affect of sperm competition risk and mating system on external sperm morphology.

I compared the sperm of seven species of bitterling (*R. amarus*, *R. ocellatus*, *R. sinensis*, *T. himantegus*, *T. limbata*, *A. barbatulus*, and *A. tabira*) and the related zebra fish (*D. rerio*), which has a more conventional mating system, to compare spermatozoa morphology. I predicted that bitterling spermatozoa would be similar in outer morphology among species due to the similarity in their mating system. However, I anticipated differences in the size of spermatozoa among bitterling species related to the predicted sperm competition risk in each species and in line

with the continuous fertilisation model. The biggest differences in spermatozoa dimensions were anticipated to be between *R. amarus*, *R. sinensis* and *T. limbata*, all species which face high risk of sperm competition, and the remaining 4 Asian species which, though there is little published research, are expected to have lower risk of sperm competition due to prolonged spawning seasons than *R. amarus*, and smaller clutch sizes than *R. sinensis* (Smith et al. 2000; Smith et al. 2004; Reichard et al. 2007).

METHODS

Sperm was stripped from 3 sexually mature males of each study species (*R. amarus*, *R. ocellatus*, *R. sinensis*, *T. himantegus*, *T. limbata*, *A. barbatulus*, *A. tabira* and *D. rerio*) and immediately fixed in a mixture of glutaraldehyde (2.2%), paraformaldehyde (4.5%), sucrose (5%) and Sorensen's phosphate buffer (pH 7.5). After fixing for 2 hours, samples were centrifuged to remove spermatozoa from the seminal fluid, washed in Sorensen's phosphate buffer (pH 7.5), dehydrated in ethanol, allowed to dry, and then sputter coated onto viewing stubs according to the methodology of Maricchiolo et al. (2002).

Prepared spermatozoa were visualised using the scanning electron microscope facilities at the University of Leicester. Images were photographed and in a minimum of 25 spermatozoa (per fish), sperm length, flagella length, head width and total sperm length were all subsequently measured from scaled photographs using Image J and then statistically compared among species.

Data Analysis

All data were tested for normality using a Kolmogorov-Smirnov test. A one-way ANOVA was used to test for differences among species in sperm length, total sperm length, head length and head width. A 2-sample *t*-test was used to compare spermatozoa dimensions between bitterling and *D. rerio*.

RESULTS

General Morphology

The spermatozoa of all bitterling species was similar in external morphology, being composed of a head, a middle piece, and a tail, but with no obvious acrosome (*R. ocellatus*; Ohta 1991) (see appendix 3a). In all bitterling spermatozoa a head and neck region was distinguishable, each with an approximately spherical shape (see appendix 3b). Spermatozoa have a large mitochondrion section and centrioles in the middle piece. As in some blenniid species (Lahnsteiner and Patzner 1990), the flagellum arises in the nuclear notch at the lateral side of the spermatozoon, at the border between the head and neck region. The midpiece encircles the root of the flagellum (Ohta 1991). The proximal portion of the flagellum is surrounded by a sleeve of plasma membrane (arising from a flattened section of the midpiece), which appears separated from the head piece, except at the centriolar fossa, and allows free range of movement (see appendix 3b).

The structure of the head, sleeve and mitochondrial sections are smooth in all species with no obvious characters. As shown in other teleost fishes, including some bitterling (Ohta 1993), I observed no acrosomal structure in the anterior portion of the sperm head.

The spermatozoa of *D. rerio* are of a primitive morphology similar to that of other externally fertilising teleost fishes. Sperm are composed of a spherical head and long flagellum. Unlike bitterling the head does not have a distinct neck region but instead is completely spherical with the flagellum protruding directly from the base of the head. There was no acrosome present and the midpiece containing mitochondria (Kessel et al. 1983) is short and narrow.

Sperm Length

The total spermatozoa length and flagellum length of bitterling was significantly different among species (sperm length; one way ANOVA, $F_{6,20} = 17.18$, $P < 0.001$, Fig 1 (*a*); flagellum length, one way ANOVA, $F_{6,20} = 17.18$, $P < 0.001$, Fig 1 (*b*)) with the longest spermatozoa at 36.305 um (SD ± 2.799) in *T. limbata*, the shortest in *T. himantegus* at 27.597 um (SD ± 3.562) and the descending size order; *T. limbata*, *R. Amarus*, *A. tabira*, *R. sinensis*, *R. ocellatus*, *A. chankaensis*, *T. himantegus*. The average length of bitterling sperm (32.21 μm ± 2.953) was significantly greater than *D. rerio* (30.03 μm ± 2.546) (t test, $t_{10} = 2.27$, $P = 0.046$).

Sperm Head

Spermatozoa width was significantly different among species (one way ANOVA, $F_{6,20} = 4.04$, $P = 0.015$, Fig 2 (*b*)). The greatest width of spermatozoa was in *A. tabira* (2.060 um ± 0.314) before, in descending order; *R. ocellatus*, *T. limbata*, *R. sinensis*, *A chankaensis*, *R. amarus*, and the smallest in *T. himantegus* (1.753 μm ± 0.122). The average width of sperm was significantly greater in *D. rerio* (2.673 μm ± 0.274) than in any of the bitterling species (1.910 μm ± 0.111) (t test, $t_{19} = 9.25$, $P < 0.001$).

Head length varied significantly between species (one way ANOVA, $F_{6,20} = 10.79$, $P < 0.001$, Fig 2 (*a*)) with *A. tabira* the greatest (3.517 μm ± 0.581) before in descending order; *T. limbata*, *R. ocellatus*, *A. chankaensis*, *R. sinensis*, *T. himantegus* and *R. amarus* (2.576 μm ± 0.035). Average head length was significantly greater in bitterling (3.046 μm ± 0.295) than in *D. rerio* (2.611 μm ± 0.220) (t test, $t_9 = 3.46$, $P < 0.007$).

DISCUSSION

I stripped sperm from the testes of seven species of bitterling (*R. amarus*, *R. ocellatus*, *R. sinensis*, *T. himantegus*, *T. limbata*, *A. barbatulus*, *A. tabira*) and zebrafish, *D. rerio*, to compare sperm among bitterling species and contrast the morphology of bitterling sperm with a species exhibiting a more typical cyprinid mating system.

I tested the prediction that similarities would exist between the external morphology of spermatozoa among bitterling species due to their common mating system. In addition, I anticipated that due to differences in sperm competition risk among bitterling species there would be corresponding differences in spermatozoa size, with the greatest differences between *R. amarus*, which is known to have a high sperm competition risk due to a short intense spawning season, and the remaining group of Asian species, which are expected to have lower risk of sperm competition due to a more prolonged spawning season (Pateman-Jones et al. unpublished data).

I observed that in each bitterling species, all spermatozoa were of a primitive morphology similar to that of other externally fertilising teleost fish species, and all lacking an acrosome (Franzen 1970; Grier 1981). Spermatozoa comprised a round

head, a large midpiece containing mitochondria, and a flagellum sleeved by an elongated and thinner section of the midpiece. In all species the flagellum projected laterally from the nuclear notch of the spermatozoon at the border between the head and neck region so that it appeared to project from one side when viewed from the tail (Ohta 1993). The spermatozoa of *D. rerio* also share a primitive morphology, comprising simply a spherical head, a short narrow midpiece and a flagellum.

While I only looked at a limited number of individuals from each species, I did identify some significant differences in sperm head length, sperm width, flagellum length and total sperm length between species. *T. limbata*, *R. amarus* and *A. tabira* had the longest flagellum and total sperm length, while *T. himantegus*, *A. chankaensis* and *R. ocellatus* were the shortest. *R. ocellatus* and *A. tabira* had the greatest sperm width, while *R. amarus* and *T. himantegus* were the thinnest. However despite a statistical difference between spermatozoa width, there was little actual difference between species. It is possible that with a greater sample size from each species the differences would have been more pronounced and this is an area I would suggest for future studies. Head length was greatest in *A. tabira*, *T. limbata* and *R. ocellatus*, and smallest in *T. himantegus* and *R. amarus*. I did not measure the dimensions of the sleeve structure surrounding the flagellum as variation between individual spermatozoa among males was high, making differences between species difficult to quantify. In some cases the sleeve structure was small, while in others it was equivalent in length to half of the sperm head. The function of the sleeve is unknown, though it is more likely to act to protect the flagellum base than perform any function in sperm competition and, therefore it was of little interest to the current study.

It is difficult to assign the observed differences in bitterling spermatozoa morphology to mating system or reproductive behaviour as mating behaviour does not differ prominently among bitterling species; all spawn onto the gills of freshwater mussels. As in blennid fishes differences in sperm morphology are unlikely to be related to reproductive behaviour due to the similarity among species, but instead, may be related to phylogeny, indicating phylogenetic differences among species (Favard and Andre 1970; Lahnsteiner and Patzner 1990) or adaptations to the risk of sperm competition.

Unlike those within the bitterling group, the differences between *D. rerio* and bitterling may be related to mating system as each species may be specifically adapted to their spawning environment. The spermatozoa of *D. rerio* have a relatively small mitochondria section (Kessel et al. 1983) and are ejaculated directly onto the spawned ova. In contrast bitterling spermatozoa are released over the inhalant siphon of a freshwater mussel and have to swim to the unfertilised ova. When sperm are released in a pre-oviposition ejaculate (Reichard et al. 2004) sperm may have to survive until fertilisation, some time after initial ejaculation (Reichard et al. 2004). Thus, differences in optimal sperm longevity between *D. rerio* and the bitterling group may explain the contrasting proportion of sperm head devoted to mitochondria, as sperm longevity and motility is thought to be positively correlated with mitochondria size and sperm competition (Dixon and Anderson 2004; Anderson et al. 2005). The time from ejaculation to sperm and egg meeting may also position the bitterling group between those fishes suited to the instantaneous fertilisation model (normally internally fertilising species), where longevity may increase with sperm competition risk, and those fishes suited to the continuous fertilisation model

(normally externally fertilising species), where the importance of longevity is thought to decrease as sperm competition increases.

While differences in spermatozoa morphology among bitterling species are unlikely to be related to mating system, morphological differences may instead be related to the intra-specific risk of sperm competition, where significant differences have been observed. Where sperm competition is intense it is expected that there will be an increased investment into spermatogenesis through both increased sperm production and testicle size (Harcourt et al. 1981; Møller 1988; Jennions and Passmore 1993; Gage 1994; Stockley et al. 1997; Snook 2005; Chapter 7). Both *R. amarus* and *T. limbata* are thought to face a high risk of sperm competition and both species possess spermatozoa with long flagellum, together having the longest spermatozoa among the investigated species. This result is expected as where sperm competition intensity is high the most successful ejaculate is one that has large spermatozoa with a fast swimming speed (Parker 1982; Stockley et al. 1996, 1997; Burness et al. 2004; Snook 2005), though while it is expected that a large flagellum will confer faster swimming speed, there is currently no empiracle evidence to support this. Where sperm size is high it is assumed that sperm numbers should be reduced due to the trade off between size and number (Stockley et al. 1997). However, previous work (Chapter 7) has shown that male *R. amarus* have disproportionately large sperm ducts which continue the development of prematurely released spermatids into spermatozoa. This mode of spermatogenesis may allow the production of larger quantities of sperm than if full spermatogenesis occurred within cysts inside the lumen. Therefore together, these adaptations may allow the production of both large spermatozoa and a large ejaculate.

Whilst both *T. limbata* and *R. amarus* have long spermatozoa, the width and head length of spermatozoa were short in proportion to the total length, especially in *R. amarus* where length and width were the lowest of the studied species. Head size usually correlates with the number of mitochondria, which reflects swimming speed and endurance and, therefore sperm longevity (Stockley et al. 1997; Dixon and Anderson 2004; Anderson et al 2005). It may be that there is a trade off between a long flagellum, fast swimming speed and the overall longevity of sperm, all of which may involve greater energy input and might lead to a reduction in overall sperm numbers (Stockley et al. 1997; Birkhead and Møller 1998). In *R. amarus* spawning is frequent (Smith et al. 2003), suggesting that when ejaculates are released pre-spawning (pre-oviposition) the time until spawning occurs is likely to be small and, therefore, longevity (or mitochondrial investment) may not be as important as gaining sperm precedence through sperm numbers or swimming velocity, a characteristic directly linked to flagellum length (Stockley et al. 1997).

Alongside *R. amarus* and *T. limbata*, *R. sinensis* also faces a high risk of sperm competition but in this study I found it to have smaller spermatozoa than either other species. Due to the intense spatial and temporal clustering of fertilisation opportunities (caused by large egg clutches) the risk of sperm competition is likely to be higher in *R. sinensis* than in either *R. amarus* or *T. limbata*. *R. sinensis* conforms to sperm competition theory as it is suggested that where risk is high the production of numerous small spermatozoa will be selected for. However there may be a trade off between size, which may confer speed, and numerical superiority, which may win in a lottery.

Research has demonstrated a positive correlation between sperm numbers and the mean number of ova released at a spawning in externally fertilising fishes (Peters

1971; Stockley et al. 1996, 1997) and also a link between sperm number and sperm size; as sperm size decreases sperm number is predicted to rise (Stockley et al. 1996, 1997). Mean clutch size is significantly greater in *R. sinensis* (15.7 (±2.6)) than the average for bitterling I studied (*R. amarus*, 2.9 (±0.2); *R. ocellatus* 4.4 (±1.3)) (Smith et al. 2000; Reichard et al. 2007)) while sperm size was small. Though ejaculate size was not quantified, sperm size was significantly greater in *R. amarus*, which has a significantly smaller clutch size, but like *R. sinensis* a high sperm competition risk (Pateman-Jones et al. unpublished data). Therefore, our data suggest that sperm numbers are also positively correlated with ova number in bitterling.

Also in contrast to *R. amarus* and *T. limbata*, *R. ocellatus*, *A. barbatulus* and *T. himantegus* all have significantly smaller spermatozoa. Small spermatozoa may be an adaptation to sperm competition, as with a fixed resource budget, smaller sperm may allow for the production of more gametes (Stockley et al. 1997) and thus may confer a higher reproductive success if sperm compete numerically (Parker 1990). However, while this may be an adaptation to sperm competition in *A. barbatulus* and *T. himantegus*, this is unlikely in *R. ocellatus* as previous work on testis structure and mating behaviour showed adaptations to low sperm competition risk, which together with a prolonged spawning season and temporally unclustered fertilisations inferred a naturally low risk of sperm competition. Therefore, the production of small sperm in this species may be a mechanism for reducing the cost of spermatogenesis and not a specific adaptation to sperm competition. Alternatively as discussed later, the size of spermatozoa may be directly related to the mussel preferentially selected by a specific species; mussel gill structure may have a strong selective effect on spermatozoa morphology and specifically sperm head width.

In spermatozoa that feature an acrosomal structure, the acrosome is usually situated at the most anterior point of the head, just opposite the position of the centriolar fossa (Yanagimachi 1988; Ohta et al. 1993). However I observed that the bitterling species I studied all had a spherical head but no acrosome. This configuration is not unexpected as most teleost fishes are known to lack an acrosomal structure (Kessel et al. 1983; Ohta and Iwamatsu 1983; Yanagimachi 1988). In teleosts, spermatozoa enter the egg through the micropile into the perivitelline space. While not possessing an acrosome, all bitterling studied here were shown to have arrays of intramembranous particles (IMPs) over the head, which have been suggested to react to the sperm stimulating factor (located in the chorion near the micropile), guiding the spermatozoa to the egg and specifically the micropile (Suzuki 1961; Ohta 1991). A similar IMP distribution has been identified and attributed the same functional role in *D. rerio* (Kessel et al. 1983).

While differences in sperm dimensions between species may be attributed to the natural risk of sperm competition, it is also possible that mussel morphology may have a selective pressure on sperm dimensions (Liu et al. 2006; Reichard et al. 2007a, b). Though still unknown, it is possible that sperm fix themselves to the mussel gill or that they are adapted to features of the mussel gill that ensures they are transported across the gill surface to the egg. Alternatively, sperm width may differ among species to allow sperm to avoid capture by the mussel gill of the host mussel species. The necessity of sperm to have a large mitochondrial section or be adapted to swim long distances may also be related to the mussel species used. Although mussel respiration rates are not greatly different (Reichard et al. 2007a,b), even the slightest differences in speed of respiration could greatly influence the distances that sperm are required to swim as well as the time before the sperm is likely to meet the egg.

Further research on mussel preferences and mussel gill structure are needed before the differences in spermatozoa size can be fully explained, but it is likely that mussel gill structure and respiration have some selective influence in the external morphology of bitterling spermatozoa.

In conclusion, the spermatozoa of all the bitterling species I studied were similar, comprising of a head, midpiece and a flagellum protruding from the nuclear notch and sleeved in the upper section by a thinned portion of the midpiece. The differences in spermatozoa dimensions I observed between species were all small, probably because each species is extremely similar in mating system, each utilising the gills of freshwater mussels as a spawning site. The differences between species could be attributed to small sample sizes as the number of fish for use in the study was limited, however more likely is that the differences are related to both the mean number of ova released per spawning and the risk of sperm competition. The longest sperm, also with the longest flagellum, belonged to *R. amarus*, a species with a high natural risk of sperm competition and frequent spawnings where faster swimming sperm may be advantageous. *R. sinensis* a species with a large clutch size and therefore a high risk of sperm competition had smaller spermatozoa than *R. amarus* and therefore I suggest that our data conforms with previous work suggesting a negative correlation with egg number and sperm size. Differences between species may also be attributed to mussel host, though as the exact mechanism of fertilisation is unknown, the impact of differences in mussel filtration, or gill structure on sperm morphology are difficult to assess.

Figures

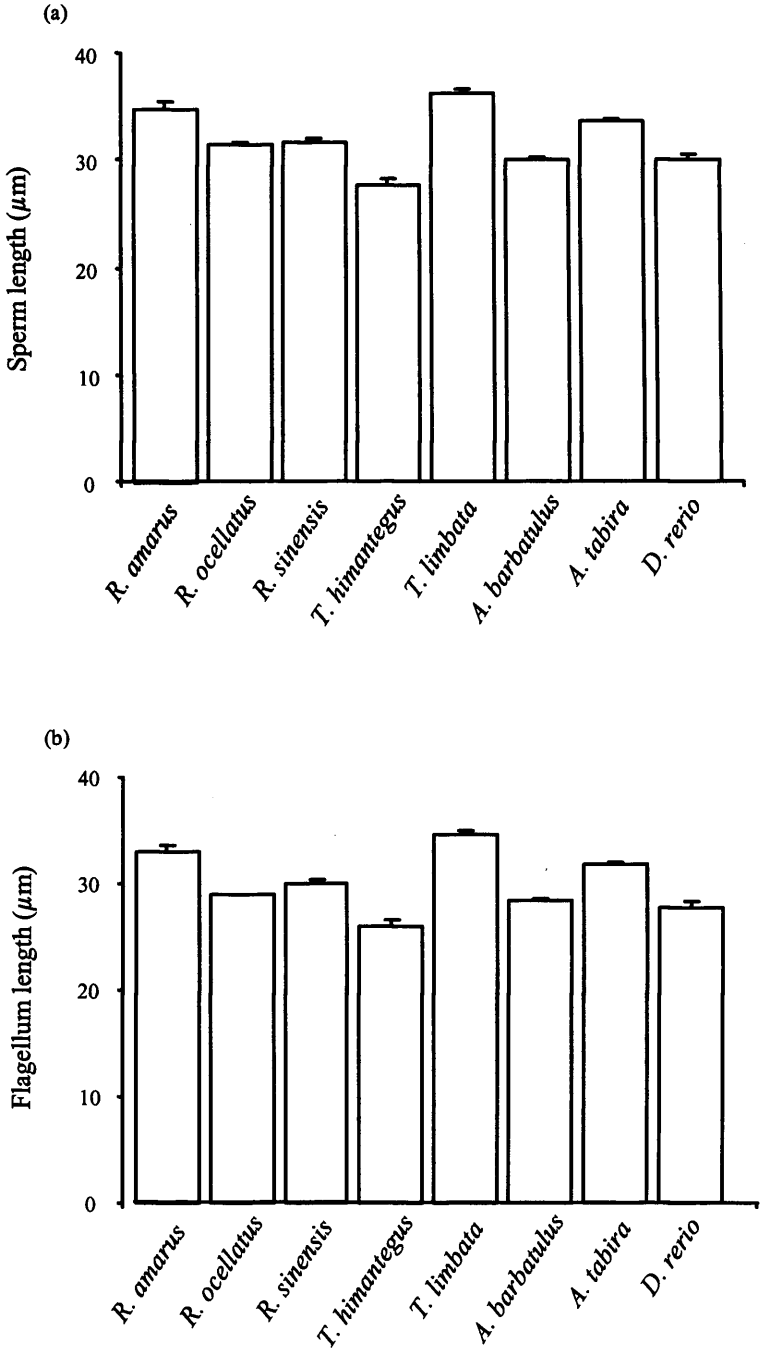

Figure 1. Total sperm length (a) and flagellum length (b) in observed bitterling species and the zebra fish (*D. rerio*). X axis: fish species. Y axis: measurement, um. Error bars are one standard error.

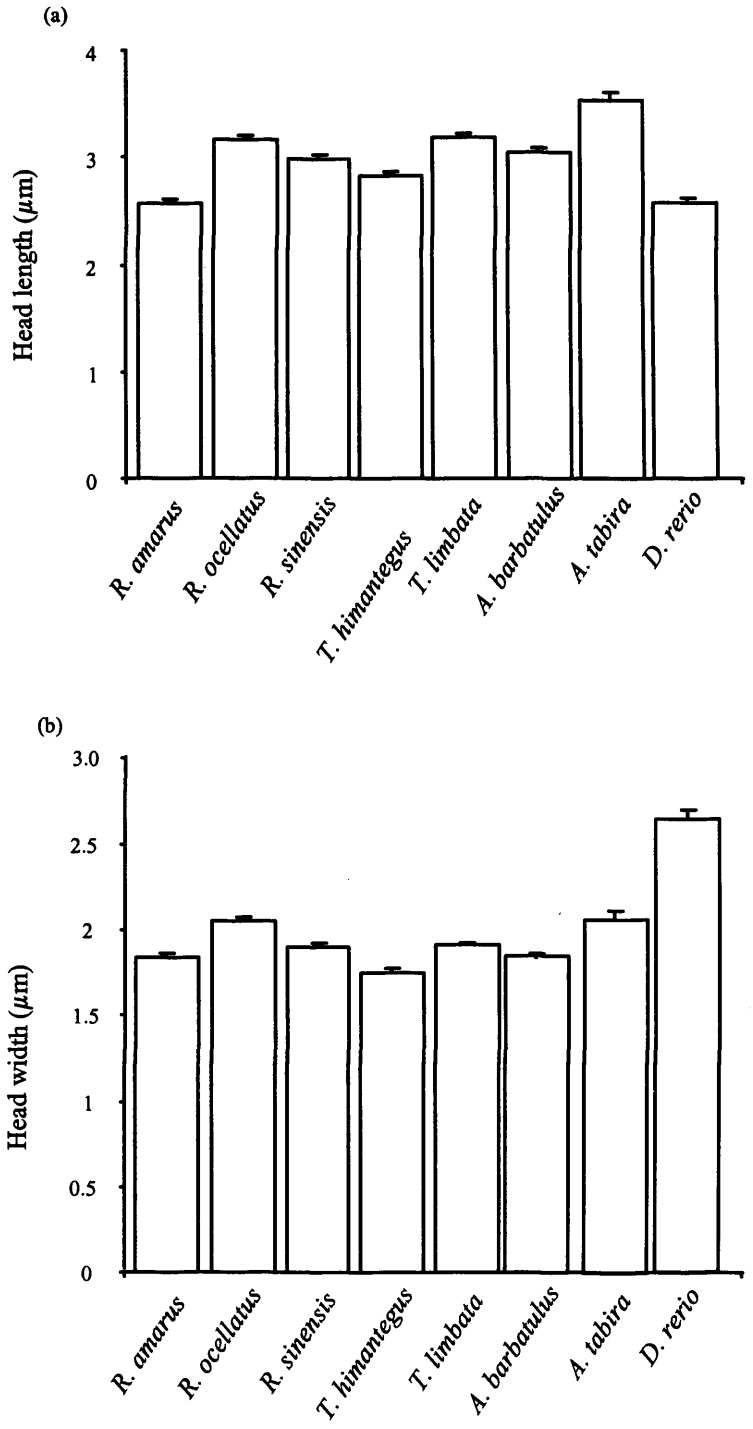

Figure 2. Spermatozoa head length (a) and Head width (b) in bitterling species and the zebra fish (*D. rerio*). X axis: fish species. Y axis: measurement, um. Error bars are one standard error.

Chapter 7. Testis structure and spermatogenesis in 3 species of bitterling; *Rhodeus amarus*, *R. sinensis* and *R. ocellatus*

Abstract

Several factors, such as sperm competition, cost of spermatogenesis, spawning frequency and egg clutch size, influence ejaculate expenditure. As a consequence, males are expected to have evolved mechanisms that act at different levels of the male reproductive apparatus, to allow modulation of ejaculate size and/or influence sperm production. Here I investigated, both qualitatively and quantitatively, the male reproductive apparatus of 3 separate but closely related species of bitterling, freshwater fishes that lay their eggs on the gills of living freshwater mussels. I used the European bitterling (*Rhodeus amarus*), the rose bitterling (*R. ocellatus*) and the Chinese bitterling (*R. sinensis*), which all have similar mating tactics and female fecundities but differ in spawning season length and clutch size and are, therefore, predicted to face different sperm competition risk.

I identified clear differences among species in testis structure, size and spermatogenic strategy, which are all linked to the natural risk of sperm competition. Our data, combined with previous experimental work, suggests that the development of semi-cystic spermatogenesis functions to allow the rapid production of large quantities of sperm without the need for costly investment into increased testis size, rather than as previously documented in gobies, an adaptation to the production of few spermatozoa.

INTRODUCTION

Sperm competition is a widespread phenomenon occurring when the ejaculates of different males compete to fertilise the same eggs (Parker 1970). Where sperm competition risk (the probability that ejaculates will overlap during a mating) is high the behaviour, physiology and reproductive anatomy of males are predicted to be adapted to compete through sperm competition (Smith 1984; Parker et al. 1996; Birkhead and Møller 1998). Empirical evidence demonstrates that high sperm competition risk drives the evolution of male adaptations across a wide range of taxa, including adaptations in testis and ejaculate size (Møller 1988; Briskie 1993; Gage and Barnard 1996; Stockley et al. 1996, 1997; Birkhead and Møller 1998; Sicotte 2002), sperm morphology (Gage 1994; Stockley 1997; Snook 2005), seminal fluid content (Scaggiante et al. 1999; Rasotto and Mazzoldi 2002), as well as fighting ability and other behavioural adaptations for sperm competition avoidance (Anderson 1994; Polak 1994; Alcock 1996; Sokolovska et al. 2000; Jones and Hutchings 2001; Preston et al. 2001; Carlini et al. 2002; Smith et al. 2002). In addition, modulation of ejaculate size and quality depending on sperm competition intensity has also been demonstrated at an intra-specific level (Shapiro et al. 1984; Candolin and Reynolds 2002; Pilastro et al. 2002; Evans et al. 2003; Pizzari et al. 2003; Zbinden et al. 2003; Burness et al. 2004; Pound and Gage 2004; Rudolfsen et al. 2006; Locatello et al. 2007; for reviews see Birkhead and Møller 1998; Simmons 2001; Wedell et al. 2002).

Despite selection to maximise the size of ejaculates when the risk of sperm competition is high, other factors, such as the cost of spermatogenesis, mating frequency, or the mode of spawning also appear to influence ejaculate expenditure, preventing males from producing and/or releasing a limitless number of sperm

(Dewsbury 1982; Wedell et al. 2002). Indeed data from different taxa indicate that sperm have nontrivial energetic costs in terms of both production and maintenance (Nakatsuru and Kramer 1982; Van Voorhies 1992; Shapiro et al. 1994; Marconato et al. 1996; Olsson et al. 1997). In addition, if males ejaculate at high frequencies over a relatively brief period, owing to repeated matings with the same female, such as in lions, or to a high degree of polygyny, as occurs in some labroid fish, sperm must be parcelled among matings to avoid rapid sperm depletion (Shapiro et al. 1994; Marconato et al. 1995; Marconato and Shapiro 1996). Moreover, where eggs of a batch are laid one by one or in small groups, such as the stickleback (Le Comber et al. 2004), the bitterling (Smith et al. 2004), gobies or blennies (Mazzoldi 1999; Giacomello et al. in press), males often divide sperm reserves between several ejaculates so that they can adjust the release of sperm in relation to the likely fertilisation return. As a consequence of the factors influencing optimal ejaculate structure, males are expected to have evolved mechanisms allowing prudent sperm allocation (Wedell et al. 2002). These mechanisms appear to influence either sperm release or sperm production and involve different parts of the reproductive apparatus. The amount of sperm released at a particular mating can be modulated by varying muscular contractions of the sperm duct or the vas deferens as demonstrated in a highly polygynous pelagic wrasse (*Thalassoma bifasciatum*) (Rasotto and Shapiro 1998) and in a rodent (*Peromyscus maniculatus*

seminal vesicles, accessory organs releasing the mucin component of the gobies viscous ejaculate (sperm trails) correlate positively with the degree of polygyny (Mazzoldi 1999; Mazzoldi et al. 2005). Among gobies, prudence in sperm allocation is particularly advantageous for polygynous species as opposed to monogamous ones, because males have to fertilize the eggs of different females, simultaneously or in rapid succession.

A mechanism affecting sperm production is the semi-cystic type of spermatogenesis in which spermatids, not spermatozoa, are released from the testis lobules, maturing into sperm within the reproductive duct system prior to reaching the genital opening (Lahnsteiner et al. 1990; Manni and Rasotto 1997; Mazzoldi 2001). This type of spermatogenesis may result in asynchronous maturation of spermatids, thereby reducing the number of simultaneously mature sperm produced. Until now semi-cystic spermatogenesis has been found in species characterized by low sperm competition risk, low fecundity and male parental care, such as jawfish, monogamous goby species and some blennies (Marconato and Rasotto 1993; Manni and Rasotto 1997; Mazzoldi 2001; Giacomello et al. in press). Among blennies the production of larger ejaculates has been experimentally linked with higher sperm competition risk and higher number of eggs per spawning (Giacomello et al. in press).

Bitterlings, a group of freshwater fish (Arai 1988) which have an unusual spawning system, utilising the gills of living freshwater mussels as spawning sites (Smith et al. 2004) represent a good model to further examine mechanisms within the male reproductive apparatus, which enable sperm economy. During the spawning season female bitterling develop long ovipositors which they use to place their eggs onto the gills of a mussel, through the mussel's exhalant siphon. Among bitterling, the spawning season length and therefore the frequency of female oviposition

(intervals between ovulation) is highly variable between species (Kondo et al. 1984; Nagata 1984; Smith et al. 2000; Reichard et al. 2007). Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel and are known to distribute sperm among ejaculates, which are frequent both pre and post-oviposition (Reichard et al. 2004). Sperm competition is known to occur in bitterling species and due to the similarity in mating system is thought to be widespread across the group (Smith et al. 2003, 2004; Reichard et al. 2005).

Some males adopt a guarder tactic (guarding a territory containing mussels and courting females) and others mate as sneakers (joining a spawning pair with no investment in territorial defence or courtship, and investing primarily into sperm competition) (Smith et al. 2003, 2004; Reichard et al. 2005). Bitterling mating tactics are opportunistic rather than fixed with individuals' often switching mating tactic to take advantage of local conditions to maximise their reproductive success (Kanoh 2000; Reichard et al. 2004; Smith et al. 2004). Mating tactic appears to be determined by among others, male size, density, operational sex ratio and the spatial clustering of mussels.

Here I analyzed, both qualitatively and quantitatively, the male reproductive apparatus of three separate, but closely related, species of bitterling, the European bitterling (*Rhodeus amarus*), the rose bitterling (*R. ocellatus*) and the Chinese bitterling (*R. sinensis*), which all have similar mating tactics and female fecundity (Kondo et al. 1984; Nagata 1984; Smith et al. 2000, 2004; Kitamura 2006), but differ in spawning season length and egg clutch size. The European bitterling has a substantially shorter spawning season (*R. amarus*; 6 weeks (Douglas 2003; Reichard et al. 2004) than the two Chinese species (*R. sinensis*; 3-4 months, *R. ocellatus*; 5-6 months (Kondo et al. 1984; Nagata 1984)). Overall seasonal fecundity is the same

among species, (Smith et al. 2000; Kitamura 2005; Reichard et al. 2007) though, while small clutches of eggs are produced several times a day during each ovulation period by female *R. amarus* (typically 2.9 (±0.2) eggs) and *R. ocellatus*, (typically 4.4 (±1.3) eggs), female *R. sinensis* lay considerably larger clutches with a mean of 15.7 (±2.6) eggs per spawning act, possibly only once or twice during a given period of receptivity (Smith et al. 2000, 2004; Reichard et al. 2007).

Subsequently, the risk of sperm competition varies among species. The intense clustering of spawning females during a short spawning season means that sperm competition risk in *R. amarus* is high, while longer spawning seasons in both *R. sinensis* and *R. ocellatus* suggest that both species face a lower risk of sperm competition. However, in *R. sinensis*, unlike *R. amarus* and *R. ocellatus*, large but infrequent clutches of eggs mean that fertilisation opportunities are both highly temporally and spatially clustered and therefore the risk of sperm competition and the intensity of male interference competition at each spawning is likely to be higher than either of the other species, where due to smaller clutch sizes fertilisations are rarely expected to become so clustered.

I expected to find differences in GSI between species related to sperm competition risk and conforming to sperm competition theory, with *R. ocellatus* having the smallest reproductive apparatus and *R. sinensis* the largest. I anticipated that there may be some kind of adaptation which allowed males to separate sperm reserves into individual ejaculates possibly similar to that found in the bluehead wrasse (*Thalassoma bifasciatum*, Rasotto and Shapiro 1998), where the sperm duct is subdivided into numerous compartments.

METHODS

Gonosomatic Index, Histology and Histochemistry

Male *R. amarus* (from an oxbow lake adjacent to the River Vistula, near the village of Soczewka (52° 32' N; 19° 34' E), central Poland), and males of each *R. sinensis* and *R. ocellatus* (from Lake Bao'an in the River Yangtze basin Central China (30° 50' N; 114° 16' E)), were collected and through behavioural experiments measuring aggressive behaviour and courtship (see Chapter 4) fish were assigned as either guarders (10 males of each species) or sneakers (10 males of each species). Following assignment to tactics fish were sacrificed with an overdose of anaesthetic (clove oil). Once dead, fish Standard length (SL) and body weight were recorded before the complete male reproductive apparatus (testes and sperm duct) was dissected and weighed. The gonadosomatic index, GSI, was calculated for each individual (gonad weight/body weight x 100). Samples were preserved in Deitrich's Liquid (900 ml distilled water, 450 ml 95% ethanol, 150 ml 40% formaldehyde, 30 ml acetic acid) for histological examination.

After fixation, samples were dehydrated in alcohol (30 minutes in 70, 80 95 and then 100%), embedded in paraplast, cross-sectioned serially at 7 μm moving posterior to anterior and mounted on slides. Sections from each specimen covering the complete sperm duct and testes were stained with Haematoxylin and Eosin as well as histochemical stains. For polysaccharide detection, sections were stained by the reaction of periodic acid-Schiff (PAS), and for the differentiation of sulphated and non-sulphated mucins by Alcian Blue at pH 1.0 and pH 2.5 (Pearse 1985). Using a Leica M216 Dissecting Microscope the area of sperm duct and testes were separately measured to provide a measure of the total proportion of each within the reproductive

apparatus. Secondly, using a Leica DMR fluorescence microscope, the thickness of epithelium cells was measured over the complete length of sperm duct to observe any changes, which may infer specific functions or areas of mucus production. Using the same microscopes the general anatomy of the testes and ducts was examined.

Data Analyses

All data were tested for normality using a Kolmogorov-Smirnov test. A Pearson's correlation was used to test for a link between male length and GSI and a *t*-test was used to compare GSI between mating tactics in each species. A one-way ANOVA with a Tukey's HSD *post hoc* test was used to compare GSI between species. A one-way ANOVA was also used to compare the proportion of the reproductive apparatus (volume) that the sperm duct occupied in each species, and to compare the thickness of the epithelium within the sperm duct.

RESULTS

Gonosomatic Index

Clear differences in the GSI were observed among species; *R. amarus* and *R. sinensis* had significantly larger GSI than *R. ocellatus* (one-way ANOVA, $F_{2,59} = 15.35$, $p < 0.001$; Fig. 1a). A Tukey's HSD *post hoc* test showed that while there was a difference in GSI between *R. amarus* and *R. ocellatus*, *R. sinensis* and *R. ocellatus* there was no significant difference between *R. sinensis* and *R. amarus*. Within *R. amarus* and *R. sinensis*, but not *R. ocellatus*, GSI was correlated with male length (Pearson's correlation, *R. amarus*, $r_{1,19} = 0.582$, $p = 0.007$; *R. sinensis*, $r_{1,19} = 0.480$, $p = 0.032$; *R. ocellatus*, $r_{1,19} = 0.419$, $p = 0.066$) and a significant difference between

mating tactics was observed (*R. amarus*, *t*-test, $t_{10} = 3.44$ $p = 0.007$, *R. sinensis*, *t*-test, $t_{10} = 3.83$ $p = 0.004$, *R. ocellatus*, *t*-test, $t_{10} = 2.31$ $p = 0.046$: Fig 1b); guarders had proportionally larger testes than sneakers.

General Morphology

The genital system is similar among each of the three species and does not differ between male mating tactics. The testes are elongated, paired bodies with two main testicular ducts continuing the sperm transport system. The ducts run the length of the testes and fuse posteriorly into a large, convoluted, common sperm duct before reaching the urogenital opening (Appendix 1a). The relative proportion of reproductive apparatus devoted to either the sperm transport system (main testicular ducts and sperm duct) or the testes varied among species (Fig. 1). In *R. amarus* a significantly higher proportion of the reproductive apparatus comprised the sperm transport system (51.73 %, ± SD, 3.85), than *R. ocellatus* (37.19 %, ± SD, 13.41), or *R. sinensis* (33.30 %, ± SD, 8.20) (one-way ANOVA, $F_{2,19} = 6.65$, $p < 0.007$; Fig 2). In *R. ocellatus*, but neither *R. amarus* nor *R. sinensis*, testis and sperm duct walls were spotted with chromatophores on the ventral side.

Gonads

In each species the testes are organised in lobules, opening onto the testicular duct. Lobules are separated by a thin layer of fibrous connective tissue rich in blood vessels and containing Leydig cells. Lobule walls are lined with a germinal epithelium containing spermatogonia and the subsequent stages of sperm development (Yasuzumi 1974; Billard 1986; Lahnsteiner and Patzner 1990a, 1990b; Suquet et al.

1994; Appendix 1b). In both *R. ocellatus* and *R. sinensis* the germinal epithelium shows all stages of spermatogenesis within cysts before fully developed sperm are eventually released into the lumen. In contrast, while the early stages of spermatogenesis appear similar in *R. amarus*, spermatids, not sperm, are released into the lobule lumen so that a mixture of spermatids and fully developed sperm are present within the lumen. The release of spermatids, which mature into sperm within the sperm transport system characterises the semi-cystic form of spermatogenesis.

Sperm Transport System

The sperm transport system comprises the two main testicular ducts and the sperm duct. Their walls show a common structure being organized in three layers; firstly, an external flat epithelium representing the coelomic wall, secondly, an intermediate layer of connective tissue, containing smooth muscle, and thirdly an internal single layered epithelium. I observed that the thickness of the internal epithelium was significantly greater in *R. amarus* than either *R. ocellatus* or *R. sinensis* (one-way ANOVA, $F_{2,357} = 841.56$, $p < 0.001$; Fig 3, 4; Appendix 1c, 1d), though there was also a significant difference between the latter two species (one-way ANOVA, $F_{2,357} = 841.56$, $p < 0.001$; Fig 3, 4). Also, in *R. amarus*, but not in *R. ocellatus* (where thickness was extremely variable) or *R. sinensis*, epithelium thickness increased along the length of the sperm transport system from posterior to anterior (*R. amarus*, one-way ANOVA, $F_{8,140} = 8.90$, $p < 0.001$; *R. ocellatus*, one-way ANOVA, $F_{11,111} = 6.85$, $p < 0.001$; *R. sinensis*, one-way ANOVA, $F_{7,95} = 0.40$, $p = 0.900$; Fig 4). In the posterior section, in close proximity to the genital pore, the sperm duct became more convoluted and the smooth muscle tissue thicker. All ducts were subdivided into chambers by thin walls of connective tissue containing smooth muscle cells. In all

three species samples reacted with Alcian blue histochemical staining at pH 2.5 and 1.0 suggesting that secretions of both sulphated and non-sulphated mucins were produced from the inner epithelium cells.

DISCUSSION

While the general organisation of male reproductive apparatus is similar among the three study species, I identified quantitative differences among species in the total investment, and the relative proportion devoted to the sperm transport system, as well as qualitative differences in the type of spermatogenesis. The total investment in reproductive apparatus, indicated by the GSI, shows that *R. amarus* and *R. sinensis* allocate significantly more than *R. ocellatus*, but not than each other. Both *R. amarus* (due to a short spawning season), and *R. sinensis* (with larger egg clutches), are thought to have a higher risk of sperm competition than *R. ocellatus* (long protracted spawning season and small clutch sizes) and invest significantly more in a large reproductive apparatus. Our data fits with sperm competition models and experimental studies indicating that testis size correlates positively with sperm competition risk (Harcourt et al. 1981; Gage 1994; Stockley et al. 1997; Byrne et al. 2002). Differences between mating tactics were observed in each species, with guarding males exhibiting a higher GSI than sneakers. Our data on intraspecific GSI variability may reflect a difference in the sperm requirements of different mating tactics. However, I observed little distinction in GSI between middle-sized males attributed to different tactics and believe the reason for this is that our GSI data probably reflects a difference in age, rather than specific morphological differences,

as younger smaller males are most often forced to act as sneakers, while older, larger individuals usually perform the role of guarders.

There was a significant difference among species in the proportion of reproductive apparatus devoted to the sperm transport system or testes; *R. sinensis* (33.30 %, ± SD, 8.20), and *R. ocellatus* (37.19 %, ± SD, 13.41), allocated significantly less (to sperm ducts) than *R. amarus* (51.73 %, ± SD, 3.85; Fig 2). It was also observed that epithelium thickness within the sperm duct was significantly greater in *R. amarus* than either *R. sinensis* or *R. ocellatus*, though there was also a significant difference between the latter two (*R. sinensis*> *R. ocellatus*). Mucus was produced in each species and did not appear restricted to a specific area of the ducts. The differences observed between species in the relative investment into testis or sperm duct may be representative of the temporal differences in sperm demand among the three species. Due to a short intense spawning season and small clutch sizes (3-4 eggs), which ensure that spawnings are frequent, *R. amarus* is expected to require a rapid production of multiple small ejaculates. However, unusually when compared to other non-bitterling species (Rinchard and Kestemont 1996; Tarkan 2006), which face a high demand for sperm, the GSI in *R. amarus* is comparatively quite low, while the development of the sperm duct relative to the testes is high. In contrast to both *R. ocellatus* and *R. sinensis*, where fully developed sperm were released from the wall of the lumen, in *R. amarus* spermatogenesis is semi-cystic; germ cells are released from cysts as spermatids rather than sperm, and continue their development to full maturity in the lumen or the ducts. A semi-cystic mode of spermatogenesis has been observed in species with low GSI investment (in these cases evaluated as an index of exclusively testis size), low fecundity and low risk of sperm competition (Marconato and Rasotto 1993; Mazzoldi 2001; Giacomello et al.

in press), and in some cases the release of small ejaculates has been experimentally documented (Giacomello et al. in press). Therefore, in *R. amarus*, considering the non-trivial cost of sperm production, semi-cystic spermatogenesis could be an adaptation to the production of small ejaculates. However, the spawning frequency and the risk of sperm competition might have influenced the development of an extremely large sperm transport system where sperm maturation is accelerated. This system may allow the rapid production of large quantities of sperm with relatively little cost in terms of reproductive tissue and may be ideal for fish species with short but intense spawning seasons.

In contrast to *R. amarus*, both *R. sinensis* and *R. ocellatus* invest more in the production of testicular tissue, possibly reflecting a more prolonged spawning season and longer intervals between spawnings. While both *R. amarus* and *R. sinensis* need large quantities of sperm, *R. sinensis* has significantly longer intervals between periods of intense sperm demand (matings) in which it can replenish sperm reserves. Therefore, with a high risk of sperm competition but longer intervals between spawnings, investing primarily into larger testes rather than sperm duct, may be the most effective strategy, allowing sperm to be produced synchronously in large quantities. In support of this hypothesis, epithelium thickness was significantly greater in *R. amarus* than either *R. sinensis* or *R. ocellatus* and in *R. amarus*, was greatest nearer the testes (anterior) rather than the genital pore. A thicker epithelium may suggest either (but not mutually exclusive), a specific adaptation to the maturation of spermatids into sperm, such as the re-absorption of residual bodies cast off by developing spermatids (Manni and Rasotto 1997), or that mucus is being produced for some purpose such as parcelling sperm into bundles or prolonging sperm longevity (Marconato et al. 1996; Scaggiante et al. 1999).

In all three species the posterior section of the sperm duct (nearest the genital pore) was well developed, highly convoluted and divided into multiple compartments, each surrounded by layers of smooth muscle tissue. A similar chambered sperm duct has been noted in the bluehead wrasse (Shapiro et al. 1994; Rasotto and Shapiro 1998) where males may be able to tailor the size of their ejaculate to the level of sperm competition at spawning or the quality of a female. Data on sperm concentrations after successive ejaculates at spawning events in *R. amarus* (Chapter 3) suggest that males may control ejaculate size, dependent on the risk of sperm competition, sperm depletion or female interest and, therefore, gain a reproductive advantage over rivals. Therefore, I suggest that as in the bluehead wrasse (Shapiro et al. 1994; Rasotto and Shapiro 1998), smooth muscle tissue surrounding duct compartments may act as a mechanism that enables the control of ejaculate size by differential chamber contraction within the sperm duct.

In conclusion our data shows clear supporting evidence for the influence of sperm competition risk and spawning frequency on the evolution of adaptations in the male reproductive apparatus of bitterling (Harcourt et al. 1981; Gage 1994; Stockley 1997), and presents an alternative hypothesis for the development of semi-cystic spermatogenesis; to allow the rapid production of large quantities of sperm without the need for costly investment into increased testis size. The key difference between our study species that appears to determine spermatogenic strategy is spawning season length and specifically, the length of the intervals between mating bouts. *R. amarus* which has a short intense spawning season has an enlarged sperm duct where the final stages of spermatogenesis take place, freeing the testes to produce the earlier stages of spermatogenesis. Similarly, *R. sinensis* has a high risk of sperm

competition, but also considerably longer intervals between spawnings and therefore, unlike *R. amarus*, full spermatogenesis is viable within the testes.

Figures

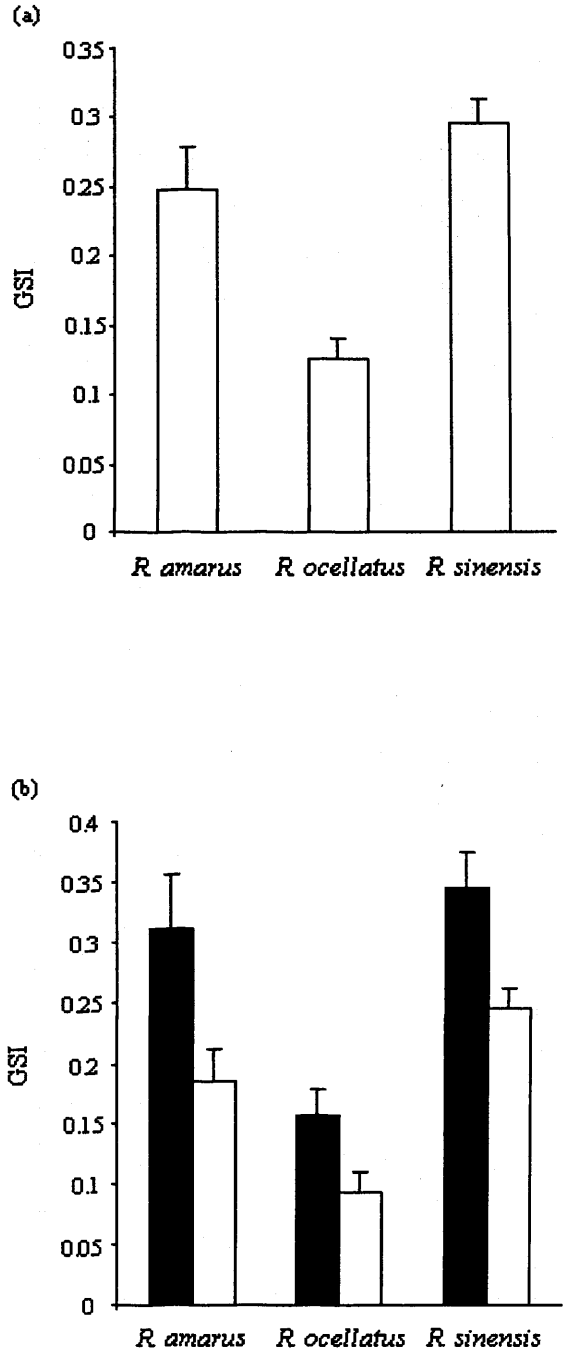

Figure 1. Mean gonosomatic index (GSI) for *R. amarus*, *R. ocellatus* and *R. sinensis*. Graph (a) presents combined GSI data for guarder and sneaker males in each species. Graph (b) shows the GSI data for both sneaker and guarders separately. White bars represent sneaker males, while black bars represent guarders. Error bars are one standard error.

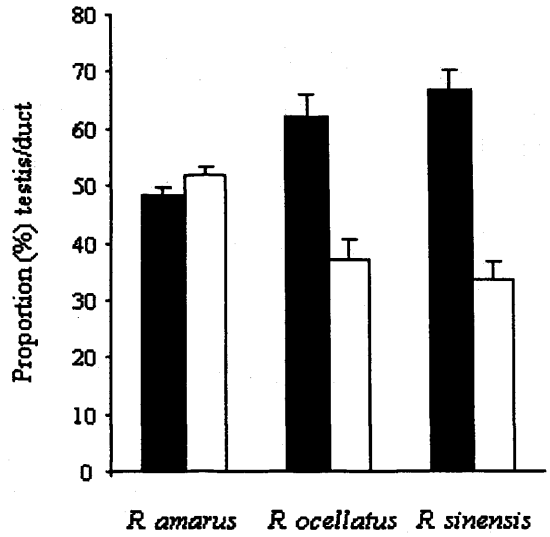

Figure 2. Mean percentage of the total reproductive apparatus devoted to sperm duct or testis in *R. amarus*, *R. ocellatus*, *R. sinensis*. Black bars represent the proportion of the reproductive apparatus devoted to testes, while white bars show the proportion devoted to sperm ducts. Error bars are one standard error.

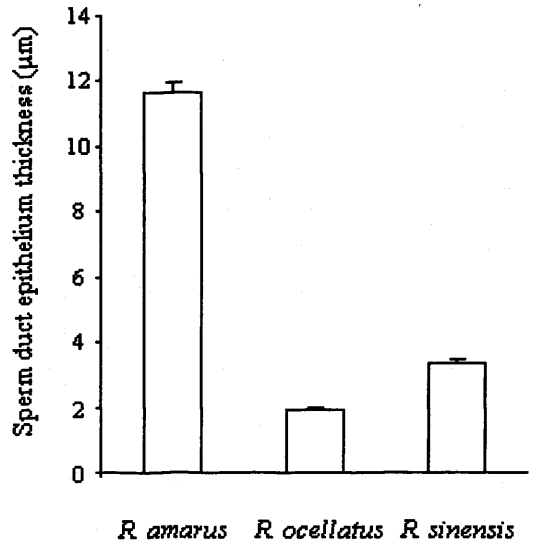

Figure 3. Average epithelium thickness (μm) within the sperm ducts of *R. amarus*, *R. ocellatus*, *R. sinensis*. Error bars are one standard error.

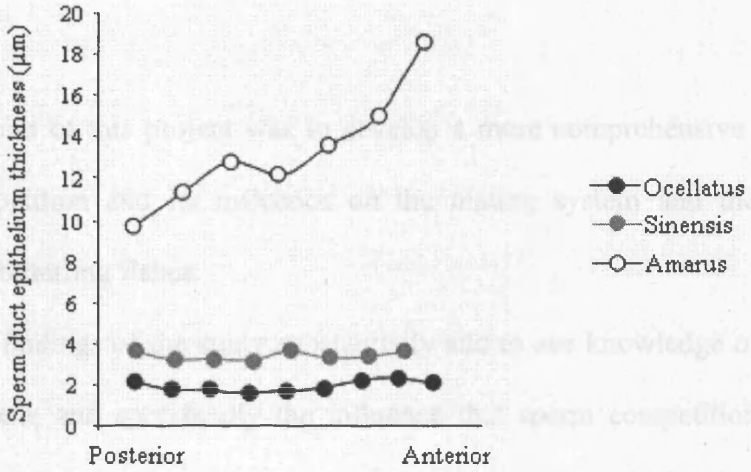

Figure 4. Epithelium thickness (μm) over the entire length of the sperm duct. Marks represent measurements taken serially, moving from posterior to anterior within the reproductive apparatus of each species; *R. amarus*, *R. ocellatus*, *R. sinensis*.

Chapter 8. Discussion

The broad aim of this project was to develop a more comprehensive knowledge of sperm competition and its influence on the mating system and the reproductive anatomy of bitterling fishes.

The findings of the study substantially add to our knowledge of the bitterling mating system, and specifically the influence that sperm competition has on male behaviour and reproductive anatomy in terms of testicular structure and spermatozoon morphology. I have shown that male bitterling are highly sensitive to the intensity of sperm competition, increasing ejaculate frequency according to sperm competition theory (Parker 1990) (Chapter 2 and 4). Males were affected by sperm depletion over both a single mating and the course of a day, and the extent to which males became depleted was more acute when a rival male was present (Chapter 3). I also identified that while the ejaculates of sneaker and guarder males were of a similar size, small males produced more sperm per unit of bodyweight than larger males, but that there was no difference in sperm quality between mating tactics (Chapter 2). Sperm competition risk also appears to affect the morphology of spermatozoa, with species that face a high risk of sperm competition having sperm with significantly longer flagella, possibly reflecting the importance of ejaculate timing, as flagella length suggests that swimming speed may be of greater importance than numerical superiority in the bitterling system (Chapter 6). Also conforming to sperm competition theory, the smallest spermatozoa were found in *R. sinensis* the species with the largest clutch size (Peters 1971; Stockley et al. 1996, 1997, Chapter 6). Reproductive anatomy is also affected by sperm competition risk, with a greater GSI found in species with higher risk of sperm competition, and the proportion of the

reproductive apparatus invested into either duct or testes directly linked to spermatogenic strategy, varying among species, and most likely dependent on the intensity of sperm demand (Chapter 7). As an adaptation to prolonged periods of intense sperm demand male *R. amarus* have semicystic spermatogenesis and a large reproductive apparatus with a significantly greater proportion of the apparatus devoted to sperm duct, where, prematurely released spermatids develop into full spermatozoa. In *R. sinensis*, a species where periods of intense sperm demand (infrequent high value spawnings) were spread over a long spawning season, allowing sperm reserves to recover between spawnings, spermatogenesis was normal and the proportion of the reproductive apparatus devoted to the sperm duct was considerably lower than in *R. amarus* (Chapter 7). To my knowledge this adaptation for semicystic spermatogenesis to allow rapid sperm production is the first known example of this type. Semicystic spermatogenesis is normally found in species where males are trying to reduce spermatozoa investment through mucus production or the addition of other seminal fluids (Marconato et al. 1996; Manni and Rasotto 1997; Scaggiante et al. 1999).

The risk of sperm competition and the ability of guarding males to control mating opportunities was directly influenced by the Operational Sex Ratio and the spatial clustering of mussels; sneaking males had significantly greater opportunity to ejaculate when mussels were spatially dispersed and the OSR at unity (Chapter 4). It appears that sneakers and guarding males behave differently in terms of their distribution of ejaculates among mussels; guarders focus their ejaculates on a small group of mussels where they can monopolise matings and maximise their overall paternity, while sneakers, which normally sire a much lower proportion of any clutch, visit a large group of mussels to release ejaculates (Chapter 5). Importantly for

sneakers, sperm remain within the mussel for long periods and the peak of sperm concentration is 30 seconds after ejaculation, suggesting that pre-oviposition ejaculations can not only sire young (Reichard 2004a), but may be more effective (in sperm competition) than ejaculates released at or just after oviposition (Chapter 2).

My finding that males often fail to release sperm when performing an ejaculating motion over the mussel suggests an intersexual conflict (Chapter 3). Where males perform "false" ejaculations, females may be encouraged to spawn, which may enable males to conserve sperm and avoid sperm depletion. Females also perform a "skimming" behaviour, which may increase the probability of sperm competition by attracting males to the spawning and thereby ensuring female fertility (Chapter 3). I suggest that further work to clarify the exact mechanisms of the sexual conflict would provide added insight into bitterling reproductive biology.

The spatial movement between mussels of male *R. ocellatus* (Chapter 5), together with other bitterling species, suggests there may be a heightened spatial cognitive memory and possibly the associated hippocampal adaptations (Gaulin and Fitzgerald 1989; Krebs et al. 1989; Sherry et al. 1989; Smulders et al. 1995; Reboreda et al. 1996; Clayton et al. 1997; Aubin-Horth et al. 2005). In species which require a spatial memory the hippocampal area of the brain is often enlarged in comparison with the same area in other closely related species that have less need of spatial memory; with further research this may also be proved the case among bitterling (Gaulin and Fitzgerald 1989; Krebs et al. 1989; Sherry et al. 1989; Real 1993; Reboreda et al. 1996; Healy and Braithwaite 2000).

Parker (1998) suggests that males may revisit a spawning site to frequently top up sperm concentrations, so that should a spawning occur in the males' absence, that male may still sire a proportion of the spawned ova. Sneaker male bitterling

frequently return to mussels to ejaculate and it may be that males are topping up sperm concentrations within the mussel (Chapter 5). Therefore the movement of males between mussels to repeatedly ejaculate, and the known depletion in sperm concentration within the mussel after ejaculation suggests that further work on cognitive ecology, hippocampal volume, and the male behaviour of topping up may prove useful in the understanding of bitterling biology.

Future work should also aim to understand the movement of sperm within the mussel as currently little is known of the mechanism of the sperm-egg collision. Work should also attempt to test the importance of timing on the success of ejaculates in sperm competition and also examine the structure of guarder and sneaker ejaculates together with pre and post-oviposition ejaculates in terms of mucus and seminal fluid content.

Bitterling are an extremely useful and undervalued model species for behavioural studies. They are ideally suited to research into sperm competition and dominance as experiments need not affect fish behaviour. The main advantage in using bitterling comes from their relationship with freshwater mussels which can be easily manipulated to provide different intensities of likely sperm competition. However a distinct disadvantage in using bitterling is the difficulty in rearing young and the differences among species in their readiness to breed, where some of the species, specifically those with long spawning seasons were almost never ready to spawn in the aquarium and therefore useless for sperm competition experiments in the UK.

In summary this project has collected data on both the behavioural and anatomical aspects of bitterling biology relating to sperm competition. It has identified behavioural differences between mating tactics in terms of spatial

movement and ejaculate frequency and size, but histological work has showed no obvious differences between guarder and sneaker males, and therefore may explain the ability of males to adapt successfully to either mating strategy. Histological analysis may have identified an adaptation to intense sperm demand; semicystic spermatogenesis in *R. amarus* may reduce the impact of sperm depletion (which I showed to be of importance to males, especially where rivals were present), and allow the production of larger volumes of sperm with a smaller investment into testis tissue. We now have a fuller understanding of anatomical adaptations (gamete and reproductive apparatus) to sperm competition and more fully understand differences in sperm competition risk and spermatozoa morphology among bitterling species.

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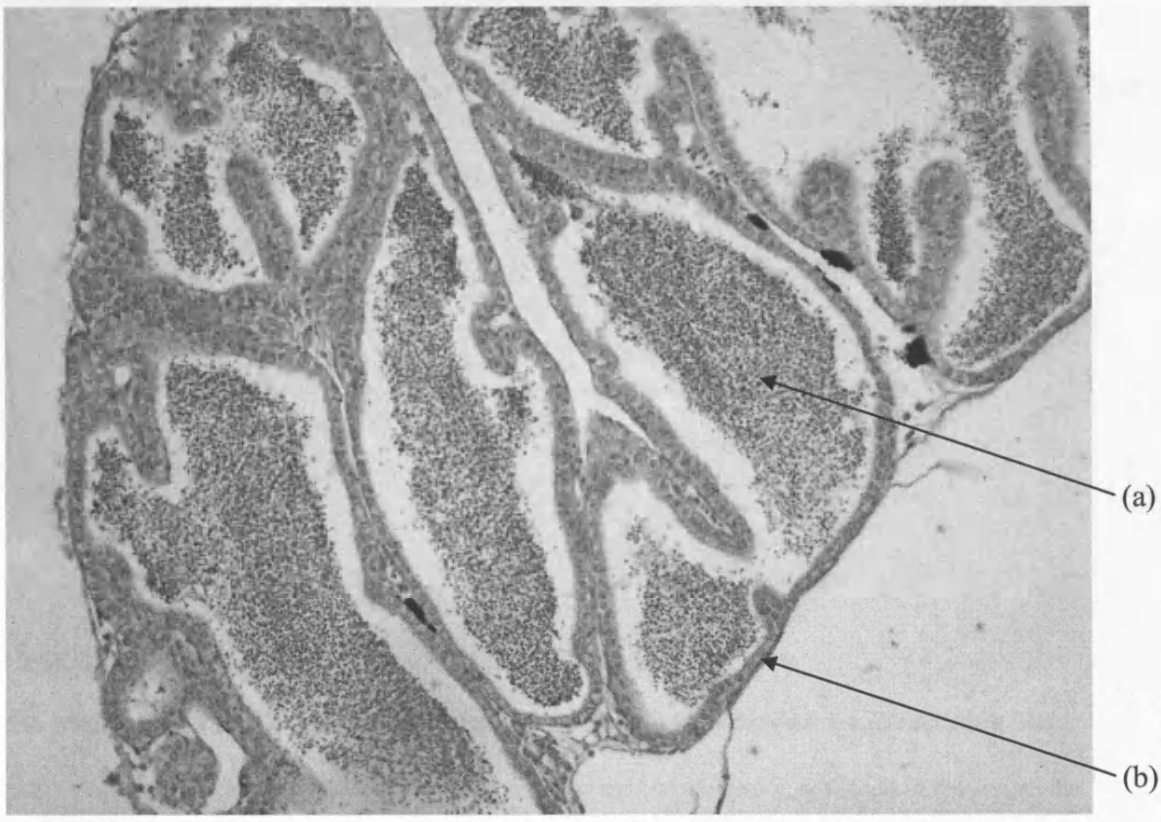
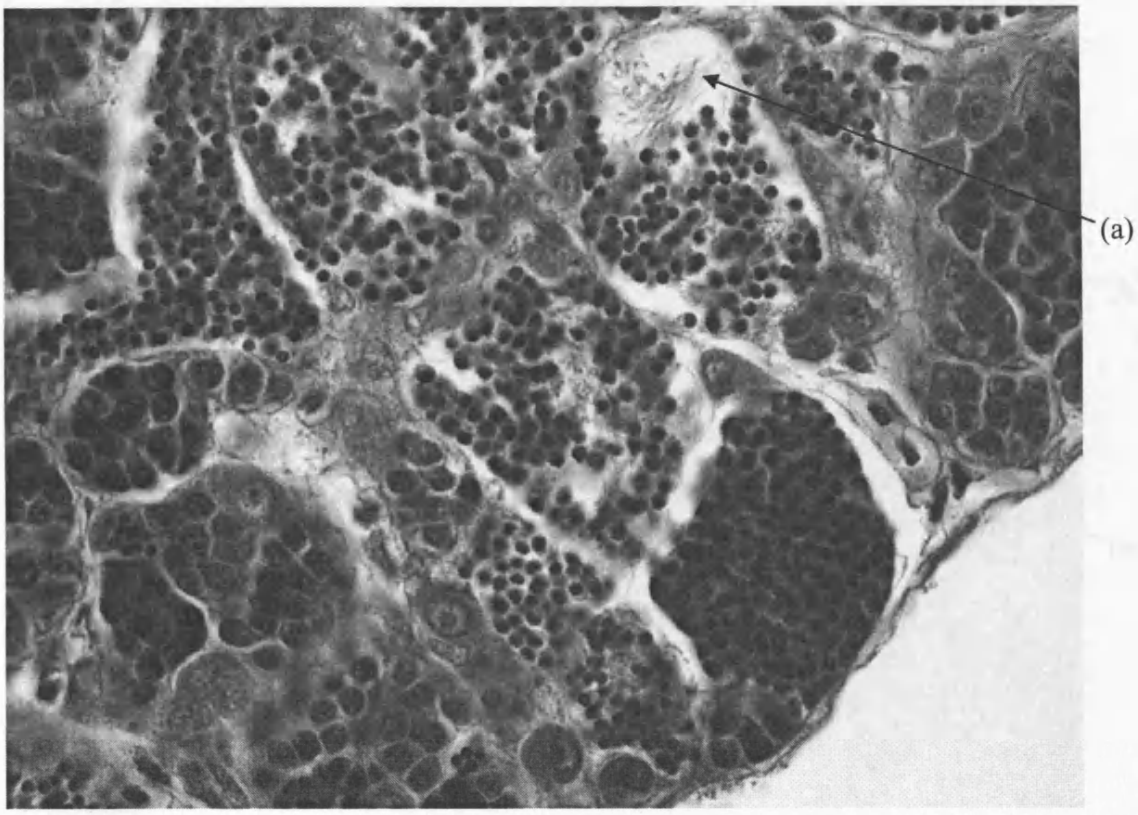
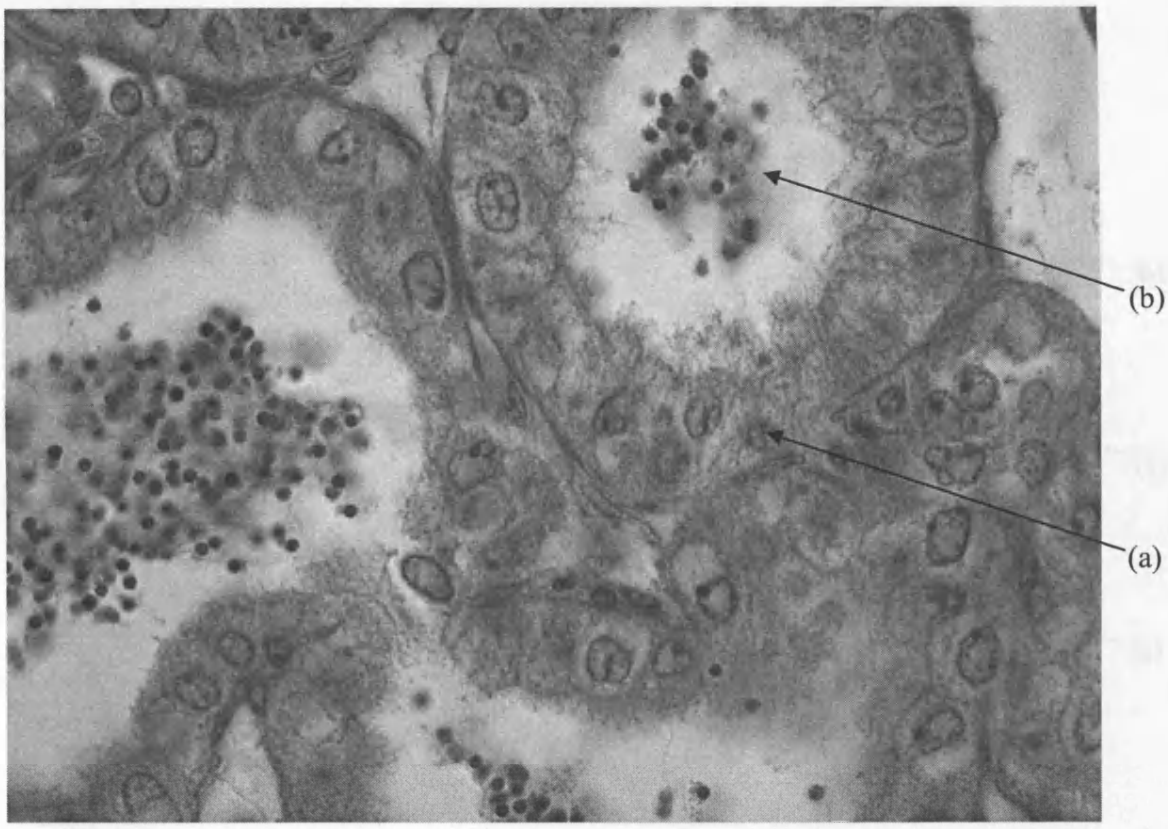
APPENDIX 1



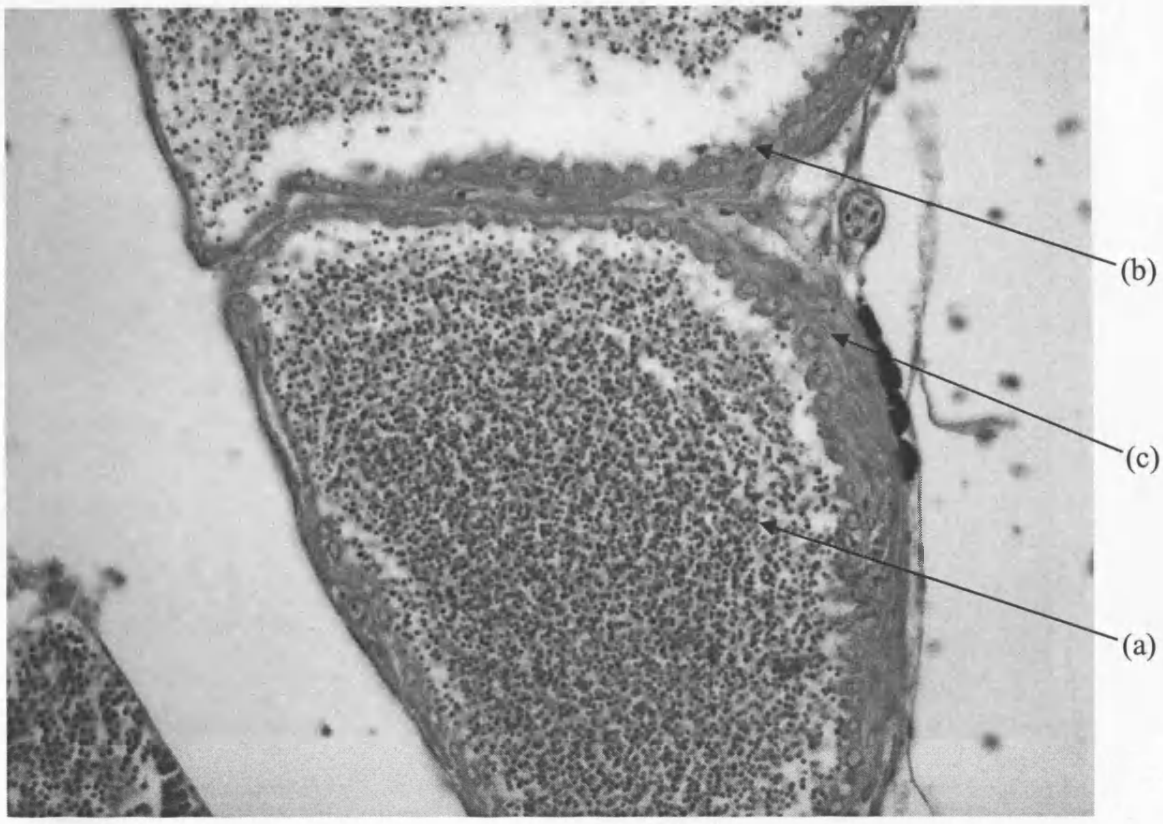
1a. Magnified (15 x 20) image of the sperm duct of *R. amarus*. The duct is highly convoluted with areas containing densely packed spermatozoa (a) separated by epithelium (b) and muscle tissue.



1b. Magnified (5 x 100) image showing a detailed view of the testis structure (in *R. ocellatus*). The image shows all four stages of spermatogenesis and just right of the centre, at the top of the image, the tails of mature spermatozoa being released from a cyst are visible (a).

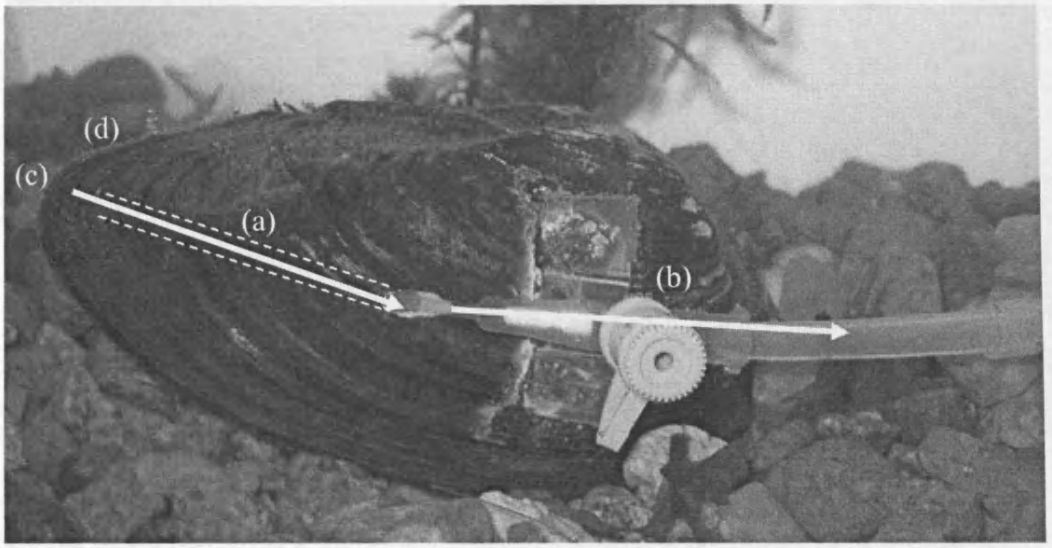


1c. Magnified (15 x100) image of the epithelium layer within the sperm duct of *R. amarus* (a). Small groups of sperm (b) are separated by the convoluted duct tissue. Here in *R. amarus* the epithelium layer is considerably thick appears to be secreting mucus.

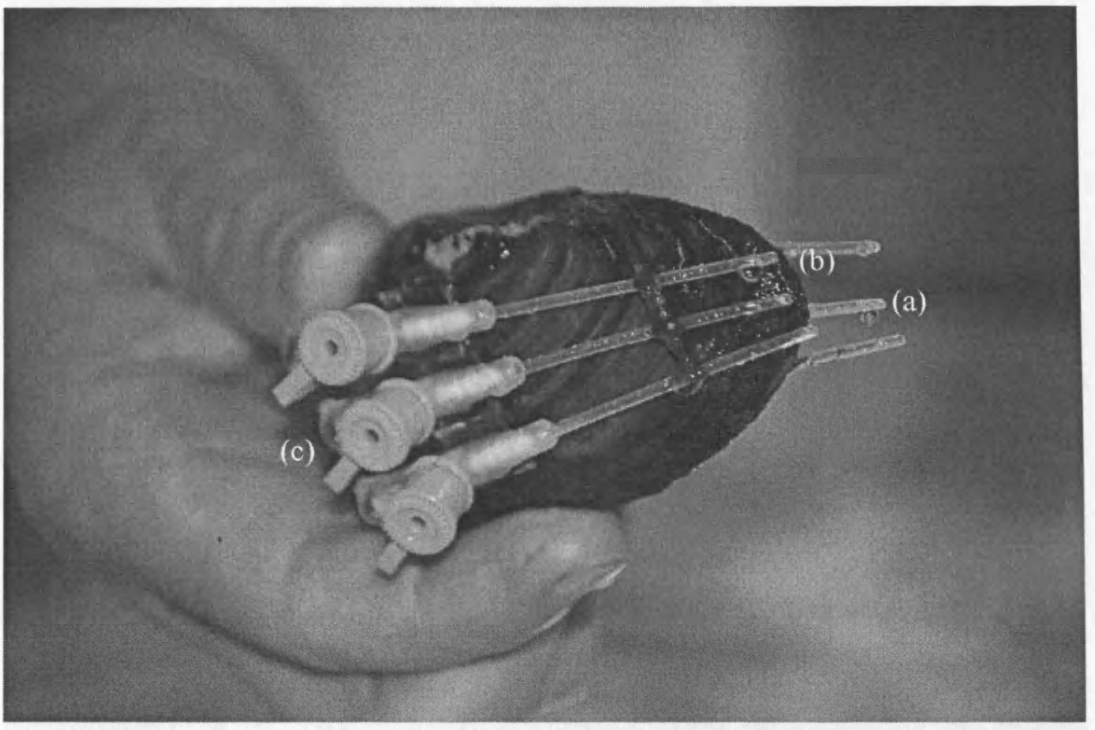


1d. Magnified (2 x 40) image of the sperm duct of *R. sinensis* (similar in structure to that of *R. ocellatus*) containing spermatozoa (a). The epithelium (b) is considerably thinner than that of *R. amarus*. Running parallel to the epithelium layer there is smooth muscle tissue (c) which may be used to contract the different compartments of the duct at ejaculation.

APPENDIX 2

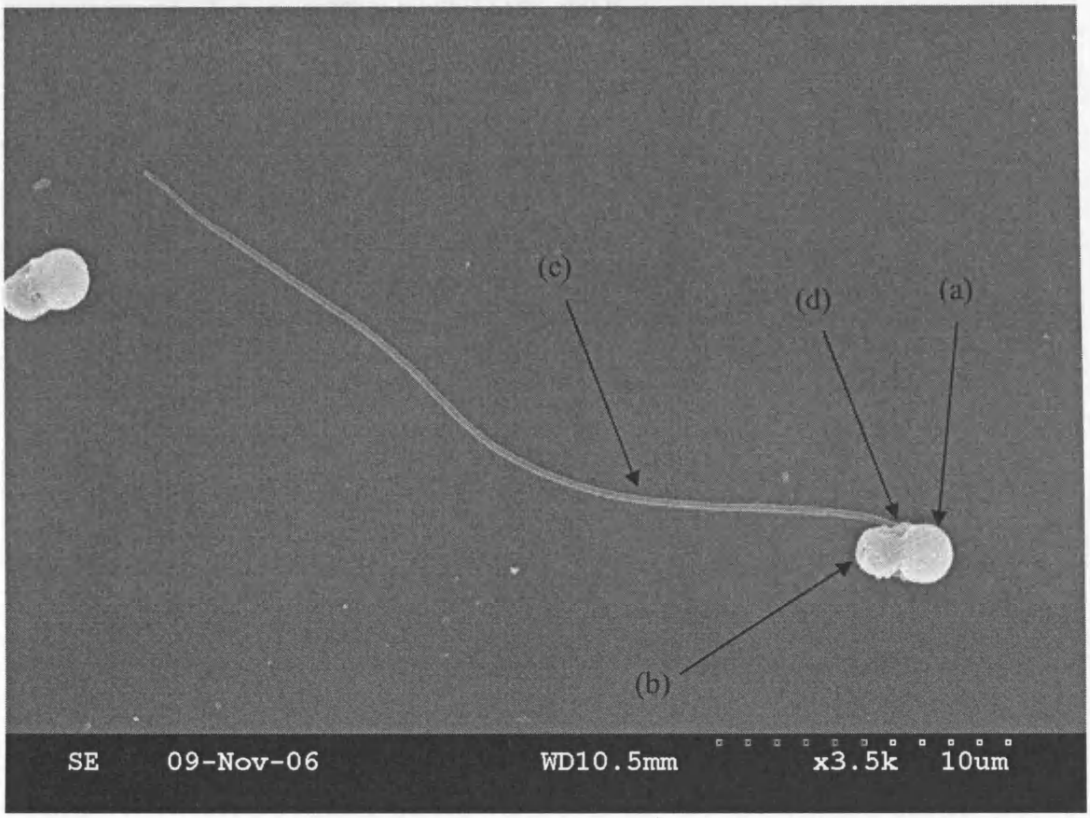


2a. Picture of a Unio pictorum mussel, illustrated to show the flow of water (white arrow) from the tip of the cannula (5mm (b)), inside of the inhalant siphon of the mussel, to the plastic tubing where the sample is removed. (a) The dashed line shows the position of the cannula tubing within the mussel shell. (c) Marks the position of the inhalant siphon and (d) the position of the exhalant siphon.

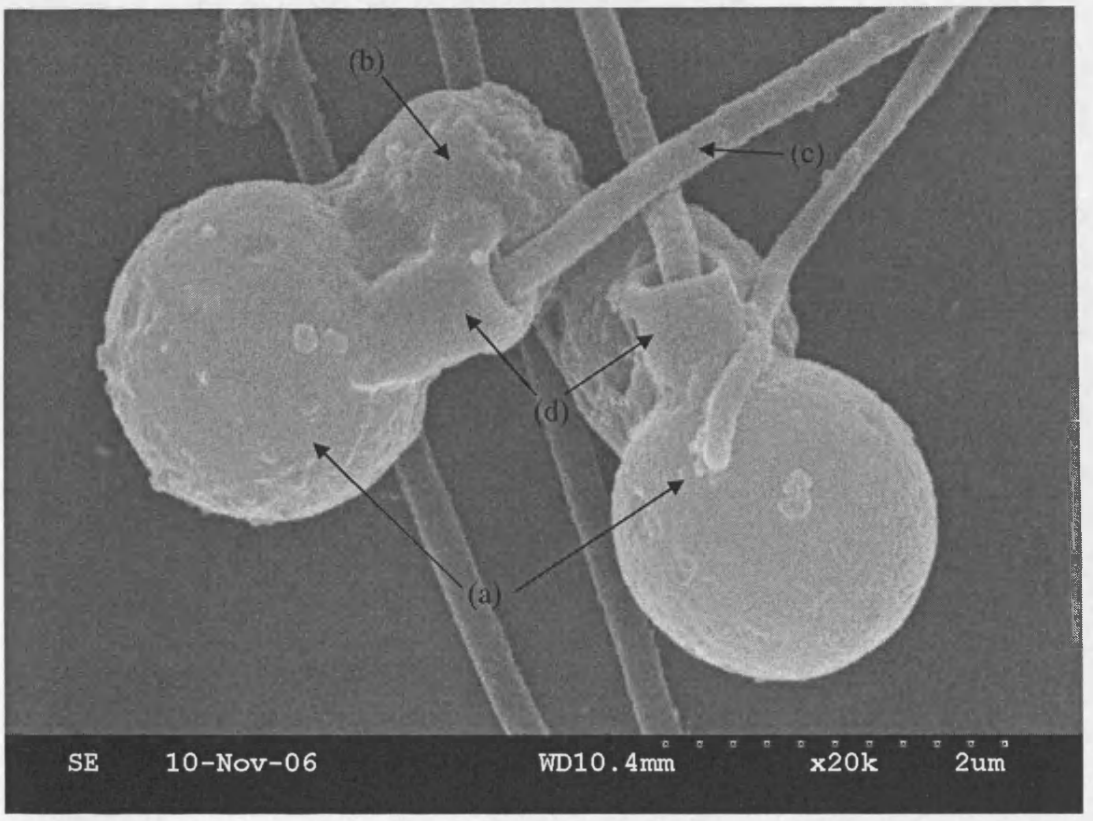


2b. Photograph of a *Unio tumidus* mussel with six cannulas attached to it. Later, the tips of the cannulas (a) were grouped together so that each rested within 5 mm of the inhalant siphon (b). In the photo the tubing used to remove the sample (as shown in 2a) is not yet attached at the base of the cannula (c).

APPENDIX 3



3a. Annotated photograph of a single spermatozoon from *R. ocellatus*, taken using the scanning electron microscope facilities at the University of Leicester. The sperm head (a) and neck regions (b) (containing the mitochondria) are both distinguishable and round in shape. The flagellum (c) arises in the nuclear notch at the lateral side of the spermatozoon and is surrounded, at the base, by a sleeve of plasma membrane (d).



3b. Annotated photograph of the head region of two *R. ocellatus* spermatozoon, taken using the scanning electron microscope facilities at the University of Leicester. The sperm head (a) and neck regions (b) (containing the mitochondria) are both distinguishable and round in shape. The flagellum (c) is clearly surrounded at the base by a sleeve like structure (d), consisting of plasma membrane.