



## Variation in male reproductive traits among three bitterling fishes (Acheilognathinae: Cyprinidae) in relation to the mating system

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Male traits that correlate with fertilization success include testis size and structure, ejaculate size, ejaculation frequency, and sperm motility. Two hypotheses potentially explain interspecific differences in these traits: sperm competition and sperm limitation. We examined variation in six traits associated with fertilization success in three closely-related species of bitterling fish; the European bitterling (*Rhodeus amarus*), the Chinese rose bitterling (*Rhodeus ocellatus*), and the Chinese bitterling (*Rhodeus sinensis*). Interspecific differences indicated that the three study species have evolved different sperm allocation strategies. *Rhodeus amarus* displayed the most developed reproductive apparatus with a number of traits associated with both high levels of sperm production and fertilization efficiency. *Rhodeus ocellatus* and *R. sinensis* appear to have more comparable sperm allocation strategies, although relative testis size and spermatozoa head : tail ratio were greater in *R. sinensis*, suggesting that sperm competition risk may be higher in this species. All three species possessed an unusually well developed sperm duct with evidence of mucin production, which greatly extends the longevity of sperm and, consequently, the period over which fertilization can occur. We discuss these findings in the context of differences in the mating systems of the species examined, and relate the results obtained to differences in the temporal and spatial clustering of fertilizations. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **103**, 622–632.

**ADDITIONAL KEYWORDS:** reproduction – sperm competition – sperm depletion – sperm economy – sperm limitation.

### INTRODUCTION

The principal determinant of the intensity of sexual selection on males is the way in which fertilizations are distributed in space and time (Emlen & Oring, 1977; Shuster & Wade, 2003). In many species, males defend patches of resources critical for female repro-

duction (such as sites for nesting or oviposition) and females are distributed around these resources during breeding. If resources are highly aggregated (spatially clustered), a relatively small number of males can monopolize them. A consequence is often an increase in the variance of male reproductive success; a few males will obtain the majority of fertilizations, whereas many may obtain none. By contrast, if resources are widely dispersed in the environment, or

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cannot be readily monopolized, many males may mate successfully, with the result that variance in male reproductive success will be low. There can also be variation in the length of reproductive seasons and the temporal distribution of female ovulation. In species in which the breeding season of females shows low synchronization or the breeding season is spread over a long period, a few males may be able to monopolize the majority of fertilizations. By contrast, if the spawning season is short and most females are in breeding condition simultaneously, the intensity of sexual selection on males is predicted to be low (Shuster & Wade, 2003). Thus high variance in male reproductive success is predicted when females are clumped in space and asynchronous in their mating receptivity. Under these conditions, the prediction is for the evolution of alternative male mating tactics and adaptations associated with sperm competition (Shuster & Wade, 2003). By contrast, if fertilizations are spatially dispersed and highly synchronous, competition among males for fertilizations may be limited, although male reproductive success may be constrained by their ability to efficiently distribute ejaculates among numerous matings.

In many animal taxa, there is variation among species in male traits, such as testis size, ejaculate size, ejaculation frequency, and sperm motility, which correlate with fertilization success (Parker, 1998). Two general hypotheses have been invoked to explain interspecific differences in such traits: sperm competition (Parker, 1970) and sperm limitation (*sensu* Wedell, Gage & Parker, 2002). Sperm competition (i.e. competition between the sperm of two or more males for the fertilization of ova) is an important mechanism of sexual selection (Andersson, 1994; Eberhard, 1996). The probability that a male's sperm will compete with the ejaculates of rival males, termed the risk of sperm competition, can explain a wide range of male adaptations. These include mate guarding, larger relative testis size, larger ejaculates, and larger sperm stores (Gage, 1994; Stockley *et al.*, 1997). Because ejaculates are energetically expensive to produce (Wedell *et al.*, 2002), males are predicted to adjust actual ejaculate expenditure in response to the intensity of sperm competition (actual number of competing ejaculates). Thus, males should maximize ejaculate expenditure in competition with a single competitor, although reduce expenditure as the number of rivals exceeds one because the probability of fertilizing eggs diminishes with the number of competing males (Parker *et al.*, 1996).

Sperm limitation occurs when males become depleted of sperm, or prudently control the size and frequency of their ejaculates to avoid becoming depleted of sperm. Sperm limitation is predicted to evolve when males mate frequently, when fertilization

efficiency is low (e.g. in the case of external fertilization), when clutch sizes are large, and when males have high reproductive costs (Emerson, 1997; Levitan, 1998; Giacomello, Neat & Rasotto, 2008; Lessells *et al.*, 2009). Male traits associated with sperm limitation include muscular control of sperm ducts to modulate ejaculate size, a high mucin component to ejaculates to slowly release sperm over an extended period, and the semi-cystic type of spermatogenesis in which spermatids, and not spermatozoa, are released from the testis lobules, which enables the asynchronous maturation of spermatids, thereby reducing the number of simultaneously mature sperm, possibly favouring the production of numerous small ejaculates (Manni & Rasotto, 1997; Mazzoldi, 2001; Giacomello *et al.*, 2008). Where sperm limitation is a feature of a mating system, a prediction is that female fertility will be constrained by sperm availability, possibly with adaptations for enhanced fertilization in females as well as males (Levitan, 1998).

In the present study, we examined behavioural and morphological adaptations linked to fertilization success among three closely-related bitterling fishes (subfamily Acheilognathinae, family Cyprinidae) that form a single phylogenetic clade (Okazaki *et al.*, 2001) but differ in the way fertilizations are distributed in space and time. The traits examined were ejaculation rate, relative testis size, reproductive apparatus morphology, and sperm morphology. Thus, high rates of ejaculation and relatively large testis size, and testis with a well-developed sperm transport system, would possess a greater capacity for sperm production. Other adaptations examined that are associated with an enhanced control of sperm production include semi-cystic spermatogenesis. We also examined testes for their capacity related to mucin production. In some teleost fishes, mucins are released with spermatozoa at ejaculation. Mucins slowly dissolve in water, liberating active spermatozoa by degrees; in some species, for several hours after ejaculation (Marconato, Rasotto & Mazzoldi, 1996; Scaggiante *et al.*, 1999). Consequently, mucins prolong the viability of spermatozoa and are associated with sperm economy. Finally, we also examined the ratio of spermatozoa tail length to head length. This ratio is predicted to provide a measure of sperm motility in species with external fertilization, which in turn correlates with sperm competitiveness and fertilization efficiency (Humphries, Evans & Simmons, 2008).

The predictions we tested were that: (1) in species with spatial clustering of fertilizations and an asynchronous pattern of spawning, the intensity of sexual selection, and therefore sperm competition, would be highest and (2) in species with more spatially dispersed fertilizations and synchronous spawning,

sperm competition would be relaxed, although the probability of sperm limitation would be enhanced. For species experiencing sperm competition, we predicted a large relative testis size and fast-swimming spermatozoa. In the case of sperm limitation, we also predicted a large relative testis size but also high rates of ejaculation and mucin production.

Bitterling fishes have a spawning relationship with freshwater mussels, which makes them especially amenable to experimental studies of reproduction. During the spawning season, males develop bright coloration and defend territories around mussels. Females develop long ovipositors that they use to place their eggs into the gills of a mussel through the mussel's exhalant siphon. Males actively court females and lead them to mussels in their territories. Females inspect mussels before spawning, basing their spawning site choices on mussel and male quality (Kitamura, 2005, 2006; Casalini *et al.*, 2009; Agbali *et al.*, 2010) with consistent preferences for certain mussel characteristics (Smith *et al.*, 2004). Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Males release sperm into the inhalant siphons of mussels both before (pre-oviposition ejaculation) and after spawning (post-oviposition ejaculation). Those males that control access to mussels enjoy high reproductive success (Reichard, Smith & Jordan, 2004a; Reichard, Jurajda & Smith, 2004b; Reichard *et al.*, 2005). Males also show flexible mating tactics, behaving as territory holders, as well as participating in mating in the territories of rivals as sneakers (Kano, 1996, 2000; Smith, Reichard & Jurajda, 2003; Smith & Reichard, 2005). Embryos remain inside the host mussel for approximately 1 month before emerging as well-developed larvae (Aldridge, 1999). Because bitterling must use mussels for spawning, it is possible to manipulate spawning site characteristics, meaning that bitterling are ideal models for investigating male mating tactics and mating system evolution.

The three species of bitterling used in the present study were European bitterling (*Rhodeus amarus*), Chinese rose bitterling (*Rhodeus ocellatus*), and Chinese bitterling (*Rhodeus sinensis*). These three species are closely related (Okazaki *et al.*, 2001; Liu *et al.*, 2006), and their reproductive biology and behaviour are superficially similar. Males of all species show territoriality, although sneaking behaviour also occurs (Kano, 2000; Smith, Douglas & Jurajda, 2002). The mean spawning season fecundities for all three species are similar, at approximately 150–250 eggs depending on female size (Nagata, 1985; An, 1995; Smith *et al.*, 2004; Kitamura, 2005). However, these species do show differences in clutch size; mean  $\pm$  SE clutch size per spawning; *R. amarus*:

$2.9 \pm 0.2$  (Smith *et al.*, 2000), *R. ocellatus*:  $4.4 \pm 1.3$  (Reichard, Liu & Smith, 2007), and *R. sinensis*:  $15.7 \pm 2.6$  eggs (Reichard *et al.*, 2007). In addition, *R. amarus* have a substantially shorter spawning season, lasting approximately 6 weeks (Smith *et al.*, 2004), in contrast to 6 months in the other two species (An, 1995; Kano, 1996; Kitamura, 2005). Thus, fertilizations are temporally clustered and spatially dispersed in *R. amarus* but spatially clustered and temporally dispersed in *R. sinensis*, with *R. ocellatus* intermediate between the two.

## MATERIAL AND METHODS

### STUDY SUBJECTS

Approximately 150 experimental fish of each species were collected. *Rhodeus sinensis* were caught using an electroshocker from a canal connected to Lake LuHu and *R. ocellatus* from Lake Bao'an (30°50'N; 114°16'E) in early April 2005. Both Lakes are in Hubei Province in the River Yangtze Basin, China. *R. amarus* were collected in early May 2005 from oxbow lake adjacent to the River Vistula, near the village of Soczewka, central Poland (52°32'N; 19°34'E) using a 5-m fine-mesh Seine net. All fish were housed in stock aquaria under a natural light regime and fed frozen chironomid larvae and commercial fish flakes before the experimental procedures.

### EJACULATION RATE

Estimates of ejaculation rate for *R. ocellatus* and *R. sinensis* were obtained during April 2005 at the Institute of Hydrobiology of the Chinese Academy of Sciences, Wuhan, China, and for *R. amarus* during May 2005 at the University of Łódź, Poland. Experiments were conducted in the aquarium facilities at the respective institutions using an identical experimental design. One of us (C.S.) oversaw and participated in data collection at both locations to ensure a common protocol was followed.

For *R. ocellatus* and *R. sinensis*, the freshwater mussels used as spawning sites in the study were *Unio douglasiae*. Mussels were collected in Lake Poyang using a long-handled dip net. Mussels used for the experiment with *R. amarus* were *Unio pictorum*, which were collected by hand from the Sulejowski Reservoir, located on the River Pilica, Poland. Both mussel species are widespread in their respective geographic area and are the preferred species used by the bitterling species with which they were tested (Smith *et al.*, 2004; Reichard *et al.*, 2007). Different mussel species do not elicit different male courtship or ejaculatory behaviour (Smith *et al.*, 2000, 2001, 2004).

Experiments began 3 days after the fish were collected and were conducted in aquaria measuring 50 cm (length)  $\times$  30 cm (height)  $\times$  40 cm (width) with a sand substrate of 5 cm in depth. The water temperature during the experiments was 23 °C for all species. For each trial, a mussel was added to the test aquarium in a sand-filled plastic cup at the centre of the aquarium. Two artificial plants were placed at the back of each aquarium as a refuge. A single male bitterling was haphazardly caught from the stock aquarium and gently released in the test aquarium. After 2 h, males had established territories around mussels and additional males were added to aquaria as potential rivals in a predetermined random order. The addition of rivals was intended to provide a measure of male response to sperm competition. Three competitor treatments were used for each bitterling species: one, two or four rival males. Rivals were haphazardly selected, although only those smaller than territorial males were used. The largest male always acts as the territory holder when mussels are limiting (Casalini *et al.*, 2009). After adding males, a female with an extended ovipositor was selected from the stock aquarium and also added. Mussels were initially covered with an upturned perforated clear plastic cup to prevent females spawning in them, although all fish were able to see and smell the mussel, which territorial males continued to guard and attempt to lead the female towards. After 30 min, the cup was removed and the frequency of ejaculation by territorial and rival males recorded for a further 30 min or until spawning occurred. On the completion of a trial, all the experimental subjects (i.e. fish and mussels) were removed and measured (fish from the tip of the snout to the origin of the base of the tail fin, mussels total shell length) and none were used again. A total of ten replicates for each treatment for each species were completed, giving 90 trials in total.

#### TESTIS SIZE AND MORPHOLOGY

A haphazardly collected subsample of 20 males of each species were killed with an overdose of anaesthetic. Once dead, fish body length and body weight were recorded to the nearest 1 mm and 1 mg, respectively, before the complete male reproductive apparatus (testes and sperm duct) was dissected and weighed. The cross-sectional area ( $\mu\text{m}^2$ ) of testis and sperm duct were measured under a Leica M216 dissecting microscope to provide a measure of the relative area occupied by the sperm duct. Testes and sperm duct were then fixed in Deitrich's liquid (900 mL of distilled water, 450 mL of 95% ethanol, 150 mL of 40% formaldehyde, and 30 mL of acetic acid) for subsequent histological examination.

After fixation, samples were dehydrated in ethanol (30 min in 70%, 80%, 95%, and then 100%), embedded in paraplast, cross-sectioned serially at 7  $\mu\text{m}$ , moving posterior to anterior, and mounted on slides. Complete sections from seven *R. amarus* and *R. ocellatus*, and six *R. sinensis*, which included 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, and, for the differentiation of sulphated and nonsulphated mucins, with alcian blue at PH 1.0 and 2.5 (Pearse, 1985). The thickness ( $\mu\text{m}$ ) of sperm duct epithelial cells was measured using a Leica DMR fluorescence microscope over the complete length of the sperm duct, which might infer a secretory function. In addition, the general anatomy of the testis and sperm ducts was examined.

#### SPERM MORPHOLOGY

Sperm was stripped from three sexually mature males of each study species by gently pressing their abdomen and immediately fixed in a mixture of glutaraldehyde (2.2%), paraformaldehyde (4.5%), sucrose (5%), and Sørensen's phosphate buffer (pH 7.5). After fixing for 2 h, samples were centrifuged to remove spermatozoa from the seminal fluid, washed in Sørensen's phosphate buffer (pH 7.5), dehydrated in ethanol, allowed to dry, and then sputter coated onto viewing stubs in accordance with the methodology of Maricchiolo *et al.* (2002). Prepared spermatozoa were visualized using a scanning electron microscope. Images were photographed and sperm flagella and head length were subsequently measured from scaled photographs using IMAGEJ (<http://rsbweb.nih.gov/ij/>). The ratio of spermatozoa flagellum length to head length was estimated as an index of sperm swimming speed in accordance with the method described by Humphries *et al.* (2008). Estimates were based on measurements from 60 individual spermatozoa for *R. amarus*, 68 for *R. ocellatus*, and 65 for *R. sinensis*, distributed equitably among the three individuals of each species from which the sperm was collected.

#### STATISTICAL ANALYSIS

All data were tested for normality using a Shapiro-Wilk test and for equality of variance using Levene's robust test. Where necessary, data that deviated from normality or homoscedasticity were transformed. The mean rate of ejaculation by territorial and subordinate males among species and number of rivals was compared using two-way generalized log-linear models (quasi-Poisson error structure). One-way analysis of variance (ANOVA) was used to compare

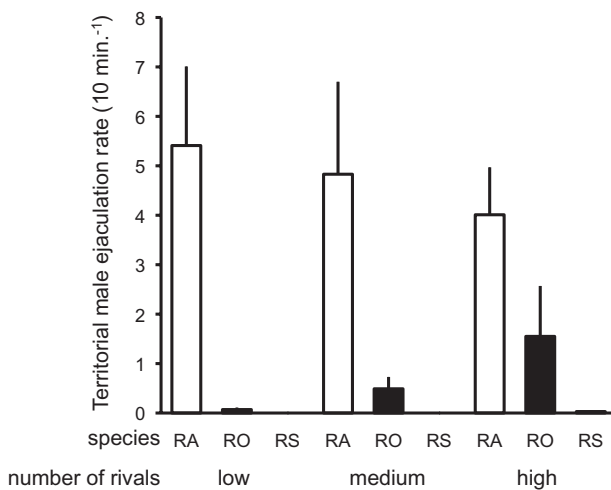


the relative proportion of the reproductive apparatus that comprised the sperm transport system, the thickness of the internal epithelium of the sperm ducts, and the ratio of spermatozoa flagellum length to head length among species. Analysis of covariance (ANCOVA), with fish body length as covariate, was used to compare testis size among species. Šidák post-hoc tests were used to identify where significant differences lay. Statistical analyses were performed using STATA, version 11.0 (Stat Corp.).

## RESULTS

### EJACULATION RATE

There was a significant difference in the mean rate of ejaculation of territorial males among bitterling species (generalized log-linear model with quasi-Poisson distribution:  $F_{2,81} = 22.42$ ,  $P < 0.001$ ), although there was no effect of the number of rivals ( $F_{2,81} = 0.01$ ,  $P = 0.994$ ) or an interaction between species and number of rivals ( $F_{4,81} = 0.60$ ,  $P = 0.664$ ; Fig. 1). Among species, the rate of ejaculation in *R. amarus* was significantly higher than both *R. ocellatus* and *R. sinensis* (Šidák post-hoc test,  $P < 0.002$ ), although there was no significant difference between *R. ocellatus* and *R. sinensis* ( $P = 0.885$ ). A similar pattern was obtained between estimates of the mean rate of ejaculation of subordinate males, with a significant difference among species ( $F_{2,81} = 5.99$ ,  $P < 0.001$ ), although no effect of the number of rivals ( $F_{2,87} = 2.39$ ,  $P = 0.098$ ) or interaction ( $F_{4,81} = 1.04$ ,  $P = 0.391$ ). Among species, the rate of ejaculation in *R. amarus* was significantly higher than *R. sinensis*



**Figure 1.** Mean territorial male pre-oviposition ejaculation rate of: *Rhodeus amarus* (RA), *Rhodeus ocellatus* (RO), and *Rhodeus sinensis* (RS); at three levels of sperm competition: one rival (low), two rivals (medium), and four rivals (high). Error bars are one SE.

(Šidák post-hoc test,  $P = 0.001$ ), although there was no significant difference between *R. ocellatus* and *R. sinensis* ( $P = 0.072$ ) or between *R. amarus* and *R. ocellatus* ( $P = 0.368$ ).

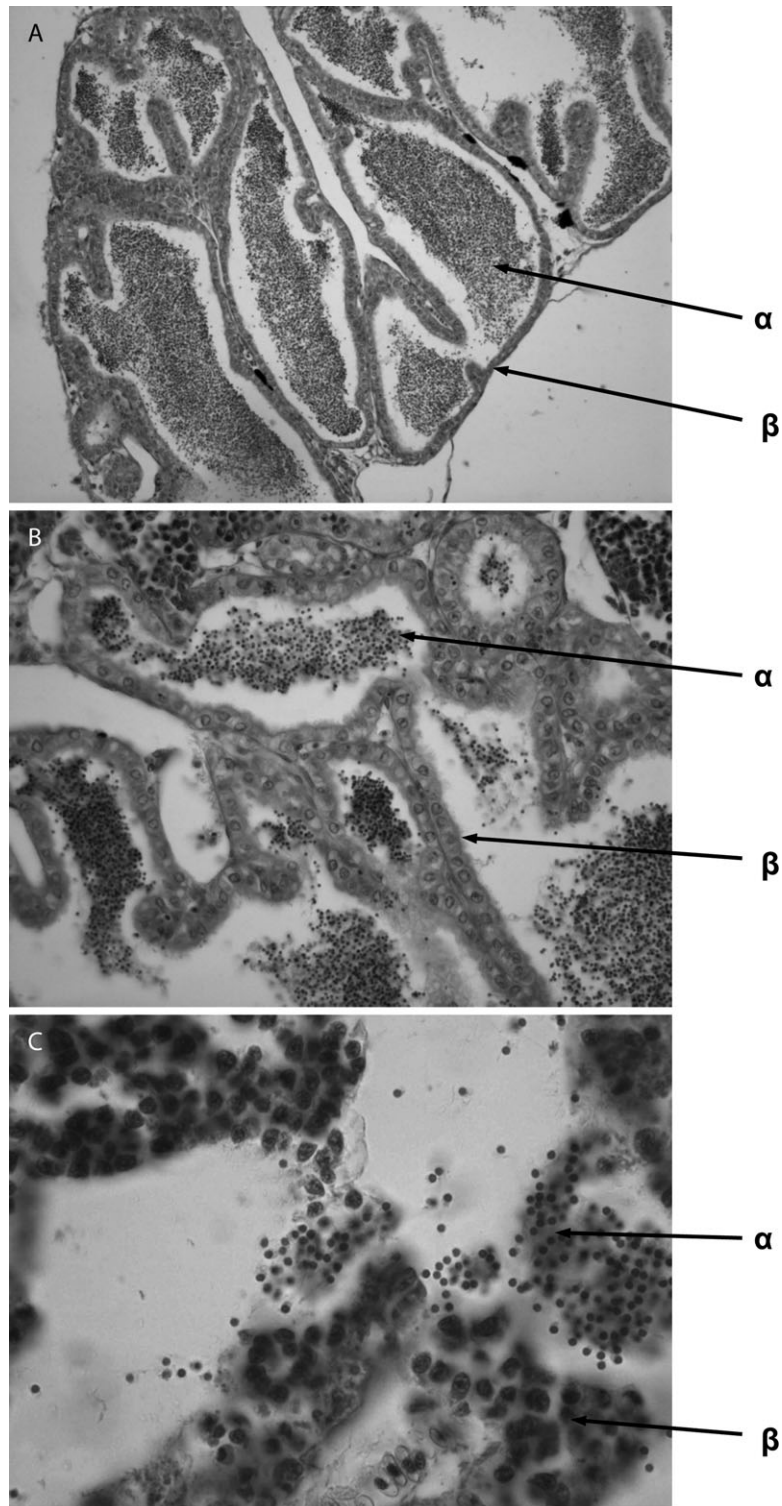
### TESTIS SIZE AND REPRODUCTIVE APPARATUS MORPHOLOGY

*Rhodeus ocellatus* had a lower mean testis weight than the other two species (ANCOVA, square-root transformed data:  $F_{2,56} = 13.03$ ,  $P < 0.001$ ; Šidák post-hoc test,  $P < 0.031$ ). There was no significant difference between *R. amarus* and *R. sinensis* ( $P = 0.184$ ).

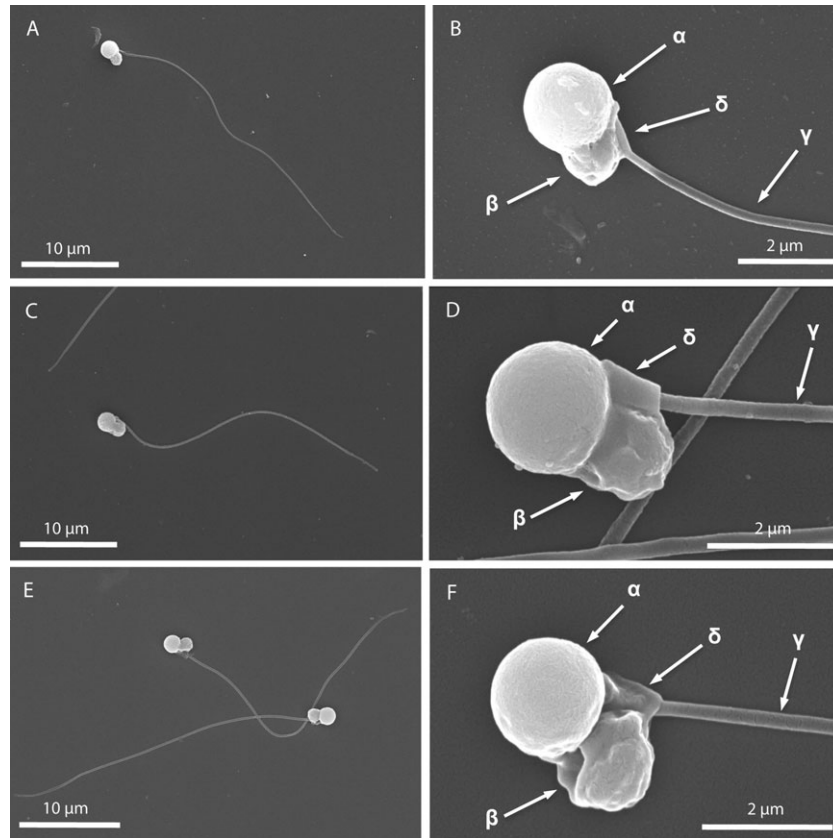
The organization of male reproductive apparatus among species was similar. The testes are elongated, paired bodies with two main testicular ducts collecting sperm from the testis lobules. These ducts run the length of the testes and fuse at their posterior into a large, convoluted, common sperm duct. 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 (Billard, 1986; Lahnsteiner & Patzner, 1990a, b). 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. By contrast, although the early stages of spermatogenesis appear similar in *R. amarus*, spermatids, and not sperm, are released into the lobule lumen so that a mixture of spermatids and fully developed sperm are present within the lumen (Fig. 2). The release of spermatids, which mature into sperm within the sperm transport system, characterizes the semi-cystic form of spermatogenesis (Giacomello *et al.*, 2008).

In all three species, the sperm transport system comprises two main testicular ducts and the sperm duct. The relative proportion of the reproductive apparatus devoted to main testicular ducts and sperm duct varied among species (one-way ANOVA, arcsine transformed data:  $F_{2,17} = 11.09$ ,  $P < 0.001$ ), ranging from  $52 \pm 0.40\%$  in *R. amarus* to  $37 \pm 0.97\%$  in *R. ocellatus* and  $34 \pm 0.81\%$  in *R. sinensis*. In particular, the sperm transport system was significantly more developed in *R. amarus* than both *R. ocellatus* (Šidák post-hoc test,  $P = 0.006$ ) and *R. sinensis* ( $P = 0.001$ ), whereas there was no significant difference between *R. ocellatus* and *R. sinensis* ( $P = 0.783$ ).

The walls of the sperm transport system ducts show a common structure, being organized in three layers; an external flat epithelium representing the coelomic wall, an intermediate layer of connective tissue containing smooth muscle, and an internal



**Figure 2.** Cross-sectional images of the sperm duct of *Rhodeus amarus* at  $\times 300$  (A),  $\times 600$  (B), and  $\times 1500$  magnification (C). The duct is highly convoluted with areas containing densely packed spermatozoa and spermatids ( $\alpha$ ) separated by thick-walled epithelium ( $\beta$ ).



**Figure 3.** Spermatozoa of three species of bitterling. A, B, *Rhodeus amarus*. C, D, *Rhodeus ocellatus*. E, F, *Rhodeus sinensis*. The spermatozoon head ( $\alpha$ ) and neck ( $\beta$ ) are relatively spherical. The flagellum ( $\gamma$ ) arises in the nuclear notch at the base of the head and is surrounded by a sleeve ( $\delta$ ) of plasma membrane.

single-layered epithelium. The thickness of the internal epithelium was significantly higher in *R. amarus* than both *R. ocellatus* and *R. sinensis* (one-way ANOVA:  $F_{2,9} = 64.18$ ,  $P < 0.001$ ; Šidák post-hoc test,  $P < 0.001$ ), although there was no significant difference between *R. ocellatus* and *R. sinensis* ( $P = 0.319$ ). The mean  $\pm$  SD internal epithelium thickness was  $11.5 \pm 2.15 \mu\text{m}$  in *R. amarus*,  $1.9 \pm 0.29 \mu\text{m}$  in *R. ocellatus*, and  $3.4 \pm 0.58 \mu\text{m}$  in *R. sinensis*. In *R. amarus*, but not in *R. ocellatus* or *R. sinensis*, the epithelium thickness increased progressively along the length of the sperm transport system from posterior to anterior. In the posterior section, in close proximity to the genital pore, the sperm duct becomes 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, indicating that secretions of both sulphated and nonsulphated mucins were produced from the inner epithelium cells. Consequently, all three species are predicted to release ejaculates rich in mucins.

#### SPERM MORPHOLOGY

The spermatozoa of all three bitterling species were similar in external morphology, being composed of a head, a midpiece, and a tail but with no obvious acrosome. The head and neck region was distinguishable, each with an approximately spherical shape. Spermatozoa have a large mitochondrion section and centrioles in the midpiece. As in some blenniid species (Lahnsteiner & 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 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. The structure of the head, sleeve, and mitochondrial sections are smooth in all species, with no obvious characters, and no acrosomal structure in the anterior portion of the sperm head was observed (Fig. 3).

The ratio of spermatozoa flagellum length to head length was significantly different among all bitterling species (one-way ANOVA:  $F_{2,191} = 92.47$ ,  $P < 0.001$ ; Šidák post-hoc test,  $P < 0.004$ ). The mean  $\pm$  SD flagellum length to head length ratio was  $12.8 \pm 1.22$  in *R. amarus*,  $9.23 \pm 1.29$  in *R. ocellatus*, and  $10.11 \pm 1.72$  in *R. sinensis*.

## DISCUSSION

In the present study, we compared male morphological and behavioural adaptations linked to fertilization success in three related bitterling fishes aiming to infer their degree of adaptation for sperm competition and/or sperm limitation (*sensu* Wedell *et al.*, 2002). The results obtained showed that the European bitterling, *R. amarus*, displayed the most developed reproductive apparatus with a number of traits associated with both high levels of sperm production and fertilization efficiency. Both territorial and subordinate male *R. amarus* ejaculated at the highest rates. Male *R. amarus* showed the highest proportion of the reproductive apparatus devoted to the sperm transport system, which had the greatest epithelium thickness. Sperm duct epithelium thickness correlates with sperm duct gland secretory activity in fishes (Cinquetti *et al.*, 1990). *Rhodeus amarus* also had a higher relative testis weight as a proportion of body weight than *R. ocellatus* and, uniquely among the species examined, showed evidence of semi-cystic spermatogenesis. Semi-cystic spermatogenesis is associated with sperm economy (Giacomello *et al.*, 2008) because it enables males to produce numerous small ejaculates. Finally, the ratio of spermatozoa flagellum length to head length, an index of spermatozoa motility, was highest in *R. amarus*. Between *R. ocellatus* and *R. sinensis*, the pattern was less striking, although with some notable differences. Both relative testis size and spermatozoa tail to head length ratio were significantly higher in *R. sinensis* than *R. ocellatus*.

A striking feature of the male reproductive apparatus of all three species is the presence of an unusually well developed sperm duct with evidence of mucin production, a feature that is particularly pronounced in *R. amarus*. The production of mucous seminal fluids is uncommon in teleost fishes, although it is reported in some gobies, toadfish, cottids, blennies, and catfish (Mazzoldi, 1999, 2001; Barni, Mazzoldi & Rasotto, 2005). The role of mucins in fertilization is to slowly release active spermatozoa over an extended period after ejaculation, which is accomplished by the gradual dissolution of the mucin in water (Marconato *et al.*, 1996; Scaggiante *et al.*, 1999). Mucins thereby greatly extend the longevity of sperm and, consequently, the period over which fer-

tilization can occur. A role for mucins may be particularly advantageous in bitterling because filtration of water by the mussel used for spawning would tend to remove sperm from the site of oviposition quickly. A role for mucous seminal fluid in bitterling may help explain the prevalence of pre-oviposition ejaculation by males, especially in *R. amarus* (Smith *et al.*, 2004). Male bitterling frequently release sperm into mussels before a female deposits her eggs, and often before a female has even approached a mussel. If the presence of mucins in sperm extends the period over which viable sperm are present in a mussel, the role of pre-oviposition ejaculation in the mating system, and especially as a sneaky mating tactic, may be of greater significance than previously anticipated in these fishes (Smith *et al.*, 2004). Notably, a previous study by Reichard *et al.* (2004a) using paternity analysis demonstrated that a male *R. amarus* successfully fertilized eggs in a mussel in which he had ejaculated a minimum of 14 min previously, a feat that could only be accomplished with an adaptation such as mucous seminal fluid because bitterling sperm loses motility through osmotic shock within less than 2 min in freshwater (Pateman-Jones, 2008).

Interspecific differences in male reproductive apparatus and reproductive behaviour suggest that the three study species have evolved different sperm allocation strategies. In *R. amarus*, males show several adaptations for sperm economy, as well as for sperm competition. Male reproductive apparatus in this species showed the highest level of investment, with the most developed sperm transport system, semi-cystic spermatogenesis, and the most marked capacity for mucin production. *Rhodeus amarus* also displayed the highest rate of ejaculation and their spermatozoa were predicted to have the fastest swimming speed. These features are associated with the production of numerous small ejaculates with the greatest fertilization efficiency. Field studies have demonstrated that male *R. amarus* experience sperm competition, with the majority of natural spawnings involving more than one male (Smith *et al.*, 2002, 2003). There is also evidence of sperm depletion over successive ejaculations in this species, with the rate of sperm depletion exacerbated by competition with rival males (Smith *et al.*, 2009). In a field study, it was estimated that territorial male *R. amarus* ejaculated on average approximately 250 times over the course of a day (Smith *et al.*, 2009).

*Rhodeus ocellatus* and *R. sinensis* appear to have more comparable sperm allocation strategies. Sperm duct morphology is similar in these two species and neither exhibits semi-cystic spermatogenesis. However, relative testis size was substantially greater in *R. sinensis*, suggesting that sperm competition risk may be higher in this species compared to *R. ocella-*



*tus*. The swimming speed of spermatozoa was also predicted to be higher in *R. sinensis*. Thus, although the mating system of these two species is similar, *R. sinensis* is tentatively predicted to experience a higher sperm competition risk than *R. ocellatus*.

The major differences in the mating systems of the three bitterling species tested comprise clutch size and the length of spawning season. *Rhodeus amarus* has the smallest clutch size and shortest spawning season. These features of *R. amarus* mean that spawnings are temporally clustered over a short, but relatively intense breeding season, although spatially dispersed, with eggs distributed among the greatest number of spawning sites. Both these features of the mating system increase the probability of male sperm depletion. A short breeding season will result in a higher frequency of matings per unit time than a more protracted breeding season, with the result that males will potentially have shorter intervals between ejaculations and less opportunity to replenish sperm reserves (Shuster & Wade, 2003). A larger number of matings distributed over a greater number of spawning sites will have the same effect because eggs laid in small clutches require a larger sperm production to fertilize them than if the entire clutch is fertilized by a single ejaculate (Emerson, 1997). As a result, males will be required to distribute ejaculations more widely and therefore sparingly. Consequently, these conditions would tend to favour the evolution of a greater level of sperm production, but also better control of sperm release and enhanced fertilization efficiency. These manifest themselves in *R. amarus* in semi-cystic spermatogenesis, which facilitates small ejaculates in conjunction with a high rate of ejaculation. Further adaptations include a greater ratio of spermatozoa flagellum length to head length, an index of spermatozoa motility, and mucous seminal fluid, both of which would tend to increase fertilization efficiency.

In *R. sinensis*, clutch size is larger than *R. ocellatus*, with the result that spawnings would tend to be more spatially clustered in the former species (Shuster & Wade, 2003). There appear to be no temporal differences in spawning in these two species. Consequently, male *R. sinensis* are predicted to experience a greater risk of sperm competition than *R. ocellatus* because fertilizations will be concentrated in fewer matings. The larger testis size and faster swimming spermatozoa seen in *R. sinensis* are adaptations typically associated with sperm competition (Wedell *et al.*, 2002; Humphries *et al.*, 2008) and may have evolved in this species in response to selection through sperm competition.

A caveat to these inferences is that the distribution of male ejaculates may be mediated, to some extent at least, by the abundance of mussel spawning sites. If spawning sites were consistently limiting in one

species, a result would be to concentrate spawnings on those spawning sites. Thus, sperm depletion might be less likely, although only in the case where females spawned in quick succession such that more than one clutch was fertilized by a single ejaculate. However, under these circumstances, the effect would also be to increase the risk of sperm competition. Among the species tested, both *R. amarus* and *R. ocellatus* are generalists and utilize a broad range of mussel species (Reichard *et al.*, 2007). By contrast, *R. sinensis* is a specialist, principally using *U. douglasiae* for spawning (Reichard *et al.*, 2007). However, at the sites from which we collected fish, mussels were widespread and abundant, including *U. douglasiae* in Lake Bao'an. Thus, mussel availability may not have shaped the evolution of male reproductive traits to the extent that clutch size and spawning season duration may have done.

Adaptations for sperm economy by males have been described in a range of taxa (Alonzo & Warner, 2000; Mazzoldi, 2001; Petersen *et al.*, 2001; Wedell *et al.*, 2002; Pizzari *et al.*, 2003) and often involve specific behaviours, as well as morphological and physiological adaptations to male reproductive apparatus, that permit enhanced control of ejaculation (Manni & Rasotto, 1997; Rasotto & Shapiro, 1998; Pound, 1999; Giacomello *et al.*, 2008). However, whether these adaptations for elevated sperm production and viability, as well as the control of ejaculation frequency and ejaculate size and distribution, have evolved primarily as a result of sperm competition or sperm limitation remains unclear, although sperm competition theory is frequently invoked to explain male adaptations of this type (Emerson, 1997). These two processes are not independent because a high risk of sperm competition would tend to result in sperm depletion by males if they respond to competitors with larger or more frequent ejaculations. The converse, however, is not necessarily the case; sperm limitation could occur in any polygynous species with female mate choice if matings are concentrated on a small number of males (Warner *et al.*, 1995; Jones, 2001). Similarly, sperm limitation may be contingent on fertilization efficiency or clutch size (Emerson, 1997; Levitan, 1998). Further studies are needed to quantify the relative importance of sperm competition and sperm limitation in different mating systems with the aim of clarifying their relative role in this aspect of sexual selection.

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C.P.J. and G.Z. conducted the experiments. C.S. analyzed the data and wrote the paper with input from M.B.R. and M.R. The study was supported by a Leverhulme Trust award to C.S. and a Polish State Committee Scientific Research award (2P04F 01529) to G.Z.

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