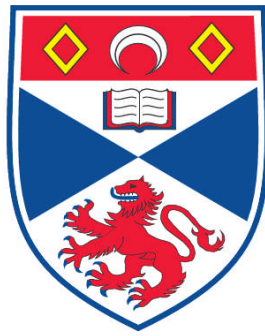


**FEMALE MATING DECISIONS IN THE ROSE BITTERLING
(*RHODEUS OCELLATUS*)**

Muna Agbali

**A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews**



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Female mating decisions in the rose bitterling (*Rhodeus ocellatus*)

by

Muna Agbali

A Thesis Submitted in Part Fulfillment of the Requirements for the Degree of Doctor of
Philosophy

from

The School of Biology

THE UNIVERSITY OF ST ANDREWS

May 2011

Declaration

I, Muna Agbali, hereby certify that this thesis, which is approximately 35,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in October 2007 and as a candidate for the degree of PhD in October 2008; the higher study for which this is a record was carried out in the University of St Andrews between 2009 and 2010.

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Co-authorship statement

Chapters 2-7 were collaborations between myself and my research supervisor, Carl Smith. Carl assisted me in experimental design, data analysis and helped edit the manuscripts. Data were collected, analysed and the manuscripts written by myself.

Chapter 2 was a collaboration between myself, Mara Casalini, Martin Reichard, Marketa Konečná, Anna Bryjová, and Carl Smith. Mara Casalini and Marketa Konečná carried out a series of behavioural studies, none presented in this thesis, that informed my own experimental design. Their work is included in the published paper that arose from this chapter. Martin Reichard contributed substantially to the overall concept behind the chapter, and Anna Bryjová conducted paternity analysis. I was responsible for the experimental design, collection and analysis of data (excluding paternity data) and writing the manuscript.

Chapter 3 was a collaboration between myself, Martin Reichard, Anna Bryjová, Josef Bryjá, and Carl Smith. All genetic analysis were conducted by Anna Bryjová, and MHC data were analysed and interpreted by Josef Bryjá. Martin Reichard estimated MHC functional differences among MHC alleles. I was responsible for the experimental design, collection and analysis of data (excluding paternity and MHC data) and writing the manuscript.

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To my son Ahmed and my daughter Eman

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Abstract

The aim of this study was to obtain an understanding of the basis to female mating decisions in the Chinese rose bitterling (*Rhodeus ocellatus*). Bitterling have a resource-based mating system that involves the female laying her eggs inside the gills of a freshwater mussel. Male bitterling perform elaborate courtship behaviour and are territorial and aggressively guard mussels in their territory from other territory holders and non-territorial males. Using a series of laboratory experiments it was shown in this study that females were choosy over the males they mated with, but females were not congruent in their preferences. Female mate preferences correlated positively with offspring growth rates and survival during early development. Female mate choice did not correspond with male dominance, and there may be an intersexual conflict between female mate preferences and male dominance as a result. Females tended to prefer males with functionally dissimilar MHC alleles. MHC alleles may influence male odour cues, and females showed a preference for mussels in which the sperm of multiple males had been released, possibly indicating that females use odour cues associated with sperm release in mating decisions. Bitterling show an innate preference for the colour red in a foraging context and there may be a receiver bias for red nuptial colouration in female mating preferences. Despite a significant role for mate preferences, direct (oviposition) mating preferences were shown to be more important in the mating system. Choice of oviposition sites has both immediate (survival) consequences for offspring, as well as longer-term fitness effects.

Chapter One – General introduction

The role of female mating decisions in sexual selection

Darwin (1871) defined sexual selection as a form of natural selection that has an influence on an individual's traits which affect its capacity to obtain a mate, rather than the traits that enhance its ability to survive. Sexual selection was proposed to explain the evolution of secondary sexual characteristics that are limited to one sex and inconsistent with natural selection. Sexual selection depends on fitness variations that arise from difference in traits that affect reproductive success (Andersson, 1994). Sexual selection is a powerful mechanism of adaptive evolution. It arises through a difference in the relative strength of selection acting on the sexes, either as a result of competition between members of the same sex, usually among males, and termed intra-sexual selection, or as a consequence of female mate choice, termed intersexual selection. Sexual selection tends to act more strongly on males because they have relatively greater variance in reproductive success than females (Shuster and Wade 2003). Intrasexual selection is associated with the evolution of large male body size and weapons, whereas inter-sexual selection is associated with elaborate male traits and courtship behaviours combined with female discrimination among males. Intra- and intersexual selection are both believed to play a role in the evolution of mating systems (Shuster and Wade 2003). However, while intrasexual selection is generally accepted, intersexual selection has proven more controversial (Andersson 1994; Andersson and Simmons 2006). Mate choice is the mechanism that leads member of one sex to non-randomly mate with certain individuals of the other sex, depending on one or more varying traits (Heisler et al. 1987). Kokko et al. (2006) stated that mate choice is the outcome of the inherent tendency of individuals to mate with particular phenotypes of

the opposite sex (i.e. mating preference or bias) and the extent to which individuals engage in mate sampling prior to deciding to mate (i.e. choosiness). However, the concept of mate choice is sometimes not clear because it is hard to separate mating bias resulting from active mate choice from other processes, such as passive mate attraction that has evolved as a side-effect of other process unrelated to mate choice, such as sensory biases, and male-male competition (Ryan and Rand 1990; Rowe et al. 1994; Kotiaho and Puurtinen 2007).

Direct and indirect benefits of female mate choice

Direct intersexual selection

Sexual selection may act on female mating decisions that benefit either the female or her offspring. If males provide a resource that yields a fitness benefit to the female or her young, selection may act directly on female preferences for males that provide those resources. In fishes the most common resource males provide that enhances female reproductive success is a nest site for oviposition. In addition, males frequently provide parental care, in some cases in conjunction with the female, but more usually as the sole care provider. There is good evidence for selection for direct mate choice benefits by females in fishes. In the river bullhead, *Cottus gobio*, males dig a cavity nest, often at the base of a rock, where they guard and aerate a series of egg clutches spawned in the nest by females. Parental care of a clutch of eggs lasts approximately four weeks and the male fans the eggs throughout this period using his pectoral fins. Males perform courtship to females, and females are choosy over which males they mate with (Morris 1955). Bisazza and Marconato (1988) showed that females had a significant mating preference for large males, though they avoided males 150% larger than their own body size, possibly because of the risk of cannibalism by the male during courtship. This

mating preference was proposed as providing a direct fitness benefit to females because larger males were more successful at defending their offspring from nest predators, particularly other bullheads. Larger males may also be more effective at fanning the developing young. Other examples of direct fitness benefits to females from mate choice include territory quality in three-spined sticklebacks (Sargent 1982), and male parental quality in the sand goby (*Pomatoschistus minutus*) (Forsgren 1997).

Elaborate male ornaments and behaviours are often linked with benefits to females through indirect selection (below). However, they may also signal direct benefits to females. In the bicolor damselfish (*Stegastes partitus*) male courtship accurately signalled male parental quality (Knapp and Kovach 1991). A similar situation was observed in a population of sand gobies (*Pomatoschistus minutus*), in which male body size signalled how much a male subsequently fanned eggs in his nest (Lindström and Hellström 1993), though this pattern was not consistent in another population (Forsgren 1997). Few studies have adequately separated the role of sexually selected male traits as indicators of indirect benefits from direct benefits, and distinguishing the two remains a challenge for understanding the mechanism of female mate choice.

Indirect intersexual selection

Indirect benefits of mate choice are obtained in the generation succeeding that in which mate choice is performed and assume a direct relationship between a male trait and his viability. Theories of indirect benefits present the evolutionary biologist with some difficulties and are, consequently, controversial. Indirect benefits have also proven more difficult to demonstrate than direct benefits, and there are fewer persuasive examples.

“Good genes”

Under a “good genes” model the prediction is that female mate preferences are under selection through the choice of males that father offspring of superior viability. Females are able to assess male viability using male ornaments or behaviour that accurately, or “honestly”, signals male genetic superiority. Good genes models of mate choice are exemplified by the much criticised “handicap principle” (Zahavi 1977). Here an honest signal is one that is costly to the male. Since ornaments are effectively a handicap, only genetically superior males are able to express them fully. A more widely accepted derivation of the handicap principle was developed by Hamilton and Zuk (1982), who argued that ornaments may serve as indicators of parasite resistance.

While intuitively appealing, good genes models of sexual selection have proven challenging to demonstrate and raise theoretical difficulties. An implicit assumption of good genes models is that male traits under selection have additive genetic effects. Thus when a male mates with a female, irrespective of female genetic constitution, offspring have greater viability as a consequence of the male contribution. This assumption has rarely been demonstrated. Another problem with this hypothesis is that additive genetic variance is predicted to deplete under selection, and it has proven challenging to explain how female mate choice could be maintained in a population as a consequence. Hamilton and Zuk’s (1982) proposal for parasite-driven selection does offer a mechanism, through the cycling of host and parasite resistance and virulence, which would tend to maintain heritability for resistance genes, and thereby the ornaments that indicate those genes, and female mate preferences for them. A weakness of many studies that purport to show good genes effects is that they often fail to exclude direct selection on male traits; simply because a male possesses an exaggerated ornament and

a female chooses to mate with that male is not robust evidence for good genes selection in operation.

Indirect selection for good genes has been demonstrated in the three-spined stickleback. Male sticklebacks develop red colouration on their throat during the breeding season. In some populations, female sticklebacks spawn more frequently with males that show the greatest intensity of red colouration. Barber et al. (2001) showed for a Scottish population that offspring fathered by males with the greatest intensity of red had greater resistance to a parasite (*Schistocephalus solidus*). Interestingly this study also showed that the offspring of redder males suffered a cost of lower growth rates. This cost of mating with redder males offers an explanation for why genetic variance for male colour is not depleted in the population, since fluctuations in food availability, size-selective predation, and rates of parasitism by *S. solidus* would have the effect of varying the strength of selection on female preference for redder males. Thus, Barber et al.'s (2001) study conforms with Hamilton and Zuk's (1982) model of selection for good genes through fluctuating resistance to parasites. This study also illustrates the contradiction in the term "good genes" to describe this form of indirect selection, since a gene (or suite of genes) that confers an indirect fitness benefit through female mate choice in one environment, may be detrimental to fitness (i.e. represent "bad genes") under different conditions.

Compatible genes

Another mechanism for indirect selection on mate choice is through genetic compatibility. Here a female enhances the viability of her offspring by mating with a genetically compatible male. In contrast to good genes models, offspring viability is enhanced only when particular male and female haplotypes are combined (Zeh and Zeh

1996), thus compatible genes are predicted to show non-additive variation (Neff and Pitcher 2005). Where a mating system is underpinned by compatible gene effects, females will not necessarily share mate preferences, the opposite prediction of the good genes model.

The genetic compatibility paradigm also raises conceptual difficulties as to how it operates. The functional basis to compatibility is unclear, but may be related to advantages associated with enhanced heterozygosity. The process also demands that a female is able to recognise her own genotype, or at least components of her genotype, as well as those of potential mates in making mate choice decisions. How a female is able to match her genotype to that of a compatible male is unclear. For genetic compatibility to be able to function as the basis to a mate choice system it is likely to be limited to specific genetic systems. One such genetic system comprises the genes of the major histocompatibility complex (MHC), for which there is evidence of a role in mate choice. The MHC is a family of highly polymorphic genes that encode a set of trans-membrane proteins. The function of these proteins is to distinguish between self and non-self antigen and present foreign peptides to T-cell receptors, thereby playing a key role in resistance to infectious and autoimmune disease (Hill 2001). Many studies have implicated MHC genes in influencing mate choice, including in fish (Forsberg *et al.* 2007; Consuegra and Garcia de Leaniz 2008; Yeates *et al.* 2009). MHC genes appear to influence odour, and thereby have the potential to function in an odour-based mate recognition system enabling females to identify mates that can provide ‘good genes’ genetic benefits through enhanced immunocompetence, or to identify genetically compatible mates (Penn and Potts 1999; Tregenza and Wedell 2000; Penn 2002).

One hypothesis that has gained ground recently is the proposal that some intermediate, rather than maximal, level of MHC sequence variability will be optimal

for offspring viability (Reusch et al. 2001; Milinski et al. 2005; Forsberg et al. 2007; Kalbe et al. 2009). This situation only applies to species with duplicated MHC genes. An advantage of intermediate MHC dissimilarity may be important in mating between animals from different populations or races where outbreeding depression can result from genetic incompatibilities, possibly the result of disruption to co-adapted gene complexes (Turelli and Orr 2000; Barton 2001; Jiggins et al. 2001).

Sexual conflict

This hypothesis claims that female preference for male traits has a negative effect on female fitness, thereby generating an antagonistic arms race between the sexes (Holland and Rice 1998). Males are predicted to express a trait that gives them a fitness advantage in mating with females. For example, males may be better able to attract females for mating, or fertilise their eggs more efficiently. A key element of this model is that when females mate with males that express these traits they suffer reduced fitness. The result is that females evolve resistance to the trait, which elicits selection in males on the trait to overcome female resistance alleles. The outcome is cyclical antagonistic coevolution that has been termed “chase-away” selection, which results in exaggerated male traits and strong mate discrimination in females. Potential mating costs to females associated with chase-away selection include exposure to toxins in seminal fluid, manipulation of their endocrine system, increased exposure to parasites and predators, and elevated time and energy expenditure in avoiding males (reviewed by Arnqvist and Rowe 2005).

An example of chase-away selection is found in the guppy (*Poecilia reticulata*). Male guppies persistently court and attempt to mate with females, which substantially disrupts foraging; male harassment was estimated by Magurran and Seghers (1994a) to

reduce food intake by 25%, which represents a potential loss in fecundity. Female guppies avoid males when possible (Magurran and Nowak 1991; Magurran and Seghers 1994b) and the evolution of female resistance to male courtship may be a general feature of many species.

Arbitrary mate choice

Mate choice decisions may not always generate a benefit for the choosing sex. Two models of intersexual selection that do not invoke fitness benefits of mate choice are Fisherian selection and selection through sensory bias.

Fisherian

This model of mate choice, developed by Fisher (1930), is based on the idea that female mate preference is influenced by male ornaments or behaviour, but without these male traits conveying any information about male quality. Thus an ornament is attractive to females for an arbitrary reason, and the greater the elaboration of the ornament the stronger the attraction it has for females. An assumption of this mechanism of mate choice is that the alleles controlling the ornament under selection in males are genetically coupled to the alleles that control female preference for the ornament. This linkage disequilibrium between male trait and female preference is maintained through sexual selection, with a result that the male trait tends to become more elaborate and female preference for the trait stronger over time, hence the name for this type of selection as Fisher's runaway process. Fisherian selection is predicted to select for ever greater elaboration of male traits until a point is reached when natural selection, for example through predation, limits selection on the male trait (Andersson 1994). Conditions for Fisherian selection are found in the three-spined stickleback. In this

species, male red nuptial colouration and female preference for red are genetically correlated (Bakker 1993). In the absence of indirect benefits of red colouration, which have rarely been demonstrated in this species, this correlation suggests the operation of Fisherian selection.

Fisher (1930, 1958) additionally argued that the origin of runaway sexual selection would be initiated through female preference for a trait that conferred a fitness benefit, either through direct or indirect selection. Later models have suggested these starting conditions need not be in place to initiate Fisherian selection (Lande 1981; Kirkpatrick 1987). The Fisherian model of intersexual selection most closely reflects Darwin's (1871) original formulation of how sexual selection through mate choice might operate. The reformulation of Fisherian sexual selection by Lande (1981) and Kirkpatrick (1987) (the so-called Lande-Kirpatrick model, or simply LK model) can be considered as a null model for intersexual selection (Kirkpatrick and Ryan 1991; Prum 2010). Thus, while few examples of Fisherian selection have been demonstrated in fishes, an alternative view is that *all* cases of intersexual selection should be viewed as arising through this process, unless evidence demonstrates otherwise (Prum 2010). This approach has the advantage of providing a null hypothesis against which alternative ideas of indirect selection can be tested.

Sensory bias

Female preferences for male traits may exist because females have existing predispositions in their sensory systems for certain stimuli, either visual, auditory, or olfactory (Ryan 1990; Fuller et al. 2005). Male traits that stimulate those existing biases may subsequently be selected through sexual selection. Female sensory biases may be arbitrary. Alternatively, they may have evolved under natural selection, for example in

the context of prey detection. Female mating preferences that arise through a sensory bias, like Fisherian selection, do not assume a fitness benefit for the female from mate choice decisions. The key element of the various sensory bias hypotheses is that the preference evolves first, with the favoured male trait evolving later as a consequence of how the bias shapes the evolution of the trait. Another feature of the hypothesis is that males as well as females should express a sensory bias, though females may subsequently evolve a stronger or more refined response than males through sexual selection. An appealing aspect of the sensory bias hypothesis is the prediction that a pre-evolved preference for a male trait will be universal, with the result that all females will share the preference for a male trait when it evolves. From a conceptual viewpoint, then, this model presents no difficulties in explaining how an arbitrary male ornament could be elaborated through sexual selection.

There are several examples of where a sensory bias has been invoked to explain the elaboration of male traits in fishes. A commonly cited example is in the livebearing swordtails and platyfishes (*Xiphororus* spp.), which possess a pronounced sword-like extension to the lower lobe of their caudal fin. Female *Xiphororus* show a mating preference for males with swords, even in species in which males do not exhibit the trait. Female preference for swords appears, at least in some analyses, to pre-date the evolution of swords, which may have evolved, therefore, through a female sensory bias (Basolo 1990).

Another proposed example of sensory bias is in the guppy (*Poecilia reticulata*). In this species females have a mating preference for males with orange spots, though the strength of this preference varies. Rodd et al. (2002) showed that both male and female guppies also have a preference for orange food items, with the strength of variation in attraction to orange food items explaining most variation in female

preference for orange in a mating context. Finally, in the three-spined stickleback (*G. aculeatus*), both males and females were shown to have a preference for red in a foraging context, while females had a preference for males with red nuptial colouration. A red preference was also shown in the nine-spined stickleback (*Pungitius pungitius*), a species without red male nuptial colouration. A phylogenetic analysis showed that the preference for red must have evolved prior to red nuptial colouration, a key prediction of the sensory bias hypothesis (Smith et al. 2004a).

Mating decisions and male dominance

Males can increase their reproductive success by maximising the number of eggs they fertilise. Hence males engage in intrasexual competition to monopolize females or resources that are important for female reproductive success. Males often display their dominance status through larger body size and armaments, or ornament traits that correlate with success in male-male competition (Andersson 1982; Berglund et al. 1996; Houde 1997). Traits associated with dominance are probably costly, either because they increase energy expenditure, predation risk or vulnerability to disease. Consequently females may use these traits as an honest signal of male quality (Qvarnström and Forsgren 1998). Males successful in intrasexual competition are predicted to provide high-quality resources, and may also confer genetic benefits that correspond to good genes on offspring. Therefore, dominant males may influence female mate preference; male-male competition may represent a reliable source of information that facilitates female mate choice and hence female adapts to choose traits that are related to dominance (Berglund et al. 1996; Wong and Candolin 2005; Neff and Pitcher 2005). A correspondance between male-male competition and female mate choice has been found in some species. For example, in many populations of three-spined sticklebacks

(*Gasterosteus aculeatus*) red nuptial colouration functions as a signal of male dominance that is also attractive to females who obtain good genes for their offspring by mating with redder males (Milinski and Bakker 1990; Candolin 1999; Barber et al. 2001). However, a difference between male dominance and female mate preferences in their reproductive interests can lead to sexual conflict (Berglund et al. 1996; Gowaty 1997; Murphy 1998; Parker and Partridge 1998; Qvarnström and Forsgren 1998). Female mate choice may or may not be associated with male-male competition; females may not always prefer dominant males if traits selected by intrasexual selection are not important cues in female mate choice (Qvarnström and Forsgren 1998). For example, female mate choice in zebrafish *Danio rerio* does not correspond with dominance, thus dominant males may override female mate preferences (Spence and Smith 2006). Dominant males may limit female reproductive success by monopolizing resources that are important for female reproduction and constraining their opportunities for mating with other males (Warner 1987; Reichard et al. 2005; Reichard et al. 2007a). Thus mating with dominant males may decrease the indirect benefits of mate choice, especially in mating systems that rely on non-additive genetic effects, as well as direct costs to female fitness through coercive male mating behaviour such as, harassment (Arnqvist and Rowe 1995; Jacob et al. 2007; Reichard et al. 2007a). Therefore, dominant males may maximise their reproductive success at the price of limiting female mate choice and the opportunity of subordinate males to engage in mating (Warner 1987; Reichard et al. 2005; Reichard et al. 2007a), ultimately leading to a conflict between the consequences of intra- and intersexual selection (Reichard et al. 2007a).

Mating decisions and alternative male mating tactics

Variance in reproductive fitness among males has led to the evolution of alternative male mating tactics; males that are disadvantaged in intrasexual competition are predicted to adopt tactics such as “sneaking” (Gross 1996; Wade and Shuster 2004). Males unsuccessful in male-male competition can achieve some mating success through stealing fertilisations from dominant males, and thereby avoiding the costs of investing in attracting females or establishing and maintaining dominance (Andersson 1994; Taborsky 1994). Female mate choice has been generally viewed as undermined by alternative male mating tactics (Taborsky 1998; Jones et al. 2001). However, it has been recently argued that male tactics such as sneaking could augment female mate choice in some cases, and alternative mating tactics could increase female reproductive success in circumstances where male dominance and female mate preferences do not coincide (Reichard et al. 2007a). In this case, the genetic benefits of female mate choice may be enhanced by males that adopt alternative mating tactics (Reichard et al. 2005, 2007a). Sperm competition arising through sneaking has been considered to be associated with increased fertility because of a greater number of sperm released during mating (Reichard et al. 2007a), but sperm competition also reduces the probability of genetic incompatibility between sperm and ova (Zeh and Zeh 1997; Reichard et al. 2007a). However, sneaking can reduce female mate choice benefits if sneakers produce low offspring quality; i.e. if additive genetic benefits are important in the mating system. Sneaker males may also negatively influence female mating success if guarder male prevent sneakers from participating in mating leading to interruption of male courtship and delayed oviposition (Alonzo and Warner 1999; Reichard et al. 2004a). Female responses to sneaking appear to vary among species. In some species female avoid spawning with sneakers and reduce their spawning rate if sneakers try to participate in a mating (Van den Berghe et al. 1989; Alonzo and Warner 2000), while others show no

sneaking-avoidance behaviour (Svensson and Kvarnemo 2005; Byrne and Roberts 1999, 2000) In others females appear to respond positively to sneakers by laying more eggs in matings associated with sneaking (Fu et al. 2001; Smith and Reichard 2005; Watters 2005).

Aims

The basis to female reproductive decisions, and their outcomes for females and their offspring, are important in understanding sexual selection (Trivers 1972; Roughgarden 2004; Andersson and Simmons 2006). The goal of this thesis was to investigate the consequences and the mechanisms of female mate choice. The aims were to obtain a fuller understanding of:

1. The consequences of female mate preferences.
2. The role of genetic benefits in female mate choice.
3. The cues used by females in mate choice.
4. The relative role of direct and indirect benefits of female mate choice.
5. The consequences of female spawning site choice.

Study species

An experimental study to understand the basis to female mate choice, the role of genetic compatibility, and the relative importance of direct and indirect female mate choice benefits requires a suitable model. The ideal candidate is one in which genetic compatibility plays a role in the mating system, shows unambiguous mate choice, and is amenable to lab manipulation. It should have external fertilisation to enable controlled experimental crosses, achieve sexual maturity within a timescale that is practical for replicable experimental protocols, and reproduce year round. A small fish, the Chinese

rose bitterling (*Rhodeus ocellatus*), is such a model. Bitterling (Family Cyprinidae, Subfamily Acheilognathinae) are small freshwater fishes found in East Asia and Europe. There are about 40 species of bitterling distributed throughout China, Japan, Korea, Laos and Vietnam belonging to three genera; *Acheilognathus*, *Tanakia*, and *Rhodeus* (Arai 1988; Okazaki et al. 2001), while in Europe there is a single species, *Rhodeus amarus* (Reichard et al. 2007c, 2010; Zaki et al. 2008).

Bitterling have a distinctive biology that makes them especially amenable material for research on reproduction and sexual selection. Uniquely, they will only lay their eggs in the gills of living freshwater unionid mussels, in which their eggs and embryos are incubated for 3 weeks. Females develop a long ovipositor that they use to place their eggs in the gills of the mussel through its exhalant siphon. Males guard mussels, vigorously court females and attempt to lead them to mussels in their territories. This dependence on mussels gives the experimenter enormous control over where and when reproduction takes place. Both intra- and intersexual selection play a role in the mating system (Kanoh 1996, 2000; Smith et al. 2003). Female oviposition decisions are based on both male and mussel quality (Candolin and Reynolds 2001; Smith et al. 2000b, 2001), with evidence that compatibility plays a role (Casalini 2007). Females spawn in several bouts lasting one day and consisting of approximately 5-10 independent spawnings in which they deposit 1-5 (typically 3) eggs in mussels (Smith et al. 2004b). Territorial males release sperm over the inhalant siphon of the mussel to fertilizes the eggs, though sneaking behaviour, in which a male releases his sperm into a rival's mussel, is common (Candolin and Reynolds 2002a,b; Reichard et al. 2005; Smith et al. 2004b, 2009). There is no evidence for a morphological or genetic distinction between territorial and sneaking males (Kanoh 1996; Reichard et al. 2004b; Smith et al. 2004b). Females appear able to undermine male dominance, to some extent

at least, by soliciting sneakers, delaying spawning and performing a spawning action but without depositing eggs (Smith and Reichard 2005; Smith et al. 2007). Details of bitterling reproductive biology are reviewed by Smith et al. (2004b). Thus, bitterling uniquely lend themselves to the aims of this study as a model system because:

1. They are small, amenable to laboratory studies and breed all year round in captivity.
2. Their biology, and particularly reproductive behaviour, are well described.
3. Fertilisation is external, making *In Vitro* Fertilisations (IVFs) for artificial crosses possible.
4. They can only use freshwater mussels for oviposition, permitting an exceptional level of control in mating experiments.
5. Females can mate with several males during a single bout of spawning, and mate choice decisions are unambiguous.
6. Sexual maturity is achieved after only 3-4 months, making it practical to measure reproductive traits in offspring derived from experimental crosses.
7. Embryo and juvenile survival is high (ca. 70%), minimising unexplained variance in mortality, and permitting balanced cross-classified experimental mating designs.
8. There is evidence that mate choice arises through compatibility.

While the bitterling represents a valuable model for the present study, the goal was to address broad questions with application to other taxonomic groups.

Structure of the thesis

The thesis comprises eight chapters, and can be summarised as follows. In **Chapter 2** the potential conflict between male dominance and female mate choice was examined, while the contribution of additive and nonadditive genetic mate choice benefits were evaluated in **Chapter 3**, including the potential role of MHC as the basis to female mate

choice. The relative importance of direct versus indirect benefits of female mate choice was tested in **Chapters 4** and in **Chapters 5** the potential role of a receiver bias in female mate choice is assessed. The long-term consequences of female oviposition decisions were investigated in **Chapter 6**, while in **Chapter 7** the role of olfactory cues in female spawning decisions is dealt with. In **Chapter 8** a final synthesis of the findings in Chapters 2-7 is presented.

Chapter Two - Male dominance, female mate choice, and intersexual conflict in the rose bitterling (*Rhodeus ocellatus*)

ABSTRACT

An intersexual conflict arises when males and females differ in their reproductive interests. Although experimental studies have shown that females often mate with dominant males, it may not always be in the interest of a female to do so. Here we investigated the impact of male dominance on female mate choice and offspring growth and survival in the rose bitterling (*Rhodeus ocellatus*), a freshwater fish with a resource-based mating system. A mating experiment was conducted using males of known dominance rank whereby females were able to choose among males that could see and smell each other, but could not directly interact. The results showed that female preferences did not correspond with male dominance, resulting in a potential intersexual conflict. The survival of offspring to independence was significantly correlated with female mate preferences, but not with male dominance.

Casalini, M., M. Agbali, M. Reichard, M. Konečná, A. Bryjová, and C. Smith. 2009. Male dominance, female mate choice and intersexual conflict in the rose bitterling (*Rhodeus ocellatus*). *Evolution* 63:366-376.

INTRODUCTION

Sexual selection arises through intrasexual variance in reproductive success, with variance usually higher in males due to their greater potential reproductive rates, a result of differences in gamete allocation between the sexes. Two main mechanisms of sexual selection are recognized. Intrasexual selection typically involves male–male competition; males actively compete for access to females or resources that are necessary to attract females. Intrasexual selection gives rise to selection for male fighting ability, often including weapons and large body size. Intersexual selection involves some aspect of mate choice, usually by females, with preferences based on direct or indirect (or both) benefits and giving rise to selection for elaborate signals and displays by males and preferences for these traits by females (Darwin 1871; Fisher 1930; Williams 1966; Andersson 1994).

The relative roles of intra- and intersexual selection and their interaction is a key question in sexual selection theory (Kokko 2005). Males that succeed in contests with other males may be attractive to females because they provide direct or indirect benefits of mate choice or both, and male contests may better resolve information that females use in mating decisions (Berglund et al. 1996; Wong and Candolin 2005). Alternatively, dominant males, and the traits associated with male dominance, may not be preferred by females (Qvarnström and Forsgren 1998). Instead females may be constrained in mate choice through male intrasexual selection, and risk a reduction in reproductive fitness when mating with dominant but nonpreferred males, particularly if mate choice is linked to nonadditive genetic benefits or direct costs to female fitness arising through male coercion (Jacob et al. 2007; Reichard et al. 2007a).

Here intra- and intersexual components of sexual selection were experimentally separated in the rose bitterling (*Rhodeus ocellatus*), a small cyprinid fish with a

resource-based mating system. Using males of known dominance rank, female mate choice was tested for males that could see and smell each other, but could not directly interact. Female and male effects on reproductive success were contrasted and matched to male morphological and behavioural traits. In addition, *in vitro* fertilizations were used to compare the fitness benefits of preferred and nonpreferred mates to females in terms of embryo growth and survival. Two predictions were tested: (1) Whether dominant males (and traits associated with dominance) would be preferred by females, or whether there was a mismatch between male dominance and female mate preference. (2) Whether female mate preference conferred a fitness benefit to females through increased offspring survival or quality.

In bitterling (*Rhodeus* spp., Acheilognathinae), both intra and intersexual selection can play a role in the mating system (Kano 2000; Candolin and Reynolds 2001; Smith et al. 2002, 2003; Reichard et al. 2005). During the breeding season, males develop red carotenoid-based nuptial coloration, most notably in the iris and on the fins, and compete for territories around living unionid mussels that females use for oviposition. The bitterling mating system is promiscuous; both males and females spawn repeatedly, with multiple partners. Males court females and attempt to lead them to mussels in their territories. Female oviposition decisions are based on both male and mussel quality (Smith et al. 2000b; Candolin and Reynolds 2001; Kitamura 2006; Casalini 2007). Females deposit one to five (typically three) eggs in the mussel gill chamber. Females spawn in several bouts lasting one or two days and consisting of approximately 5–10 independent spawnings each day (Nagata 1985; Smith et al. 2004b). Territorial males release sperm over the inhalant siphon of the mussel so that sperm drawn into the gills fertilizes the eggs. Sneaking behavior, in which a rival male (an adjacent territory holder or a male that does not possess a territory) releases his

sperm into a rival's mussel, is common in bitterling (Kano 1996, 2000; Smith et al. 2002, 2003; Reichard et al. 2004a,b). Male mating behavior is largely opportunistic and there is no evidence of a morphological or genetic distinction between territorial and sneaking males (Kano 2000). Females appear able to undermine male dominance, to some extent at least, by soliciting sneakers, delaying spawning, and performing a spawning action but without depositing eggs (Smith and Reichard 2005; Smith et al. 2007). For further details on bitterling reproductive biology see Smith et al. (2004b).

MATERIAL AND METHODS

Fish for experimental work were wild-caught *R. ocellatus* from the River Yangtze Basin, China. They were raised in captivity and were between 30–34 months of age during the experiments. Prior to experiments fish were kept in a single aquarium measuring 120 (length) × 40 (width) × 45 (depth) cm and fed a mixture of frozen bloodworm (*Tubifex* spp.) and commercial dried fish flake food twice each day and live zooplankton three times each week. Stock and experimental aquaria were on a recirculating system with water temperature in all aquaria at 23 °C. Each aquarium contained a 20 mm layer of sand substrate and fish were exposed to a 16 h: 8 h light/dark regime. *Rhodeus ocellatus* readily spawn in *Unio pictorum* (Casalini 2007), and this species of mussel was used in all experiments. Experimental mussels were collected from the River Cam and stored in a 100-L tank and fed live phytoplankton daily.

EXPERIMENT 1: FEMALE MATE CHOICE

Before each trial, groups of experimental males were ranked for dominance. To do this, four males were haphazardly selected from the stock aquarium and placed in an

experimental aquarium measuring 60 (length) \times 40 (width) \times 40 (depth) cm with a female in spawning condition (with a fully extended ovipositor) and a mussel in a sand-filled flower pot covered with a perforated transparent plastic cup to allow visual and olfactory inspection of the mussel but not spawning. Two artificial plants were placed in the aquarium as refuges. The four males remained together for 1 h to enable dominance to establish; the dominant male aggressively excluded other males from approaching the mussel. Once dominance was established the dominant male was removed. The same procedure was repeated to identify the second, third, and fourth-ranked males. Dominance in bitterling, once established, is highly stable (Reichard et al. 2005) and can last for at least six weeks under natural conditions (Smith et al. 2000b).

After ranking, males were placed in an experimental aquarium measuring 60 (length) \times 40 (width) \times 40 (depth) cm divided into four compartments, each measuring 30 (length) \times 20 (width) cm, separated with walls of thin wire mesh measuring 5 \times 5 mm. Males were placed alone in compartments with a size-matched mussel in a sand-filled pot and an artificial plant. Male *R. ocellatus* are larger and deeper bodied than females and the mesh walls prevented males passing into adjacent compartments but enabled smaller females to pass through freely. Males could see and smell individuals in neighboring compartments. The following morning, after covering all mussels with a transparent perforated plastic cup, a female in spawning condition was placed in a randomly selected compartment of the experimental aquarium. After 2 h the mussels were uncovered and fish behavior was scored for 30 min using a palm computer with the FIT-system behavior recording software (Held and Manser 2005). The behaviors scored were: (1) frequency of female inspection of mussels (the fish positions its snout close to the exhalant siphon of the mussel), and (2) rate of male courtship (the male swims toward a female then approaches a mussel while “standard length (quivering)”

(Smith et al. 2004b). At the end of each trial the number of eggs laid by the female was quantified using a mussel-opening device. Experimental mussels were measured (maximum shell length) to the nearest 1 mm and moved to large tanks with phytoplankton to allow the embryos to complete development and were not used again in the experiment.

EXPERIMENT 2: CONSEQUENCES OF MATE CHOICE

After completion of the mate choice trial (experiment1) the female was isolated until she ovulated another batch of eggs, an interval of 5–7 days (Smith et al. 2004b). Eggs were stripped from the female by gently squeezing her abdomen and divided into four batches of equivalent size. The eggs were fertilized within 5 min. using sperm stripped from the same four males used in the mate choice trial. Stripping sperm from males yields an excess of sperm and all eggs were successfully fertilized, developed a perivitelline space, and began initial development. The eggs were incubated in a 70-mm-diameter Petri dish in freshwater and a record was kept of their survival over a period of four weeks, after which time the larvae achieve independence. After four weeks larvae were photographed under standard light conditions next to a scale bar and their length was estimated to the nearest 0.1 mm from digital images.

The Standard Length (SL; measured from the tip of the snout to the base of the tail) of all males and females was measured to the nearest 1 mm. In addition, the left and right eye and tail fin spot of every male were photographed under standard light conditions. The area of red color in the iris of males was measured using a protocol modified from that of Barber et al. (2000). Images were analyzed using Photoshop 2.0 (Adobe Systems Inc., San Jose, CA). Using the magic wand facility, a single pixel was chosen from the area of red in the iris and successive portions of the image were

selected based on color similarities until all the pixels within the red-colored area had been captured. The total area of the iris, excluding the pupil was similarly estimated and the red area was expressed as a proportion of the total. The extent and brightness of red colour of the caudal fin were measured using an ordinal scale from Casalini et al. (2009). After measurements were completed, fish were returned to stock aquaria and were not used again.

STATISTICAL ANALYSIS

All data were tested for normality using a Kolmogorov–Smirnov test and for equality of variance using Bartlett’s test. Data that did not meet assumptions of normality and homoscedasticity were transformed. If data transformation did not improve the data distribution to meet assumptions of parametric tests, nonparametric equivalents (Kruskal-Wallis test, Spearman correlation) were used. One-way ANOVAs and Pearson’s correlations were used to test differences and associations between morphological and behavioral data, dominance, and reproductive success.

RESULTS

Male body size (one-way ANOVA: $F_{3, 28} = 5.84$, $P = 0.003$; Fig. 2.1) and extent of tail spot redness (Kruskal–Wallis test: $H = 8.81$, $df = 3$, $P = 0.032$) were positively related to male dominance rank. There was no difference among male ranks in the extent of red area of the iris ($F_{3, 28} = 0.83$, $P = 0.491$). There was no difference between the number of eggs spawned by females among male ranks (one-way NOVA, square-root transformation: $F_{3, 28} = 0.84$, $P = 0.482$), and no correlation between female mate preference and male color (Pearson’s correlation: red area of the iris $r_{30} = 0.042$, $P = 0.820$; Spearman’s correlation: tail spot redness $r_{30} = 0.066$, $P = 0.722$). However, there

was a correlation between male courtship frequency and the number of eggs spawned by females with a male (Pearson's correlation, square-root transformation: $r_{30} = 0.537$, $P = 0.002$; Fig. 2.2). The survival of eggs was positively correlated with female preference for males, one (Pearson's correlation, rank transformation: $r_{30} = 0.455$, $P = 0.009$), two ($r_{30} = 0.379$, $P = 0.032$), three ($r_{30} = 0.378$, $P = 0.033$) and four weeks after fertilization ($r_{30} = 0.449$, $P = 0.010$; Fig. 2.3). There was no correlation between female mate preference and embryo size after four weeks (Pearson's correlation, rank transformation: $r_{30} = -0.195$, $P = 0.487$). There was no difference in offspring survival among male dominance ranks after four weeks (one-way ANOVA: $F_{3,28} = 0.84$, $P = 0.482$) and no correlation with male color (Pearson's correlation: red area of the iris $r_{30} = -0.023$, $P = 0.902$; Spearman's correlation: tail spot redness $r_{30} = -0.018$, $P = 0.922$).

DISCUSSION

The aim of this study was to investigate the interaction between male intrasexual competition with intersexual mate choice in the rose bitterling, a fish with a resource-based mating system. It was shown that when females were able to select males for mating without interference through male-male competition, they demonstrated preferences for specific males, measured in terms of the number of eggs they deposited. However, female mate choice did not correspond with male dominance rank, size, or coloration (extent of red in the iris or on the tail fin). The only male trait that correlated with female choice was the vigor of male courtship behavior. Female preference for males correlated positively with embryo survival to independence, but there was no effect of male dominance on offspring survival. No effect of male dominance, color, or female preference on offspring size was detected, although density effects on embryo growth rates cannot be excluded from the experimental design used.

Ostensibly these results suggest a conflict between intra and intersexual selection, with female mate choice overridden by male dominance. A conflict between these two forms of sexual selection has been argued previously (Moore and Moore 1999; Arnqvist and Rowe 2005). Reichard et al. (2005) used the related European bitterling (*R. amarus*) to show that traits selected in intrasexual selection did not correspond with those selected through intersexual selection. Spence and Smith (2006) demonstrated that male dominance did not match female mate choice in the zebrafish (*Danio rerio*), and comparable results have been obtained for water striders (*Aquarius remigis*) (Sih et al. 2002) and brown trout (*Salmo trutta*) (Petersson et al. 1999). However, in some instances where females are free to choose mates they do select dominant males (reviewed by Wong and Candolin 2005). Consequently, there appears a disparity between mating systems in which female resistance to male dominance has evolved (through so-called "chase-away" selection) and the conventional view of sexual selection that arises through direct, but especially indirect, benefits to mate choice. However, although a distinction between these forms of sexual selection has been drawn, the functional difference between them has been questioned (Kokko 2005).

Despite constraints placed on them by male dominance, females may still find opportunities to mate with subordinate males, for example through extra-pair copulations (Mulder et al. 1994; Forstmeier et al. 2002), avoidance of dominant males (Kangas and Lindström 2001; Bro-Jørgensen 2003), or by increasing mating opportunities for subordinates by delaying oviposition (Smith and Reichard 2005; Smith et al. 2007). Females may also directly demonstrate preferences for subordinates. In coho salmon, *Oncorhynchus kisutch*, females performed more digging behaviour (a measure of mate choice) when accompanied by sneaker males and oviposited for longer (and therefore, perhaps, laid more eggs) when sneaker males took part in spawning

(Watters 2005). Females appeared to prefer mating with sneaker males to avoid direct costs of mating that are imposed on them when spawning with aggressive guarder males (Watters 2005). Similarly, female bluegill sunfish, *Lepomis macrochirus*, laid three times more eggs when sneaker males participated in spawning (Fu et al. 2001). Whether female behavior played a role in influencing the success of subordinate males cannot be determined from our experimental design, although previous studies in the related *R. amarus* suggests it can (Smith and Reichard 2005; Smith et al. 2007). Here female rose bitterling did show a preference for specific males. Notably, eggs fertilized by preferred males showed significantly higher survival to independence, providing evidence for a fitness benefit to female mate choice.

There is an ongoing controversy over the importance of additive benefits of female mate choice (Kirkpatrick and Barton 1997; Møller and Alatalo 1999; Head et al. 2005), and only a limited number of studies provide substantial evidence for an effect (e.g. three-spined stickleback, *Gasterosteus aculeatus*, Barber et al. 2001; gray tree frog, *Hyla versicolor*, Welch et al. 1998). Zeh and Zeh (1996) argued that genetic incompatibilities between potential mates, arising from intragenomic conflict, could substantially decrease fertilization rate and offspring viability, which could generate strong selection for mate choice based on compatibility. In a study of the Alpine whitefish, *Coregonus* sp., Wedekind et al. (2001) demonstrated that offspring sired by males with the most extensive breeding ornamentation had higher survival rates following bacterial infection at the egg stage regardless of female identity, suggesting additive genetic benefits to mating. However, offspring mortality arising from developmental problems appeared related to specific male–female combinations, suggesting incompatibilities between parental haplotypes (Wedekind et al. 2001). In the related Arctic charr (*Salvelinus alpinus*), the fertilization success of sires correlated

positively with their genetic similarity, measured using microsatellites, with dams (Liljedal et al. 2008); a result consistent with a role for genetic compatibility.

Recent work suggests that females may be capable of choosing genetically compatible mates through olfactory cues based on MHC-specific odors (Jordan and Bruford 1998); a substantial body of empirical evidence for MHC-based mate choice has accumulated (Neff and Pitcher 2005; Piertney and Oliver 2006). In this case mate choice operates through a self-referential process involving detection of MHC peptide ligands (Milinski et al. 2005). MHC preferences may manifest themselves as pre-mating behavioural decisions but also as postmating fertilization or developmental incompatibilities (Tregenza and Wedell 2000; Skarstein et al. 2005).

The mechanism of female mate choice in rose bitterling is not known. Olfactory cues play a key role in mate choice in many fish, including poeciliids (McLennan and Ryan 1997) and zebrafish (Spence et al. 2008), and there is evidence that the same is true in bitterling (Chapter 7). Chemical cues in fish may be released from specialized exocrine glands, or if water-soluble compounds are released, from the gills or in the urine (Rosenthal and Lobel 2006). The release of chemical compounds by fish is often associated with fin or body movements, because the diffusion of compounds in water is relatively slow (Atema 1996). In the case of bitterling, male courtship behavior involves the male swimming in front of the female and undulating his body, and particularly the anal and tail fin, at high frequency and low amplitude (Smith et al. 2004b), which may assist in directing male odor toward courted females. Female mate choice correlated significantly with male courtship rate in the present study, an observation that is consistent with previous field and laboratory studies on bitterling (Smith et al. 2002; Reichard et al. 2005). In our experiment, the correlation between male reproductive success and courtship was a consequence of male dominance; dominant males

prevented subordinate rivals from courting or mating, except through sneaking, resulting in a strong relationship, but not one derived from female mate choice. Courtship behavior in bitterling may be associated with the release of olfactory cues by males, possibly in the urine (M. Agbali, unpubl. data). Thus, one hypothesis is that females may choose preferred mates through olfactory cues based on MHC-specific odors. Ongoing work will explore the role of MHC gene compatibility in offspring fitness, and the role of MHC-specific odors in mate choice decisions of bitterling.

The findings of this study suggest differences in the outcome of sexual selection for dominant and subordinate males, depending on the social or environmental context in which they compete. For example, in an environment in which male contests for mating opportunities are limited, and females are able to exercise mate choice without constraint, otherwise subordinate males that court vigorously are predicted to enjoy high reproductive success. In contrast, in an environment that results in strong intrasexual selection the reverse will be true with dominant males able to largely monopolize matings. The bitterling offers an excellent model for these studies; previous work has shown that bitterling occur in lakes in which their population size varies 90-fold and mussel abundance 500-fold (Smith et al. 2000a). Consequently female choice may be more important in populations in which bitterling densities are low and mussel densities are high; all male bitterling could potentially hold territories and interference between males is predicted to be limited. In contrast, when population densities are high and mussel abundances are low, conditions will favour competition for spawning sites through male dominance.

Male nuptial coloration weakly predicted male dominance. In the experiment, dominance matched the degree of tail spot redness. However, although male coloration appeared to function, albeit to a limited extent, in intrasexual competition, there was no

evidence that it played a role in intersexual selection; male color failed to predict female mate preference. These findings match those for a study on a related species, the European bitterling, *R. amarus*. Reichard et al. (2005) found no effect of the intensity of male eye or fin color on female mate choice, although the intensity of eye color predicted male reproductive success in a competitive environment. Similarly, Smith et al. (2002) found the extent of eye redness to be greater among dominant males under natural conditions of *R. amarus*. Candolin and Reynolds (2001) did detect a significant effect of the intensity of fin color on female spawning decisions, although this finding has never been repeated. Thus, although the extent and intensity of male coloration may have some limited function as a badge of status (Andersson 1994), further research will be needed to establish the functional role of male coloration in bitterling fish.

In conclusion, it was demonstrated that intersexual selection through female mate choice and intrasexual selection through aggressive male contests do not correspond in *R. ocellatus*; females appear to suffer a net fitness loss as a consequence of intrasexual selection suggesting an intersexual conflict.

Figure 2.1. Mean (\pm SE) body length of males in relation to dominance rank. Lower case Greek letters above error bars denote significantly different groups as determined by *post hoc* pairwise comparisons; values with the same letter did not differ significantly.

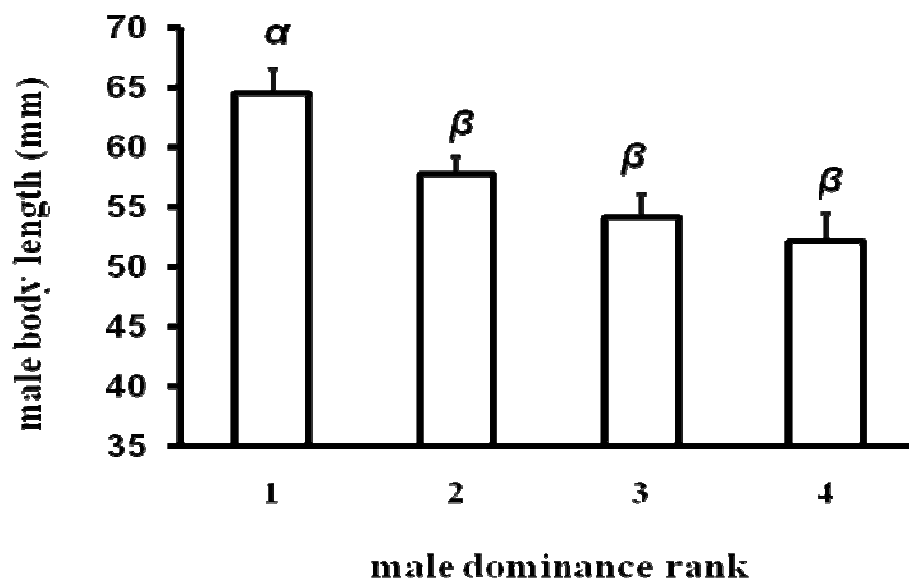


Figure 2.2. Correlation between the square-root male courtship rate and number of eggs spawned by females.

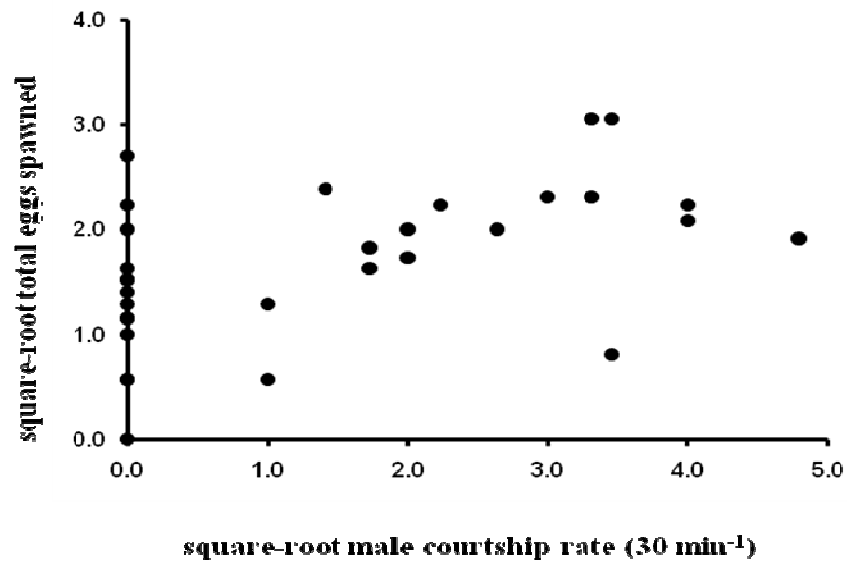
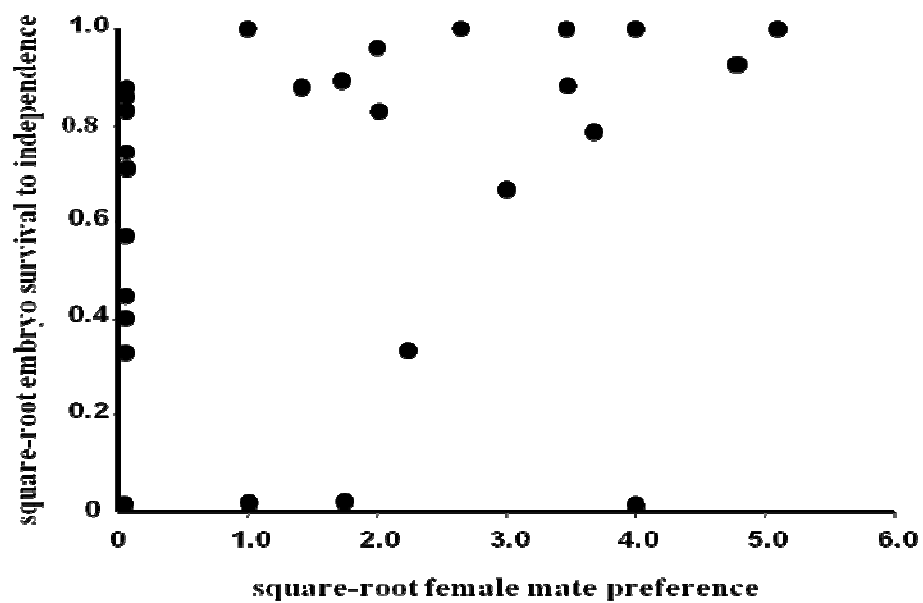


Figure 2. 3. Correlation between square-root female mate preference (number of eggs spawned with males in mate choice trials) and egg survival to independence.



Chapter three – Mate choice for nonadditive genetic benefits correlate with MHC dissimilarity in the rose bitterling (*Rhodeus ocellatus*)

ABSTRACT

Good genes models of mate choice predict additive genetic benefits of choice whereas the compatibility hypothesis predicts nonadditive fitness benefits. Here the Chinese rose bitterling, *Rhodeus ocellatus*, a freshwater fish with a resource-based mating system, was used to separate additive and nonadditive genetic benefits of female mate choice. A sequential blocked mating design was used to test female mate preferences, and a cross-classified breeding design coupled with in vitro fertilizations for fitness benefits of mate choice. In addition, the offspring produced by the pairing of preferred and nonpreferred males were reared to maturity and their fitness traits were compared. Finally, the MHC DAB1 gene was typed and male MHC genotypes were correlated with female mate choice. Females showed significant mate preferences but preferences were not congruent among females. There was a significant interaction of male and female genotype on offspring survival, rate of development, growth rate, and body size. No significant male additive effects on offspring fitness were observed. Female mate preferences corresponded with male genetic compatibility, which correlated with MHC dissimilarity. It is proposed that in the rose bitterling genetic compatibility is the mechanism by which females obtain a fitness benefit through mate choice and that male MHC dissimilarity, likely mediated by odor cues, indicates genetic compatibility.

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INTRODUCTION

Females may obtain a fitness benefit under sexual selection by choosing to mate with a male who provides direct, nongenetic, benefits, such as protection from predators or harassment by other males, access to resources or nuptial gifts, or parental care of offspring (Andersson 1994; Qvarnström and Forsgren 1998; Andersson and Simmons 2006). Female mate preference may also be selected through indirect benefits, based on the genetic quality of potential mates (Kirkpatrick 1982; Eberhard 1996). Genetic quality can be considered as the combined effects of good and compatible genes (Neff and Pitcher 2005). In good genes models of mate choice, a male with high genetic quality is predicted to confer a fitness advantage on offspring, with male beneficial alleles acting independently of maternal genotype. Consequently, good genes are predicted to show additive genetic variation (Wedekind et al. 2001; Neff and Pitcher 2005). In populations in which mate choice is driven by good genes, female mating decisions will tend to be congruent, and selection will be directional. Good genes effects are associated with signals of male quality, typically in the form of ornamentation or display (Maynard Smith and Harper 2003). In contrast, compatible genes are predicted to impart enhanced fitness to offspring only when particular male and female genotypes are combined (Zeh and Zeh 1996, 1997; Penn 2002), thus compatible genes are predicted to show nonadditive variation (Neff and Pitcher 2005; Piálek and Albrecht 2005). In populations in which compatible gene effects operate, females will not necessarily share mate preferences, and selection will not be strongly directional (Tregenza and Wedell 2000; Neff and Pitcher 2005; Ivy 2007). The genetic basis to compatibility is poorly understood, but the process requires an individual to recognize its own genotype as well as those of potential mates, while also allowing for

the impact of recombination, asymmetric inheritance patterns, and sex-specific effects (Charlesworth et al. 1987).

The genes of the major histocompatibility complex (MHC), and their role in the functioning of the vertebrate immune system, have received attention as the potential targets of sexual selection through mate choice (Jordan and Bruford 1998; Penn 2002). The MHC is a family of highly polymorphic genes that encode a set of transmembrane proteins. The function of these proteins is to distinguish between self and nonself antigen and present foreign peptides to T-cell receptors, thereby playing a key role in resistance to infectious and autoimmune disease (Hill 2001).

Many studies indicate MHC genes can influence odor, thus MHC genes have the potential to function in an odor-based recognition system enabling females to identify mates that can provide additive genetic benefits through enhanced immunocompetence, or to identify genetically compatible mates (Penn and Potts 1999; Tregenza and Wedell 2000; Penn 2002). Several studies have demonstrated MHC-related mate choice. In inbred lines of mice (*Mus musculus*) both sexes show a strong preference for MHC-dissimilar mates (Potts et al. 1991), and in humans women favour the odor of men with different MHC alleles to their own (Wedekind et al. 1995; Wedekind and Furi 1997). A role for an MHC-based mate choice system has also been proposed in fish (Landry et al. 2001; Reusch et al. 2001; Arkush et al. 2002; Forsberg et al. 2007; Consuegra and Garcia de Leaniz 2008; Yeates et al. 2009), birds (Richardson et al. 2005; Bonneaud et al. 2006), and reptiles (Olsson et al. 2005).

A mate choice system mediated by MHC genes that generates additive genetic benefits could arise through negative frequency-dependent selection (Clarke and Kirby 1966). Here, the prediction is that rare MHC sequence variants confer an advantage on their carrier, although this advantage may eventually be lost through the coevolution of

pathogens. An outcome of this model of mate choice is a turnover of advantageous genetic variants in the form of an evolutionary arms race (van Valen 1973). In contrast, a mechanism of mate choice that would confer nonadditive benefits is through heterozygote advantage (Penn 2002). Here, high numbers of MHC sequence variants carried by an individual are predicted to maximize the probability of mounting immune responses against a large number of foreign peptides, and thereby enable an individual to resist a broader range of pathogens. A constraint on maximal MHC diversity may arise through negative T-cell selection during thymic development (Nowak et al. 1992), which would tend to favor an optimum rather than maximum MHC sequence variant diversity (Kalbe et al. 2009). These two models of mate choice evolution are not mutually exclusive.

In the present study, the basis and mechanism of female mate preference was experimentally tested in the rose bitterling (*Rhodeus ocellatus*), a small fish with a resource-based mating system. A sequential blocked mating design was used to test female mate preferences, and a North Carolina Type II breeding design (Lynch and Walsh 1998), utilizing in vitro fertilizations (IVFs) to generate crosses to test for additive and nonadditive genetic benefits of mate choice. The offspring produced by the pairing of preferred and nonpreferred males were reared to maturity and their fitness traits were compared. In addition, rose bitterling MHC genes were analyzed and experimental fish genotyped. Four predictions were tested: (1) Offspring sired by preferred males show enhanced fitness in comparison with nonpreferred males, with the genetic benefits of mate choice either additive or nonadditive. (2) Females show significant mate preferences, which are either congruent among females, suggesting additive genetic effects, or divergent, indicating nonadditive effects. (3) Female mate preferences are based on MHC genotype, with females showing a preference for mating

with males that are MHC dissimilar to maximize MHC diversity in the offspring if mate choice is for nonadditive benefits, or a preference for males possessing specific MHC alleles in case of additive benefits. (4) Male offspring that are the products of preferred pairings should have higher mating success than those from nonpreferred pairings in the case of additive benefits, but not if benefits are nonadditive.

Bitterling (Cyprinidae, Acheilognathinae) lay their eggs in the gills of living freshwater unionid mussels, in which their eggs and embryos are incubated for approximately 1 month. During the breeding season, males develop carotenoid-based nuptial coloration, most notably in the iris and on the tail fin, and compete for territories around mussels. Female bitterling develop a long ovipositor that they use to deposit their eggs inside the gills of the mussel through its exhalant siphon. Males vigorously court females and attempt to lead them to mussels in their territories. Both intra- and intersexual selection play a role in the mating system (Kano 2000; Candolin and Reynolds 2001; Smith et al. 2002, 2003; Reichard et al. 2005, 2008; Casalini et al. 2009). Female oviposition decisions are based on both male and mussel quality (Smith et al. 2000b; Candolin and Reynolds 2001; Smith and Reichard 2005; Kitamura 2006, 2007; Casalini 2007; Casalini et al. 2009). Females spawn in several bouts lasting one day and consisting of approximately 5–10 independent spawning (Nagata 1985; Smith et al. 2004b). Females deposit one to five (typically three) eggs in the mussel gill chamber. Territorial males ejaculate over the inhalant siphon of the mussel so that sperm is drawn into the gills of the mussel and fertilizes the eggs. Neighbouring territorial males, and males that do not own a territory, also fertilise some eggs by stealing fertilisations in the territory of other males (Kano 1996, 2000; Smith et al. 2002, 2003; Reichard et al. 2004a, 2008, 2009; Smith et al. 2009). Sneaking behaviour is common in bitterling and is not genetically determined (Kano 2000; Pateman-Jones 2008). Females can avoid dominant males and

may influence the outcome of contests among males by soliciting sneakers, delaying spawning, or adopting skimming behaviour (Smith and Reichard 2005; Smith et al. 2007). A review on bitterling mating system is in Smith et al. (2004b).

MATERIAL AND METHODS

Experimental *R. ocellatus* were collected from the River Yangtze Basin, China and were the first-generation bred in captivity, derived from an original stock of 200 fish imported in 2005. Prior to experiments, fish were held in stock aquaria measuring 120 (length) \times 40 (width) \times 45 (depth) cm. Stock and experimental aquaria were on a recirculating system at 23°C, exposed to 16:8 h light: dark cycle, and provided with a 20 mm layer of sand substrate. Fish were fed a mixture of commercial dried fish flake food and bloodworm (*Chironomus* spp.) twice daily and live zooplankton (*Daphnia* spp.) three times each week. Freshwater mussels used in mate choice and courtship trials were *Unio pictorum*. This mussel occurs across Eurasia and is readily used as a spawning site by *R. ocellatus* (Casalini 2007). Mussels were collected from the River Cam and kept in a 100 L tank and fed with live phytoplankton daily. At the start of experiments all fish were individually marked using colored (blue and white) elastomer tags (Northwest Marine Technology) injected under the skin on the dorsum.

EXPERIMENT 1: FEMALE MATE CHOICE

Using the isolated sequential mating design of Spence and Smith (2006), four marked males were individually assigned to four aquaria measuring 25 (length) \times 40 (width) \times 30 (depth) cm in a random order with one *U. pictorum* mussel that did not already contain bitterling eggs. This arrangement was replicated in four independent blocks. This design permits female choice to be measured in the absence of male dominance,

which conflicts with female mate choice decisions in rose bitterling (Casalini et al. 2009). Opaque barriers prevented visual contact between adjacent fish. Males were left alone overnight to establish territoriality and the following morning marked females in spawning condition (with a fully extended ovipositor) were randomly paired with each male for 1 h. A mussel-opening device (Kitamura 2005) was used to nondestructively pry the valves of mussels apart and inspect the gills for eggs that had been deposited during pairing. The number of clutches laid was estimated by dividing the total number of eggs by a mean clutch size of three (Nagata 1985; Smith et al. 2004b). Females were moved to a second aquarium within blocks with a different male according to a predetermined randomized order. This process was repeated until each female had been paired once with each of the four males in each of four independent blocks. Thus, a total of 16 males and females was used in 64 independent pairings. The same mussel was used with the same female to remove any effect of oviposition site quality on female spawning preference. Although the presence of eggs in the gills of a mussel can affect female spawning decisions (Smith et al. 2000b), the eggs already deposited by a single female were not expected to influence her subsequent spawning decisions, because a mussel can host over 250 eggs in its gills; considerably more than the 30 or so that one female can deposit in a single day (Smith et al. 2001, 2004b; Kitamura 2005, 2007). Female mate preference was measured as the number of eggs laid with individual males within blocks (Spence and Smith 2006); that is, that females showed a preference for particular males by mating more frequently with them, a standard practice for measuring mate choice (Wagner 1998; Smith and Reichard 2005).

After completion of trials the body length of every adult fish was measured from the tip of the snout to the base of tail fin and a small amount of fin tissue removed for MHC typing. The right and left eye of males and red caudal fin spot were photographed

under standard light conditions. The red area in the iris of each male eye was analyzed using Photoshop Elements 2.0 following the protocol of Barber et al. (2000). The extent and brightness of the red caudal fin spot were measured using the ordinal scale of Casalini et al. (2009).

EXPERIMENT 2: *IN VITRO* FERTILIZATIONS

To measure the relative contribution of additive and nonadditive genetic effects on offspring fitness traits, a North Carolina Type II breeding design (Lynch and Walsh 1998; Dziminski et al. 2008) using IVFs was adopted to generate a series of replicated halfsibling families. The same fish used in the female mate choice experiment (above) were used for crosses, within the same experimental blocks as for the mate choice experiment. Thus, four blocks, each with a set of 4×4 male \times female factorial crosses were used with females of known mate preferences within blocks. Within each block, each four males were crossed with four females, with a replicate of each cross. Therefore, this design generated two replicates of 16 families of maternal and paternal halfsiblings in each block, with a total of 64 replicated families in the final combined design; a total of 128 families overall.

To generate crosses, experimental females were isolated until they ovulated a batch of eggs; obvious from the female's extended ovipositor. The eggs were gently squeezed from the female and divided into approximately four equal groups in separate 70 mm-diameter Petri dishes containing freshwater (mean = 8.0 ± 3.10 SD eggs per group). Sperm was stripped from the four experimental males by gently pressing their abdomens and mixed in 9 mL of teleost saline (Yokoi et al. 2008). A 1-mL subsample of this sperm solution was diluted with a further 9 mL of saline. The concentration of sperm in each diluted suspension was quantified by performing a count of individual

spermatozoa. The sample was gently mixed and a subsample pipetted onto a haemocytometer (Neubauer improved, VWR International, Vienna, Austria). A count was made of the number of spermatozoa in the sample using a binocular microscope (Nikon Eclipse E200; Nikon, Tokyo, Japan) with a 40× objective. Counts were made of sperm cells in five $1 \times 1 \times 0.1$ mm squares to obtain an estimate of mean sperm density. Sperm suspensions were pipetted over the eggs and the covered petri dishes were left on the laboratory bench for 30 min. The fertilized eggs were washed with freshwater and incubated at 23°C in an environmentally controlled room until the yolk sac was absorbed and the larvae began exogenous feeding, a period of approximately 30 days. Each family of developing embryos was photographed alongside a scale bar under standard light conditions every day during development using a Canon EOS 300D camera with 60-mm macro lens (Canon, Tokyo, Japan). A daily record was also kept of embryo survival. Egg, embryo and larval sizes were estimated from digital images, as was the rate at which embryos reached the key stage of developing pigmented eyes (Kim and Park 1985).

EXPERIMENT 3: OFFSPRING FITNESS TRAITS

Free-swimming and exogenously feeding juveniles were retained as separate family groups and raised to maturity in aquaria measuring 35 (length) × 25 (width) × 20 (depth) cm on a recirculating system at 23°C. They were fed twice daily to satiation with a mixture of dried fish flake food and live brine shrimp (*Artemia salina*). A record was kept of the age and size at maturity of all individuals of both sexes. Sexual maturity was attained after a mean of 130 (± 20.0 SD) days and at a mean length of 27 (± 3.1 SD) mm. In females, maturity was recognized by the first extension of the ovipositor, and in males by the development of red pigmentation in the iris. In addition, male dominance and reproductive success was tested. In bitterling, female body size correlates strongly

with lifetime reproductive success (Reichard et al. 2008, 2009), whereas male lifetime reproductive success is determined by dominance and mating success (Reichard et al. 2008, 2009; Casalini et al. 2009). Only nine groups of offspring derived from the same female, but fathered by a preferred and nonpreferred male and containing at least two male offspring, survived to sexual maturity; a total of 71 individuals. To measure male dominance and reproductive success, size-matched male offspring from each of these groups were paired together once both reached sexual maturity. Distinguishing features of each male were noted to enable them to be individually identified and pairs were placed together in isolated experimental aquaria measuring 60 (length) × 40 (width) × 40 (depth) cm. Pairs were provided with a single *U. pictorum* and allowed to settle overnight. The following morning an unrelated female in spawning condition was released in the test aquarium and male behaviour (establishment of dominance, rate of aggression, rate of courtship, and rate of leading female to the mussel) (see Smith et al. 2004b for details) was recorded for 20 min. After the female completed spawning, the mussel was dissected and the eggs were incubated in a Petri dish for five days. After this time, the embryos were fixed in ethanol for parentage analysis. A total of 153 embryos were fixed for genotyping. The procedure was repeated with the same pair of males with a second female. After completion of trials, the right and left eye and caudal fin spot of males were photographed under standard light conditions. The red area in the iris of each male eye and the extent and brightness of the caudal fin spot were analyzed using the same methods for the mate choice experiment. A small portion of the tail fin of all experimental fish was removed and fixed in ethanol to assign parentage.

Parentage analysis was conducted by colleagues at the Institute of Vertebrate Biology, Brno, Czech Republic. Methods are retained here for completeness but were not conducted by the candidate. For parentage

analysis, DNA was extracted from ethanolpreserved tissue using the methods of Reichard et al. (2008). A subset of samples from adult fish was initially genotyped for 12 variable microsatellite loci developed for the closely related *Rhodeus amarus*; Rser01 – 06, Rser08 – Rser12 (Dawson et al. 2003), and Rser13 (Reichard et al. 2008; Casalini et al. 2009). Based on their informative value and compatibility, eight loci were combined in two multiplex PCR reactions (Set I – Rser03, 04, 09, 13 and Set II – Rser01, 02, 05, 12), with a mean of 11 (range: 5–17) alleles per locus. For details see Casalini et al. (2009). The length of the DNA fragments was analyzed using GeneMapper version 3.7 (Applied Biosystems, Foster City, CA) software. Observed heterozygosities enabled parental assignment by exclusion of incompatible paternal and maternal genotypes using Cervus 3.0 (Kalinowski et al. 2007). A total of 130 embryos were collected from nine experimental groups and analyzed for parentage analysis, with a mean of 15 (range: 9–24) embryos per replicate. Paternity was assigned with 95% confidence in all offspring.

EXPERIMENT 4: MHC TYPING AND ANALYSIS

MHC analysis was conducted by colleagues at the Institute of Vertebrate Biology, Brno, Czech Republic. Results are retained here for completeness but were not conducted by the candidate. Analysis was focused on MHC Class II, which is known to be associated with mate choice in many vertebrate species. In most cyprinid fish, there is at least one functional gene (named DAB) encoding the MHC class II β chain of the protein (Sambrook et al. 2005). This gene can be duplicated, resulting in the expression of DAB1 and DAB3 genes (e.g., Ottová et al. 2005). We sequenced the complete exon 2 encoding the β 1 domain, which is the most polymorphic fragment of MHC Class II molecules responsible for antigen binding. Because of the highly polymorphic nature of MHC genes there is often a problem with null alleles; sequence variants that are not amplified because of

substitutions in primer sites. To overcome this problem, for all individuals, the DAB gene was amplified using three combinations of primers located in various introns and exons. The primers were designed on the basis of homology with known DAB1 sequences downloaded from GenBank and all combinations amplified the fragment of genomic DNA that included the complete exon 2. Polymerase chain reactions (PCRs) were performed in the following conditions: 3 mM MgCl₂, 0.2 µM of each primer, 0.2 mM dNTPs, 0.5 U Taq polymerase (Fermentas) in appropriate 1 × PCR buffer and 1 µl of extracted DNA. Deionized water was added to a 10-µl reaction volume. The amplification consisted of an initial denaturation at 94°C (2min) followed by 35 cycles of denaturation at 94°C (20 s), annealing at 56°C or 59°C (30 s), and extension at 72°C (1 min), with a final extension step at 72°C (10 min). The reactions were run on a Mastercycler ep (Eppendorf, Hauppauge, NY).

All PCR products were purified by ExoSAP-IT (USB Affymetrix, Santa Clara, CA) and directly sequenced using the BigDye Terminators Sequencing Kit version 1.1 and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Homozygous sequences had no double peaks in electrophoretograms and 10 of 17 alleles (see Results) were confirmed by independent PCRs using at least two different sets of primers. Heterozygotes showed double peaks in variable sites and had to be cloned to separate individual alleles. Hence, the PCR products were purified using the MinElute PCR purification kit (Qiagen, Valencia, CA), ligated to the vector and transformed to bacteria using the Gene- JET™ PCR Cloning Kit (Fermentas, Burlington, ON, Canada) and JM109 Competent Cells (Promega, Madison, WI) according to the manufacturer's protocols. Positive transformants containing inserts of appropriate length were identified by PCR screening using primers on the vector and agarose gel electrophoresis. We randomly chose eight clones, amplified the inserts with specific primers and sequenced the PCR products as described above. The presence of PCR artifacts in cloned sequences (either substitutions or PCR recombinations; Bryja et al.

2005) was checked by comparison with the heterozygous sequence obtained directly from genomic DNA.

To avoid the possibility of analyzing pseudogenes, which can be common in MHC Class II in fish (Sambrook et al. 2005), we compared the genotypes of the DAB gene from six individuals obtained from complementary DNA (cDNA) and genomic DNA (gDNA). Total RNA was extracted from the spleen, stored in RNA later (Qiagen) by RNeasy PlusMini Kit (Qiagen) and cDNA was prepared by reverse transcription of 5 µg of total RNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers as primers (Roche, Indianapolis, IN). Subsequently, we amplified exon 2 using primer combination III, sequenced purified PCR products, and compared them with sequences obtained from genomic DNA.

The sequences of exon 2 of the DAB gene derived from *R. ocellatus* were edited and aligned in SeqScape version 2.5 (Applied Biosystems). To determine whether all sequences represented functional classical Class II alleles, the sequences were examined by eye for the presence of insertions or deletions (indels) causing a shift of the reading frame and/or stop codons. The sites encoding the peptide-binding region (PBR) of functional MHC Class II are known to be under positive selection; that is, the number of nonsynonymous substitutions exceeds the number of synonymous substitutions. The presence of selection on specific codons was analyzed separately for each allele using the random effects likelihood (REL) approach (Kosakovsky Pond and Frost 2005). This method involves fitting a distribution of substitution rates across sites with inference of the rate at which individual sites evolve. Inference of selection was made using an empirical Bayes approach. REL analysis implemented in the HyPhy software package (Kosakovsky Pond et al. 2005) and a web-based interface running on a cluster of computers at <http://www.datamonkey.org> was employed (Kosakovsky Pond and Frost 2005). As recommended by these authors, positively selected sites were considered first with a Bayes factor value of >50. Because this strict identification of positively selected sites revealed only 10 amino acid sites under

positive selection (hereafter referred to as strongly positively selected sites), we also used less-stringent conditions for identification of positively selected sites defined as a Bayes factor of >30 (referred to as positively selected sites with a total of 24 amino acid sites under selection) (see also Schwensow et al. 2008).

To calculate dissimilarity, phylogenetic distances between amino acid sequences were calculated using molecular evolutionary genetic analysis (MEGA) software, version 4.0 (Tamura et al. 2007) using a Poisson correction and different evolutionary rates with a gamma parameter of 1. We calculated three distance matrices for amino acids coded by exon 2; that is, potentially included in antigen binding: (1) based on all amino acids coded by exon 2 (92 amino acids), (2) based on positively selected sites (24 amino acids), and (3) based on strongly positively selected sites (10 amino acids).

The functional differences between alleles were further analyzed using the approach of Schwensow et al. (2007, 2008). Unlike phylogenetic analysis, functional difference between amino acids does not treat all differences in amino acids equally, but uses their functional properties (hydrophobicity, steric bulk, polarity and electronic effects). Each variable amino acid site was described by five z-descriptors (Sandberg et al. 1998) as quantitative measures of difference that are important for differences in antigen-binding motifs. A matrix of Euclidean distances between alleles was calculated for all amino acids (59 variable sites out of 92 amino acids), positively selected sites (13 of 24), and strongly positively selected sites (9 of 10).

STATISTICAL ANALYSIS

All data were tested for normality using a Kolmogorov–Smirnov test and for equality of variance using Bartlett’s test. Data that did not meet assumptions of normality and homoscedasticity were transformed. To demonstrate female mate choice the distribution of spawnings among male–female pairings was tested for significant deviation from a

Poisson distribution. Congruence in female mate choice decisions was examined using a one-way block analysis of variance (ANOVA) with a significant male effect within blocks indicating congruent female choice. A one-way block ANOVA was also used to test for an effect of order of pairing on the number of eggs spawned by females.

For IVFs, dependent variables were the proportion of eggs fertilized, proportion surviving 72 h, 1 week, 2 weeks, and 4 weeks, and to the stage at which the eyes developed pigmentation and size at 72 h, 1 week, and 2 weeks. Because the amount of yolk invested in each offspring can significantly affect offspring fitness (Wootton 1998), egg size was used as a covariate in all analyses. A Spearman correlation was used to test the relationship between sperm density, a potentially confounding variable, and fertilization success. For each 4×4 factorial block, analysis of covariance (ANCOVA) was used to compare effects of sire, dam, and their interaction on each dependent variable. Sums of squares were combined to calculate mean squares and degrees of freedom for all blocks combined in accordance with Lynch and Walsh (1998). Because dependent variables tended to correlate with each other, subsequent analyses were focused only on key parameters. A Spearman correlation was used to test the relationship between female mate preference from the mate choice experiment (measured in terms of number of eggs spawned with a male) and proportion of larvae surviving to independence at 4 weeks, as well as the relationship between male color traits (eye and tail) and egg fertilization success and larval survival to 4 weeks. A paired t-test was used to compare the survival of larvae derived from IVFs in which a female's clutch was split and the eggs fertilized with sperm from males with which the female had previously spawned in mate choice trials (preferred males) and those with which they failed to spawn (nonpreferred). Data were paired because replicate clutches were derived from a single female.

For measures of offspring fitness traits, pairwise comparison of the male offspring fathered by preferred and nonpreferred males were performed using paired t-tests, and the number of males belonging to each group that achieved dominance was compared using a chi-square test. In each case, the expectation was that male offspring of preferred males would perform better than nonpreferred males in the case of additive benefits of mate choice, or would perform equally well in the case of nonadditive benefits.

For the analysis of MHC variability on mate choice, two measures of dissimilarity between pairs were calculated for phylogenetic distances between amino acid sequences (Landry et al. 2001; Forsberg et al. 2007). The summation method calculated a sum of all pairwise distances between male and female MHC alleles (up to four individual distances in the case of two heterozygous partners sharing no alleles, thereby accounting for individual heterozygosity). The maximum distance method used only the largest distance from all pairwise comparisons (hence controlling for the level of heterozygosity). The same approach was used to calculate two measures of functional dissimilarity between partners, using a matrix of Euclidean distances from pairwise combinations. Dissimilarity values were tested between preferred and nonpreferred partners using a t-test. An association between parental MHC dissimilarity and offspring survival was tested using a Spearman correlation.

RESULTS

FEMALE MATE CHOICE

Females spawned a total of 233 eggs, distributed among an estimated 78 spawnings (assuming a mean of three eggs per spawning). At least one spawning occurred in 18 of 64 separate pairings. The distribution of spawnings among males deviated significantly

from a Poisson distribution (chi-square test, $\chi^2 = 53.2$, $df = 3$, $P < 0.001$); a significantly greater proportion of pairings than expected did not result in a spawning, with a greater than expected proportion of eggs being concentrated on a limited number of males. However, there was no significant male effect on number of eggs received by males within experimental blocks (one-way blocked ANOVA, $F_{12,48} = 1.50$, $P = 0.156$), indicating that female mate preferences were not congruent within blocks. There was no significant effect of the temporal order of pairings within a day on the number of eggs spawned by females (one-way blocked ANOVA, $F_{12,48} = 0.72$, $P = 0.726$).

IN VITRO FERTILIZATIONS

There was no significant correlation between sperm concentration and the fertilization success of IVFs (Spearman correlation, $r_{126} = -0.115$, $P = 0.195$). There was a significant effect of egg size on offspring survival and growth and this variable has been used as a covariate in analyses (Tables 3.1 and 3.2). The density of embryos during rearing was not significant for any measured offspring parameters.

There was no significant male effect on offspring survival, growth, or development (Tables 3.1 and 3.3); variance attributable to males was typically zero or negative (and hence set to zero). Maternal effects, excepting egg size, were negligible (Tables 3.1 and 3.2). However, for all measured offspring variables there was a highly significant male \times female interaction. On average, parental interaction effects explained 31% of variance in fertilization success and survival (Table 1), and 37% of growth and development effects, with the biggest impact on survival at 4 weeks (59%) when larval bitterling depart their mussel host and begin exogenous feeding.

There was a highly significant positive correlation between the survival of larvae at 4 weeks, derived from IVFs, and female mate preference measured as number

of eggs spawned with a male, from the mate choice experiment (Spearman correlation, $r_{62} = 0.501$, $P < 0.001$). Correspondingly, there was a significant difference in survival at 4 weeks of offspring derived from pairings with preferred males (males with which females spawned in mate choice trials), and those with nonpreferred males (with which females failed to spawn) (two-sample t-test, $t_{62} = 2.81$, $r_{14} = 0.007$). The extent of eye redness in males was not significantly correlated with fertilization success (Spearman correlation, $r_{14} = -0.275$, $P = 0.302$) or offspring survival after 4 weeks ($r_{14} = -0.276$, $P = 0.301$), nor was there a correlation with the extent of male tail redness: fertilization success (Spearman correlation, $r_{14} = 0.130$, $P = 0.632$), survival after 4 weeks ($r_{14} = 0.061$, $P = 0.823$).

OFFSPRING FITNESS TRAITS

There was no significant difference in the offspring of preferred and nonpreferred males in either body size at maturity (two sample t-test, $t_{29} = 0.82$, $P = 0.421$) or age of at maturity ($t_{25} = 0.56$, $P = 0.578$). In pairwise comparisons of the behavior of preferred and nonpreferred male offspring, there was no significant difference in the rate of courtship behavior (paired t-test, squareroot transformation, $t_8 = 0.50$, $P = 0.628$) or the rate that they led females to mussels (paired t-test, square-root transformation, $t_8 = 0.58$, $P = 0.581$). There was also no significant difference in the aggression rate between male offspring of preferred and nonpreferred males (paired t-test, $t_8 = 0.98$, $P = 0.355$) or of which male was dominant (chi-square test, $\chi^2 = 0.11$, $df = 1$, $P = 0.740$). Neither the extent of eye (paired t-test, $t_8 = 0.12$, $P = 0.906$) or tail redness (paired t-test: $t_8 = 0.35$, $P = 0.738$) differed between the offspring of preferred and nonpreferred males. Finally, male offspring from preferred matings did not sire a higher proportion of embryos than

males from nonpreferred matings in paired competitive trials (paired t-test: $t_8 = 0.19$, $P = 0.858$).

MHC TYPING

MHC analysis was conducted by colleagues at the Institute of Vertebrate Biology, Brno, Czech Republic. Results are retained here for completeness but were not conducted by the candidate.

By cloning and sequencing the complete exon 2 (276 bp), 17 MHC sequence variants were obtained that could be translated into 17 different MHC class II β chains of the protein. Using a neighbor-joining phylogenetic analysis, all bitterling sequences were shown to cluster with DAB1 sequences of other cyprinid fish (Ottová et al. 2005) and hence were designated as alleles Rhoc- DAB1* 01–17 in accordance with recommended nomenclature (Klein et al. 1990). They were submitted to GenBank under the accession numbers GU080071-87.

A maximum of two variants per one individual were obtained with the primer combinations used, indicating only one copy of DAB1 (i.e., no duplication) in the rose bitterling. From a total of 32 individuals, 15 were homozygous, 15 were heterozygous, and in two individuals no DAB1 was amplified. A Hardy–Weinberg probability test (GENEPOP 4.0, Rousset 2008) indicated strong deviation from Hardy–Weinberg Equilibrium ($P < 0.001$), which can indicate null alleles. However, given that all homozygote individuals were reamplified by all three independent combinations of primers and reamplification confirmed their homozygosity, the presence of null alleles was unlikely. More plausibly the DAB1 gene is not present on all chromosomes in our study population of the rose bitterling with two experimental parents homozygous for DAB1 deletion.

No sequence contained insertions, deletions, or stop codons, suggesting that all MHC alleles were functional. Sequencing of cDNA from six individuals revealed five

alleles that are transcribed to mRNA and in all cases the genotype identified from cDNA was identical to that from gDNA, indicating that analyzed loci were fully functional.

MHC DISSIMILARITY AND MATE CHOICE

Females preferred mating with more dissimilar males; that is, with partners whose MHC composition resulted in a higher number of MHC alleles in the offspring, for positively selected sites (two-sample t-test, $t_{62} = 2.00$, $P = 0.0496$) (Table 3.3). Female preferences approached significance for strongly positively selected sites ($P = 0.057$) and effect sizes were large-medium for both strongly positively selected and positively selected sites ($r_{ES} = 0.262$ and 0.270 respectively) (Table 3.3). Functional distances in amino acids explained the pattern of mate choice marginally better (mean $r_{ES} = 0.227$) than phylogenetic distances (mean $r_{ES} = 0.217$). A summation method (mean $r_{ES} = 0.242$) explained the mate choice pattern better than the maximum distance method (mean $r_{ES} = 0.201$) (Table 3.3). The use of positively selected sites and strongly positively selected sites in the analysis consistently explained more variability than the complete set of amino acid sequences (Table 3.3).

MHC DISSIMILARITY AND EMBRYO SURVIVAL

Embryo survival was higher for matings between more MHC dissimilar partners (Table 3.4). The summation method; that is, inclusion of the effect of individual heterozygosity, explained the mate choice pattern better than the maximum distance method (Table 3.4), and functional dissimilarity at positively selected sites and strongly positively selected sites was the best descriptor of embryo survival among the measures used (Table 3.4). The effect of MHC dissimilarity on embryo survival was within the

range of the effect on mate choice decisions (r between 0.22 and 0.26 for embryo survival, r_{ES} between 0.20 and 0.27 for mate choice using the summation method).

DISCUSSION

The goal of this study was to understand the adaptive basis of female mate choice in the rose bitterling, a fish with a resource based mating system. In mate choice trials females showed preferences for males, though these were not congruent; preferences for males varied among females. In a further experiment, using a North Carolina Type II breeding design and IVFs, significant differences in offspring growth and survival among families were detected that depended on specific combinations of parental genotypes, indicating significant nonadditive genetic effects. Notably there were no significant additive paternal or maternal (excluding egg size) effects on offspring fitness and relatively minor unexplained environmental effects (error variance).

The performance of surviving offspring (distinct from fitness), measured in males as reproductive success in mating contests and in females on the basis of size and age at maturity, did not differ between those sired by preferred and nonpreferred males. This result indicates that although the survival rates of offspring resulting from pairings of females with preferred and non-preferred males was greater for preferred males, the performance of surviving offspring was equivalent.

Notably, female mate preferences and offspring survival were correlated; females chose males that conferred a nonadditive fitness benefit through enhanced offspring development and survival to independence. Further, both female mate preference and offspring survival to independence were correlated with MHC dissimilarity between paired males and females. Females showed a significantly greater likelihood of choosing MHC dissimilar males (based on functional distances between

positively selected amino acids) as mates and the offspring of dissimilar matings had significantly higher survival rates. Mate choice based on MHC dissimilarity is, again, indicative of a nonadditive genetic basis to the rose bitterling mating system. Thus, overall this study shows: (1) Female mate choice is adaptive through an indirect nonadditive genetic fitness benefit. (2) Female mate preferences and offspring survival correlate with the degree of MHC dissimilarity of a mating pair.

Selection for genetic compatibility requires an individual to reference its own genotype, or at least components of its genotype, as well as those of potential mates. As such the genetic compatibility paradigm raises some conceptual difficulties as to how it operates. The functional basis to compatibility is also less than transparent, but may be related to advantages associated with heterozygosity, for example through dominance or overdominance (Tregenza and Wedell 2000). For genetic compatibility to be able to function as the basis to a mate choice system, it is likely to be limited to specific genetic systems, because complex interactions of male and female genotypes across many genes would place severe constraints on any such system (Puurtilinen et al. 2005). One such genetic system comprises the genes of the MHC (Jordan and Bruford 1998; Tregenza and Wedell 2000), for which there is growing evidence of a role in mate choice (Landry et al. 2001; Arkush et al. 2002; Richardson et al. 2005; Bonneaud et al. 2006; Forsberg et al. 2007; Consuegra and Garcia de Leaniz 2008; Yeates et al. 2009). The results of the present study lend support to the hypothesis that mate choice will tend to maximize MHC diversity in the offspring by selection of MHC dissimilar mates, representing a nonadditive benefit of mate choice.

One hypothesis that has gained ground recently is the proposal that some intermediate, rather than maximal, level of MHC sequence variability will be optimal for offspring viability (Reusch et al. 2001; Milinski et al. 2005; Forsberg et al. 2007;

Kalbe et al. 2009). This idea has not been tested in the present study because the number of alleles per individual was relatively low in rose bitterling; an intermediate level of MHC sequence variation may only be applicable in species with duplicated MHC genes. An advantage of intermediate MHC dissimilarity may be important in mating between animals from different populations or races in which outbreeding depression can result from genetic incompatibilities, possibly the result of disruption to co-adapted gene complexes through epistasis, a state recognized in a range of taxa (Turelli and Orr 2000; Barton 2001; Jiggins et al. 2001), including humans (Getahun et al. 2005; Rosenberg et al. 2005; Nystrom et al. 2008).

The findings of this study provoke further questions, most notably the problem of how females perceive MHC dissimilarity in prospective mates. In many taxa, olfactory cues appear to play a key role in mate choice (Eggert et al. 1999; Penn 2002; Piertney and Oliver 2006). Genes of the MHC may influence odor through production of proteins that may be water soluble or linked to volatile compounds, or by affecting an individual's gut flora (Tregenza and Wedell 2000). Olfactory cues linked to MHC have been demonstrated to play a role in mate choice (Milinski et al. 2005), dominance (Almeida et al. 2005), and kin recognition (Gerlach et al. 2008) in fish, and this may also be the case in the rose bitterling (Casalini et al. 2009; M. Agbali, unpubl. data). The origin of MHC-specific odors in bitterling has yet to be established, although it has been proposed that cues may be present in urine (Casalini et al. 2009) or the products of ejaculation (Y. Kanoh, unpubl. data). There is also a potential role of male courtship in disseminating odor; courtship behavior involves the male swimming in front of the female and undulating his body and fins, which may assist in broadcasting potential chemical cues. Significantly, female mate choice correlates with male courtship in

laboratory and field studies (Smith et al. 2002; Reichard et al. 2005; Casalini et al. 2009).

No evidence was found for a role of male coloration in female mate choice, or as a signal of male quality as a mate. This finding mirrors several previous studies that have shown the same in both *R. amarus* and *R. ocellatus* (e.g., Smith et al. 2002; Reichard et al. 2005; Casalini et al. 2009). One study, that of Candolin and Reynolds (2001), did record a significant effect of male fin color on female mate choice in *R. amarus*, although this finding has never been repeated. The consistent failure to reliably demonstrate a role for male color in intersexual selection suggests it may play either a trivial role or may have a primary function in intrasexual competition (Reichard et al. 2005; Casalini et al. 2009). Alternatively, the relatively crude measures of male coloration used in the present study and in others (Candolin and Reynolds 2001; Smith et al. 2002; Reichard et al. 2005; Casalini et al. 2009) may be too imprecise to identify color differences among males that influence female mating preferences.

Although the present study shows a role for female mate choice in the mating system of the rose bitterling, two other key features need to be considered; the role of intrasexual competition and spawning site quality in female mating decisions. In the case of the former, there appears to be an intersexual conflict between male aggressive dominance of spawning sites and female mate choice; females do not always prefer dominant males, but dominant males have higher reproductive success than subordinate males (Casalini et al. 2009). Females may be able to circumvent male dominance to some degree by increasing the mating success of subordinates by delaying oviposition and thereby increasing their opportunity to sneak fertilizations (Smith and Reichard 2005; Smith et al. 2007). In respect to the relative significance of mate choice and oviposition choice to female mating decisions, while both appear to play a role in the

bitterling mating system, to compare their relative importance it will be necessary to contrast the benefits of each using a common currency related to female reproductive output (reviewed by Kotiaho and Puurtinen 2007). Thus, although female mate choice appears to play a role in the mating system of the rose bitterling, this result still needs to be placed in the context of the other selective forces that contribute to female reproductive success.

A caveat to the results of the present study is the relative weakness of the effect of MHC dissimilarity on mate choice and, to a lesser degree, with offspring survival; no results for MHC dissimilarity were strikingly strong (Tables 3.3 and 3.4). Thus, although some studies (this one included) appear to lend credence to the idea that MHC genes may play a role in mate choice, with direct functional significance for offspring fitness, few are entirely convincing. A weakness of these studies is that there is no standard practice for the analysis of MHC data, with the result that multiple variables may be tested by experimenters, though not all may be reported in some studies. We have fully reported all the tests we performed on our own data; the procedures we used were selected on the basis of previous studies of MHC-based mate choice. Note that we chose procedures based on theoretical reasoning and previous evidence from other mating systems, but more measures of MHC dissimilarity can be found in other studies.

An explanation for the relatively weak results for MHC dissimilarity might be because sequencing of rose bitterling MHC Class II genotypes was not complete; data for DAB3 were missing, though it is probably present in *R. ocellatus* because the gene has been recorded for the related European bitterling, *R. amarus* (M. Vyskocilova, unpubl. data). More complete data including DAB3 may support a stronger link between genetic distance, mate preference, and offspring growth and survival, although the converse may also be the case. It was notable that DAB1 genes were not detected

for some individuals, although this is not unusual with MHC genes, for example there is considerable variation in the number of expressed DAB genes in the common carp, *Cyprinus carpio* (van Erp et al. 1996). Those individuals with missing DAB1 genes may have expressed DAB3 genes, and a more comprehensive study of MHC Class II structure in the bitterling would be likely to increase the variability explained.

The weak relationship between MHC dissimilarity and mate choice may be related to the accuracy and precision with which females are able to discriminate the MHC dissimilarity of males, which may be limited. Females may also use additional cues to measure male compatibility or to make mate choice decisions. Rose bitterling have a significant visual bias for red (M. Agbali unpubl. data). Male bitterling display striking red nuptial coloration, and like in other fish, a receiver bias may play a role in the mating system of this species (Rodd et al. 2002; Smith et al. 2004a). Further research will help to clarify the functional and mechanistic basis of mate choice for indirect genetic benefits in bitterling.

In conclusion, it was demonstrated that female rose bitterling show significant mate preferences, but are not congruent in their preferences. A significant interaction of male and female genotype on offspring fitness traits was shown. There was no evidence for male additive effects. Females preferred mating with genetically compatible males, and both female mate preference and offspring survival correlated with MHC dissimilarity. It is proposed that genetic compatibility is the mechanism by which females obtain a fitness benefit through mate choice and that male MHC dissimilarity indicates genetic compatibility, probably detected through odor cues.

Table 3.1. ANCOVAs for fertilization success and offspring performance variables for in vitro fertilizations. Mean and standard deviation of variables are in parentheses.

| Source | df | SS | MS | <i>F</i> | <i>P</i> | Variance | Variance (%) |
|--------------------------------------------------------------|----|---------|-------|----------|----------|----------|--------------|
| Proportion of eggs fertilised(0.98±0.059) | | | | | | | |
| Egg size | 1 | 0.880 | 0.880 | 109.10 | <0.001 | 0.44 | 91.0 |
| Male (M) | 4 | 0.069 | 0.017 | 0.223 | 0.912 | 0 | 0 |
| Female (F) | 4 | 0.557 | 0.142 | 1.820 | 0.288 | 0 | 0.4 |
| M X F | 4 | 0.311 | 0.078 | 9.641 | <0.001 | 0.03 | 7.3 |
| Error | 64 | 0.516 | 0.008 | 8.162 | 0.033 | 0.01 | 1.7 |
| Proportion of offspring surviving after 72 h (0.90±0.208) | | | | | | | |
| Egg size | 1 | 849.0 | 849.0 | 20.2 | <0.001 | 403.45 | 67.1 |
| Male (M) | 4 | 264.1 | 66.0 | 0.2 | 0.937 | 0 | 0 |
| Female (F) | 4 | 1943.1 | 485.8 | 1.3 | 0.393 | 3.80 | 0.6 |
| M X F | 4 | 1456.2 | 364.1 | 8.6 | <0.001 | 160.98 | 26.8 |
| Error | 64 | 2694.0 | 42.1 | 4.7 | 0.040 | 42.09 | 7.0 |
| Proportion of offspring surviving after 1 week (0.88±0.211) | | | | | | | |
| Egg size | 1 | 913.2 | 913.2 | 19.2 | <0.001 | 432.82 | 66.9 |
| Male (M) | 4 | 293.8 | 73.5 | 0.1 | 0.954 | 0 | 0 |
| Female (F) | 4 | 2539.6 | 634.9 | 1.3 | 0.407 | 4.41 | 0.7 |
| M X F | 4 | 1975.3 | 493.8 | 10.4 | <0.001 | 223.14 | 34.5 |
| Error | 64 | 3042.1 | 47.5 | 8.6 | 0.030 | 47.53 | 7.3 |
| Proportion of offspring surviving after 2 weeks (0.82±0.266) | | | | | | | |
| Egg size | 1 | 13291.1 | 13291 | 14.07 | <0.001 | 6173.3 | 64.2 |
| Male (M) | 4 | 6894.3 | 1724 | 0.30 | 0.865 | 0 | 0 |
| Female (F) | 4 | 50853.9 | 12713 | 2.21 | 0.231 | 217.3 | 2.3 |
| M X F | 4 | 23035.1 | 5759 | 6.10 | <0.001 | 2407.2 | 25.0 |
| Error | 64 | 60443.8 | 944 | 7.38 | 0.039 | 944.4 | 9.8 |

Proportion of offspring
surviving after 4 weeks
(0.42±0.369)

| | | | | | | | |
|------------|----|---------|------|-------|--------|--------|------|
| Egg size | 1 | 4679.1 | 4679 | 5.70 | 0.020 | 1929.0 | 28.5 |
| Male (M) | 4 | 19710.2 | 4928 | 0.56 | 0.708 | 0 | 0 |
| Female (F) | 4 | 32808.4 | 8202 | 0.93 | 0.529 | 0 | 0 |
| M X F | 4 | 35406.7 | 8852 | 10.78 | <0.001 | 4015.4 | 59.4 |
| Error | 64 | 52543 | 821 | 1.66 | 0.317 | 820.98 | 12.1 |

Table 3.2. ANCOVAs for offspring growth and development variables for in vitro fertilizations. Mean and standard deviation of variables are in parentheses.

| Source | df | SS | MS | <i>F</i> | <i>P</i> | Variance | Variance (%) |
|-------------------------------------------------------------------------|----|---------|---------|----------|----------|----------|--------------|
| Mean body size (mm) after 72 h (7.56±1.905) | | | | | | | |
| Egg size | 1 | 819692 | 819692 | 26.3 | <0.001 | 394243.9 | 71.2 |
| Male (M) | 4 | 160880 | 40220 | 0.1 | 0.964 | 0 | 0 |
| Female (F) | 4 | 901155 | 225289 | 0.7 | 0.618 | 0 | 0 |
| M X F | 4 | 1240806 | 310202 | 9.9 | <0.001 | 139498.6 | 25.2 |
| Error | 64 | 1997070 | 31204 | 5.6 | 0.062 | 31204.2 | 5.6 |
| Mean body size (mm) after 1 week (10.8±2.70) | | | | | | | |
| Egg size | 1 | 12857.7 | 12857.7 | 15.7 | <0.001 | 6018.4 | 56.4 |
| Male (M) | 4 | 6220.9 | 1555.2 | 0.2 | 0.937 | 0 | 0 |
| Female (F) | 4 | 53282.3 | 13320.6 | 1.5 | 0.342 | 146.7 | 1.4 |
| M X F | 4 | 34503.8 | 8625.9 | 10.5 | <0.001 | 3902.5 | 36.6 |
| Error | 64 | 52537.3 | 820.9 | 8.6 | 0.031 | 820.9 | 7.7 |
| Mean body size (mm) after 2 weeks (11.3±3.76) | | | | | | | |
| Egg size | 1 | 11998.8 | 11998.8 | 11.8 | 0.001 | 5490.7 | 58.8 |
| Male (M) | 4 | 8247.1 | 2061.8 | 0.3 | 0.856 | 0 | 0 |
| Female (F) | 4 | 52160.5 | 13040.1 | 1.9 | 0.261 | 202.5 | 2.2 |
| M X F | 4 | 26243 | 6560.8 | 6.4 | <0.001 | 2771.7 | 29.7 |
| Error | 64 | 65110.1 | 1017.3 | 6.3 | 0.051 | 1017.4 | 10.9 |
| Proportion of offspring with pigmented eyes at 144 h (0.57±0.381) | | | | | | | |
| Egg size | 1 | 12830.7 | 12831 | 17.8 | <0.001 | 6055.1 | 67.7 |
| Male (M) | 4 | 9949.2 | 2487 | 0.5 | 0.721 | 0 | 0 |
| Female (F) | 4 | 44439.6 | 11110 | 2.4 | 0.211 | 201.5 | 2.3 |
| M X F | 4 | 18653 | 4663 | 6.5 | <0.001 | 1971.4 | 22.0 |
| Error | 64 | 46110.5 | 720 | 4.5 | 0.088 | 720.5 | 8.1 |

Table 3.3. Dissimilarity of amino acids coded by exon 2 of MHC DAB1 between preferred and nonpreferred partners in *R. ocellatus*, measured as phylogenetic and functional distances between amino acids. Distance matrices were derived for strongly positively selected sites (Bayes factor value >50; 10 amino acids), positively selected sites (Bayes factor value >30; 24 amino acids), and all amino acids (92 amino acids). Estimates of effect size (r_{ES}) are given in addition to conventional statistical results. Values of $r_{ES} > 0.371$, 0.243, and 0.100 are considered to represent large, medium, and small effects, respectively.

| | <i>t</i> -value | <i>p</i> | <i>r</i> _{ES} | Preferred | | | Non-preferred | | |
|-------------------------------------------|-----------------|--------------|------------------------|-----------|------|----|---------------|------|----|
| | | | | mean | SD | n | mean | SD | n |
| Functional distances | | | | | | | | | |
| <u>Strongly positively selected sites</u> | | | | | | | | | |
| sum of distances | 1.94 | 0.057 | 0.262 | 37.5 | 20.2 | 18 | 26.4 | 20.8 | 46 |
| maximum distances | 1.33 | 0.188 | 0.197 | 15.6 | 4.3 | 18 | 13.4 | 6.6 | 46 |
| <u>Positively selected sites</u> | | | | | | | | | |
| sum of distances | 2.00 | 0.049 | 0.270 | 35.8 | 19.2 | 18 | 24.8 | 19.9 | 46 |
| maximum distances | 1.44 | 0.155 | 0.212 | 14.9 | 4.2 | 18 | 12.6 | 6.3 | 46 |
| <u>All amino acid sites</u> | | | | | | | | | |
| sum of distances | 1.77 | 0.082 | 0.242 | 50.8 | 26.9 | 18 | 37.0 | 28.6 | 46 |
| maximum distances | 1.20 | 0.234 | 0.178 | 21.8 | 6.3 | 18 | 18.9 | 9.5 | 46 |
| Phylogenetic distances | | | | | | | | | |
| <u>Strongly positively selected sites</u> | | | | | | | | | |
| sum of distances | 1.80 | 0.076 | 0.244 | 2.60 | 2.62 | 18 | 2.60 | 2.61 | 46 |

| | | | | | | | | | |
|-------------------|------|-------|-------|------|------|----|------|------|----|
| maximum distances | 1.82 | 0.074 | 0.243 | 2.13 | 1.27 | 18 | 1.51 | 1.22 | 46 |
|-------------------|------|-------|-------|------|------|----|------|------|----|

Positively selected sites

| | | | | | | | | | |
|------------------|------|-------|-------|------|------|----|------|------|----|
| sum of distances | 1.74 | 0.087 | 0.235 | 0.86 | 0.51 | 18 | 0.61 | 0.51 | 46 |
|------------------|------|-------|-------|------|------|----|------|------|----|

| | | | | | | | | | |
|-------------------|------|-------|-------|------|------|----|------|------|----|
| maximum distances | 1.54 | 0.129 | 0.221 | 0.39 | 0.13 | 18 | 0.32 | 0.17 | 46 |
|-------------------|------|-------|-------|------|------|----|------|------|----|

All amino acid sites

| | | | | | | | | | |
|------------------|------|-------|-------|------|------|----|------|------|----|
| sum of distances | 1.43 | 0.158 | 0.201 | 0.59 | 0.31 | 18 | 0.45 | 0.37 | 46 |
|------------------|------|-------|-------|------|------|----|------|------|----|

| | | | | | | | | | |
|-------------------|------|-------|-------|------|------|----|------|------|----|
| maximum distances | 1.06 | 0.295 | 0.156 | 0.28 | 0.10 | 18 | 0.24 | 0.14 | 46 |
|-------------------|------|-------|-------|------|------|----|------|------|----|

Table 3.4. Spearman correlation between embryo survival and MHC dissimilarity in *R. ocellatus*, measured as phylogenetic and functional distances between amino acids coded by exon 2 of MHC DAB1. Distance matrices were derived for strongly positively selected sites (Bayes factor value>50; 10 amino acids), positively selected sites (Bayes factor value>30; 24 amino acids), and all amino acids (92 amino acids). Significant results are in bold.

| | Phylogenetic distances | | Functional distances | |
|-------------------------------------------|------------------------|--------------|----------------------|--------------|
| | r_{62} | p | r_{62} | p |
| <u>strongly positively selected sites</u> | | | | |
| sum of distances | 0.252 | 0.044 | 0.253 | 0.044 |
| maximum distance | 0.250 | 0.046 | 0.143 | 0.261 |
| <u>positively selected sites</u> | | | | |
| sum of distances | 0.238 | 0.058 | 0.256 | 0.041 |
| maximum distance | 0.227 | 0.072 | 0.155 | 0.221 |
| <u>all amino acid sites</u> | | | | |
| sum of distances | 0.216 | 0.087 | 0.232 | 0.065 |
| maximum distance | 0.134 | 0.292 | 0.087 | 0.492 |

Chapter four: Direct and indirect benefits of mate choice in the rose bitterling (*Rhodeus ocellatus*)

ABSTRACT

Research on sexual selection has focused on the role of indirect benefits of female mate choice to the exclusion of direct benefits. Few studies have attempted to examine the relative role of direct and indirect mate choice benefits on sexual selection; to do so it is necessary to compare the benefits of each using a common currency related to female reproductive output. Here we compared direct and indirect benefits in the mating system of the rose bitterling (*Rhodeus ocellatus*), a fish with a resource-based mating system, using experimental treatments with identical consequences for female reproductive success. The response of female bitterling to potential direct benefits of oviposition choice, and indirect benefits of mate choice were measured in terms of offspring survival rates to independence, with female mating preferences measured in terms of reproductive investment. Direct benefits were varied using high and low quality sites for oviposition, and male quality using males of known genetic compatibility. The results show a significant effect for direct benefits, in the form of oviposition site quality, in mate choice decisions in the rose bitterling. Indirect benefits also played a role, but accounted for a relatively minor component of variance in female oviposition rate. The significance of oviposition decisions on fitness is discussed.

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INTRODUCTION

Sexual selection is a powerful mechanism of adaptive evolution. It arises through variance in reproductive success, either as a result of competition between members of the same sex, generally males, and termed intra-sexual selection, or as a consequence of female mate choice, termed inter-sexual selection. In some species mate choice decisions are associated with direct benefits to the female; females may obtain nutrients or items of food, assistance with parental care, access to breeding sites, and protection from harassment or predation. Alternatively, and more controversially, females may acquire indirect benefits from mate choice. In this case females accrue a benefit from selecting mates through producing offspring of enhanced heritable viability (reviewed by Andersson 1994).

Much recent research on sexual selection has focused on the role of indirect benefits of female mate choice, as well as on alternative mechanisms of sexual selection, such as receiver bias (Ryan 1998; Smith et al. 2004a; Fuller et al. 2005). The significance of direct benefits in animal mating systems has, consequently, been somewhat overlooked (Kotiaho and Puurtinen 2007). However, while they have attracted less attention, the effects of direct benefits on mating decisions are no less significant as a mechanism of intersexual selection. Indeed, despite a wealth of empirical studies seeking to demonstrate the significance of indirect benefits, it is striking that there is so little compelling evidence for a role of indirect mate choice benefits in sexual selection (Kotiaho and Puurtinen 2007).

Few studies have attempted to examine the relative role of direct and indirect mate choice benefits on sexual selection. To do so it is necessary to compare the benefits of each using a common currency related to female reproductive output (Kotiaho 2001; Kotiaho and Puurtinen 2007). Here we compare direct and indirect

benefits in the mating system of the rose bitterling (*Rhodeus ocellatus*), a fish with a resource-based mating system, using experimental treatments with identical consequences for female reproductive success. The aim of the present study was to compare the relative role of direct and indirect benefits on female mating decisions. The response of female bitterling to potential direct benefits of oviposition choice, and indirect benefits of mate choice were measured in terms of offspring survival rates to independence, with female mating preferences measured in terms of reproductive investment.

Bitterling are freshwater cyprinid fishes, belonging to the sub-family Acheilognathinae, which use living fresh water mussels for oviposition (Wiepkema 1961; Smith et al. 2004b). Males compete for territories around unionid mussels and lead females to spawn (Kano 2000; Casalini et al. 2009). Female bitterling have long ovipositors that they use to place their eggs onto the gills of a mussel through the exhalant siphon of the mussel (Wiepkema 1961; Shirai 1962; Smith et al. 2004b). Female *R. ocellatus* place one to five eggs in the gills chamber of the mussel in one spawning act and can spawn up to five clutches each day (Nagata 1985). Males release sperm over the inhalant siphon of the mussel to fertilise the eggs (Wiepkema 1961; Kano 1996; Smith et al. 2004b). Fertilised eggs develop inside the gill of the mussel, which provides protection for the embryos from predators (Nagata 1985). Embryos emerge from mussels as independently swimming larvae after approximately one month (Kim and Park 1985; Aldridge 1999; Smith et al. 2004b).

Females make mating decisions relating to mussel quality and male genetic compatibility, which represent direct and indirect benefits of mating respectively. Females prefer certain mussel species, and those that do not already host large numbers of bitterling eggs and embryos (Smith et al. 2000b, 2001; Reichard et al. 2007b). These

oviposition decisions are adaptive; egg and embryo mortality rates in mussels are strongly density dependent and the strength of density dependence varies among mussel species (Smith et al. 2000b, 2001; Reichard et al. 2004a, 2005). The oxygen conditions inside the mussel gill is the probable determinant of bitterling embryo mortality, with developing embryos dying over the first 7 days of development by asphyxiation (Smith et al. 2001; Kitamura 2005), and dissolved oxygen the most likely cue used by females to assess mussel quality (Smith et al. 2001; Reichard et al. 2007b). Mate choice decisions are based on olfactory cues, which identifies male genetic compatibility, with significant differences in embryo survival rates between preferred and non-preferred males (Smith et al. 2002; Reichard et al. 2005; Smith and Reichard 2005; Casalini 2007; Casalini et al. 2009; Agbali et al. 2010).

MATERIAL AND METHODS

QUANTIFYING DIRECT AND INDIRECT BENEFITS OF FEMALE MATING DECISIONS

Direct and indirect benefits of female mating decisions were each varied at two levels; high and low. Direct benefits of mating decisions were controlled by altering the number of eggs in mussels available to females for oviposition, from zero (high quality) to between 80-100 eggs (low quality). Differences in the number of eggs in mussels affects egg mortality during incubation and, therefore, on the quality of mussels as sites for oviposition. The mortality rate of embryos in mussels was estimated from Smith et al. (2000b), who derived a function for the mortality rate of European bitterling (*R. amarus*) eggs in four species of mussel. For *Unio pictorum*, the mussel species used in the present study, the mortality of eggs during incubation for a single clutch of eggs was 29%, for one containing 80-100 eggs the estimate was 76%. These estimates were

supported by a field study on *R. ocellatus* that showed a mortality rate of eggs during incubation varying between 25% at the start of the spawning season, when mussels contained few eggs (typically a single clutch), to approximately 70% later in the year, when egg numbers in mussels reached levels of up to 60 eggs per mussel (Kitamura 2005; Kitamura personal communication).

Indirect benefits were controlled by presenting females with males of known genetic compatibility and for which female preferences had previously been measured. These data were obtained in a study (Agbali et al. 2010) in which female mate preferences were measured in a mate choice test combined with a cross-classified breeding design that used *in vitro* fertilisations to test for additive and non-additive benefits of mate choice. The results, none of which are repeated here, showed a significant interaction of male and female haplotype on offspring survival during the period of incubation in mussels, with females demonstrating significant mate preferences corresponding with male genetic compatibility (Agbali et al. 2010). The mean mortality of offspring to independence fathered by preferred males was 28%, and for non-preferred was 74%.

In the present study we used the same group of fish tested by (Agbali et al. 2010) to examine female responses to direct and indirect benefits of mating decisions. Thus, high-quality direct benefits (high mussel quality) conferred an offspring mortality rate of 29% on females and a low-quality direct effect (low mussel quality) of 76%, while high-quality indirect benefits (male genetic compatibility) conferred on females an offspring mortality rate of 28%, and low-quality indirect benefits (male genetic incompatibility) a rate of 74%. There was no significant difference in estimated offspring mortality rates between high-quality direct and indirect benefits ($t_{10} = 0.52$, $p = 0.616$) or low-quality direct and indirect benefits ($t_{10} = 0.22$, $p = 0.834$). In contrast,

there were highly significant differences between estimated low and high-quality ($t_{10} = 6.39$, $p < 0.001$) and high and low-quality ($t_{10} = 8.11$, $p < 0.001$) direct and indirect benefits respectively. Consequently, the benefits of female mating decisions for direct and indirect benefits could be compared unambiguously using a common currency of offspring survival to independence.

EXPERIMENTAL PROTOCOL

Experimental fish (*R. ocellatus*) were the first generation of stock originally from the River Yangtze Basin, China. Stock and experimental aquaria were maintained on a recirculating system at 23 °C and exposed to 16:8 h light/dark regime. Fish were fed twice daily a mixture of commercial dried fish flake food and bloodworm (*Tubifex spp.*) supplemented with live zooplankton three times each week.

Freshwater mussels used in trials were *U. pictorum*. This mussel occurs across Eurasia, overlapping in its distribution with *R. ocellatus*, which readily uses it as a spawning site (Casalini 2007). Mussels were collected from the River Cam and kept in a 100 L stock tank on the roof of the Department of Biology and fed every day live phytoplankton. Experimental mussels were haphazardly selected from the stock tank and randomly assigned to either low or high quality treatment groups. Those assigned to the low-quality group were exposed to groups of approximately 40 fish that were allowed to use the mussels for spawning until they had accumulated between 80-100 eggs. To confirm egg numbers a mussel-opening device (Kitamura 2005) was used to gently prise open the valves of the mussel and the eggs counted. Those assigned to the high quality mussel group received no spawnings, but were housed in aquaria with identical water quality.

FEMALE MATING DECISIONS

Female mating decisions were tested using an isolated sequential mating design with males of known genetic compatibility. Two males were assigned to each female; one genetically compatible the other genetically incompatible (Agbali et al. 2010). Males were housed singly in aquaria measuring 25 (length) x 40 (width) x 30 (depth) cm with a sand substrate and two artificial plants. Opaque barriers prevented visual contact between adjacent fish. High and low quality mussels were randomly assigned to the two males which were able to inspect the mussel and establish territoriality around it. The female was paired with each male in random order for one hour and allowed to spawn. A mussel-opening device was used to gently open the mussel and any eggs that had been spawned were counted. Recently deposited eggs could readily be identified by their stage of development in mussels already containing developing eggs, which were at least 24 h older. Mussels were swapped between males and the mate choice trial repeated so that each female was paired with a compatible and non-compatible male with a high and low-quality mussel. After completion of a trial mussel maximum length was measured and fertilised bitterling eggs allowed to complete development. A total of 11 independent replicates were completed.

STATISTICAL ANALYSIS

The normality of data were examined using a Shapiro-Wilk test and equality of variance using Bartlett's test. Data that did not meet assumptions of normality or homoscedasticity were transformed. The effect of direct and indirect benefits, and their interaction, on the number of eggs spawned by females was analysed using a balanced two-way ANOVA. The proportion of variance explained by each variable was

compared by calculating eta squared (η^2). A paired t-test was used to compare mussel lengths between mussel treatment groups.

RESULTS

There was a significant effect of direct benefits (mussel quality) on female mating decisions (ANOVA, square-root transformation: $F_{1,40} = 11.05$, $P = 0.002$, $\eta^2 = 0.21$); females spawned significantly more eggs in high quality mussels (Fig. 4.1). In addition, there was a significant effect of indirect benefits ($F_{1,40} = 4.33$, $P = 0.044$, $\eta^2 = 0.07$), with females spawning more eggs with genetically compatible than incompatible males (Fig. 4.1). There was no interaction between direct and indirect benefits ($F_{1,40} = 0.700$, $P = 0.407$, $\eta^2 = 0.01$; Fig. 4.1). There was no significant difference in mussel size between high and low quality treatments (paired t-test $t_{10} = 1.40$, $P = 0.192$).

DISCUSSION

The aim of the present study was to examine the effects of direct and indirect benefits on female mating decisions in the rose bitterling, *R. ocellatus*, a fish with a resource-based mating system. The study was designed such that high quality and low quality direct and indirect benefits yielded comparable fitness gains to females, measured in terms of offspring survival to independence. The results showed a stronger effect of direct than indirect benefits on female mating decisions; direct benefits explained approximately three times more variance in oviposition rates than indirect benefits, though both variables were significant. These findings support the argument of Kotiaho and Puurtinen (2007) that direct benefits may be more important for sexual selection than the current preoccupation with indirect benefits might suggest.

For oviparous species, oviposition decisions have important consequences for offspring fitness. Indeed, for species without parental care oviposition decisions are one of the principal means by which a female can influence offspring survival, and these decisions represent important maternal effects (Roitberg 1998). Sites for depositing eggs can vary in quality, which might affect their suitability for developing embryos. In some species a female may also need to decide whether to deposit all her eggs in one site or distribute them among several. The fitness consequences of oviposition decisions have been studied for invertebrates (Chew 1977; Jaenike 1978; Smith and Lessells 1985; Godfray 1994; Cope and Fox 2003), but are less well understood in vertebrates. A series of studies with bitterling fishes has shown that they are able to discriminate the quality of spawning sites precisely. Female bitterling avoid spawning eggs in mussels already containing developing embryos and also certain species of mussel (Smith et al. 2000b). These choices are adaptive; embryo mortality in mussels is density dependent and the strength of density dependence varies among host mussel species (Smith et al. 2000b, 2001).

Although the experimental design used here enabled a direct comparison between direct and indirect benefits of mate choice by controlling for offspring mortality, other more subtle aspects of offspring fitness may also play a role in female mating decisions through alterations to development. Conditions during sensitive periods of embryo development can have a pronounced effect on the adult owing to epigenetic effects, either by irreversible disruption to normal development or through inducing alternative adult phenotypes (Gluckman et al. 2005). Another outcome is ‘coping’ whereby challenges to development are compensated in the short-term, but with a longer-term fitness cost in the adult organism (Gluckman and Hanson 2004; Gluckman et al. 2005). In bitterling, embryos that develop at high densities; i.e. in poor

quality sites, do so at a much lower rate than those at low density, and this may have fitness costs later in life (Agbali and Smith in review). Consequently, the importance of direct benefits of mating decisions may be underestimated in the current study, though if the case, this supports the argument that direct benefits, not indirect, impose stronger sexual selection.

In contrast, there appear to be no significant fitness costs to offspring sired by non-preferred males over and above the significantly increased mortality costs associated with mating with such males. Agbali et al. (2010) raised the surviving offspring of preferred and non-preferred males to maturity. The reproductive success of males was compared in pairwise mating contests using parentage analysis, while females were compared in body size, a reliable predictor of female lifetime reproductive success (Reichard et al. 2009). In neither case were the offspring of preferred males superior to non-preferred (Agbali et al. 2010).

Despite the importance of spawning site choice for female mating decisions, indirect benefits were demonstrated to play a significant role in female mating decisions, albeit a minor one. Female bitterling are able to discriminate among males with respect to their genetic compatibility, with indirect benefits accruing through enhanced offspring survival to independence. In *R. ocellatus* genetic compatibility appears to be linked to genetic distance between parents in the functional region of the major histocompatibility gene complex (Agbali et al. 2010).

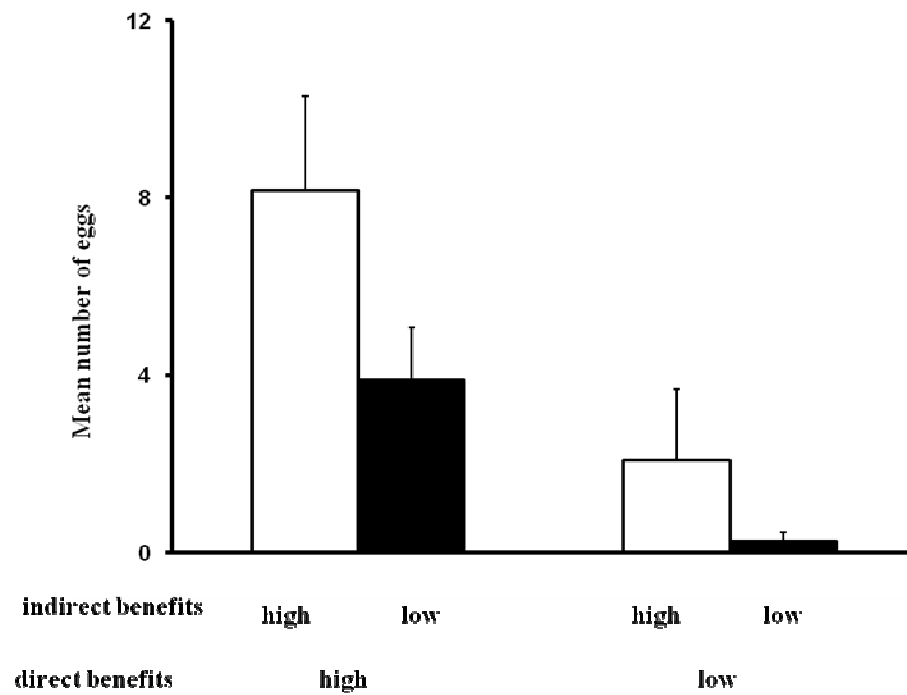
There was no significant interaction between direct and indirect benefits of female mate choice. This finding contradicts a field-based study by Smith et al. (2002) that showed an interaction between male traits and mussel quality in the related European bitterling, *R. amarus*. However, in Smith et al. (2002) male traits, such as size and colour, were thought to function in intersexual selection, but probably function only

in intrasexual selection (Reichard et al. 2005, 2009; Casalini et al. 2009). In addition, Smith et al. (2002) failed to control for the role of male dominance in their experimental design, which has been demonstrated to conflict with female mate choice decisions (Casalini et al. 2009)

The principal cues used in both oviposition and mate choice are probably olfactory. Choice of mussels is partly based on the presence of the amino acid glycine (Y. Kanoh, unpublished data), as well as the oxygen conditions inside the mussel gill (Smith et al. 2001; Reichard et al. 2007b). In mate choice, olfactory cues released from the body surface or in the urine are most likely to play a role (Casalini et al. 2009; Agbali et al. 2010).

In summary, this study demonstrates a leading role for direct benefits, in the form of oviposition site quality, in mate choice decisions in the rose bitterling. Indirect benefits also played a role, but accounted for a relatively minor component of variance in female oviposition rate. Oviposition decisions may have immediate effects on offspring mortality rate, as well as longer-term impacts on fitness.

Figure 4.1. Effect of direct and indirect benefits of mate choice on the oviposition rate of female rose bitterling, *Rhodeus ocellatus*.



Chapter five: A role for receiver bias in rose bitterling mate choice?

ABSTRACT

The evolution of costly secondary sexual traits in males may arise as a result of male-male competition or female mate choice. Female mating decisions may be associated with either direct or indirect benefits, and may evolve as a consequence of selection for signal detection in a non-mating context. Male rose bitterling (*Rhodeus ocellatus*) display red, carotenoid-based nuptial colouration, though its function in the mating system is unclear. There is little evidence that male colour plays a role in female mating decisions, although there is a weak relationship between colour and dominance among males. We tested for a sensory bias for red in a non-mating context and found that bitterling show a strong response to red food items, irrespective of age or sex. This correlation between a sexually selected trait and an intrinsic attraction to red appears to support the receiver-bias hypothesis for the evolution of red nuptial colouration in rose bitterling.

INTRODUCTION

Sexual selection arises through variance in reproductive success, either as a result of competition between members of the same sex, generally males, and termed intra-sexual selection, or as a consequence of female mate choice, termed inter-sexual selection. In some species female mating decisions are associated with direct benefits to females; they may obtain nutrients or items of food, assistance with parental care, access to breeding sites, and protection from harassment or predation. Alternatively, females may acquire indirect benefits from mate choice, by selecting mates through producing offspring of enhanced viability (reviewed by Andersson and Simmons 2006).

Alternative mechanisms of sexual selection have also been suggested, prominent among these is that of receiver bias (Endler and Basalo 1998; Ryan 1990; Smith et al. 2004a; Fuller et al. 2005). Receiver-bias models propose that the origin of a preference and the origin of a sexually dimorphic trait are evolutionarily decoupled (Arnqvist 2006). Consequently the perceptual or cognitive system in the receiver undergoes selection for other functions prior to the evolution of the sender's trait. Selection pressures other than as a mating response to a trait must have contributed to the evolution of the receiver's sensory, perceptual or cognitive system. In the context of mate choice, the receiver-bias hypothesis predicts that sexually selected traits are those most conspicuous to the perceptual or cognitive systems of females. Receiver bias may have played a central role in the evolution of mate choice (Kokko et al. 2003; Arnqvist 2006). In fish, a mating preference in female guppies (*Poecilia reticulata*) for orange-coloured males, and in female three-spined sticklebacks (*Gasterosteus aculeatus*) for males with red throats may have arisen in a foraging context (Rodd et al. 2002; Smith et al. 2004a). The basis for a preference for red and orange food items may be associated with a requirement for carotenoids (Ringelberg 1980; Ibrahim and Huntingford 1989),

which are essential for certain physiological functions and must be obtained in the diet (Olson and Owens 1998).

The aim of the present study was to test for a receiver bias for red in a non-mating context in the rose bitterling, *Rhodeus ocellatus*. Bitterling are cyprinid fish belonging to the sub-family Acheilognathinae that use fresh water mussels as a spawning site (Wiepkema 1961; Kanoh 1996, 2000; Aldridge 1999; for review see Smith et al. 2004b). All bitterling species, including *R. ocellatus*, show striking sexual dimorphism. Males are larger and deeper-bodied than females. Mature males and females also have different coloration; females are silver ventrally and brownish dorsally, whereas males develop a red carotenoid-based nuptial coloration in the iris, at the base of the tail fin, and near the base of the pectoral fins. In addition, males are blue-black dorsally with an iridescent green-blue tail fin stripe (Tadayuki and Kohsuke 2001; Chen and Chang 2005). In the breeding season, males compete for territories around unionid mussels and actively court females (Kanoh 2000; Smith et al. 2004b; Casalini et al. 2009). Females develop a long ovipositor that they use to place their eggs onto the gills of a mussel through the exhalant siphon of mussel (Wiepkema 1961; Shirai 1962; Smith et al. 2004b). Territorial males release sperm over the inhalant siphon of the mussel which is drawn into the gills of the mussel and fertilizes the eggs. Rival males (an adjacent territory holder or a male that does not possess a territory) also release their sperm into a rival's mussel by sneaking fertilisation (Kanoh 1996, 2000; Smith et al. 2002, 2003; Reichard et al. 2004a). Fertilised eggs develop inside the mussel gill and emerge as free swimming larvae after about 30 days (Kim and Park 1985; Aldridge 1999; Smith et al. 2004b). Females show a strong preference for spawning sites (Smith et al. 2002; Casalini 2007; Casalini et al. 2009), preferring certain mussel species and mussels with a low number of bitterling eggs already inside their

gills (Smith et al. 2000b; Smith et al. 2001). Females also choose specific males, with significant non-additive benefits linked to genetic compatibility appearing to play the principle role in female mate choice (Agbali et al. 2010). Notably, there appear to be negligible additive benefits of mate choice in rose bitterling, and no correlation between male colour and offspring quality (Casalini et al. 2009; Agbali et al. 2010).

Male bitterling appear unable to discriminate mussel quality and, consequently, male size and colour do not appear to signal direct mate choice benefits to females (Casalini, unpublished data). Consequently, the role, if any, of male nuptial colouration in the bitterling mating system is unclear. The extent of male colour may play a role in intrasexual selection as a badge of status (Andersson 1994), though a consistent role for colour in intrasexual selection has not been established (Reichard et al. 2005; Casalini et al. 2009). The only study to show a role for male colour is that of Candolin and Reynolds (2001), which claimed to show a significant effect of male eye and fin color on female mate choice in the European bitterling (*R. amarus*), although this finding has never been replicated. In addition, both Smith et al. (2002) and Casalini et al. (2009) detected a correlation between male courtship behaviour and the probability of a female following a male, though not of spawning with him. During courtship the male rapidly undulates his red-coloured anal fin and tail directly in front of the female (Smith et al. 2004b). A possibility is that the display of red pigmentation by males during courtship serves to attract the attention of a female and advertise the location of a territorial male and his mussel spawning site, but without conveying information about either direct or indirect mating benefits, which would be consistent with the sensory-exploitation hypothesis (Ryan 1990; Ryan and Rand 1990; Endler 1992; Endler and Basalo 1998). Thus, male colouration might be favoured through sexual selection, even without reliably signalling male or resource quality, by attracting

females to inspect males and mussels with the sensory bias for the trait maintained in a different context through natural selection.

Here we tested a key prediction of the receiver-bias hypothesis in the rose bitterling. Specifically, we tested whether male and female rose bitterling show a sensory bias for red colouration in a foraging context. To limit the confounding effect of learning on responses we raised fish in the laboratory fed only on green coloured food and subsequently tested their foraging preferences by presenting them with plastic strips of different colours and recording the number of bites directed at each. We conducted separate tests on juveniles and sexually mature fish, and compared the responses of males and females. If juvenile as well as adult fish showed a bias towards red, this result would lend support to the pre-existing bias hypothesis. Any differences between sexually mature and immature fish and between males and females might suggest that sexual selection is also involved in shaping the bias. We further predicted that there might be an effect of conditioning on preferences through associative learning, which has been demonstrated in the related zebrafish (*Danio rerio*) (Spence and Smith 2008).

MATERIAL AND METHODS

Experimental fish were generated using *in vitro* fertilisations in order to control for any effects of parentage. Parental fish were first generation offspring of wild-caught fish collected from the River Yangtze Basin, China. A total of 30 sexually mature males and 30 females were haphazardly selected from stock aquaria and the eggs were gently squeezed from the females into a 70 mm diameter Petri dishes containing fresh water. Sperm was stripped from the males by gently pressing their abdomens and mixed in 100 ml of teleost saline (Yokoi et al. 2008). Sperm suspensions were pipetted over the eggs and the covered Petri dishes were left on the laboratory bench for 30 min. The fertilized

eggs were washed with freshwater and incubated at 23 °C in an environmentally controlled room until they developed into free-swimming larvae after approximately one month.

Larvae were reared in plastic aquaria measuring 35 (length) x 25 (width) x 20 (depth) cm with a 20 mm layer of fine gravel and artificial plants as refuges. Aquaria were on a recirculating system with water at 23 °C and fish were kept under a 16:8 h light: dark cycle. The sides of the aquaria were covered using opaque barriers in order to isolate fish from possible confounding visual stimuli. For the first 4 weeks after becoming free swimming larvae were fed three times each day on green infusoria-grade commercial food (ZM Systems, ZM000 grade). Thereafter, they were fed solely on green commercial dried fish flake food (Tetramin Complete) twice each day.

The first colour preference tests were conducted on juvenile fish at the age of 12 weeks post fertilisation. Tests were conducted in plastic aquaria identical to those in which the fish were reared. Tests were conducted under natural daylight at a water temperature of 20 °C. Fish were tested in pairs, as pilot studies showed isolated bitterling did not bite freely at the test strips. Two sides of each experimental aquarium was covered to prevent visual contact between adjacent pairs of fish. Fish were released in the experimental aquarium and allowed to settle overnight. The following morning fish were fed 5 h before trials began to standardise their motivation to feed. To perform tests each pair was consecutively presented with five differently coloured (green, blue, red, white and black) strips of plastic (10 mm length, 1.4 mm diameter) in a random order. The coloured strips were identical to those used by Smith et al. (2004a) and were of known reflectance. The strips of plastic were tied to a 160 mm length of nylon monofilament line (0.1 mm diameter) and presented to the fish suspended, stationary, in the upper half of the aquarium. Multiple strips of the same colour were used to avoid

any consistent bias with a particular plastic strip. The total of number of bites directed at each strip by both fish were recorded for 3 min. After testing, the Standard Length (measured from the tip of the snout to the origin of the tail fin) of all fish were measured to the nearest 1 mm. The mean \pm SD length of juvenile fish was 16.2 ± 0.56 mm. Fish were used in trials only once and a total of 15 pairs of juvenile fish were tested, each pair representing a single replicate.

The remaining juvenile fish were reared until sexually mature (at approximately 20 weeks of age), male and female *R. ocellatus* being easily distinguished because males develop red colouration in the iris and tail fin while females develop a long ovipositor. Pairs of each sex were tested separately for colour preference using the same protocol as that for the juveniles. Thus the adult fish, like juveniles, had no prior experience of red, white, black and blue colours. A total of 12 pairs of males and 8 pairs of females were tested, each pair representing a single replicate. After testing all fish were measured for body length. The mean \pm SD body lengths of males and females were 25.08 ± 0.771 and 25.19 ± 0.586 respectively, with no significant difference in length between the sexes (unpaired t-test: $t_{38} = 0.098$, $P = 0.922$).

STATISTICAL ANALYSIS

The normality of data were examined using a Shapiro-Wilk test and equality of variance using Bartlett's test. Data that did not meet assumptions of normality or homoscedasticity were transformed. Analysis of variance was used to test for a colour preference, with bite rate as the dependent variable, colour of plastic strips and order of presentation as independent variables. For adult fish, sex was used as an additional independent variable. A Bonferroni test was used for *post hoc* pairwise comparisons between bite rates towards different colours.

RESULTS

There was a significant difference in the biting rate of juveniles among the five test colours (ANOVA, square-root transformation: $F_{4,66} = 7.38$, $P < 0.001$). There was no significant effect of order of colour presentation on biting rate ($F_{4,66} = 1.36$, $P = 0.257$). The highest rate of biting was directed at red strips, followed by green, black, blue and white (Fig. 5.1). *Post hoc* pairwise comparisons showed that there was no significant difference in bite rate between red and green (the colour of the food on which the fish had been reared) but red was preferred to all the other colours (white: $P < 0.001$, black: $P = 0.013$, blue: $P = 0.001$). Green was preferred to white ($P = 0.026$) but there was no difference in preference between green, blue and black.

There was a significant effect of colour on the biting rate of mature fish (ANOVA, square-root transformation: $F_{4,86} = 17.19$, $P < 0.001$). There was no effect of order of presentation ($F_{4,86} = 0.79$, $P = 0.534$), or sex ($F_{4,86} = 1.88$, $P = 0.174$), neither was there a significant interaction between colour and sex ($F_{4,86} = 0.12$, $P = 0.974$). The rank order of colour preference was the same as that for juveniles and did not differ between the sexes (Fig. 5.2). *Post hoc* pairwise comparisons showed that for each sex red was preferred over all other colours (males, green: $P = 0.003$, white, black and blue: $P < 0.001$; females, green: $P = 0.005$, white, black and blue: $P < 0.001$).

DISCUSSION

Rose bitterling showed a significant innate preference for biting red plastic strips. This effect was not masked by first conditioning fish on green food. The same effect was observed irrespective of stage of maturity or sex. The behaviour of the fish when biting the plastic strips was consistent with feeding behaviour; the fish swam directly to the

strips and bit them immediately. The visual system of bitterling has not been described. However, it is likely to be comparable to many shallow-water fish species, including closely related species such as the zebrafish and goldfish (*Carassius auratus*). These species possess tetrachromatic colour vision, with peak absorbance in the ultraviolet (Robinson et al. 1993), blue, green and red wavelengths (Hughes et al. 1998). Thus, bitterling hue perception is likely to be comparable to that of humans (Boynton 1979).

A possible ecological explanation for the observed red bias in rose bitterling is that the fish may be adapted to seek red food items that are rich in carotenoids, in common with guppies, three-spined sticklebacks and zebrafish (Rodd et al. 2002; Smith et al. 2004a; Spence and Smith 2008). Carotenoids are essential for several important physiological functions, including immune response, liver function, and growth (Olson and Owens 1998; Lozano 1994; Chew and Park 2004). Animals are unable to synthesize carotenoids and thus depend on obtaining them in their diet (Goodwin 1984; Schiedt 1989). The diet of bitterling is rich in diatoms, green and red algae and planktonic microcrustaceans (copepods, cladocerans and ostracods) (Solomon et al. 1985; Przybylski 1996; Smith et al. 2004b). Bitterling are primarily visual feeders, and the potential benefits of a diet rich in carotenoids (Olson and Owens 1998) may have favoured the evolution of a preference for red food items, as it appears to have done in other related species. Notably, the main component of the nuptial coloration on the body and fins of rose bitterling are the carotenoid-based pigments zeaxanthin, β -carotene and diatoxanthin (Kim et al. 1999).

The correlation demonstrated here between a sexually-selected trait and an intrinsic attraction to red lends support to the receiver bias model for the evolution of red nuptial colouration in bitterling. Although a role for carotenoid-based nuptial coloration in mate choice has been demonstrated in other species (Andersson 1994;

Barber et al. 2001; Bourne et al. 2003), the extent of red in the iris and fins of male *R. ocellatus* has no obvious role in female mate choice (Casalini et al. 2009) and is not clearly linked to any indirect mating benefits (Agbali et al. 2010). Similarly, there is no evidence for a substantial role for red pigmentation in the mating system of the related species *R. amarus* (Smith et al. 2002; Reichard et al. 2005). Candolin and Reynolds (2001) claimed to show red nuptial colouration in *R. amarus* as a signal to females of male genetic quality, though not mussel quality since males did not alter their extent or intensity of colouration in relation to mussel quality. However, in *R. ocellatus* (and possibly also *R. amarus*) indirect mating benefits are related to non-additive effects, not additive benefits as Candolin and Reynolds (2001) infer. MHC dissimilar mates confer fitness advantages on their offspring and MHC-specific odour may play a role the principle role mate choice (Agbali et al. 2010).

Male colouration may function in other contexts, possibly intrasexual selection (Evans and Norris 1996). In many vertebrates, bright colours signal male dominance and fighting ability (Rohwer 1982; Whitfield 1987). However, the function of red colour in male-male competition has yet to be adequately demonstrated in bitterling (Reichard et al. 2005; Casalini et al. 2009). In *R. amarus*, the intensity of male red colour is a general indicator of male dominance status (Smith et al. 2002; Reichard et al. 2005), though in *R. ocellatus* the association with male dominance is weak (Casalini et al. 2009). Further research on the role of male colouration in intrasexual selection, particularly including field-based studies, may be illuminating. Thus, while there appears to be a significant bias in the visual system of rose bitterling for red colouration, and male nuptial colouration is predominantly red based, a link between male colour and fitness is lacking.

An alternative explanation for the results of this study is that females are attracted to red objects as a side effect of a female mate preference for red coloration. However, this interpretation raises two difficulties. First, both males and females, rather than only females, were attracted to red objects, though male attraction to red objects could be the result of a genetic correlation between the sexes. A second difficulty is that in zebrafish, which are related to bitterling (Spence et al. 2008), both sexes also show a strong attraction to red objects (Spence and Smith 2008). Given the lack of red nuptial coloration of male zebrafish, the prediction would be for an attraction to male nuptial colours of black and yellow and not red in this species if the response evolved in a mating context. Consequently, the most straightforward interpretation of our results is that a sensitivity to red functions in a feeding context in both bitterling and zebrafish.

Although the design of this study did not allow the effects of innate and conditioned responses to different coloured foods to be partitioned, there was a noticeable attraction to green objects in addition to red, but less so to black, white and blue. Experimental fish were only exposed to a diet of green food prior to testing. Consequently, the significant attraction of bitterling to green may have resulted through learning. Zebrafish also show a strong learned component to diet choice (Spence and Smith 2008) and the same may be true in rose bitterling. Research on other species indicate that innate preferences tend to steer general foraging responses while learning may be more important in discriminating among similar stimuli (Gumbert 2000; Gamberale-Stille and Tullberg 2001; Dyer and Chittka 2004).

In conclusion, although, bitterling *R. ocellatus* do not utilize male red carotenoid-based nuptial coloration in mate choice, this study suggests that they possess a visual bias for red that has arisen through natural selection as an adaptive response to

foraging. These results are consistent with the predictions of the receiver-bias hypothesis for the evolution of red nuptial colouration in males.

Figure 5.1. Mean \pm SE bite rate (3 min^{-1}) at coloured plastic strips (red, green, black, blue, white) by juvenile *R. ocellatus*.

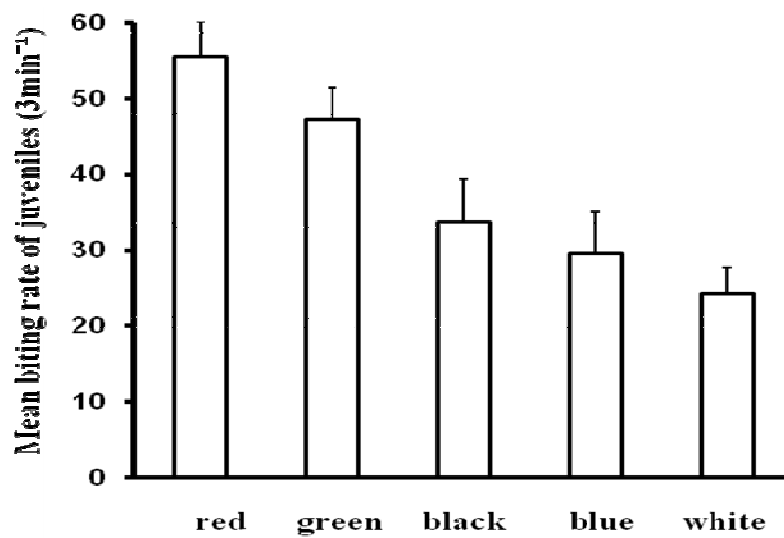
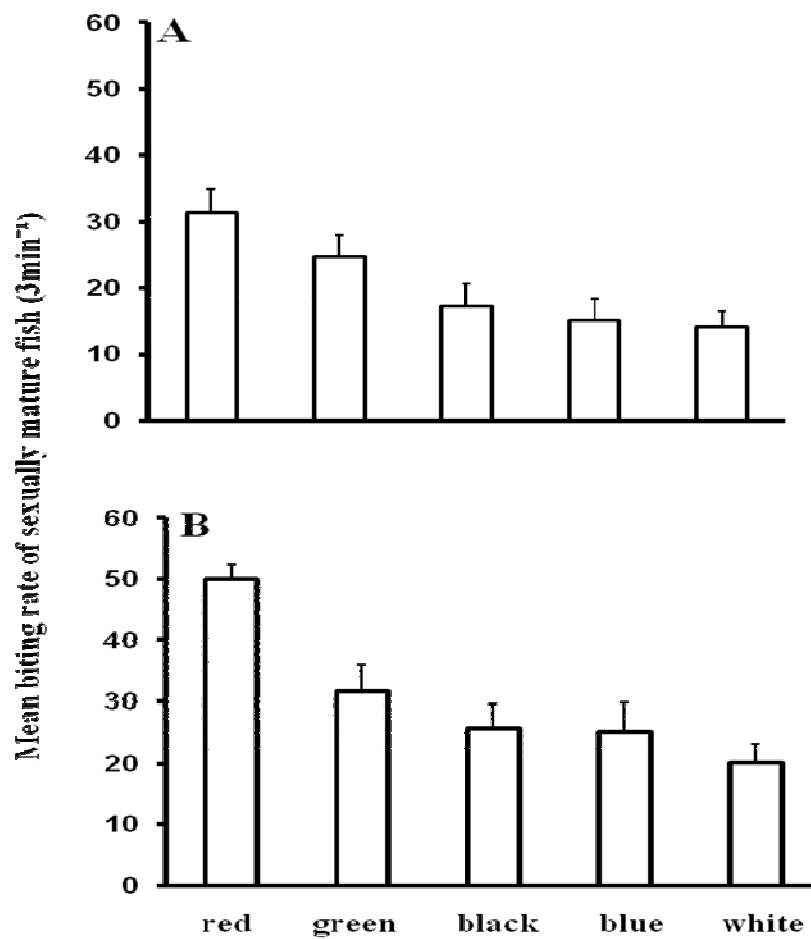


Figure 5.2. Mean \pm SE bite rate (3 min^{-1}) at coloured plastic strips (red, green, black, blue, white) by sexually mature *R. ocellatus* (A) females, (B) males.



Chapter six: Female oviposition decisions have long-term fitness consequences for offspring in the rose bitterling (*Rhodeus ocellatus*)

ABSTRACT

In oviparous species oviposition decisions can have immediate consequences for offspring fitness. In addition there may be long-term effects, either through irreversible disruption to normal development, inducing alternative adult phenotypes, or ‘coping’ whereby challenges to development are compensated in the short-term, but carry a fitness cost in the adult. Here we investigated the long-term impacts of oviposition decisions on offspring fitness parameters in a fish, the rose bitterling (*Rhodeus ocellatus*). Bitterling lay their eggs in the gills of living freshwater mussels and the eggs and embryos complete development inside the mussel gill chamber. Female bitterling avoid using mussels that already contain high numbers of eggs and embryos. Embryo mortality in mussels is strongly density dependent and, consequently, there are immediate costs associated with using mussels containing high numbers of eggs and embryos. Here we investigated the long-term consequences of female oviposition decisions by raising embryos at high and low densities for the first four weeks after fertilisation, the period that corresponds with incubation of embryos in a mussel gill, then at a common low density until sexual maturity. The results showed no significant effect of density on offspring survival or growth. However, there was a significant impact of density on age and size at maturity, with embryos raised at a high density becoming sexually mature earlier and at a significantly smaller size than those raised at low density. This effect may arise through embryos coping with low oxygen conditions during development. In addition, at a low density the sex ratio of offspring was

significantly male biased while at high density it was at unity. The results are discussed in the context of the bitterling mating system.

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INTRODUCTION

The eggs of oviparous species are rarely deposited haphazardly, more usually they are placed at pre-selected sites that afford protection or a better-quality environment for development. In some species a female may also need to choose whether to deposit all her eggs in one site or distribute them among several. Thus, oviposition decisions can have important consequences for offspring fitness and, for species without parental care, oviposition decisions are one of the principal means by which a female can influence offspring survival; these decisions represent important maternal effects (Roitberg 1998). The fitness consequences of oviposition decisions have been studied for invertebrates (Chew 1977; Jaenike 1978; Smith and Lessells 1985; Godfray 1994; Cope and Fox 2003), but are less well understood in vertebrates.

Here we investigated the impact of oviposition decisions on offspring fitness parameters in a fish, the rose bitterling (*Rhodeus ocellatus*). Bitterling lay their eggs in the gills of living freshwater mussels and the eggs and embryos complete development within the mussel gill chamber, a temperature-dependent process that lasts 3-4 weeks. Bitterling are parasites of mussels, the presence of their eggs halts the growth of mussels and high densities of eggs can kill the host (Reichard et al. 2006). Female bitterling are choosy about which mussels they use for oviposition. In rose bitterling, and the related European bitterling (*R. amarus*), females avoid mussels that already contain the eggs and developing embryos of other females, and there is a negative relationship between the density of embryos in a mussel and the propensity of a female to use it for spawning. The mortality rate of bitterling embryos in mussels is density dependent, with mortalities during incubation in the mussel linked to ejection of eggs by the host and asphyxiation of embryos through competition with other embryos and their host. Both rose and European bitterling use a range of mussel species, but show clear

preferences for certain species. These preferences are linked to the respiration rates of mussels, with both species preferring mussels with low rates (Smith et al. 2001; Reichard et al. 2006, 2007b). The mechanism by which female bitterling measure mussel quality, both in terms of eggs already in the mussel gill (analogous to ‘superparasitism’ in parasitoids) and species of mussel, appears to be linked to the oxygen conditions inside the mussel (Smith et al. 2001; Kitamura 2005). Females may be able to perceive conditions inside the mussel by sampling the exhalant current of water emerging from the mussel gill. Prior to spawning females ‘inspect’ a mussel by positioning herself over the exhalant siphon, at approximately 70° from horizontal for several seconds before either spawning or departing to seek a different mussel (Smith et al. 2004b).

Depositing eggs in a mussel that already contains a high density of eggs and embryos has an immediate fitness cost for a female through egg mortality, either because of ejection of the eggs because they are not properly placed inside the gill, or from suffocation. However, there may be additional fitness costs that arise through alterations to normal development at high offspring densities, which may contribute to shaping female oviposition decisions. It is recognised that conditions during sensitive periods of embryo development can have a pronounced effect in the adult, either through irreversible disruption to normal development or through inducing alternative adult phenotypes (Gluckman et al. 2005). Another outcome is ‘coping’ whereby challenges to development, such as low oxygen conditions, are compensated in the short-term, but carry a longer-term fitness cost in the adult (Gluckman and Hanson 2004; Gluckman et al. 2005). In bitterling, embryos that develop at high densities do so at a significantly slower rate than those at low densities, and this may have fitness costs

later in life (Kitamura 2005). Consequently, the importance of oviposition decisions for females may be underestimated if egg mortality alone is considered.

The aim of the present study was to quantify the impact on offspring fitness of developing at low and high densities. Our prediction was that at high densities egg mortality rate would be elevated, and development would be slower, and also that there would be fitness costs to survivors following incubation. To address this question we reared rose bitterling eggs and embryos at two densities for the period over which they would be incubated in a mussel. The juveniles were raised to sexual maturity at a common density and two fitness traits examined; age and size at maturity. Most bitterling populations are annual, and the age at reproduction starts is an important determinant of reproductive success. Body size in bitterling also correlates strongly with both male and female lifetime reproductive success (Reichard et al. 2009). To circumvent effects of parental and mussel quality in our experimental design we generated embryos using *in vitro* fertilisations and split clutches into two densities, thus individual replicates were full siblings and mussel quality played no role in embryo development.

MATERIAL AND METHODS

EXPERIMENTAL CONDITIONS

In vitro fertilizations were used to generate a total of 20 replicates of 2 levels of egg density. Experimental *R. ocellatus* were from the River Yangtze Basin, China and were the first generation bred in captivity. Females and males were isolated in aquaria with a mussel covered with a perforated plastic cup so that they could see and smell the mussel but the female was unable to spawn. Once the female had ovulated a batch of eggs, evident from her extended ovipositor. The eggs were gently squeezed from the female

and divided in a 1 to 5 ratio in two 70 mm diameter Petri dishes containing freshwater. Sperm was stripped from the male and mixed with 9 ml of teleost saline (Yokoi et al. 2008). A 1 ml subsample of sperm solution was pipetted over the eggs and the Petri dishes left on the laboratory bench for 30 min. The fertilized eggs were subsequently washed with freshwater and incubated at 23 °C in an environmentally controlled cabinet until the yolk sac was absorbed, the swim bladder inflated, and the larvae began active swimming, a period of approximately four weeks. There was a highly significant difference in the number of eggs in high and low density treatments (t-test on square-root transformed data, $t_{38} = 10.58$, $P < 0.001$). The mean \pm SE number of eggs in the high density treatment was 19.4 ± 1.86 , while in the low density treatment was 3.9 ± 0.38 .

Every group of eggs and developing embryos was photographed daily, from fertilisation until yolk-sac absorption, under standard light conditions alongside a scale bar using a Canon EOS 300D camera with 60 mm macro lens. A record was kept of the survival rates of developing embryos and the age at which they began to move. Egg, embryo and larval sizes were estimated from digital images.

To measure offspring fitness traits at maturity, two free-swimming and exogenously feeding juveniles were haphazardly selected from every low and high offspring density treatment, and each pair housed in separate aquaria measuring 21 (length) x 14 (width) x 14 (depth) cm on a recirculating system at 23 °C. Fish were fed *ad libitum* twice daily with dried fish flake food and once with finely chopped *Tubifex* spp. A record was kept of the age and body length (measured from the tip of the snout to the base of the tail) at which every individual achieved sexual maturity. In males maturity was recognised by the appearance of red pigment in the iris and at the base of the tail fin, which correspond with the onset of territoriality, courtship behaviour and

participation in spawning (Chen and Chang 2005). In the female maturity was determined by the first extension of the ovipositor, which corresponds with the first ovulation of mature eggs (Smith et al. 2004b). In addition, the right and left eye of all males were photographed under standard light conditions and the red area of the iris measured using the protocol of Barber et al. (2000).

STATISTICAL ANALYSIS

All data were tested for normality using a Shapiro-Wilk test and for equality of variance using Levene's robust test. Appropriate transformations were applied to those data that deviated from normality or homoscedasticity. Paired *t*-tests were used to compare the proportion of embryos that were moving after an arbitrary period of 120 h, the proportion and size of larvae that survived to 4 weeks of age, and the size and age of fish at sexual maturity. An unpaired *t*-test was used to compare the proportion of the iris in sexually mature males that was pigmented, and a Chi-squared test was used to examine the deviation of the sex ratio from 1:1 in high and low density treatments. Data analyses were performed using *Stata* 11.0 for Macintosh.

RESULTS

There was a significant difference in the proportion of embryos showing movement 120 h after fertilisation between high and low density treatments, with a higher proportion of embryos in the low density treatment motile than those raised at a high density (Table 6.1; Figure 6.1). However, there was no significant difference between treatments in either the survival rate or size of larvae after 4 weeks (Table 6.1), the stage at which they would normally depart their mussel host.

There was a significant difference in the age and size at maturity between high and low offspring density treatments (Table 6.1). Offspring that were exposed to high density over their first 4 weeks of life were significantly younger and smaller at maturity than those exposed to a low density (Table 6.1; Figure 6.2, 6.3). Data for age and size at maturity for males and female offspring were pooled since there was no difference between the sexes in age or size at maturity (unpaired t-test on square-root transformed data, age: $t_{23} = 0.69$, $P = 0.496$; body length $t_{23} = 0.24$, $P = 0.810$). There was no significant difference in the extent of eye redness of males in high and low density treatments (Table 6.1). There was a significant deviation from a 1:1 sex ratio of fish in the low density treatment, which was male-biased ($\chi^2 = 4.77$, $df = 1$, $P = 0.029$). There was no significant sex bias in the high density treatment ($\chi^2 = 1.47$, $df = 1$, $P = 0.225$).

DISCUSSION

The aim of the present study was to examine the effects of female oviposition decisions on offspring fitness in the rose bitterling, *R. ocellatus*, a fish with a resource-based mating system. The results showed no difference in offspring survival between high and low density treatments. However, a significant difference in offspring growth and development was detected; embryos commenced movement sooner at low densities, while adults achieved sexual maturity sooner and at a smaller size at high densities. At a low density the sex ratio was significantly male biased, at high density it was at unity.

Our prediction was that at a high density the rate of embryo development would be limited by a reduced availability of oxygen and possibly also exposure to elevated levels of metabolic waste (Kamler 1992). This prediction was partly satisfied. The proportion of embryos that were motile after 120 h was significantly higher at low

density than high, suggesting that the rate of development may have been hindered at high density. Despite this effect the mortality rate of embryos over the period they were exposed to a high density, and the size they achieved, did not differ between treatments. This result suggests that either the effects of the high density treatment were insufficient to generate an effect, or that the developing embryos were able to ‘cope’ sensu Gluckman and Hanson (2004), and complete development despite the environmental challenges generated by high conspecific density.

Under natural conditions in a mussel gill bitterling embryos routinely, but unpredictably, encounter high densities of other eggs and embryos during development. In populations where the mussel population is large and the bitterling populations small, mussels may contain fewer than 10 eggs. In contrast, where the ratio is reversed the number of eggs and embryos in a mussel can exceed 250 (Smith et al. 2000b). Consequently, the evolution of coping tactics to enable embryos to develop, particularly with limited oxygen, might be anticipated. Bitterling embryos display a number of unique adaptations for surviving the low oxygen conditions they encounter in the mussel gill. These include early hatching and an unusually well developed larval respiratory system (Suzuki and Jeon 1988; Dmitrijeva et al. 1985). In common with certain other cyprinid species, they also appear able to utilise anaerobic glycolysis in anoxic conditions (Hochachka 1980; Waarde et al. 1993). However, the longer-term effects of embryo development while oxygen is restricted are unclear.

Here, we detected a significant effect of high density on maturation, in both males and females. Fish that developed over their first four weeks at a high density matured earlier (by a mean of 14 days) and were significantly smaller (Table 6.1). It should be noted that density treatments only applied over the first four weeks of development, and that fish were subsequently housed at the same density. Thus, despite

completing embryo and larval stages in comparable conditions, density treatments manifested themselves later during development. From a functional perspective it is unclear whether these responses to high embryo and larval densities are plastic responses that would confer a fitness advantage, or costly by-products of coping.

Adult male bitterling can adopt two mating tactics; that of a territorial guarder or a sneaker. Territorial males guard mussels and court females, while also occasionally sneaking fertilisations in the territories of neighbouring males. Sneakers reproduce only by stealing fertilisation by releasing sperm into mussels during courtship by territorial males (Kano 1996, 2000; Smith et al. 2002, 2004b). Male role is determined by size, with large males aggressively monopolising mussels through territoriality, and small males forced to adopt sneaking (Casalini et al. 2009). At low adult densities territorial males have higher reproductive success than sneakers (Reichard et al. 2004a, b). However, at high densities the frequency of sneaking erodes territorial male success to the extent that the reproductive success of males adopting each tactic is identical. Under these conditions territoriality breaks down (Reichard et al. 2004a). Consequently, at high fish densities, or more strictly at high levels of ‘crowding’; the ratio of fish to mussels (Casalini et al. 2010), small males might not be significantly disadvantaged in their reproductive fitness compared to larger males. Indeed, early sexual maturity in an annual fish like the bitterling may confer a fitness advantage, since the onset of reproduction is earlier. A caveat to this conclusion, however, is that since size and fecundity are highly correlated in females, there is no obvious fitness advantage to females of maturing at a small size at high levels of density/crowding. In addition, the lifetime reproductive success of male and female bitterling has been demonstrated to be greater in larger individuals, irrespective of mussel availability (Reichard et al. 2009). Finally, in bitterling populations with historically high levels of crowding, mean male

size and aggression are significantly higher than those with low levels of crowding (M. Casalini and C. Smith, unpubl. data). Thus, there is no substantial evidence for a fitness advantage to maturing at a smaller size in response to high density during embryo incubation in bitterling, and some evidence for the converse. Consequently, our tentative conclusion is that the shift in age and size at maturity we observed in response to elevated density reflects a long-term fitness cost of coping in response to an environmental challenge during embryonic development.

We detected a modest, but significant, deviation from unity in the sex ratio of offspring at low rearing density. There is an array of adaptive hypotheses that have been invoked to explain variation in sex ratios, some with credible empirical support (reviewed in Hardy 2002). The sex-determining system in the rose bitterling is female heterogametic (ZW) (Kawamura 1998), but the genetic and physiological sex of fishes does not always coincide as it does in other vertebrates, permitting substantial flexibility in sex determination (Devlin and Nagahama 2002). Consequently, fish embryos may be responsive to their environment with respect to sex and further research on adaptive sex ratio variation in this species is justified.

In conclusion, we investigated the effect of embryo density in rose bitterling during the first four weeks of development as a measure of the long-term impact of female oviposition decisions. The results showed no significant effect of density on offspring survival or growth. However, there was a significant impact of density on age and size at maturity, with embryos raised at a high density becoming sexually mature earlier and at a smaller size than those raised at low density. This effect probably imposes a fitness cost that arises from embryos coping with low oxygen conditions during development. At a low density the sex ratio of offspring was significantly male

biased while at high density it was at unity. This impact on sex ratio warrants further investigation.

Figure 6.1. The proportion of onset of movement by embryos of high and low density after 120h from fertilisation.

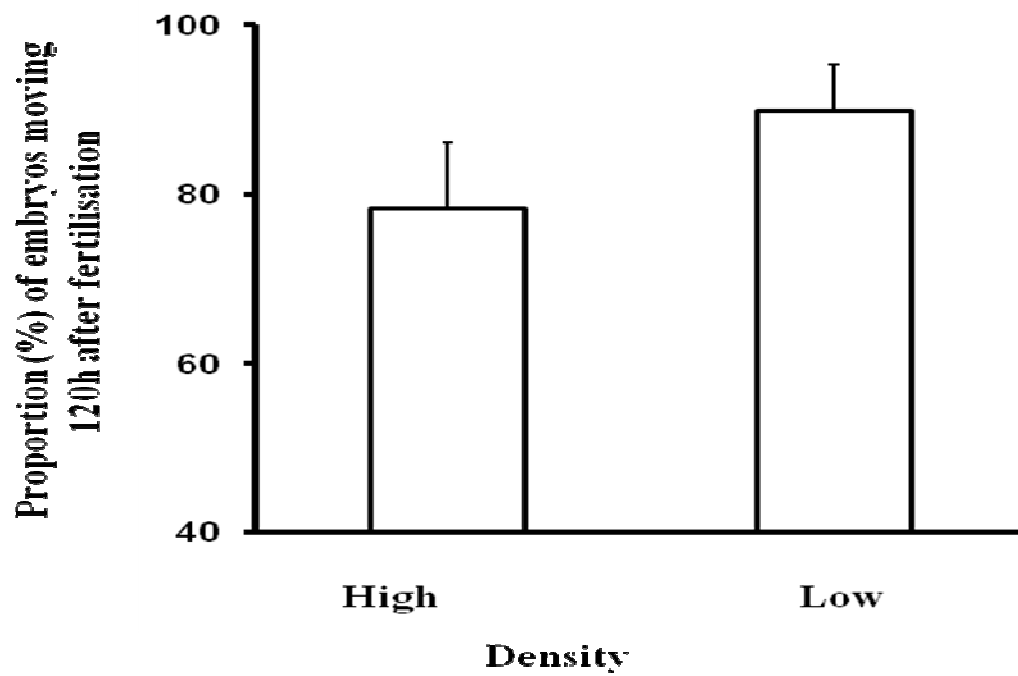


Figure 6.2. Mean \pm SE body length (mm) of offspring of high and low density at maturity.

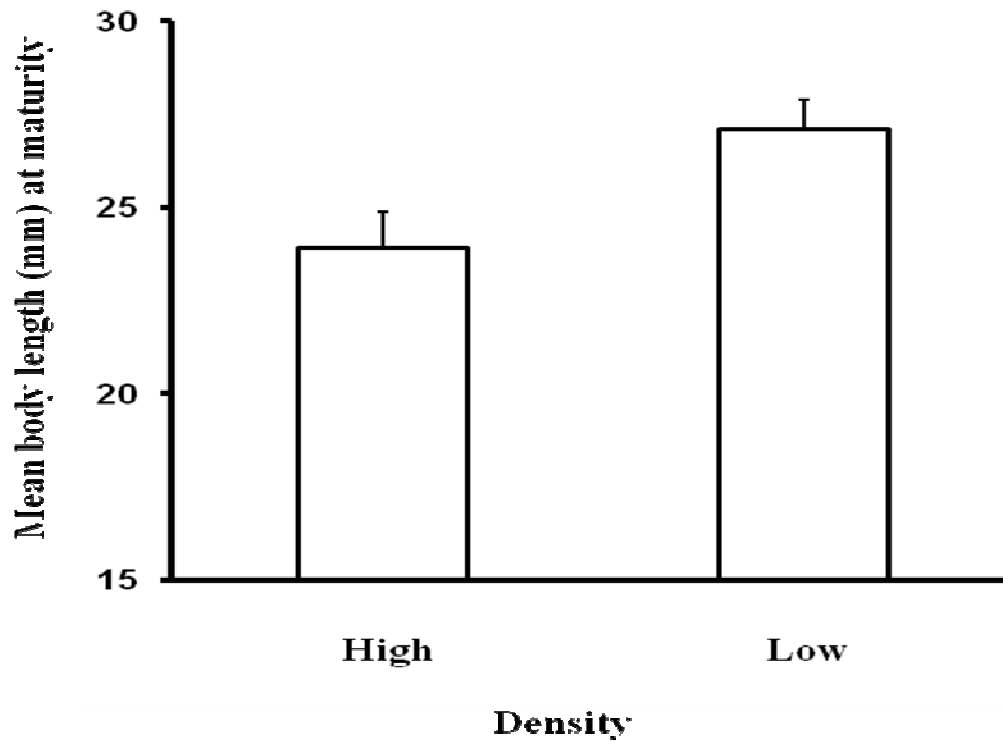


Figure 6.3. Mean \pm SE age (days) of offspring of high and low density at maturity.

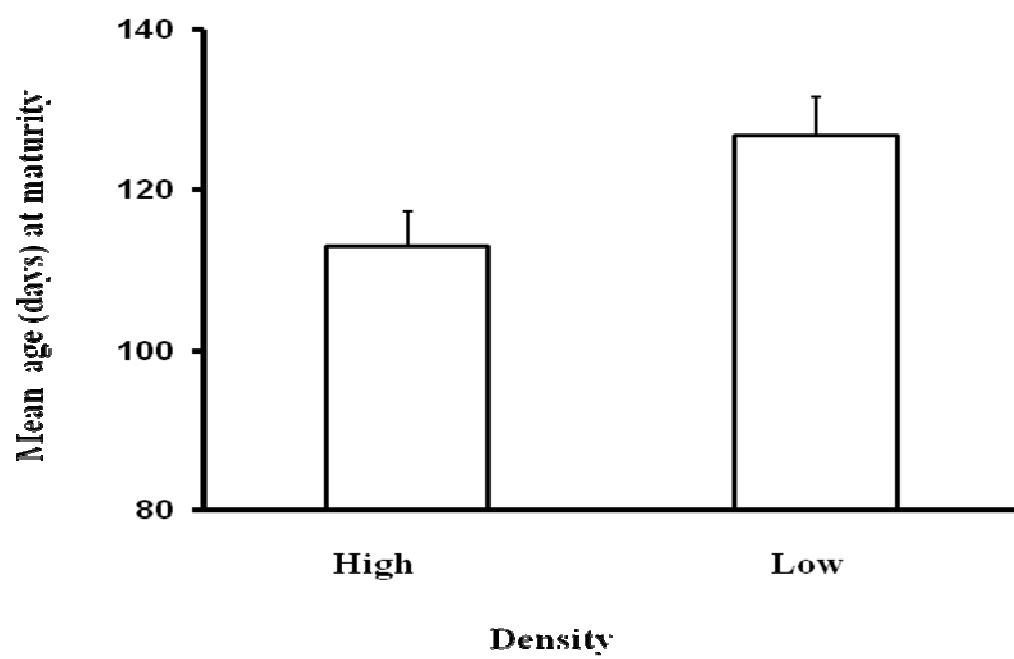


Table 6.1. Summary of paired t-values for offspring fitness parameters. DF = 19.

| Variable | Mean (se) low density | Mean (se) high density | <i>t</i> -value | <i>P</i> |
|------------------------------------|-----------------------|------------------------|-----------------|----------|
| Proportion moving after 120 h | 0.90 (0.054) | 0.78 (0.077) | 2.44 | 0.024 |
| Proportion surviving after 4 weeks | 0.63 (0.083) | 0.51 (0.061) | 1.31 | 0.207 |
| Size after 4 weeks | 14.2 (1.12) | 14.1 (0.77) | 0.09 | 0.927 |
| Size at maturity (mm) | 27.4 (0.93) | 24.6 (1.13) | 2.50 | 0.031 |
| Age at maturity (days) | 130 (5.6) | 116 (4.9) | 4.34 | 0.002 |
| Male eye colour at maturity* | 5.0 (0.70) | 4.0 (0.92) | 0.92 | 0.374 |

*Unpaired t-test with 15 degrees of freedom.

Chapter seven: The role of olfactory cues in female spawning decisions in the rose bitterling (*Rhodeus ocellatus*)

ABSTRACT

In many fish species conspecifics release chemical cues that function in communication, including in reproduction. In this study the role of olfactory cues in the spawning decisions of the rose bitterling, *Rhodeus ocellatus*, a fish with a resource-based mating system was investigated. In this species mate choice is based on genetic compatibility, and correlates with the functional dissimilarity of major histocompatibility complex (MHC) alleles. Female responses to the sperm of multiple males released over spawning sites were investigated. Female responses were measured in terms of oviposition and rate and duration of inspection of mussel spawning sites. The results showed that spawning rate was significantly higher with sperm added to mussels in comparison with a water control. However, there was no significant difference in the rate or duration of female inspection of spawning sites. The results suggest that female may be able to evaluate the presence of sperm inside a mussel, and this response may be linked to olfactory cues.

INTRODUCTION

The choice of a specific mate over alternative individuals has a powerful effect on adaptive evolution through the process of sexual selection (Kirkpatrick 1982; Andersson 1994). Mate choice, which is usually performed by females, often depends on specific cues or signals to distinguish among possible mates (Johansson and Jones 2007). Female mate choice for indirect benefits of choice depends on the genetic quality of potential mates, with female choice for high mate quality tending to result in elevated fitness of offspring (Kirkpatrick 1982; Eberhard 1996; Neff and Pitcher 2005). Thus, in species in which indirect genetic benefits of mate choice are implicated there must be some mechanism that conveys information about mate genetic quality to a female (Sommerfeld et al. 2008). The mechanism of information transfer is a particular problem in the case of non-additive genetic benefits. Here, in contrast to additive effects of good genes, compatible genes are predicted to confer an enhanced fitness to offspring only when particular male and female genotypes are combined (Zeh and Zeh 1996, 1997; Colegrave et al. 2002; Neff and Pitcher 2005). A genetically compatible mate is not necessarily a high quality one, in which case signals of male health, vigour or condition are redundant. Genetic compatibility demands that an individual is able to recognise its own genotype as well as those of potential mates and use this as the basis to mate choice (Charlesworth et al. 1987).

In rose bitterling (*Rhodeus ocellatus*) the genetic benefits of mate choice arise predominantly through genetic compatibility (Agbali et al. 2010). Compatibility in this species correlates with the functional dissimilarity of major histocompatibility complex (MHC) alleles, which implicates MHC genes in mate compatibility recognition. A number of studies suggest MHC genes can influence odour, thus MHC genes have the potential to mediate an odour-based recognition system that enables females to

recognise genetically compatible mates (Penn and Potts 1999; Tregenza and Wedell 2000; Penn 2002). In many fish species, olfactory cues have key functions in behaviours such as predator avoidance, shoaling, kin recognition, parental care, foraging and migration (Quinn and Hara 1986; Brown and Brown 1992; Reeb 1994; Ostroumov 1997; Gerlach et al. 2008), and their role in mate choice is also well recognised (Wootton 1998).

Here the aim was a preliminary investigation of the role of olfactory cues in female spawning decisions in the rose bitterling, a small cyprinid fish with a resource-based mating system in which female mate choice arises through non-additive genetic benefits linked to MHC dissimilarity (Agbali et al. 2010). During spawning males consistently release sperm into a mussel prior to a female spawning, termed pre-oviposition ejaculation. The function of pre-oviposition ejaculation in bitterling has been proposed as a means of obtaining sperm precedence (Smith et al. 2004b). However, it may also operate in mate choice, with male olfactory cues present in sperm reaching the female when she inspects the exhalant siphon of a mussel prior to spawning. We specifically tested female responses to sperm collected from multiple males and released into mussels in comparison with a water control. By presenting sperm from multiple males the probability of at least one sperm donor being a compatible male would be increased. Our prediction was that females would be capable of detecting sperm in the mussel and would consequently show a greater behavioural response, principally through mussel inspection, and spawn more eggs in response.

MATERIALS AND METHODS

SPERM COLLECTION

Rose bitterling used in the experiment were the first generation bred in the laboratory and were from the River Yangtze Basin, China. Six males were haphazardly selected from stock aquaria, and kept together for one day in a separate aquarium measuring 120 (length) x 40 (width) x 45 (depth) cm with six females in spawning condition and one *U. pictorum* fresh water mussel. *R. ocellatus* readily spawn in *Unio pictorum* (Casalini 2007), thus this species of mussel was used in trials. The mussel was covered with perforated plastic cup that allowed the fish to inspect the mussel, but prevented spawning or sperm release. On the following morning sperm were collected from the six males. Sperm was stripped separately from each male in 5 ml of water by gently pressing their abdomens. A 3 ml subsample of each sperm solution was combined and mixed with 2 ml of fresh water to give a sperm solution of 20 ml obtained from six males.

OLFACTION TEST

Test females were haphazardly selected from stock aquaria. Female response to sperm was tested in the presence of a single free-swimming male since isolated females do not readily inspect mussels, even with a bottled male present. A male was randomly chosen from a different stock aquarium to that of the female and placed in the experimental tank, which measured 25 (length) x 40 (width) x 30 (depth) cm and contained a single *U. pictorum* mussel, and left alone overnight to establish territoriality. Opaque barriers prevented visual contact with adjacent fish. On the following morning a female with an extended ovipositor was released in the tank after covering the mussel with a transparent perforated plastic cup to prevent the female spawning, and allowed to settle. When the female began inspecting the exhalant siphon of the mussel it was uncovered and the female randomly exposed to either the sperm solution or a water control. To

release either treatment a plastic tube measuring 65 cm was suspended 5 cm above the inhalant siphon of the mussel and connected to 20 ml plastic syringe containing sperm solution or fresh water (see Fig. 7.1). Once the female was inspecting the mussel the liquid was gently released by depressing the syringe at a rate of 4 ml per minute. The response of the female was videoed during the release of either test liquid for 5 min. and female behaviour later scored using a palm computer with the FIT-system behaviour recording software (Held and Manser 2005). The behaviours scored were: frequency and duration of mussel inspection (the fish positioned with her snout close to the exhalant siphon of the mussel), female skimming behaviour (female performs spawning action without depositing eggs in the mussel) (Smith et al. 2004b), and frequency of territorial male ejaculation. After the first trial the mussel was again covered and the pair of fish left for 1 h. After this period the mussel was uncovered and the process repeated using the alternative treatment. If a female spawned during a trial the number of eggs that had been deposited inside the mussel was counted using a mussel-opening device (Kitamura 2005).

STATISTICAL ANALYSIS

A total of 12 independent replicates were completed. The normality of data were examined using a Shapiro-Wilk test and equality of variance using Bartlett's test. Data that did not meet assumptions of normality or homoscedasticity were transformed. Paired t-tests were used to compare the number of eggs laid, male and female inspection rate and duration, female skimming rate, and male sperm release rate between sperm solution and fresh water treatments. An unpaired t-test test was used to test the effect of order of spawning on the number of eggs laid. The association between female size and the total number of eggs laid was tested using Pearson's correlation.

RESULTS

There was a significant difference in the number of eggs laid by females in mussels between the addition of a sperm solution and water (paired t-test, $t_{11} = 3.15$, $P = 0.009$; Fig. 7.2). There was no effect of order of spawning on the number of eggs laid (unpaired t-test, $t_{22} = 1.00$, $P = 0.326$). There was also no correlation between female size and the total number of eggs laid (Pearson's correlation, $r_{10} = 0.23$, $p = 0.472$).

There was no significant difference in the rate of mussel inspection by females when the sperm solution was added compared to a water control (paired t-test, $t_{11} = 1.64$, $p = 0.130$). The mean \pm SE of female inspection rate was 10.0 ± 1.35 (10 min^{-1}). There was also no significant difference in the duration of mussel inspection by females (paired t-test, $t_{11} = 1.51$, $p = 0.160$). The mean \pm SE duration of inspection was 13.4 ± 1.75 s. Similarly, there was no significant difference in the rate (paired t-test, $t_{11} = 0.41$, $p = 0.692$) or duration (paired t-test, $t_{11} = 1.01$, $p = 0.336$) of mussel inspection by males between treatments, with the mean \pm SE rate of inspection 15.3 ± 1.38 (10 min^{-1}) and duration 9.9 ± 1.17 s. In addition, there was no significant difference in the rate of skimming by females between treatments (paired t-test, $t_{11} = 0.34$, $p = 0.740$; mean \pm SE rate 1.2 ± 0.48 10 min^{-1}). Finally, there was no significant difference in the rate of ejaculation by males between treatments (paired t-test, $t_{11} = 0.68$, $p = 0.510$). The mean \pm SE rate of ejaculation was 3.8 ± 0.95 (10 min^{-1}).

DISCUSSION

The goal of the present study was to evaluate the effect of chemical cues in spawning decisions in the rose bitterling, a fish that shows non-additive genetic benefits of mating that are linked to MHC dissimilarity in mate choice. The prediction tested was that

females would be more predisposed to spawn in mussels in which the odour of the sperm of compatible males was present. By presenting sperm from multiple males the probability of at least one donor being a compatible male was magnified. Female response to odours was measured in terms of number of eggs laid and mussel-orientated behaviours. The results showed that female spawning rate was significantly higher into mussels with sperm derived from multiple males added. Similarly in a rare field behavioural study using *R. ocellatus* conducted by Kanoh (2000), females were more predisposed to spawn when a group of males released sperm into a mussel than when a female spawned alone with a male. However, with the exception of this result, there was no significant effect on female or male behaviour in the present study.

Female preferences for the scent of specific males can play a role in mate choice (Krackow and Matuschak 1991; Coopersmith and Lenington 1992). Here the results suggest that female bitterling may use olfactory cues to detect and distinguish the presence of spermatozoa from compatible males released into a mussel by inspecting its exhalant siphon. In some fish species, male-derived chemical cues are critical for ovulation. For example, in the zebra fish (*Danio rerio*) and African walking catfish (*Clarias gariepinus*) females must be exposed to olfactory cues derived from conspecific males prior to spawning (Resink et al. 1989a, 1989b; Van den Hurk et al. 1987; Spence et al. 2008). Although it has been proposed that a variety of chemicals may function as sexual pheromones in teleosts, only steroids and prostaglandins, which are produced by the gonads, have been found to regulate reproduction and serve as sexual signals (Karlson and Lüscher 1959; Sorensen and Stacey 2004). In the black goby, *Gobius niger*, and walking catfish, males release steroid-based pheromones from specialized vesicles that attract females (Bruton 1979; Colombo et al. 1980; Resink et al. 1989a; Lambert and Resink 1991). Perhaps the best studied fish model for research

on sex pheromones is the goldfish (*Carrasius auratus*). In this species males release a large quantity of androstenedione with a small amount of other related steroids and these play a key a role in intrasexual and intersexual selection (Sorensen et al. 2005).

Mate choice for compatibility may depend on a form of self-referent genotype matching, perhaps based around a highly variable gene complex such as MHC (Jordan and Bruford 1998; Penn and Potts 1999; Tregenza and Wedell 2000; Penn 2002). Female Atlantic salmon (*Salmo salar*) appear to prefer mating with males with a more dissimilar MHC genotype, which thereby increases heterozygosity (Landry et al. 2001; Consuegra and Garcia de Leaniz 2008). Female rose bitterling mate preference, which has the effect of elevating offspring fitness, corresponds with MHC dissimilarity (Agbali et al. 2010). MHC genes have been linked with olfactory cues that function in mate choice in several species (Milinski et al. 2005). In fish, chemical cues may be released in the urine, faeces, gonadal fluids and with movement of slime through the gills (Stacey and Sorenson 2006; Rosenthal and Lobel 2006). Thus, if female bitterling detect compatible mates using MHC-specific odours males may actively release and disseminate them. A suggestion was that males might release olfactory cues during courtship in their urine (Casalini et al. 2009), though evidence that they do so has hitherto been lacking (Agbali, unpublished pilot data). Alternatively, males may release olfactory cues with sperm products when they ejaculate over the inhalant siphon of a mussel (Y. Kanoh, personal communication), with females able to detect MHC-specific cues by inspecting the water flow from the exhalant siphon of mussel.

In the present study neither females nor males displayed a significant difference in the rate or duration of mussel inspection when sperm from a group of males was released over a mussel. This result mirrors a field experiment by Smith et al. (2003) who investigated the effect on mussel choice of territorial male European bitterling

(*Rhodeus amarus*) when sperm was released over the inhalant siphon of a mussel in the presence of potential competitor. In this study females did not show a significant difference in inspection rate between treatments. Territorial males did inspect mussels more frequently if a rival's sperm was released into a mussel, but they did not alter the rate of other behaviours, including leading females to spawn. Thus, although male European bitterling appeared to detect experimentally released sperm they showed no adaptive response to it. In the present study, territorial male rose bitterling routinely released sperm into experimental mussels, though the rate was not different between treatments.

Female bitterling are highly selective with respect to their choice of mussels for spawning, with mussel quality related to mussel species and number of bitterling eggs already inside the mussel (Smith et al. 2000b, 2001; Reichard et al. 2007b). High numbers of eggs in a mussel correlate significantly with embryo mortality rate during development (Smith et al. 2000b, 2001). High egg density also has significant long-term effects on offspring fitness (Agbali and Smith in review). Female mussel choice appears to be based on an assessment of dissolved oxygen in water emerging from the exhalant siphon of a mussel (Smith et al. 2001; Mills and Reynolds 2002; Reichard et al. 2007b). In addition, female bitterling appear to respond to specific amino acids in making spawning decisions, particularly glycine, alanine, tryptophan and glutamine, which are all amino acids released naturally by mussels and which appear to serve in mussel species recognition (Y. Kanoh, unpublished data). Thus females make a series of decisions during spawning based on olfactory cues that enable them to determine whether the mussel is an appropriate species as a site for oviposition and whether oxygen conditions inside the mussel gill are suitable for embryo development. In addition, the results of the present study hint that females may use olfactory cues linked

to sperm release that may indicate the degree of compatibility of males. However, these results are preliminary and require further verification.

In conclusion, although there was no difference in the rate or duration of female inspection of mussels between treatments in which sperm from multiple males was added to a mussel compared to only a single male, female rose bitterling showed a greater predisposition to spawn in the former case. This result suggests that females are able to detect the presence of sperm inside a mussel and the mechanism is most likely linked to olfactory cues. However, the role olfactory cues in reproductive biology of bitterling require more extensive study to identify the identities of the cues used, their source and their mechanism of release.

Figure 7.1. Stylised design of olfaction test. A female (α) and male (β) rose bitterling where presented with a freshwater mussel (γ). Using a syringe (δ) attached to a length of silicon tubing (ϵ) either sperm (ζ) or water could be gently released over the inhalant siphon of the mussel.

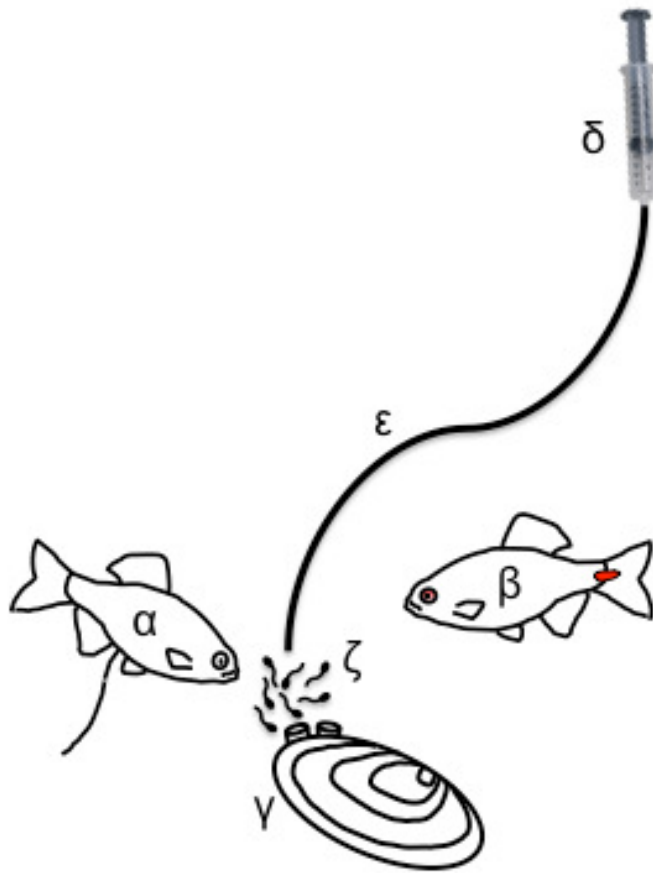
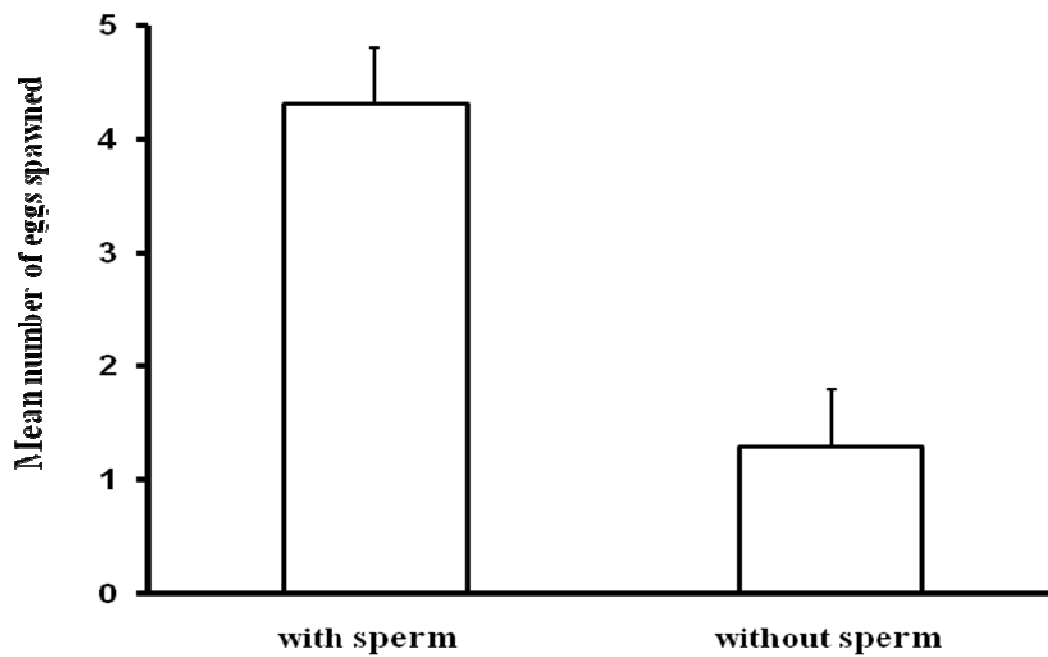


Figure 7.2. Mean \pm SE number of eggs spawned by female rose bitterling over a period of 5 min. into mussels into which sperm or a water control were added.



Chapter eight: Female mating decisions in the rose bitterling (*Rhodeus ocellatus*)

Synthesis

The overall objective of this study was to obtain an understanding of mating decisions of female rose bitterling. The specific aims of this thesis were to understand: 1. the consequences of female mate preferences; 2. the role of genetic benefits in female mate choice; 3. the cues used by females in mate choice; 4. the relative role of direct and indirect benefits of female mate choice; 5. The consequences of female spawning site choice.

Male dominance has traditionally been believed to be a reliable indicator of male quality; in nature dominant *R. ocellatus* males may monopolize territories with more high-quality spawning sites. Therefore, mate preference for dominant males may provide females with direct and indirect benefits. In many taxa female mate preferences correspond with sexual secondary traits of dominance, such as body size and brighter colouration (Møller et al. 1998; Evans et al. 2004; Charlton et al. 2007). However, I demonstrated that an intersexual conflict occurs in the mating system of the rose bitterling because female mate preferences do not correspond with male dominance (**Chapter 2**). Offspring survival to independence was correlated with female mate preferences but not with male dominance. This result is consistent with recent studies on other related species (Reichard et al. 2005; Spence and Smith 2006).

Casalini et al. (2009) showed a lack of congruence in female mate preferences in *R. ocellatus* for males, which suggests a role for parental genetic compatibility in mate choice. This finding was further investigated using a sequential blocked mating design

combined with a cross-classified breeding design coupled with *in vitro* fertilizations to measure the fitness benefits of mate choice (**Chapter 3**). Females again were shown to have significant mate preferences, but preferences were not congruent among females. The cross-classified breeding design showed a significant interaction of male and female genotype on offspring survival, rate of development, growth rate, and body size, while no significant male additive effects on offspring fitness were observed. Offspring produced by the pairing of preferred and nonpreferred males were reared to maturity but no differences in fitness traits were shown. Female mate preferences corresponded with male genetic compatibility, which correlated with MHC dissimilarity. These findings suggested that genetic compatibility is the mechanism by which females obtain a fitness benefit through mate choice and that male MHC dissimilarity, probably mediated by odour cues, indicates genetic compatibility. The results of **Chapter 3** reinforces previous findings that proposed genetic benefits of female mate choice through genetic compatibility mediated by MHC genes in fish (Forsberg et al. 2007; Consuegra and Garcia de Leaniz 2008; Yeates et al. 2009).

Both direct and indirect benefits of mate choice have a role in the bitterling mating system. In **Chapters 2 and 3** female bitterling showed a preference for specific males, and mate choice correlated with indirect benefits to females. Moreover, female bitterling are also choosy about which freshwater mussels they use for oviposition, which represent direct effects for fitness, and display strong selection for mussel quality, which is essential for egg and embryo survival in the absence of parental care (Smith et al. 2000b, 2001; Reichard et al. 2004a, 2005). However, the relative role of direct and indirect benefits in mate choice in bitterling was not known, and also in few other species. Using data from Chapter 3 a study to compare direct and indirect benefits in the mating system was undertaken (**Chapter 4**). The response of female bitterling to

potential direct benefits of oviposition choice, and indirect benefits of mate choice were measured in terms of offspring survival rates to independence, with female mating preferences measured in terms of reproductive investment. Direct benefits were varied using high and low quality sites for oviposition, and male quality using males of known genetic compatibility. The results showed a significant effect for direct benefits, in the form of oviposition site quality, in mate choice decisions in the rose bitterling. Indirect benefits also played a role, but accounted for a relatively minor component of variance in female oviposition rate. These are the first results support the idea that direct benefits impose a greater effect on female mating decision, which may refocus the current intense interest in the role of indirect benefits. The explanation for these findings is that oviposition decisions may have direct consequences for offspring survival rate, but also longer-term effects on fitness. Therefore, the long-term consequences of female oviposition decisions were further examined by raising embryos at high and low densities for the first four weeks after fertilisation, the period that corresponds with incubation of embryos in a mussel gill, then at a common low density until sexual maturity (**Chapter 6**). The results showed no significant effect of density on offspring survival or growth. However, there was a significant impact of density on age and size at maturity, with embryos raised at a high density becoming sexually mature earlier and at a significantly smaller size than those raised at low density. This effect may arise through embryos coping with low oxygen conditions during development.

The possible mechanisms of mate choice were investigated in two studies. The first examined a possible role of a receiver bias (**Chapter 5**). I tested for a sensory bias for red in a non-mating context and found that bitterling show a strong response to red food items, irrespective of age or sex. This correlation between a sexually selected trait and an intrinsic attraction to red appears to support the receiver-bias hypothesis for the

evolution of red nuptial colouration in rose bitterling. Red nuptial coloration in bitterling does not signal mussel quality (Casalini, unpublished data), nor is there an association between male colour and offspring quality (Casalini et al. 2009; Agbali et al. 2010). However, a possible role for red colour in mate choice is through an innate attraction to females, which may signal the presence of a spawning site, but without signaling information about direct or indirect benefits, which would be consistent with the receiver-bias hypothesis (Ryan 1990; Ryan and Rand 1990; Endler 1992; Endler and Basalo 1998).

In a further study I examined the role of olfactory cues in the spawning decisions of the rose bitterling (**Chapter 7**). Given that in this species mate choice is based on genetic compatibility, and correlates with the functional dissimilarity of MHC, a role for olfactory cues is likely. An hypothesis is that olfactory cues are released by males during ejaculation. Female responses to the sperm of multiple males released over spawning sites were investigated. Female responses were measured in terms of oviposition and rate and duration of inspection of mussel spawning sites. The results showed that spawning rate was significantly higher with sperm added to mussels in comparison with a water control. However, there was no significant difference in the rate or duration of female inspection of spawning sites. The results suggest that female may be able to evaluate the presence of sperm inside a mussel, and this response may be linked to olfactory cues. This response to the presence of sperm from multiple males has also been observed in the related European bitterling (Smith et al. 2003). Although, the results suggest female use olfactory cues as a mechanism to detect compatible sperm through MHC-specific odour, these results are also consistent with a role for cues associated with other functional genes, and thus warrants further investigation.

In summary, the implications of the findings in this study for the mating system of rose bitterling are:

- Females perform mate choice, but are not congruent in their preferences. Female mate preferences correlate positively with offspring growth rate and survival.
- Female mate choice does not correspond with male dominance and there may be an intersexual conflict as a result.
- Females prefer males with functionally dissimilar MHC alleles. MHC alleles may influence male odour cues, and females show a preference for the sperm of multiple males based on odour cues.
- There may be a receiver bias for red nuptial colouration in female mate choice.
- Direct (oviposition) mating preferences are more important than indirect (mate choice) preferences.
- Choice of oviposition sites may have immediate (survival) consequences for offspring, as well as longer-term fitness effects for offspring.

Future research directions

There are some areas of research that this study has identified as potentially worthwhile pursuing in the future. In this thesis I demonstrated a significant interaction of male and female genotype on offspring fitness, and mate preferences of rose bitterling also corresponded with male genetic compatibility, which correlated with MHC dissimilarity. However, our results showed only a weak effect of MHC dissimilarity on mate choice and offspring survival. Therefore, a more complete analysis of MHC class II including both the DAB1 and DAB3 genes could demonstrate a stronger influence of MHC dissimilarity on mate choice and offspring fitness.

A second point for future studies would be to examine the mechanisms that female uses to detect genetic compatibility. The results of the current study strongly suggest a role for olfactory cues in mate choice that may be released with sperm over the mussel. Here, it would interesting to compare female response for the odours of compatible and incompatible sperm, and also to test whether male release chemical cues linked to mate choice in their urine. Mate choice by female *R. ocellatus* appears to be dependent more on courtship behaviour than on secondary sexual traits related to male dominance. Thus further study might also examine the role of courtship behaviour in female mate choice, which may have a role in dispersing male odours to females. In bitterling, males display red carotenoid-based colouration in the iris and on the caudal fin. Male secondary sexual traits are typically expected to signal direct and indirect benefits of female mate choice. However, in this thesis there was no evidence that male colouration had a role in female mate choice. Thus it would be interesting to investigate the functional role of red colour in male–male competition.

Mussels had an important role in female mating decision as direct benefits and females are precisely able to measure mussel quality. Therefore, another avenue of research pursuing is in examining how females determine mussel quality. Future studies should try to test the function of amino acids that are released by the mussel in female oviposition choice and mating decision.

REFERENCES

- Agbali, M., M. Reichard, A. Bryjova, J. Bryja, and C. Smith. 2010. Mate choice for non-additive genetic benefits correlate with MHC dissimilarity in the rose bitterling (*Rhodeus ocellatus*). *Evolution* 64:1683-1696.
- Aldridge, D. 1999. Development of European bitterling in the gills of freshwater mussels. *J. Fish Biol.* 54:138-151.
- Almeida, O. G., A. Miranda, P. Frade, P. C. Hubbard, E. N. Barata, and A.V. M. Canário. 2005. Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). *Chem. Senses* 30(Suppl 1):i309-i310.
- Alonzo, S. H., and R. R. Warner. 1999. A trade-off generated by sexual conflict: Mediterranean wrasse males refuse present mates to increase future success. *Behav. Ecol.* 10:105-111.
- Alonzo, S. H., and R. R. Warner. 2000. Dynamic games and field experiments examining intra- and intersexual conflict: explaining counterintuitive mating behavior in a Mediterranean wrasse, *Symphodus ocellatus*. *Behav. Ecol.* 11:56-70.
- Andersson, M. 1982. Female choice selects for extreme tail length in a widow bird. *Nature* 299:818-820.
- Andersson, M. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ.
- Andersson, M., and L. W. Simmons. 2006. Sexual selection and mate choice. *Trends Ecol. Evol.* 21:296-302.
- Arai, R. 1988. Fish systematics and cladistics. In *Ichthyology Currents*, 4-33. (Uyeno, T. and Okiyama, M. Eds.), Tokyo: Asakura Shoten.
- Arkush, K. D., A. R. Giese, H. L. Mendonca, A. M. McBride, G. D. Marty, and P. W. Hedrick. 2002. Resistance to three pathogens in the endangered winter-run chinook salmon (*Oncorhynchus tshawytscha*): effects of inbreeding and major histocompatibility complex genotypes. *Can. J. Fish. Aquat. Sci.* 59:966-975.
- Arnqvist, G., and L. Rowe. 1995. Sexual conflict and arms races between the sexes: a morphological adaptation for control of mating in a female insect. *Proc. R. Soc. Lond. B* 261:123-127.
- Arnqvist, G., and R. Rowe. 2005. Sexual conflict. Princeton Univ. Press, Princeton, NJ.
- Arnqvist, G. 2006. Sensory exploitation and sexual conflict. *Phil. Trans. R. Soc. B* 361:375-386.
- Atema, J. 1996. Eddy chemotaxis and odor landscapes: exploration of nature with animal sensors. *Biol. Bull.* 191:129-138.
- Bakker, T. 1993. Positive genetic correlation between female preference and preferred male ornament in sticklebacks. *Nature* 363:255-257.

- Barber, I., S. A. Arnott, V. A. Braithwaite, J. Andrew, and F. A. Huntingford. 2000. Carotenoid-based sexual coloration and body condition of nesting male sticklebacks. *J. Fish Biol.* 57:777-790.
- Barber, I., S. A. Arnott, V. A. Braithwaite, J. Andrew, and F. A. Huntingford. 2001. Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasite infections. *Proc. R. Soc. Lond. B* 268:71-76.
- Barton, N. 2001. The role of hybridization in evolution. *Mol. Ecol.* 10:551-568.
- Basolo, A. 1990. Female preference predates the evolution of the sword in swordtail fish. *Science* 250:808-810.
- Berglund, A., A. Bisazza, and A. Pilastro. 1996. Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biol. J. Linn. Soc.* 58:385-399.
- Bisazza, A., and A. Marconato. 1988. Female mate choice, male-male competition and parental care in the river bullhead, *Cottus gobio* L. (Pisces, Cottidae). *Anim. Behav.* 36:1352-1360.
- Bonneaud, C., O. Chastel, P. Federici, H. Westerdahl, and G. Sorci. 2006. Complex *Mhc*-based mate choice in a wild passerine. *Proc. R. Soc. Lond. B* 273:1111-1116.
- Bourne, G., F. Breden, and T. Allen. 2003. Females prefer carotenoid colored males as mates in the pentamorphic livebearing fish, *Poecilia parae*. *Naturwissenschaften* 90:402-405.
- Boynton, R. M. 1979. Human Color Vision. Holt, Rinehart and Winston, New York.
- Bro-Jørgensen, J. 2003. The significance of hotpots to lekking topi antelopes (*Damaliscus lunatus*). *Behav. Ecol. Sociobiol.* 53:324-331.
- Brown, G., and J. Brown. 1992. Do rainbow trout and Atlantic salmon discriminate kin? *Can. J. Z.* 70:1636-1640.
- Bruton, M. 1979. The breeding biology and early development of *Clarias gariepinus* (Pisces: Clariidae) in Lake Sibaya, South Africa, with a review of breeding in the subgenus *Clarias* (Clarias). *Trans. Zool. Soc. Lond.* 35:1-45.
- Bryja, J., M. Galan, N. Charbonnel, and J. F. Cosson. 2005. Analysis of major histocompatibility complex class II gene in water voles using capillary electrophoresis-single stranded conformation polymorphism. *Mol. Ecol. Notes* 5:173-176.
- Byrne, P. G., and J. D. Roberts. 1999. Simultaneous mating with multiple males reduces fertilization success in the myobatrachid frog *Crinia georgiana*. *Proc. R. Soc. Lond. B* 266: 717-721.
- Byrne, P. G., and J. D. Roberts. 2000. Does multiple paternity improve fitness of the frog *Crinia georgiana*?. *Evolution* 54:968-973.
- Candolin, U. 1999. Male-male competition facilitates female choice in sticklebacks. *Proc. R. Soc. Lond. B* 266:785-789.

- Candolin, U., and J. D. Reynolds. 2001. Sexual signaling in the European bitterling: females learn the truth by direct inspection of the resource. *Behav. Ecol.* 12:407-411.
- Candolin, U., and J. D. Reynolds. 2002a. Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (*Rhodeus sericeus*). *Proc. R. Soc. Lond. B* 269: 1549-1553.
- Candolin, U., and J. D. Reynolds. 2002b. Why do males tolerate sneakers? Tests with the European bitterling, *Rhodeus sericeus*. *Behav. Ecol. Sociobiol.* 51:146-152.
- Casalini, M. 2007. Mate choice and oviposition decisions in the rose bitterling (*Rhodeus ocellatus*). MSc thesis, Univ. of Padua, Padua, Italy.
- Casalini, M., M. Agbali, M. Reichard, M. Konečna, A. Bryjova, and C. Smith. 2009. Male dominance, female mate choice and intersexual conflict in the rose bitterling (*Rhodeus ocellatus*). *Evolution* 63:366-376.
- Casalini, M., M. Reichard, and C. Smith. 2010. The effect of crowding and density on male mating tactics in the rose bitterling (*Rhodeus ocellatus*). *Behaviour* 147:1035-1050.
- Charlesworth, B., J. A. Coyne, and N. H. Barton. 1987. The relative rates of evolution of sex chromosomes and autosomes. *Am. Nat.* 130:113-146.
- Charlton, B., D. Reby, and K. McComb. 2007. Female red deer prefer the roars of larger males. *Biol. Lett.* 3:382-385.
- Chen, I-S., and Y-C. Chang. 2005. A photographic guide to the inland fishes of Taiwan. Vol.1. SueiChan Press, Keelung, Taiwan.
- Chew, B., and J. Park. 2004. Carotenoid Action on the Immune Response. *J. Nutr.* 134:257S-261S.
- Chew, F. S. 1977. Coevolution of pierid butterflies and their cruciferous foodplants. II. The distribution of eggs on potential foodplants. *Evolution* 31:568-579.
- Clarke, B., and D. R. S. Kirby. 1966. Maintenance of histocompatibility polymorphisms. *Nature* 211:999-1000.
- Colegrave, N., J. S. Kotiaho, and J. L. Tomkins. 2002. Mate choice or polyandry: reconciling genetic compatibility and good genes sexual selection. *Evol. Ecol. Res.* 4:911-917.
- Colombo, L., A. Marconato, P. Belvedere, and C. Frisco. 1980. Endocrinology of teleost reproduction: a testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* 47: 355-367.
- Consuegra, S., and C. Garcia de Leaniz. 2008. MHC-mediated mate choice increases parasite resistance in salmon. *Proc. R. Soc. Lond. B* 275:1397-1403.
- Coopersmith, C., and S. Lenington. 1992. Female preferences based on male quality in house mice: interactions between male dominance rank and t-complex genotype. *Ethology* 90:1-16.

- Cope, J., and C. Fox. 2003. Oviposition decisions in the seed beetle *Callosobruchus maculatus* (Coleoptera: Bruchidae): effects of seed size on superparasitism. *J. Stor. Prod. Res.* 39: 355-365.
- Darwin, C. 1871. *The descent of man, and selection in relation in sex*. John Murray, London.
- Dawson, D. A., T. M. Burland, A. E. Douglas, S. C. Le Comber, and M. Bradshaw. 2003. Isolation of microsatellite loci in the freshwater fish, the bitterling *Rhodeus sericeus* (Teleostei: Cyprinidae). *Mol. Ecol. Notes* 3:199-202.
- Devlin, R., and Y. Nagahama. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208:191-364.
- Dmitrijeva, E., M. Peňaz, M. Prokeš, and I. R'abov. 1985. Ecological groups of fishes of the family Cyprinidae in Czechoslovakia and the European part of the USSR. *Top. Probl. Ichthyol.* 2:19-36.
- Dyer, A. G., and L. Chittka. 2004. Fine colour discrimination requires differential conditioning in bumblebees. *Naturwissenschaften* 91:224-227.
- Dziminski, M. A., D. J. Roberts, and L. W. Simmons. 2008. Fitness consequences of parental compatibility in the frog *Crinia georgiana*. *Evolution* 62:879-886.
- Eberhard, W. G. 1996. *Female control: sexual selection by cryptic female choice*. Princeton Univ. Press, Princeton, NJ.
- Eggert, F., W. Müller-Ruchholtz, and R. Ferstl. 1999. Olfactory cues associated with the major histocompatibility complex. *Genetica* 104:191-197.
- Endler, J. A. 1992. Signals, signal conditions, and the direction of evolution. *Am. Nat.* 139: S125-S153.
- Endler, J., and A. Basolo. 1998. Sensory ecology, receiver biases and sexual selection. *Trends Ecol. Evol.* 13:415-420.
- Evans, J., A. Bisazza, and A. Pilastro. 2004. Female mating preferences for colourful males in a population of guppies subject to high predation. *J. Fish Biol.* 65:1154-1159.
- Evans, M., and K. Norris. 1996. The importance of carotenoids in signaling during aggressive interactions between male firemouth cichlids (*Cichlasoma meeki*). *Behav. Ecol.* 7:1-6.
- Fisher, R. A. 1930. *The genetical theory of sexual selection*. Oxford Univ. Press, Oxford.
- Fisher, R. 1958. *The genetical theory of natural selection*. Dover Publications, New York.
- Forsgren, E. 1997. Female sand gobies prefer good fathers over dominant males. *Proc. R. Soc. Lond. B* 264:1283-1286.
- Forsberg, L. A., J. Dannewitz, E. Petersson, and M. Grahn. 2007. Influence of genetic dissimilarity in the reproductive success and mate choice of brown trout - females fishing for optimal MHC dissimilarity. *J. Evol. Biol.* 20:1859-1869.

- Forstmeier, W., B. Kempenaers, A. Meyer, and B. Leisler. 2002. A novel song parameter correlates with extra-pair paternity and reflects male longevity. *Proc. R. Soc. Lond. B* 269: 1479-1485.
- Fu, P., B. D. Neff, and M. R. Gross. 2001. Tactic-specific success in sperm competition. *Proc. R. Soc. Lond. B* 268:1105-1112.
- Fuller, R., D. Houle, and J. Travis. 2005. Sensory bias as an explanation for the evolution of mate preferences. *Am. Nat.* 166:437-446.
- Gamberale-Stille, G., and B. S. Tullberg. 2001. Fruit or aposematic insect? Context-dependent colour preferences in domestic chicks. *Proc. R. Soc. Lond. B* 268:2525-2529.
- Gerlach, G., A. Hodgins-Davis, C. Avolio, and C. Schunter. 2008. Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proc. R. Soc. Lond. B* 275:2165-2170.
- Getahun, D., C. V. Ananth, N. Selvam, and K. Demissie. 2005. Adverse perinatal outcomes among interracial couples in the United States. *Obstet. Gynecol.* 106:81-88.
- Gluckman, P., and M. Hanson. 2004. Maternal constraint of fetal growth and its consequences. *Semin. Fetal Neonat. Med.* 9:419-425.
- Gluckman, P., M. Hanson, H. Spencer, and P. Bateson. 2005. Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. *Proc. R. Soc. B* 272:671-677.
- Godfrey, H. 1994. *Parasitoids: behavioural and evolutionary ecology*. Chichester, UK: Princeton Univ. Press.
- Goodwin, T. 1984. *The biochemistry of the carotenoids*. Chapman and Hall, London.
- Gowaty, P. A. 1997. Sexual dialectics, sexual selection, and variation in mating behavior. Pp. 351-384 in P. A. Gowaty, ed. *Feminism and evolutionary biology*. Chapman and Hall, New York.
- Gross, M. R. 1996. Alternative reproductive strategies and tactics: diversity within sexes. *Trends Ecol. Evol.* 11:92-97.
- Gumbert, A. 2000. Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. *Behav. Ecol. Sociobiol.* 48:36-43.
- Hamilton, W., and M. Zuk. 1982. Heritable true fitness and bright birds: a role for parasites? *Science* 218:384-387.
- Hardy, C. 2002. *Sex ratios: concepts and research methods*. Cambridge: Cambridge Univ. Press.
- Head, M. L., J. Hunt, M. D. Jennions, and R. Brooks. 2005. The indirect benefits of mating with attractive males outweigh the direct costs. *PLoS Biol.* 3:e33.
- Held, J., and T. Manser. 2005. A PDA-based system for online recording and analysis of concurrent events in complex behavioral processes. *Behav. Res. Methods* 37:155-164.
- Heisler, L., M. Andersson, S. Arnold, C. Boake, G. Borgia, G. Iiausfater, M. Kirkpatrick, R. Lande, J. Maynard Smith, P. O'donald, A. Thornhill, and F. Weissing. 1987. *The*

- evolution of mating preferences and sexually selected traits. Pp. 96-118 *in* Group Report. J. Bradbury, and M. Andersson, eds. Sexual selection: testing the alternatives. John Wiley, New York.
- Hill, A. V. S. 2001. Immunogenetics and genomics. *The Lancet* 357:2037-2041.
- Hochachka, P. 1980. Living without oxygen. Cambridge: Harvard Univ. Press.
- Holland, B., and W. Rice. 1998. Chase-away sexual selection: antagonistic seduction versus resistance. *Evolution* 52: 1-7.
- Houde, A. 1997. Sex, color and mate choice in guppies. Princeton Univ. Press, Princeton.
- Hughes, A., S. Saszik, J. Bilotta, Jr. DeMarco, and W. Patterson. 1998. Cone contributions to the photopic spectral sensitivity of the zebrafish ERG. *Visual Neurosci.* 15:1029-1037.
- Ibrahim, A., and F. Huntingford. 1989. Laboratory and field studies on diet choice in three-spined sticklebacks, *Gasterosteus aculeatus* L., in relation to profitability and visual features of prey. *J. Fish Biol.* 34:245-257.
- Ivy, T. M. 2007. Good genes, genetic compatibility and the evolution of polyandry: use of the diallel cross to address competing hypotheses. *J. Evol. Biol.* 20:479-487.
- Jacob, A., S. Nusslé, A. Britschgi, G. Evanno, R. Müller, and C. Wedekind. 2007. Male dominance linked to size and age, but not to 'good genes' in brown trout (*Salmo trutta*). *BMC Evol. Biol.* 7:207.
- Jaenike, J. 1978. On optimal oviposition behaviour in phytophagous insects. *Theor. Popul. Biol.* 14:350-356.
- Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. *Nature* 411:302-305.
- Johansson, B., and T. Jones. 2007. The role of chemical communication in mate choice. *Biol. Rev.* 82:265-289.
- Jones, A. G., D. E. Walker, C. Kvarnemo, K. Lindström, and J. C. Avise. 2001. How cuckoldry can decrease the opportunity for sexual selection: data and theory from genetic parentage analysis of the sand goby, *Pomatoschistus minutus*. *P NAS* 98:9151-9156.
- Jordan, W. C., and M. W. Bruford. 1998. New perspectives on mate choice and the MHC. *Heredity* 81:127-133.
- Kalbe, M., C. Eizaguirre, I. Dankert, T. B. H. Reusch, R. D. Sommerfeld, K. M. Wegner, and M. Milinski. 2009. Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity. *Proc. R. Soc. Lond. B* 276:925-934.
- Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* 16:1099-1006.
- Kamler, E. 1992. Early life history of fish: an energetic approach. Chapman and Hall, London.

- Kangas, N., and K. Lindström. 2001. Male interaction and female mate choice in the sand goby, *Pomatoschistus minutes*. *Anim. Behav.* 61:425-430.
- Kanoh, Y. 1996. Pre-oviposition ejaculation in externally fertilizing fish: how sneaker male rose bitterlings contrive to mate. *Ethology* 102:883-899.
- Kanoh, Y. 2000. Reproductive success associated with territoriality, sneaking and grouping in male rose bitterlings, *Rhodeus ocellatus* (Pisces: Cyprinidae). *Environ. Biol. Fish.* 57: 143-154.
- Karlson, P., and M. Lüscher. 1959. 'Pheromones': a new term for a class of biologically active substances. *Nature* 183:55-56.
- Kawamura, K. 1998. Sex determination system of the rosy bitterling, *Rhodeus ocellatus ocellatus*. *Environ. Biol. Fish.* 52:251-260.
- Kim, U., and S. Park. 1985. Eggs development and larvae of the rose bitterling *Rhodeus ocellatus* (KNER). *Bull. Kor. Fish. Soc.* 18:586-593.
- Kim, H., Y. Kim, J. Jo, G. Yoon, and B. Ha. 1999. Nuptial color component of the Korean rose bitterling (*Rodeus uyekii*). *J. Korean Fish. Soc.* 32:520-524.
- Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. *Evolution* 36:1-12.
- Kirkpatrick, M. 1987. Sexual selection by female choice in polygynous animals. *Annu. Rev. Ecol. Syst.* 18:43-70.
- Kirkpatrick, M., and N. H. Barton. 1997. The strength of indirect selection on female mating preferences. *Proc. Natl. Acad. Sci. USA* 94:1282-1286.
- Kirkpatrick, M., and M. J. Ryan. 1991. The evolution of mating preferences and the paradox of the lek. *Nature* 350:33-38.
- Kitamura, J. 2005. Factors affecting seasonal mortality of rosy bitterling (*Rhodeus ocellatus kurumeus*) embryos on the gills of their host mussel. *Popul. Ecol.* 47:41-51.
- Kitamura, J. 2006. Adaptive spatial utilization of host mussels by the Japanese rosy bitterling *Rhodeus ocellatus kurumeus*. *J. Fish Biol.* 69:263-271.
- Kitamura, J. 2007. Reproductive ecology and host utilization of four sympatric bitterling (Acheilognathinae, Cyprinidae) in a lowland reach of the Harai River in Mie, Japan. *Environ. Biol. Fish.* 78:37-55.
- Klein, J., R. E. Bontrop, R. L. Dawkins, H. A. Erlich, U. B. Gyllensten, E. R. Heise, P. P. Jones, P. Parham, E. K. Wakeland, and D. I. Watkins. 1990. Nomenclature for the major histocompatibility complexes of different species: a proposal. *Immunogenetics* 31:217-219.
- Knapp, R., and J. Kovach. 1991. Courtship as an honest indicator of male parental quality in the bicolor damselfish, *Stegastes partitus*. *Behav. Ecol.* 2:295-300.
- Kokko, H. 2005. Treat 'em mean, keep 'em (sometimes) keen: evolution of female preferences for dominant and coercive males. *Evol. Ecol.* 19:123-135.

- Kokko, H., R. Brooks, M. Jennions, and J. Morely. 2003. The evolution of mate choice and mating biases. *Proc. R. Soc. Lond. B* 270:653-664.
- Kokko, H., M. Jennions, and R. Brooks. 2006. Unifying and testing models of sexual selection. *Annu. Rev. Ecol. Evol. Syst.* 37:43-66.
- Kosakovsky Pond, S. L., and S. D. W. Frost. 2005. Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Mol. Biol. Evol.* 22:1208-1222.
- Kosakovsky Pond, S. L., S. D. W. Frost, and S. V. Muse. 2005. HyPhy: hypothesis testing using phylogenies. *Bioinformatics* 21:676-679.
- Kotiaho, J. S. 2001. Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. *Biol. Rev.* 76:365-376.
- Kotiaho, J., and M. Puurtinen. 2007. Mate choice for indirect genetic benefits: scrutiny of the current paradigm. *Funct. Ecol.* 21:683-644.
- Krackow, S., and B. Matuschak. 1991. Mate choice for nonsiblings in wild house mice: Evidence from a choice test and a reproductive test. *Ethology* 88:99-108.
- Lambert, J., and J. Resink. 1991. Steroid glucuronides as male pheromones in the reproduction of the African catfish *Clarias gariepinus*: a brief review. *J. Steroid Biochem. molec. Biol.* 40:549-556.
- Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. *Proc. Natl. Acad. Sci. USA* 78:3721-3725.
- Landry, C., D. Garant, P. Duchesne, and L. Bernatchez. 2001. "Good genes as heterozygosity": the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). *Proc. R. Soc. Lond. B* 268:1279-1285.
- Liljedal, S., G. Rudolfson, and I. Folstad. 2008. Factors predicting male fertilization success in an external fertilizer. *Behav. Ecol. Sociobiol.* 62:1805-1811.
- Lindström, K., and M. Hellström. 1993. Male size and parental care in the sand goby, *Pomatoschistus minutus*. *Ethol. Ecol. Evol.* 5:97-106.
- Lozano, G. 1994. Carotenoids, parasites, and sexual selection. *Oikos* 70:309-311.
- Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland, MA.
- Magurran, A., and M. Nowak. 1991. Another battle of the sexes: the consequences of sexual asymmetry in mating costs and predation risk in the guppy, *Poecilia reticulata*. *Proc. R. Soc. B* 246:31-38.
- Magurran, A., and B. Seghers. 1994a. A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proc. R. Soc. B* 258:89-92.
- Magurran, A., and B. Seghers. 1994b. Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proc. R. Soc. B* 255:31-36.
- Maynard Smith, J., and D. Harper. 2003. Animal signals. Oxford Univ. Press, Oxford.

- McLennan, D. A., and M. J. Ryan. 1997. Responses to conspecific and heterospecific olfactory cues in the swordtail *Xiphophorus cortezi*. *Anim. Behav.* 54:1077-1088.
- Milinski, M., and T. Bakker. 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature* 344:330-333.
- Milinski, M., S. Griffiths, K. M. Wegner, T. B. H. Reusch, A. Haas-Assenbaum, and T. Boehm. 2005. Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. *Proc. Natl. Acad. Sci. USA* 102:4414-4418.
- Mills, S., and J. Reynolds. 2002. Mussel ventilation rates as a proximate cue for host selection by bitterling, *Rhodus sericeus*. *Oecologia* 131:473-478.
- Møller, A. P., and R. V. Alatalo. 1999. Good-genes effects in sexual selection. *Proc. R. Soc. Lond. B* 266:85-91.
- Møller, A., N. Saino, G. Taramino, P. Galeotti, and S. Ferrario. 1998. Paternity and multiple signaling: effects of a secondary sexual character and song on paternity in the barn swallow. *Am. Nat.* 151:236-242.
- Moore, A. J., and P. J. Moore. 1999. Balancing sexual selection through opposing mate choice and male competition. *Proc. R. Soc. Lond. B* 266:711-716.
- Morris, D. 1955. The reproductive behaviour of the river bullhead (*Cottus gobio* L.) with special reference to the fanning activity. *Behaviour* 7:1-32.
- Mulder, R. A., P. O. Dunn, A. Cockburn, K. A. Lazenby-Cohen, and M. J. Howell. 1994. Helpers liberate female fairy wrens from constraints on extra-pair mate choice. *Proc. R. Soc. Lond. B* 255:223-229.
- Murphy, C. G. 1998. Interaction-independent sexual selection and the mechanisms of sexual selection. *Evolution* 52:8-18.
- Nagata, Y. 1985. Spawning period and migration of rose bitterling, *Rhodeus ocellatus*, in a small pond. *Jap. J. Ichthyol.* 32:79-89.
- Neff, B. D., and T. E. Pitcher. 2005. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. *Mol. Ecol.* 14:19-38.
- Nowak, M. A., K. Tarczy-Hornoch, and J. M. Austyn. 1992. The optimal number of major histocompatibility complex molecules in an individual. *Proc. Natl. Acad. Sci. USA* 89:10896-10899.
- Nystrom, M., A. Caughey, D. Lyell, M. Druzin, and Y. El-Sayed. 2008. Perinatal outcomes among Asian-white interracial couples. *Am. J. Obstet. Gynecol.* 199:385.e1-385.e5.
- Okazaki, M., K. Naruse, A. Shima, and R. Arai. 2001. Phylogenetic relationships of bitterling based on mitochondrial 12S ribosomal DNA sequences. *J. Fish Biol.* 58:89-106.
- Olson, V., and I. Owens. 1998. Costly sexual signals: are carotenoids rare, risky or required?. *Trends Ecol. Evol.* 13:510-514.

- Olsson, M., T. Madsen, E. Wapstra, B. Silverin, B. Ujvari, and H. Wittzell. 2005. MHC, health, color, and reproductive success in sand lizards. *Behav. Ecol. Sociobiol.* 58:289-294.
- Ostroumov, V. 1997. The role of chemical signals in the regulation of fish maturation and reproductive behavior. *J. Ichthyol.* 37:103-109.
- Ottová, E., A. Šimková, J. F. Martin, J. Goüy de Bellocq, M. Gelnar, J. F. Allienne, and S. Morand. 2005. Evolution and trans-species polymorphism of MHC class II β genes in cyprinid fish. *Fish Shellfish Immunol.* 18:199-222.
- Parker, G. A., and L. Partridge. 1998. Sexual conflict and speciation. *Phil. Trans. R. Soc. Lond. B* 353:261-274.
- Pateman-Jones, C. 2008. Sperm competition and male mating tactics in the bitterling fishes. Ph.D. thesis, Univ. of Leicester.
- Penn, D. 2002. The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology* 108:1-21.
- Penn, D., and W. K. Potts. 1999. The evolution of mating preferences and major histocompatibility complex genes. *Am. Nat.* 153:145-164.
- Petersson, E., T. Järvi, H. Olsén, I. Mayer, and M. Hedenskog. 1999. Male-male competition and female choice in brown trout. *Anim. Behav.* 57:777-783.
- Piálek, J., and T. Albrecht. 2005. Choosing mates: complementary versus compatible genes. *Trends Ecol. Evol.* 20:63.
- Piertney, S. B., and M. K. Oliver. 2006. The evolutionary ecology of the major histocompatibility complex. *Heredity* 96:7-21.
- Potts, W. K., C. J. Manning, and E. K. Wakeland. 1991. Mating patterns in seminatural populations of mice influenced by MHC genotype. *Nature* 352:619-621.
- Prum, R. 2010. The Lande-Kirkpatrick mechanism is the null model of evolution by intersexual selection: implications for meaning, honesty and design in intersexual signals. *Evolution* 64: 3085-3100.
- Przybylski, M. 1996. The diel feeding pattern of bitterling *Rhodeus sericeus amarus* (Bloch) in the Wieprz-Krzna canal Poland. *Pol. Arch. Hydrobiol* 43:203-212.
- Puurtinen, M., T. Ketola, and J. S. Kotiaho. 2005. Genetic compatibility and sexual selection. *Trends Ecol. Evol.* 20:157-158.
- Quinn, T., and T. Hara. 1986. Sibling recognition and olfactory sensitivity in juvenile coho salmon (*Oncorhynchus kisutch*). *Can. J. Zool.* 64:921-925.
- Qvarnström, A., and E. Forsgren. 1998. Should female prefer dominant males? *Trends Ecol. Evol.* 13:498-503.
- Reebs, S. 1994. Nocturnal mate recognition and nest-guarding by female Convict Cichlids (Pisces, Cichlidae: *Cichlasoma nigrofasciatum*). *Ethology* 96:303-312.

- Reichard, M., P. Jurajda, and C. Smith. 2004a. Male-male interference competition decreases spawning rate in the European bitterling (*Rhodeus sericeus*). *Behav. Ecol. Sociobiol.* 56:34-41.
- Reichard, M., C. Smith, and W. C. Jordan. 2004b. Genetic evidence reveals density-dependent mediated success of alternative mating behaviours in the European bitterling (*Rhodeus sericeus*). *Mol. Ecol.* 13:1569-1578.
- Reichard, M., J. Bryja, M. Ondračková, M. Dávidová, P. Kaniewska, and C. Smith. 2005. Sexual selection for male dominance reduces opportunities for female mate choice in the European bitterling (*Rhodeus sericeus*). *Mol. Ecol.* 14:1533-1542.
- Reichard, M., S. C. Le Comber, and C. Smith. 2007a. Sneaking from a female perspective. *Anim. Behav.* 74:679-688.
- Reichard, M., H. Liu, and C. Smith. 2007b. The co-evolutionary relationship between bitterling fishes and freshwater mussels: insights from interspecific comparisons. *Evol. Ecol. Res.* 9: 239-259.
- Reichard, M., M. Przybylski, P. Kaniewska, H. Liu, and C. Smith. 2007c. A possible evolutionary lag in the relationship between freshwater mussels and European bitterling. *J. Fish Biol.* 70:709-725.
- Reichard, M., C. Smith, and P. Bryja. 2008. Seasonal change in the opportunity for sexual selection. *Mol. Ecol.* 17:642-651.
- Reichard, M., M. Ondračková, A. Bryjova, C. Smith, and P. Bryja. 2009. Breeding resource distribution affects selection gradients on male phenotypic traits: experimental study on lifetime reproductive success in the bitterling fish (*Rhodeus amarus*). *Evolution* 63:377-390.
- Reichard, M., M. Ondračková, M. Przybylski, H. Liu, and C. Smith. 2006. The costs and benefits in an unusual symbiosis: experimental evidence that bitterling fish (*Rhodeus sericeus*) are parasites of unionid mussels in Europe. *J. Evol. Biol.* 19: 788-796.
- Reichard, M., M. Polačik, A. S. Tarkan, R. Spence, O. Gaygusuz, E. Ercan, M. Ondračková, and C. Smith, C. 2010. The bitterling mussel coevolutionary relationship in areas of recent and ancient sympatry. *Evolution* 64:3047-3056.
- Resink, J., W. Schoonen, P. Albers, D. File, C. Notenbloom, R. Van Den Hurk, and P. Van Oordt. 1989a. The chemical nature of sex attracting pheromones from the seminal vesicle of African catfish, *Clarias gariepinus*. *Aquaculture* 83:137-151.
- Resink, J., T. Van den Berg, R. Van den Hurk, E. Huisman, and P. Van Oordt. 1989b. Induction of gonadotropin release and ovulation by pheromones in the African catfish, *Clarias gariepinus*. *Aquaculture* 83:167-177.

- Reusch, T. B. H., M. A. Haberli, P. B. Aeschlimann, and M. Milinski. 2001. Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. *Nature* 414:300-302.
- Richardson, D. S., J. Komdeur, T. Burke, and T. von Schanz. 2005. MHC based patterns of social and extra pair mate choice in the Seychelles warbler. *Proc. R. Soc. Lond. B* 272:759-767.
- Ringleberg, J. 1980. Aspects of red pigmentation in zooplankton, especially copepods. Pp. 91-97 in W. Kerfoot, ed. *Evolution and ecology of zooplankton communities*. American Society for Limnology and Oceanography Special Symposium 3. Univ. Press of New England, Hanover, N.H.
- Robinson, J., E. A. Schmitt, F. I. Harosi, R. J. Reece., and J. E. Dowling. 1993. Zebrafish ultra-violet visual pigment: absorption spectrum, sequence, and localization. *Proc. Natl. Acad. Sci. U.S.A.* 90: 6009-6012.
- Rodd, F. H., K. A. Hughes, G. F. Grether, and C. T. Baril. 2002. A possible non-sexual origin of mate preference: are male guppies mimicking fruit? *Proc. R. Soc. Lond. B* 269:475-481.
- Rohwer, S. 1982. The evolution of reliable and unreliable badges of fighting ability. *Am. Zool.* 22:531-546.
- Roitberg, B. 1998. Oviposition decisions as maternal effects: conundrums and opportunities for conservation biologists. Pp. 67-79 in T. Mousseau, and C. Fox, eds. *Maternal effects as adaptations*. Oxford Univ. Press, New York.
- Rosenberg, T. J., S. Garbers, H. Lipkind, and M. Chiasson. 2005. Maternal obesity and diabetes as risk factors for adverse pregnancy outcomes: differences among 4 racial/ethnic groups. *Am. J. Public Health* 95:1545-1551.
- Rosenthal, G. G., and P. Lobel. 2006. Communication. Pp. 39-78 in K. Sloman, S. Balshine, and R. Wilson, eds. *Behaviour and physiology of fish* (vol. 24). Academic Press, London.
- Roughgarden, J. 2004. *Evolution's rainbow: Diversity, gender and sexuality in nature and people*. Univ. of California Press, Berkeley and Los Angeles.
- Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour.* 8:103-106.
- Rowe, L., G. Arnqvist, A. Sih, and J. Krupa. 1994. Sexual conflict and the evolutionary ecology of mating patterns: water striders as a model system. *Trends Ecol. Evol.* 9:289-293.
- Ryan, M. J. 1998. Sexual selection, receiver biases, and the evolution of sex differences. *Science* 281:1999-2003.
- Ryan, M. 1990. Sexual selection, sensory systems, and sensory exploitation. Pp. 157-195 in D. Futuyma, and J. Antonovics, eds. *Oxford surveys in evolutionary biology*. Oxford Univ. Press, New York.

- Ryan, M. J., and A. S. Rand. 1990. The sensory basis of sexual selection for complex calls in the túngara frog, *Physalaemus pustulosus* (sexual selection for sensory exploitation). *Evolution* 44:305-314.
- Sambrook, J. G., F. Figueroa, and S. Beck. 2005. A genome-wide survey of Major Histocompatibility Complex (MHC) genes and their paralogues in zebrafish. *BMC Genomics* 6:152.
- Sandberg, M., L. Eriksson, J. Jonsson, M. Sjöström, and S. Wold. 1998. New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *J. Med. Chem.* 41:2481.
- Sargent, R. 1982. Territory quality, male quality, courtship intrusions, and female nest choice in the threespine stickleback, *Gasterosteus aculeatus*. *Anim. Behav.* 30:364-374.
- Schiedt, K. 1989. New aspects of carotenoid metabolism in animals. Pp. 247-268 in N. Krinsky, M. Mathews-Roth, and R. Taylor eds. *Carotenoids: chemistry and biology*. Plenum Press, New York.
- Schwensow, N., J. Fietz, K. H. Dausmann, and S. Sommer. 2007. Neutral versus adaptive genetic variation in parasite resistance: importance of major histocompatibility complex super-types in a free-ranging primate. *Heredity* 99:265-277.
- Schwensow, N., M. Eberle, and S. Sommer. 2008. Compatibility counts: MHC-associated mate choice in a wild promiscuous primate. *Proc. R. Soc. Lond. B* 275:555-564.
- Shirai, K. 1962. Correlation between the growth of the ovipositor and ovarian conditions in the Bitterling, *Rhodeus ocellatus*. *Bull. Fac. Fish. Hokkaido Univ.* 13:137-151.
- Shuster, S. M., and M. J. Wade. 2003. *Mating systems and strategies*. Princeton Univ. Press, Princeton.
- Sih, A., M. Lauer, and J. Krupa. 2002. Path analysis and the relative importance of male–female conflict, female choice and male–male competition in water striders. *Anim. Behav.* 63:1079-1098.
- Skarstein, F., I. Folstad, S. Liljedal, and M. Grahn. 2005. MHC and fertilization success in the Arctic charr (*Salvelinus alpinus*). *Behav. Ecol. Sociobiol.* 57:374-380.
- Smith, R., and C. Lessells. 1985. Oviposition, ovicide and larval competition in granivorous insects. Pp.423-448 in R. Sibley, and R. Smith, eds. *Behavioural ecology: ecological consequences of adaptive behaviour*. Blackwell Scientific, Oxford.
- Smith, C., and M. Reichard. 2005. Females solicit sneakers to improve fertilisation success in the bitterling (*Rhodeus sericeus*). *Proc. R. Soc. Lond. B* 272:1683-1688.
- Smith, C., J. D. Reynolds, and W. J. Sutherland. 2000a. The population consequences of reproductive decisions. *Proc. R. Soc. Lond. B* 267:1327-1334.

- Smith, C., J. D. Reynolds, W. J. Sutherland, and P. Jurajda. 2000b. Adaptive host choice and avoidance of superparasitism in the spawning decisions of bitterling (*Rhodeus sericeus*). Behav. Ecol. Sociobiol. 48:29-35.
- Smith, C., K. Rippon, A. Douglas, and P. Jurajda. 2001. A proximate cue for oviposition site choice in the bitterling (*Rhodeus sericeus*). Freshwater Biol. 46:903-911.
- Smith, C., A. Douglas, and P. Jurajda. 2002. Sexual conflict, sexual selection and sperm competition in the spawning decisions of bitterling (*Rhodeus sericeus*). Behav. Ecol. Sociobiol. 51:433-439.
- Smith, C., M. Reichard, and P. Jurajda. 2003. Assessment of sperm competition by European bitterling, *Rhodeus sericeus*. Behav. Ecol. Sociobiol. 53:206-213.
- Smith, C., I. Barber, R. J. Wootton, and L. Chittka. 2004a. A receiver bias in the origin of three-spined stickleback mate choice. Proc. R. Soc. Lond. B 271:949-955.
- Smith, C., M. Reichard, P. Jurajda, and M. Przybylski. 2004b. The reproductive ecology of the European bitterling (*Rhodeus sericeus*). J. Zool. 262:107-124.
- Smith, C., Y. Zhu, H. Liu, and M. Reichard. 2007. Deceptive female oviposition behaviour elicits male ejaculation in the European bitterling. J. Fish Biol. 71:1841-1846.
- Smith, C., C. Pateman-Jones, G. Zieba, M. Przybylski, and M. Reichard. 2009. Sperm depletion as a consequence of increased sperm competition risk in the European bitterling (*Rhodeus amarus*). Anim. Behav. 77:1227-1233.
- Solomon, G., M. Shimizu, and Y. Nosey. 1985. The Feeding-habits of rose bitterling in the Shin Tone River. Bull. Jap. Soc. Sci. Fish. 51:711-716.
- Sommerfeld, R., T. Boehm, and M. Milinski. 2008. Desynchronising male and female reproductive seasonality: dynamics of male MHC-independent olfactory attractiveness in sticklebacks. Ethol. Ecol. Evol. 20:325-336.
- Sorensen, P., M. Pinillosb, and A. Scottb. 2005. Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. Gen. Comp. Endocr. 140:164-175.
- Sorensen, P., and N. Stacey. 2004. Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. N. Z. J. Mar. Fresh. Res. 38:399-417.
- Spence, R., and C. Smith. 2006. Mating preference of female zebrafish, *Danio rerio*, in relation to male dominance. Behav. Ecol. 17:779-783.
- Spence, R. and C. Smith. 2008. Innate and Learned Colour Preference in the Zebrafish, *Danio rerio*. Ethology 114:582-588.
- Spence, R., G. Gerlach, C. Lawrence, and C. Smith. 2008. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol. Rev. 83:13-34.

- Stacey, N., and P. Sorensen. 2006. Reproductive pheromones. Pp.359-400 in K. Sloman, R. Wilson, and S. Balshine, eds. Behaviour and physiology of fish (vol. 24). Elsevier Academic Press, London.
- Suzuki, N., and S. Jeon. 1988. Development of eggs, larvae and juveniles of *Rhodeus ocellatus* from Ansong river, Korea (Pisces: Cyprinidae), with a note on minute tubercles on the skin surface. Korean J. Limnol. 21:1-15.
- Svensson, O., and C. Kvarnemo. 2005. The importance of sperm competition risk and nest appearance for male behavior and female choice in the sand goby, *Pomatoschistus minutus*. Behav. Ecol. 16:1042-1048.
- Taborsky, M. 1994. Sneakers, satellites, and helpers: parasitic and cooperative behaviour in fish reproduction. Adv. Stud. Behav. 23:1-100.
- Taborsky, M. 1998. Sperm competition in fish: bourgeois males and parasitic spawning. Trends Ecol. Evol. 13:222-227.
- Tadayuki, O., and N. Kosuke. 2001. A nuptial color of the rose bitterling. Aichi Kyoiku Daigaku Kenkyu Hokoku. Shizen Kagaku 50:31-73.
- Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.
- Tregenza, T., and N. Wedell. 2000. Genetic compatibility, mate choice and patterns of parentage. Mol. Ecol. 9:1013-1027.
- Trivers, R. L. 1972. Parental investment and sexual selection. Pp. 136–179 in B. Campbell, ed. Sexual selection and the descent of man 1871-1971. Aldine Publishing Company, Chicago, IL.
- Turelli, M., and H. A. Orr. 2000. Dominance, epistasis and the genetics of postzygotic isolation. Genetics 154:1663-1679.
- van den Berghe, E. P., F. Wernerus, and R. R. Warner. 1989. Female choice and the mating cost of peripheral males. Anim. Behav. 38:875-884.
- van Erp, S. H. M., E. Egberts, and R. J. M. Stet. 1996. Characterization of major histocompatibility complex class II A and B genes in gynogenetic carp clone. Immunogenetics 41:1-17.
- van den Hurk, R., W. Schoonen, G. Van Zoelen, and J. Lambert. 1987. The biosynthesis of steroid glucuronides in the testis of the zebrafish, *Brachydanio rerio*, and their pheromonal function as ovulation inducers. Gen. Comp. Endocr. 68:179-188.
- van Valen, L. 1973. A new evolutionary law. Evol. Theor. 1:1-30.
- Waarde, A., V. Thillart, and M. Verhagen. 1993. Ethanol formation and pH-regulation in fish. Pp. 157–170 in P. Hochachka, P. Lutz, T. Sick, M. Rosenthal, and G. Thillart, eds. Surviving hypoxia: mechanisms of control and adaptation. CRC Press, INC.

- Wade, M. J., and S. M. Shuster. 2004. Sexual selection: harem size and the variance in male reproductive success. *Am. Nat.* 164: E83-E89.
- Wagner, W. E. 1998. Measuring female mating preferences. *Anim. Behav.* 55:1029-1042.
- Warner, R. 1987. Female choice for sites versus mates in a coral reef fish *Thalassoma bifasciatum*. *Anim. Behav.* 35:1470-1478.
- Watters, J. V. 2005. Can alternative male tactics 'fighter' and 'sneaker' be considered 'coercer' and 'cooperator'? *Anim. Behav.* 70:1055- 1062.
- Wedekind, C., and S. Furi. 1997. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? *Proc. R. Soc. Lond. B* 264:1471-1479.
- Wedekind, C., T. Seebeck, F. Bettens, and A. J. Paepke. 1995. MHC dependent mate preferences in humans. *Proc. R. Soc. B* 260:245-249.
- Wedekind, C., R. Muller, and H. Spicher. 2001. Potential genetic benefits of mate selection in whitefish. *J. Evol. Biol.* 14:980-986.
- Welch, A. M., R. D. Semlitch, and H. C. Gerhardt. 1998. Call duration as an indicator of genetic duality in male gray tree frogs. *Science* 280:1928-1930.
- Whitfield, D. 1987. Plumage variability, status signalling and individual recognition in avian flocks. *Trends Ecol. Evol.* 2:13-18.
- Wiepkema, P. 1961. An ethological analysis of the reproductive behaviour of the bitterling (*Rhodeus amarus bloch*). *Arch. Neerl. Zool.* 14:103-199.
- Williams, G. C. 1966. Adaptation and natural selection. Princeton Univ. Press, Princeton, NJ.
- Wong, B. B. M., and U. Candolin. 2005. How is female mate choice affected by male competition? *Biol. Rev.* 80:559-571.
- Wootton, R. J. 1998. The ecology of teleost fishes. Kluwer, Dordrecht.
- Yeates, S. E., S. Einum, I. A. Fleming, H. -J. Megens, R. J. M. Stet, K. Hindar, W. V. Holt, K. J. W. Van Look, and M. J. G. Gage. 2009. Atlantic salmon eggs favour sperm in competition that have similar major histocompatibility alleles. *Proc. R. Soc. Lond. B* 276:559-566.
- Yokoi, K., H. Ohta, and K. Hosoya. 2008. Spermmotility and cryopreservation of spermatozoa in freshwater gobies. *J. Fish Biol.* 72:534-544.
- Zahavi, A. 1977. The cost of honesty (further remarks on the handicap principle). *J. Theor. Biol.* 67: 603-605.
- Zaki, S. A. H., W. C. Jordan, M. Reichard, M. Przybylski, and C. Smith. 2008. A morphological and genetic analysis of the European bitterling species complex. *Biol. J. Linn. Soc.* 95:337-347.
- Zeh, J. A., and D. W. Zeh. 1996. The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proc. R. Soc. Lond. B* 263:1711-1717.

Zeh, J. A., and D. W. Zeh. 1997. The evolution of polyandry II: Post-copulatory defences against genetic incompatibility. *Proc. R. Soc. Lond. B* 264:69-75.