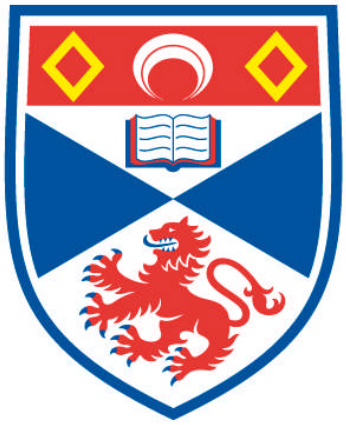
MALE MATING TACTICS IN THE ROSE BITTERLING (RHODEUS OCELLATUS) AND EUROPEAN BITTERLING (RHODEUS AMARUS)

Mara Casalini

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



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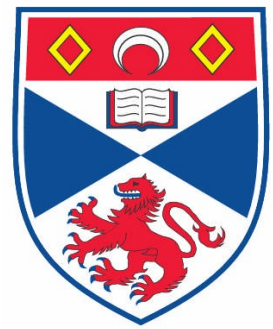

This thesis is submitted in partial fulfilment for the degree of PhD

at the

University of St Andrews

September 2012

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I, Mara Casalini, hereby certify that this thesis, which is approximately 40,000 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 July 2008 at the University of Leicester and as a candidate for the degree of PhD in July 2010 at the University of St Andrews; the higher study for which this is a record was carried out in the University of Leicester and of St Andrews between 2008 and 2012.

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To Matteo.

The fish called ventig in the common language is named aristosus in Latin because of the numerous fish bones. It is the least palatable of all edible fish. It inhabits fresh waters as well as those turned brackish by ebb and flood. The meat of the fish is so full of bones that these form an internal harness, so that it is difficult to chew. In addition, its taste is vile and rather repulsive, which makes it food only suitable for paupers and outcasts.

This fish is caught in the following manner: nets are stretched along or across the stream and behind the net an arch-like (hollow) instrument is placed in the water with a little bell suspended in the upper part. When the fish hear the sound of this bell they stupidly rush towards it, being trapped together by the lowered nets. The above proves that this fish possess the sense of hearing.

Those fish also like to carry out a kind of group dance, blushing with shame while dancing together. This is something they do (possibly) because it is a way to feed, or because it is a habit of theirs, or because this behaviour is triggered by the presence of others.

From the anonymous Flemish bestiary *Liber de naturis rerum creatarum* (early 15th century). Translation and figure from van Damme *et al*. 2007.

Hence it is the males that fight together and sedulously display their charms before the females.

Charles Darwin. From: *The Descent of Man and Selection in Relation to Sex* (1871).

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Abstract

The aim of this study was to investigate the basis to male mating decisions in two related species of bitterling: *Rhodeus ocellatus* and *R. amarus*. Bitterling have a resource-based mating system; females lay eggs in the gills of live freshwater mussels and males fertilize the eggs by releasing sperm into the inhalant syphon of the mussel. Male bitterling perform courtship behaviour and aggressively defend mussels in a territory from which they exclude other males. Using laboratory and field experiments it was shown that male aggressive behaviour is inherited through additive maternal genes. Male aggression is also influenced by the number of conspecific males encountered in competition for a mussel, and by the degree of clustering of mussels. Limited availability of mussels results in stronger selection on traits males use in mating context: hence they are more aggressive, larger and more colourful. The differences in mating behaviours in different environments may indicate a conflict between male dominance and female choice, but have not led to reproductive isolation. Resource availability during ontogenesis and male density during embryogenesis, however, do not exert an effect on male aggressive behaviour. Red carotenoid-based nuptial coloration functions as an inter- and intra-sexual signal and undergoes rapid variation in response to changes in mating context. Male bitterling do not modulate their courtship and aggressive behaviour in response to variation in female size, and their choice of mussel species is influenced by, and consistent with, female oviposition choice.

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Chapter 1. General introduction

1.1. Introduction

Darwin's (1859) theory of natural selection focused on the presence of heritable variations in traits that, in a given environment and over successive generations, can lead to differential reproductive success. This analysis of the mechanisms involved in the adaptive evolution of traits was later broadened by Darwin (1871) to underline the importance of sexual selection that "depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring" (Darwin 1859). The combined effects of natural and sexual selection were predicted to favour traits that maximize the fitness of individuals measured in terms of the number and viability of offspring they produced.

Sexual selection results from differential mating success among individuals within a population and can favour the evolution of traits such as body size, signals or weapons males use in direct competition against other males (intra-sexual selection), or ornaments, signals and courtship behaviours that influence female choice (inter-sexual selection). The opportunity for sexual selection is stronger on males due to anisogamy (Trivers 1972) and, consequently, to male greater variance in reproductive success (Shuster & Wade 2003). Intra- and inter-sexual selection influenced by ecological constraints and by the spatial and temporal distribution of receptive mates (Emlen & Oring 1977), are thought to contribute to the evolution of different mating systems (Andersson 1994; Shuster & Wade 2003).

Population density, operational sex ratio, and the availability of mating resources are key factors in determining male mating behaviours, and these variables can interact in complex ways. Thus, in analysing the effect of density on

male reproductive behaviour one must take into consideration not simply the number of individuals present per unit area but the ratio of competitors to available resources (Grant *et al.* 2000), since this will influence male aggression rate and reproductive success. Ahnesjö *et al*. (2001) underlined the essential role of the possession of mating resources in influencing reproductive fitness, and proposed a more comprehensive definition of the ratio of males and females: the sex ratio of individuals that are qualified to mate as they are sexually mature and have acquired the resources that are essential to mating. The qualified sex ratio also permits the prediction of the intensity of competition for resources.

The strength of sexual selection on traits associated with variance in resource competition, such as weapons, body size, or possibly coloration, will be stronger if males can monopolize mating resources (Emlen & Oring 1977), and if these traits influence female choice. Female choice for dominant, more colourful males has been demonstrated in several animal taxa (reviewed in Wong & Candolin 2005; Clutton-Brock & McAuliffe 2009), but females may also base their choice on cues such as territory or mating resource quality (Alatalo *et al*. 1986; Candolin 2003), parental care (Forsgren 1997), and intensity of courtship (Stapley 2008; Casalini *et al*. 2009).

Fighting and the maintenance of traits associated with dominance are costly in terms of energy expenditure (Parker 1983), higher risk of predation (Jakobsson *et al*. 1995; Herdman *et al*. 2004), and loss of potential future matings. Therefore, males can adopt a conditional strategy that can be influenced by the number of competitors and the strategies they adopt, by female mating preferences, or by environmental factors (Maynard Smith 1974). Theory predicts that males will engage in contests over females and resources critical to reproduction only if the fitness costs involved are lower than the fitness benefits they obtain (Brown 1964).

Resource availability and their spatial distribution can play an important role in shaping male behaviour; males can defend resources that are essential for their fitness and female and offspring survival or, in resource-based mating systems, resources that are essential for reproduction (Verner & Wilson 1966). For example, the availability of food and its spatial distribution can influence male behaviour and male phenotypic traits. If food is scarce or its distribution is clumped, the intensity of aggressive defence increases (Grant & Guha 1993). Similarly, if mating resources are scarce, selection on male aggression rate and phenotypic traits associated with dominance can be stronger (Borgia 1982), and a clumped distribution of mating resources can lead to strong directional selection on traits associated with intra-sexual competition for fertilization (Reichard *et al*. 2009).

Male mating decisions about the tactics to adopt in reproductive contests involve an assessment of an individual's own, and opponents', resource holding potential (RHP) and resource value. Parker (1974) summarised this point by saying: "outcomes of aggressive disputes should be decided by each individual's fitness budget available for expenditure during a fight (determined by the fitness difference between adoption of alternative strategies, escalation or withdrawal without escalation) and on the rate of expenditure of the fitness budget if escalation occurs (determined by the RHPs of the combatants)".

Sexually selected signals can play a role in measuring the RHP of rivals and to influence male decisions about whether to escalate a contest or to withdraw. To function in this role, signals must be perceived by the receiver as reliable indicators of male strength or willingness to fight and, to be "honest", signals must be costly (Grafen 1990). Under these limitations, only those individuals that are able to express costly signals can express them, thus they often signal individual fitness

(Zahavi 1975). Signals are often complex and can be visual (Møller 1987; Penteriani *et al*. 2007), auditory (Clutton-Brock & Albon 1979; Davies & Halliday 1978), and olfactory (Katoh *et al*. 2008). They can provide information about different aspects of the signaller's quality or condition (Johnstone 1995), and males exhibiting stronger or brighter signals are less frequently challenged by rivals, and can often settle contests without escalating to real fights (Evans & Norris 1996).

Carotenoid-based coloration is one of the most common visual signals in vertebrates and increases male conspicuousness during the reproductive season. Carotenoids are thought to serve as an honest signal of male quality because they are necessary for immune response, parasite resistance, and growth (Olson & Owens 1998; Lozano 1994; Chew & Park 2004). In addition, carotenoids must be obtained in the diet because they cannot be produced endogenously (Goodwin 1984; Schiedt 1989). Red or orange carotenoid coloration is present, typically in males, in many animal taxa (reviewed in Svensson & Wong 2011) and is thought to exert a role in inter- and intra-sexual communication (Andersson 1994; Houde 1997; Pryke *et al*. 2002; Griggio *et al*. 2007; Gomez *et al*. 2009).

The conventional view of sexual selection based on male-male competition and female choice (Darwin 1871; Andersson & Simmons 2006) has been challenged, in recent years, by an increasing body of experimental works that have demonstrated the role of male choosiness over mates or resources that can attract females. Male choosiness is expected to evolve if the costs involved in mate searching do not exceed the benefits of mating with fewer, preferred females. Dominant males may increase their reproductive fitness by mating with fewer larger females if female fecundity and body length are correlated (Wootton 1990; Jones *et al*. 2001), or if the chance of repeated matings is limited by a high intensity of

sperm competition (Schwagmeyer & Parker 1990). The existence of contrasting male and female choice can lead either to an inter-sexual conflict with female choice constrained by dominant males (Casalini *et al*. 2009), or to size-assortative mating as a consequence of male and female preference for larger mates and of larger males' monopolization of preferred females (Olsson 1993; Preston *et al*. 2005). In the latter case selective pressures can lead to two different scenarios: an adaptive assortative mating of small males with smaller females, or an increased adoption of alternative tactics by smaller males who sneak fertilization with larger females.

Male mating behaviours can evolve adaptively under the influence of environmental variables but, as Darwin (1859) underlined, for selection to act, traits must be heritable. Advances in genetics have permitted the investigation of the genetic basis to some behaviours in several animal taxa (Barton *et al*. 2007). Even if genes do not directly specify behaviours, they do encode molecular products that govern the functioning of the brain that, in turn, can regulate the expression of behaviour (Robinson *et al*. 2008). Behaviour itself is regulated by the complex interplay of genetic and environmental factors. Phenotypic plasticity permits rapid changes to phenotypic expression that is mediated by the environment (Wang *et al*. 2008).

The existence of genetically inherited behaviour was demonstrated in the 60s when lines of highly aggressive and non-aggressive mice were artificially selected and raised in the laboratory (Lagerspetz 1964). Since then, selected lines of mice (van Oortmerssen & Sluyter 1994) and *Drosophila* spp. (Edwards *et al.* 2006) have been extensively used to identify candidate genes influencing aggressive behaviour. The genetic heritability of behaviours and the influence of the

environment in shaping them have been demonstrated, among others, for aggression in deer mice, *Peromyscus maniculatus sonoriensis* (Wilson *et al*. 2009), migratory behaviour in European blackcaps, *Sylvia atricapilla* (Helbig 1996), song learning preferences in domestic canary birds, *Serinus canaria* (Mundinger 1995), foraging behaviour in the honey bee, *Apis mellifera* (Page *et al*. 1995), sexual attractiveness in male guppies, *Poecilia reticulata* (Brooks 2000), and anti-predator behaviour in European minnows, *Phoxinus phoxinus* (Magurran 1990). The genetic basis to female mating preference has also been demonstrated, for example, in the two-spot ladybird, *Adalia bipunctata* (Majerus *et al*. 1982; O' Donald & Majerus 1985), and in the arctiid moth, *Utetheisa ornartix* (Iyengar *et al*. 2002).

1.2. Study species

Bitterling fishes (Family Cyprinidae, Subfamily Acheilognathinae) are small freshwater fishes distributed in lotic and lentic ecosystems (van Damme *et al*. 2007) in Asia and Europe. In East Asia three genera (*Acheilognathus, Tanakia*, and *Rhodeus*) comprise approximately 40 species (Arai 1988), some at present critically endangered (Ohta *et al*. 2001; Kubota & Watanabe 2003; Kitamura *et al*. 2009). They are distributed East of the Mekong River and Lake Baikal, Laos, Vietnam, China, Japan, Korea and South-eastern Russia. One species, the European bitterling (*Rhodeus amarus*), is present throughout Europe (Reichard *et al*. 2007, van Damme *et al.* 2007; Zaki *et al*. 2008; Bryja *et al*. 2010). After a considerable decrease during the 1970s and 1980s, in recent years *R. amarus* West European populations have increased in number and their distribution now exceeds their original range (Kozhara *et al*. 2007; van Damme *et al.* 2007).

Bitterling have a promiscuous, resource-based mating system. During the spawning season females develop an extended ovipositor that they use to insert

their eggs in the gills of living freshwater unionid mussels through their exhalant siphon. Males fertilize the eggs by releasing sperm over the inhalant siphon of the mussel (Duyné de Wit 1955). Embryos develop inside the mussel gills and emerge as free swimming larvae able to feed exogenously after 20-30 days (Wiepkema 1961; Kim & Park 1985; Smith *et al*. 2004). They compete with their mussel host for oxygen, so hindering its growth rate (Reichard *et al*. 2006), and may also compete with the mussel for nutrients (Spence & Smith 2011).

In Europe, both in sites of ancient and recent sympatry, no evidence has been found for the presence of host-specific lineages of bitterling (Reichard *et al*. 2011). However, in sites of ancient mussel-bitterling sympatry, mussels seem to have evolved defences to avoid being parasitized; they close the siphon more quickly, which could interfere with the correct insertion of females' ovipositor, and the distance between the gill and mantle tissues is larger, which could result in a greater number of eggs being laid in the mantle cavity where they cannot complete their development (Reichard *et al*. 2010). In Asia, where the number of mussels and of bitterling species is far higher than in Europe and mussels and bitterling have coexisted for millions of years (Tomoda *et al*. 1977), bitterling show a high specialization in their use of host species (Liu *et al*. 2006; Reichard *et al*. 2007; Kitamura *et al*. 2009; Reichard *et al*. 2010). *Rhodeus ocellatus* males, however, tend to be more generalist in mussel choice (Reichard *et al*. 2007).

The spawning season in nature is influenced by temperature and photoperiod (Asahina & Hanyu 1983; Smith *et al*. 2004) and lasts from late March to September for *R. ocellatus* (Asahina *et al*. 1980), and from April to August with a peak spawning in May for *R. amarus* (Smith *et al*. 2004). Females spawn in several bouts lasting one or two days and consisting of at least five spawnings each day (Reichard

et al. 2005) in which they lay 1-5 (typically 3) eggs (Smith *et al*. 2004), though this pattern varies among species (Pateman-Jones *et al*. 2011). Both male and mussel quality influence female spawning decisions (Smith *et al*. 2000b, 2001; Candolin & Reynolds 2001). Females are influenced by the intensity of male courtship (Reichard *et al*. 2007; Casalini *et al*. 2009) and mate preferentially with genetically compatible MHC-dissimilar males (Agbali *et al*. 2010). Their choice for a mussel is based on its oxygen content and ventilation rate (Smith *et al.* 2001; Mills & Reynolds 2002).

Males and females spawn repeatedly with several different partners and both intra- and inter-sexual selection play a role in shaping mating tactics and strategies (Kanoh 1996, 2000; Smith *et al*. 2003; Agbali 2011). Dominant males, generally the largest individuals, defend a territory around one or more mussels and become aggressive towards other males performing parallel swimming, often extending their dorsal and ventral fins, or directly attacking opponents pushing them away with quick movements of their body (Smith *et al*. 2004). If a female in spawning condition enters their territory, males court her and attempt to lead her to the mussel they defend; during fights and courtship their nuptial coloration intensifies (Smith *et al*. 2004).

In bitterling, alternative mating tactics are conditional and not genetically determined and sneaking behaviour, in which males release sperm into a rival's mussel, is common (Candolin & Reynolds 2002a,b; Reichard *et al*. 2005; Smith *et al*. 2004, 2009) and may be adopted by males unable to obtain and defend a territory, but also by territorial males who steal fertilization in the territory of other territorial males (Casalini 2007).

At high male density, sneaking can confer the same reproductive success as

territoriality (Reichard *et al*. 2004a), and provoke spawning disruption as males abandon courtship to attack rivals (Reichard *et al*. 2004b). If many competitors gather around the mussel and attempt to steal fertilization, dominant males can abandon their territory (Smith *et al*. 2002) as the risk of sperm competition and the cost of aggressive behaviour can make resource defence uneconomical (Brown 1964).

Male aggressive behaviour and dominance can constrain female mate choice (Casalini *et al*. 2009) by monopolizing access to females (Reichard *et al*. 2005); however, females may increase mating opportunities for subordinates by soliciting sneakers to increase fertilization success and delay oviposition (Smith & Reichard 2005; Smith *et al*. 2007) by performing a "skimming" action touching the mussel with the base of their ovipositor without spawning. For a detailed review of bitterling mating system see Smith *et al*. (2004).

Experimental work for this thesis concentrated on two closely related species: *R. amarus*, and *R. ocellatus*. *R. amarus* is the best studied bitterling, at least in terms of its behaviour and ecology, and both field and lab studies were conducted on this species. Lab studies on *R. ocellatus* took advantage of the fact that this species spawns all year round in captivity, whereas *R. amarus* is more seasonal in its reproduction and offers a smaller temporal window for research, both in the field and lab. These two species are phylogenetically close (Okazaki *et al*. 2001), and their reproductive biology is similar (Smith *et al*. 2004; Pateman-Jones *et al*. 2011).

1.2.1. Bitterling as model species

Bitterling peculiar mating system makes them an ideal model to understand the basis to mating decisions. They show unambiguous mate choice, their dependence on mussels as a spawning site allows to test accurately male and female mating

behaviours, and male and mating resource quality can be independently manipulated. Furthermore, they reach sexual maturity in 3-6 months, so allowing experimental studies on successive generations, have external fertilization, which facilitates *in vitro* experimental crosses, and readily adapt to laboratory conditions.

Even if bitterling mating system is unique, they do share mating behaviours with other taxonomic groups. The results of research performed with this species can be compared in different organisms to provide a broader understanding of the evolutionary significance of the heritability of behaviours, and of the role played by sexual selection in shaping mating systems.

1.3. Aims

Ecological, genetic, and physiological factors can shape male mating behaviours and influence the evolution of mating systems and the strength of sexual selection (Emlen & Oring 1977; Brown 1964; Avise *et al*. 2002; Schuster & Wade 2003). The aim of this thesis was to investigate mechanisms and consequences of male mating decisions and obtain a deeper understanding of:

1. The heritability of male aggressive behaviour and sexually selected traits
2. The role of crowding and density on male mating tactics
3. The effect of resource availability on male mating behaviour
4. The effect of density during embryogenesis on male aggressive behaviour
5. The role of male colour signals
6. Male preference for resource quality
7. Male mate choice

1.4. Thesis structure

The thesis comprises eight chapters. In **Chapter 2** the heritability of male aggressive

behaviour and of sexually selected traits was assessed in *R. ocellatus*. The role of crowding and density in shaping male *R. ocellatus* mating tactics was examined in **Chapter 3**. In **Chapter 4** three experiments are presented, performed with wild populations of *R. amarus*. In the first, the role of mussel abundance on male aggression rate was evaluated in four populations, isolated for different lengths of time. In the second, compatibility among the four study populations was tested using a combination of behavioural observations and *in vitro* fertilization. In the third experiment, the role of mussel abundance during ontogeny was evaluated. In **Chapter 5** the role of density (individuals per unit area) during embryogenesis on male aggressive behaviour was evaluated in *R. ocellatus*. In **Chapter 6** a behavioural experiment was performed to test whether male red carotenoid-based coloration functions as an inter- and/or intra-sexual signal in *R. ocellatus*. In **Chapter 7** male choice for different mussel species and for differently sized females was tested in *R. ocellatus*. **Chapter 8** summarises and discusses the results of the experiments presented in Chapters 2-7.

Chapter 2. Heritability of male aggressive behaviour and sexually selected traits in the rose bitterling, *Rhodeus ocellatus*

2.1. Abstract

The complex interplay between genetic and environmental factors in shaping animal behaviours and their relevance in enhancing reproductive fitness has been addressed in theoretical and experimental works. The heritability of phenotypic and behavioural traits has been demonstrated in many animal taxa, but the exact mechanism of inheritance is still often unknown. In the present study, the parental contribution to aggressive behaviour, red coloration, survival, age and size at maturity were investigated in the rose bitterling, *Rhodeus ocellatus*. A North Carolina II design, *in vitro* fertilization (IVF), and behavioural observations were used to partition variation in these traits among paternal and maternal genetic effects. The results demonstrate that aggressive behaviour has a large heritable component, with a significant maternal additive effect; a maternal additive effect influenced also embryo survival and size at three weeks of age, as well as age and size at maturity. A significant non-additive effect of the interaction of male and female genotypes was detected on male red coloration, and both age and size at maturity. Egg size strongly influenced offspring age and size at maturity and size at three weeks after *in vitro* fertilization, but not survival that was associated to a maternal additive effect.

2.2. Introduction

Theory predicts that the sex with the highest reproductive rate, generally males, maximizes its reproductive fitness by controlling access to mates of the limiting sex; the greater the opportunity for resources or mates to be monopolised, the greater the strength of selection on male advantageous phenotypic and behavioural traits (Emlen & Oring 1977). In many animal taxa males, to increase their reproductive fitness, adopt aggressive behaviour to monopolize a territory, resources, or females themselves to prevent other males from mating. However, fighting can be energetically expensive and time consuming, and can expose males to a greater risk of predation and injuries. As a consequence, only the fittest individuals are expected to be able to cope with the challenges associated with aggression (Shuster & Wade 2003).

Offspring morphological and behavioural phenotypes can be influenced by a complex interplay of genetic and environmental factors; genetic additive and non-additive effects, maternal effects, and the environment experienced during ontogeny all can contribute to shape them. Maternal effects are defined as non-genetic influences derived from parental phenotypes that have an impact on offspring phenotypes (Heath & Blouw 1998). Maternal effects exert their role, particularly in the early stages of embryonic development, in many animal taxa (mammals: McAdam *et al*. 2002; birds: Hayward & Wingfield 2004; Groothuis *et al*. 2005; insects: Mousseau & Dingle 1991; amphibians: Pakkasmaa *et al*. 2003; and fish: Brooks *et al*. 1997; McCormick 1999; Zhang *et al*. 2012). They can be mediated by egg size, and hence a higher content of nutrients in the yolk sac, but also by the presence in the yolk sac of maternal hormones and proteins, transported from ovaries to eggs (Zhang *et al*. 2012). Furthermore, large amounts of maternal T3 and

T4 are present into the eggs of fish and they are believed to affect growth and development of embryos and larvae before they are able to produce endogenous thyroid hormone (Lam 1994).

The relationship between genes and complex behaviours is not straightforward (Sokolowski 2001). Researchers have investigated the genetic basis to behaviour, including the inheritance of anti-predator behaviour in European minnows, *Phoxinus phoxinus* (Magurran 1990), of risk-taking behaviour (van Oers *et al*. 2004) and exploratory behaviour (Dingemanse *et al.* 2002) in great tits, *Parus major*, and of differences in agonistic behaviour in juvenile coho salmon, *Oncorhynchus kisutch* (Rosenau & McPhail 1987). The successful selection, in a few generations, of mice with different levels of aggression also suggests a heritable component to this behaviour (Ferrari *et al*. 2005).

The aim of this study was to assess the genetic contribution to male aggressive behaviour, red nuptial coloration, survival and size during development, and age and size at maturity in the rose bitterling, *Rhodeus ocellatus*, and to test what proportion of the genetic component of these traits could be attributed to the sire, the dam or the interaction of parental haplotypes. A North Carolina Type II design (Lynch & Walsh 1998) was used in the study as it permits a comprehensive approach to measuring and partitioning the variation among paternal or maternal genetic effects, and other environmental effects (Neff & Pitcher 2005).

2.3. Methods

Experiments were conducted in the aquarium facility of the Department of Biology at the University of Leicester. Males and females for experimental work were the first generation of *R. ocellatus* derived from wild caught fish from the River Yangtze Basin, China. During the experiment they were about 30 months old. Prior

to experiments fish were held in stock aquaria measuring 120 (length) x 40 (width) x 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 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.) twice each week. Freshwater mussels used in 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 Grand Union canal in Leicestershire, UK, and kept outdoors in 100 L tanks.

At the start of the experiment twenty male and twenty female *R. ocellatus* were individually marked with coloured elastomer tags (Northwest Marine Technology) and haphazardly assigned to five blocks, each comprising four males and four females. Fish were housed in five experimental aquaria measuring 25 (length) x 40 (width) x 30 (depth) cm, each with a *U. pictorum* mussel in a sand-filled plastic cup and artificial plants as refuges.

2.3.1. *In vitro* fertilizations

To measure the proportion of the genetic component in offspring aggressive behaviour and sexually selected traits that could be attributed to the sire, the dam or the interaction of parental haplotypes, a North Carolina Type II breeding design (Lynch & Walsh 1998) was adopted to generate a series of replicated half-sib families. A set of 4 × 4 male x female factorial crosses were used to generate offspring with *in vitro* fertilization: each four males were crossed with each four females within each of the five blocks of fish, with a replicate of each cross (Figure 2.1). Thus, two replicates of 16 families of maternal and paternal half-siblings were

generated in each block, 80 replicated families in the combined design and a total of 160 families.

To perform *in vitro* fertilizations, the eggs were gently stripped from experimental females in spawning condition (with an extended ovipositor) and divided into approximately four equal groups in separate 90 mm diameter Petri dishes containing freshwater. Sperm was stripped from each of the four experimental males by gently pressing their abdomens and individually mixed in 9 ml of Tris-HCl NaCl buffer (pH 7.4). The sperm suspension of each male, which contained an excess of spermatozoa (Agbali *et al*. 2010), was pipetted over the eggs in one of the four Petri dishes and the covered Petri dishes were left on the laboratory bench for 30 min. A total of 1104 eggs were obtained by stripping the females of the five blocks of parental fish. The fertilized eggs were washed twice with freshwater and incubated at 23°C in an environmentally controlled room for about 30 days, until the larvae absorbed the yolk sac and were able to feed exogenously.

2.3.2. Rearing juveniles

The survival of developing embryos was recorded daily, and water in Petri dishes changed every other day. To ensure development was unaffected by embryo density the maximum number of embryos per Petri dish was limited to 7 and, if necessary, families were split into more than one Petri dish to accommodate them all. All the embryos were photographed against a scale bar at 3 weeks of age and their body length (from the tip of the snout to the origin of the tail fin) was measured to the nearest 0.1 mm using UTHSCSA Image Tool 3.0. When embryos had absorbed their yolk sacs and started to swim freely, families were moved to plastic tanks measuring 22 (length) x 16 (width) x 15 (depth) cm on a recirculating system at

23ºC and fed ZM 000 granular fish food (ZM Ltd., Winchester) twice a day. After 2 weeks juveniles were moved to bigger tanks measuring 30 (length) x 20 (width) x 21 (depth) cm with a layer of sand substrate and fed a mixture of crushed commercial fish flake food, ZM 100 and ZM 200 (ZM Ltd., Winchester) twice each day, live Artemia twice each week and frozen blood worms (*Chironomus* spp.) once each week.

Males reached sexual maturity, evident from the development of red pigment in the eye and on the tail fin, after a mean of 147 (± 1.7 SE) days and at a mean length of 27 (± 0.2 SE) mm. Between 1 and 3 days after the onset of sexual maturity males were placed in individual experimental aquaria together with a mussel in a sand-filled plastic cup, and, to ascertain their sexual motivation, a female with extended ovipositor was placed in the aquarium with them. If the male started courting the female he was assayed for aggression (below). Any fish that failed to achieve maturity by 6 months were not tested. A total of 174 males reached sexual maturity and were assayed for aggression.

2.3.3. Male aggression assay

To assay male aggression, a test male was placed alone in a test aquarium measuring 60 (length) x 40 (width) x 45 (depth) cm, with a *U. pictorum* mussel in a sand-filled plastic cup and allowed to settle overnight. The following morning a female in spawning condition, was haphazardly chosen from stock aquaria, placed in a perforated transparent bottle, and transferred to the aquarium with the test male. Once the male began to court her (typically after about ten minutes), the female was removed and a stimulus male, haphazardly chosen from stock aquaria, was placed in a perforated transparent bottle adjacent to the mussel in the test aquarium. After 10 minutes, a 10-minute observation of the focal male's aggressive behaviour

towards the captive male began. The behaviours scored were: (i) head butting (the male hits the bottle with his head trying to strike the constrained male), (ii) bouts of side jerking (the male attempts to push or hit the constrained male with quick movements of the body or tail), and (iii) fin spreading (the male swims around the bottle, without touching it, extending his dorsal and anal fins). After completion of each observation the body length of the stimulus male and female and the maximum shell length of the test mussel were measured to the nearest 1 mm. Stimulus males, females and mussels were not used again in the study.

After assaying for aggression, males were photographed under standard light conditions next to a scale bar and their body length estimated to the nearest 0.1 mm from digital images. The area of red colour in the iris of males was measured using a protocol modified from that of Barber *et al*. (2000). Digital images were analysed 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 colour similarities until all the pixels within the red-coloured 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 (Casalini *et al*. 2009). The extent and brightness of red colour of the caudal fin were measured using an ordinal scale from Casalini *et al*. (2009)(Table 5.1). After measurements were completed, fish were returned to stock aquaria and were not used again.

2.3.4. Data 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. Non parametric tests were used

if data did not respond to transformation. For each 4 × 4 factorial block, analysis of variance was used to compare effects of sire, dam, and their interaction on aggressive behaviour, red coloration, survival, size at three weeks, and age and size at maturity. Sums of squares were combined to calculate mean squares and degrees of freedom for all blocks combined in accordance with Lynch & Walsh (1998). The amount of egg yolk can significantly affect offspring fitness (Wootton 1990); therefore, egg size was used as a covariate in analyses of survival and growth. A Spearman correlation was used to test the relationship between male aggression rate and age at maturity, body length at maturity, extent of red in the iris, and size of stimulus males.

2.4. Results

There was no correlation between male aggression rate and age at maturity (Spearman correlation: r_{172} = -0.143, P = 0.059; Figure 2.2), body length at maturity (r_{172} = 0.095, P = 0.208; Figure 2.3), or extent of red in the iris (r_{172} = 0.062, P = 0.410). The body length of stimulus males was not correlated with male aggression rate (r_{172} = -0.023, P = 0.758).

There was a significant female additive effect on offspring survival at three weeks of age, though no male additive effect, and no significant male × female interaction (Table 2.1). Female additive effects explained 10.4% of variation in survival rate, and were independent of egg volume, which did not have a significant effect on offspring survival. A type 2 error cannot be excluded to explain the lack of egg size influence on embryo survival; alternatively, environmental effects as water quality or temperature can have affected survival at this stage. An average of 68% of variance in survival was not explained by either additive or non-additive genetic effects. In contrast to survival, offspring size was significantly associated with eggs

size, with 65% of variance in larval size explained by egg size (Table 2.1). There were additional significant female and male additive effects on offspring size at three weeks, though these effects accounted for only 7.5% and 4.4% of variance in size respectively (Table 2.1).

A highly significant effect of egg size on male age and size at maturity was detected (Figure 2.4 and 2.5; Table 2.2). In the case of age at maturity, 41% of variation was explained by egg size, and 38% of variation in size at maturity. For both these variables there was also a significant non-additive genetic effect, with 29% and 24% of variation explained by a male × female interaction respectively (Table 2.2). In the case of age at maturity, there was also a minor, but significant, female (7% variance) and male (12%) additive contribution (Table 2.2). There was no significant male additive effect on male size at maturity, though there was a minor female additive effect in addition to egg size, explaining 9% of variance in size at maturity (Table 2.2). Only 12% of variance in age at maturity was not explained by either additive or non-additive genetic effects, and 30% of variance in body length.

Male eye colour at sexual maturity showed a highly significant male × female interaction. On average, parental genetic interaction effects explained 42% of variation in the extent of red coloration (Table 2.3). There was also a significant, but minor (2% of variation), female additive effect (Figure 2.6; Table 2.3). In contrast, for male aggression rate female additive genetic effects were highly significant, explaining 32% of variation in aggression rates (Table 2.3). There was no significant male additive effect or male × female interaction. An average of 56% of male eye redness and 55% of male aggression was unexplained by additive and non-additive genetic effects (Table 2.3).

2.5. Discussion

The aim of the experiment was to test and partition the parental contribution to offspring aggressive behaviour, nuptial coloration, survival and size during embryonic development, and age and size at maturity.

The variation in offspring morphological and behavioural phenotypes can be determined by the environment they experience during development and by a genetic component. The genetic component, in turn, can be ascribable to a heritable paternal or maternal additive effect or to a non-additive effect due to the combination of a particular male and female haplotype (Zeh & Zeh 1996).

The results of the present experiment demonstrate that aggressive behaviour has a large heritable component with a significant maternal additive effect. In *R. ocellatus* the genetic sex determination system is a female heterogametic system (ZW) (Kawamura 1998; Kawamura & Hosoya 2000), but females do not show an overt inter- or intra-sexual aggressive behaviour. However, behaviour is influenced by many genes that can interact with other genes in the genome, can exert pleiotropic effects, and whose expression can vary during ontogeny (Sokolowski 2001) and it can respond to different demands made by environmental factors (see Chapter 4). So far, notwithstanding the extensive research on animals (reviewed in van Oers *et al*. 2005) and men (reviewed by Ferguson 2010), which candidate genes determine aggressive behaviour is still unclear.

The data of the present study underline a role for maternal and paternal additive effect and a significant non-additive variance on male age at maturity and for an additive maternal effect and a significant non-additive variance on male size at maturity. In bitterling, the reproductive season is influenced by temperature and photoperiod and is relatively short. Juvenile size and age at maturity can confer an

increase in reproductive fitness as juveniles that reach maturity at an earlier age and at a bigger size can start to reproduce earlier in the spawning season and have a chance to win a contest. The maintenance of variability in size can be adaptive as the reproductive fitness of smaller males who adopt alternative tactics can equate to the one of dominant males at high male density (Reichard *et al*. 2004a, b). Besides, females solicit sneakers to improve their fertilization success (Smith & Reichard 2005), and they often base their choice of a mate on traits not correlated to male size (Casalini *et al*. 2009; Agbali *et al*. 2010).

A significant non-additive genetic effect, accounting for 42% of the total variance, was present also on male extent of red in the iris. Male nuptial red carotenoid-based coloration is thought to confer a reproductive advantage as, being costly, it can signal male fitness (Zahavi 1975), and can be used as an intra- and inter-sexual signal to deter opponents or influence female mating choice (Berglund *et al*. 1996). Even if female bitterling do not show a preference for more colourful males (Casalini *et al*. 2009), males do use and increase the extent of red in the iris when fighting against a rival or courting a female (see Chapter 6).

In contrast with a previous experiment (Agbali *et al*. 2010) larvae survival at three weeks of age was influenced only by an additive maternal effect but not by egg size, possibly because of an individual response to the environment experienced during early embryonic development. Size at three weeks of age was influenced by egg size, sire, and dam, while size at maturity by egg size, dam and by a non-additive influence of male x female.

During the early stages of embryonic development paternal additive effects can result in differential efficiency of the embryos in metabolizing available resources (Weigensberg *et al*. 1998), and maternal effects by means of transferred

hormones and vestigial proteins in the yolk sac, can enhance offspring defence, protect the embryos from pathogens present in the environment, and prevent the vertical transfer of maternal pathogens before the development of their immune system (Zhang *et al*. 2012). Thus, the interaction of genetic, parental and environmental effects can confer a higher fitness to the developing embryos. These effects can last beyond the early phases of development as confirmed by the influence of eggs size, dam, and the crossing of sire and dam on male age at maturity.

In conclusion, it was demonstrated that aggressive behaviour has a large heritable component, with a significant maternal additive effect, that embryo size at three weeks of age is influenced by an additive effect of sire and dam, and that male size and age at maturity are influenced by additive maternal and paternal effect and by a non-additive effect of the combination of a particular male and female haplotype. A maternal effect influenced embryo size at three weeks of age and, mediated by egg size, size and age at maturity. Finally a significant non-additive effect was present also on male red coloration.

Figure 2.1. Experimental design for *in vitro* fertilizations. Each male was crossed with each female within each of the five blocks of parental fish. The eggs stripped from each female were divided into four equal groups in separate Petri dishes; each group of eggs was then fertilized with sperm suspension of one of the four males from the same block.

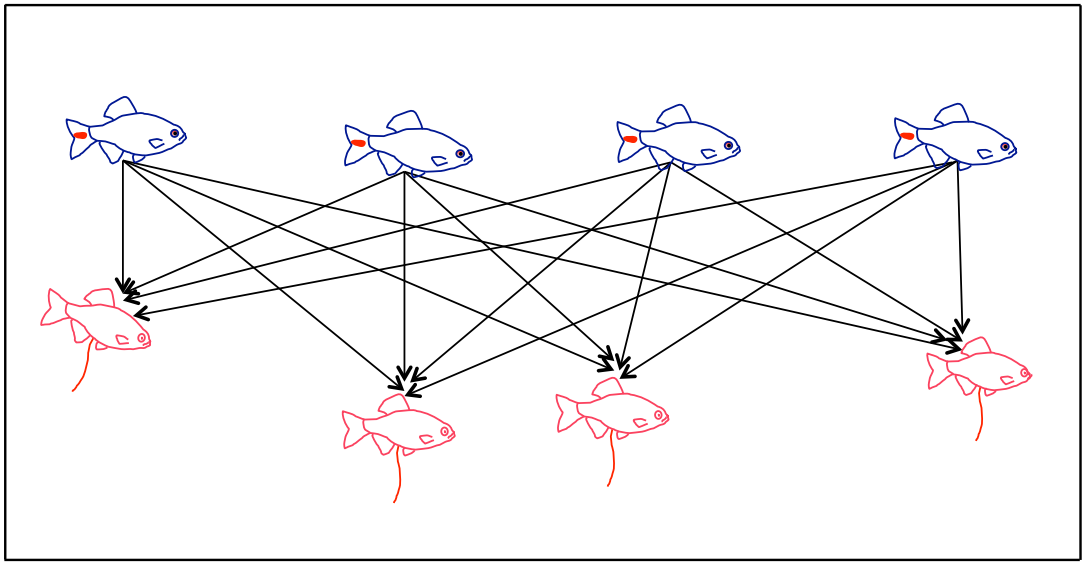


Figure 2.2. Correlation between juvenile age at maturity (d) and mean aggression rate (10 min $^{-1}$).

Figure 2.3. Correlation between juvenile size at maturity (mm) and mean aggression rate (10 min $^{-1}$).

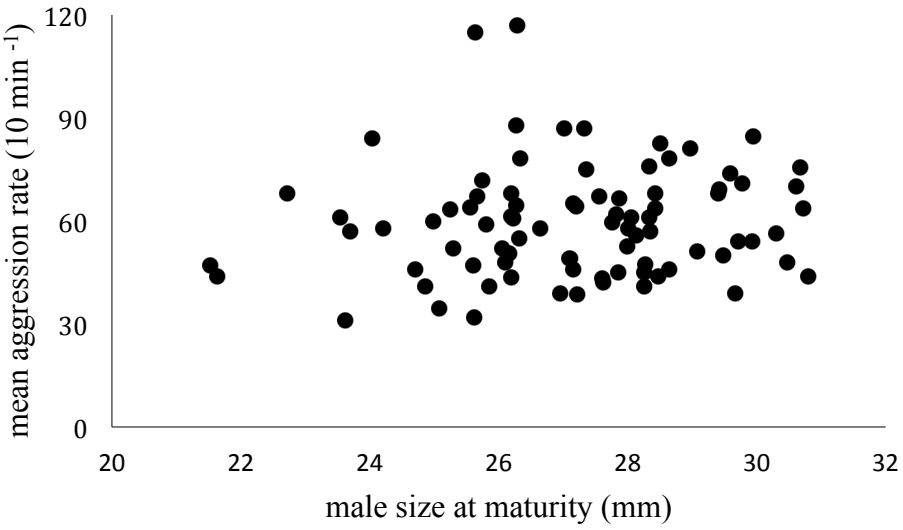


Figure 2.4. Proportion of variance in male age at maturity explained by egg size, an additive female effect, an additive male effect, and a non-additive effect of the interaction of parental haplotypes.

Figure 2.5. Proportion of variance in male size at maturity explained by egg size, an additive female effect and a non-additive effect of the interaction of parental haplotypes. (Only statistically significant results are shown in the figure).

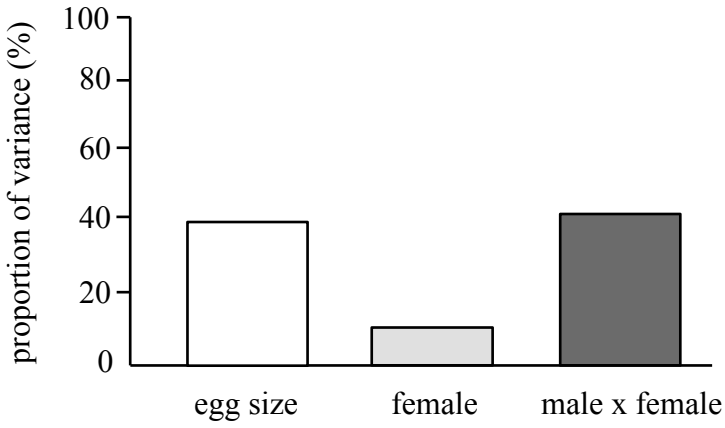


Figure 2.6. Proportion of variance in male extent of red in the iris explained by an additive female effect and a non-additive effect of the interaction of parental haplotypes. (Only statistically significant results are shown in the figure).

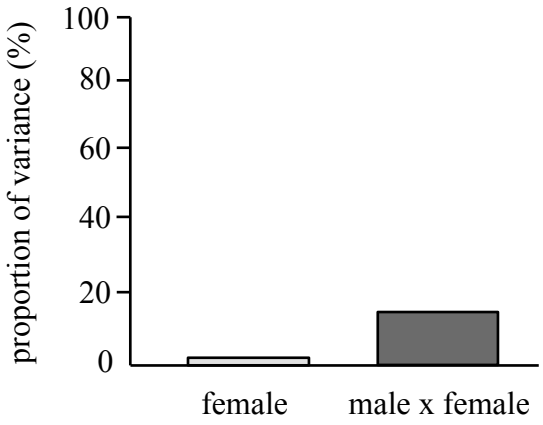


Table 2.1. ANCOVAs for offspring survival (proportion) at 3 weeks and body length (mm) at 3 weeks for *in vitro* fertilizations. Mean and standard error of variables are in parentheses.

Source	df	SS	MS	F	P	Variance	Variance (%)
Proportion of offspring surviving after 3 weeks (0.71 ± 0.02)							
Egg size (mm^3)	5	707	141.4	1.56	0.182	25.32	18.9
Female (F)	15	3365	224.3	2.64	0.006	13.94	10.4
Male (M)	15	1892	126.1	1.49	0.152	4.12	3.1
M X F	45	3822	84.9	0.94	0.589	0	0
Error	80	7261	90.8			90.76	67.7
Mean body length (mm) after 3 weeks (6.7 ± 0.02)							
Egg size (mm^3)	5	1.32	0.3	8.00	<0.001	0.12	65.0
Female (F)	15	2.73	0.2	3.69	<0.001	0.01	7.5
Male (M)	15	1.91	0.1	2.58	0.007	0.01	4.4
M X F	45	2.22	0.0	1.49	0.058	0.01	4.6
Error	80	2.64	0.0			0.03	18.6

Table 2.2. ANCOVAs for male age at sexual maturity (d) and body length (mm) at maturity for *in vitro* fertilizations. Mean and standard error of variables are in parentheses.

Source	df	SS	MS	F	P	Variance	Variance (%)
Mean male age (d) at sexual maturity (146.8 ± 1.71)							
Egg size (mm^3)	5	747	149.4	7.94	<0.001	65.29	40.6
Female (F)	15	3429	228.6	2.05	0.033	11.70	7.3
Male (M)	15	4486	299.1	2.68	0.005	18.74	11.6
M X F	45	5024	111.6	5.93	<0.001	46.41	28.8
Error	80	1506	18.8			18.83	11.7
Mean male body length (mm) at sexual maturity (27.2 ± 0.22)							
Egg size (mm^3)	5	14.2	2.8	3.52	0.006	1.02	37.6
Female (F)	15	66.9	4.5	2.14	0.025	0.24	8.8
Male (M)	15	51.0	3.4	1.63	0.104	0	0
M X F	45	94.0	2.1	2.59	<0.001	0.64	23.7
Error	80	64.6	0.8			0.81	29.9

Table 2.3. ANCOVAs for the extent of red coloration in the iris (proportion) and male aggression rate (attacks 10 min^{-1}) for *in vitro* fertilizations. Mean and standard error of variables are in parentheses.

Source	df	SS	MS	F	P	Variance	Variance (%)
Mean male extent of redness (%) in the iris (16.7 ± 0.51)							
Female (F)	15	499.5	33.3	2.96	0.002	0.15	1.9
Male (M)	15	160.5	10.7	0.95	0.517	0.01	0.1
M X F	45	505.6	11.2	2.52	<0.001	3.39	42.3
Error	80	357.0	4.5			4.46	55.7
Mean male aggression rate (attacks 10 min^{-1}) (59.7 ± 1.77)							
Female (F)	15	31.9	2.1	5.72	<0.001	0.15	32.1
Male (M)	15	4.7	0.3	0.84	0.635	0.01	1.2
M X F	45	16.7	0.4	1.42	0.087	0.05	11.5
Error	80	21.0	0.3			0.26	55.3

Chapter 3. The effect of crowding and density on male mating behaviour in the rose bitterling, *Rhodeus ocellatus*·

3.1. Abstract

Female density and resource availability are two key variables that shape mating systems. Theory predicts that reproductive skew will amplify with increased male density and decreasing availability of resources, though limited empirical evidence suggests that this may not always be the case. Here we tested mean crowding, defined as the number of males per unit of resource, and density per se, defined as the number of individuals present per unit area, to investigate their effect on the mating system of *Rhodeus ocellatus*, a fish with a promiscuous, resource-based mating system. Males were exposed to combinations of high and low levels of crowding and density, while the operational sex ratio was held constant. High levels of crowding significantly affected the proportion of mussel spawning sites defended by males and the proportion of mussels into which sperm was released. In contrast to theoretical predictions, neither density nor crowding influenced overall male aggressive behaviours. Density, but not crowding, had a significant effect on male courtship rate, which arose as a possible trade-off between intra-sexual competition and inter-sexual behaviour. We discuss the results in the context of mating system evolution.

· Casalini, M., Reichard, M. & Smith, C. 2010. The effect of crowding and density on male mating behaviour in the rose bitterling (*Rhodeus ocellatus*). *Behaviour* **137**: 1035- 1050.

3.2. Introduction

Differences in potential reproductive rate, resulting from anisogamy, have driven the evolution of sex role differentiation, whereby the sex limited by mate availability, almost always males, competes for fertilizations (Clutton-Brock & Parker 1992). Thus, males are subject to intra-sexual selection for characteristics that enhance their competitive ability, and inter-sexual selection for traits that enhance their attractiveness to females (Andersson 1994).

Kokko & Rankin (2006) argued the case for an impact of population density on the strength of sexual selection. Using a modelling approach they examined how density-dependent effects might operate on sexual selection, and how density might thereby impinge on population dynamics, proposing that sexual selection could operate as a self-limiting process mediated by density. For example, at low population density males may experience little competition, while at higher densities reproductive skew might be greater as males with greater resource holding potential monopolise matings to the exclusion of inferior males. However, while theoretical studies may predict that reproductive skew will increase asymptotically with density, empirical evidence suggests that this may not always be the case, for example because of switches in male behaviour (Jirotkul 1999), the breakdown of resource defence (McLain 1992; Reichard *et al*. 2004a,b), variation in resource availability (Wootton *et al*. 1995), changes in sex ratio (Pröhl 2002), or because the effects of density may be weak (Head *et al*. 2008). The impact of alternative male mating tactics in particular can have a marked effect on reducing variance in male mating success (Reichard *et al.* 2004a,b; Taborsky 2008).

Economic defendability theory predicts that the intensity of competition for a resource will correlate positively with population density and negatively with

resource availability (Brown 1964). However, below a certain level of resource availability the intensity of competition may moderate as defence of the resource becomes uneconomical (Grant *et al*. 2000). Experimental studies have confirmed the role of high male density (de Boer 1981), low resource availability (Almada *et al*. 1995), and a combination of high male density and low resource availability on increasing male aggressive behaviour (Kanoh 2000). On the other hand, to avoid a lower reproductive success males may have to face a trade off between aggressive defence of a territory and behaviours necessary to attract females. In *R. ocellatus* the intensity of courtship influences female mate choice (Casalini *et al*. 2009) and courtship rate has been demonstrated to increase when male density is low (de Boer 1981; Kanoh 2000; Spence & Smith 2005).

In addition, density itself, defined as the number of males present per unit area, may not be the most appropriate measure of competition for resources. Lloyd (1967) proposed a measure termed 'mean crowding'; the number of conspecifics an individual encounters in competition for a resource. The mean crowding of competitors is a more meaningful measure than density or resource availability, since it encapsulates a measure of absolute density, the degree of clustering of individuals, and in the context of competition over fertilizations, the degree of skew among reproducing males and females (Pomfret & Knell 2008).

Breeding resource availability has a major influence on sex roles and other features of animal mating systems (Emlen & Oring 1977). For example, when nest sites are scarce it is often females and not males that initiate courtship (Almada *et al*. 1995; Borg *et al*. 2002). The evolution of polyandry in particular is associated with limited breeding resources for females, including the availability of paternal care (Maynard Smith 1977; Clutton-Brock 1989; Ligon 1999). Inter-sexual

competition is also predicted to occur predominantly within the sex that is 'qualified to mate' (i.e., the sex that has achieved sexual maturity and has the resources necessary for mating) and whose reproductive success is restricted by a lower number of qualified mates (Ahnesjö *et al*. 2001).

Here mean crowding, defined as the number of males per unit of resource, and density, defined as the number of individuals present per unit area, were independently manipulated to investigate their effect on male reproductive competition using the rose bitterling, *Rhodeus ocellatus*. Bitterling are ideal candidates for examining the effects of crowding and density on male mating behaviour and reproductive success as their spawning site can be readily quantified and manipulated and they readily adapt to laboratory conditions. To do so males were exposed to combinations of high and low levels of crowding and density, while the operational sex ratio (OSR), the ratio of males and females ready to mate and an important determinant of mating competition (Jirotkul 1999) was held constant. It was predicted that (1) male aggression would increase at high density and crowding, while courtship rate would decrease and (2) the frequency of alternative mating tactics would increase at high density and crowding. Crowding and density were expected to influence the proportion of mussels defended since at high crowding and low density territorial males are more likely to be able to monopolise a higher proportion of resources. Thus, since a change in the availability of resources and the number of rivals might affect male behaviour, a final prediction was that (3) there would be an interaction in the effects of crowding and density.

3.3. Methods

Fish for experimental work were first generation offspring of 200 wild caught *R.*

ocellatus collected from the River Yangtze Basin, China, in 2005. Experimental fish were raised in captivity and were 18–24 months old when experiments were conducted. Prior to experiments fish were kept in stock aquaria measuring 60 (length) x 40 (width) x 40 (depth) cm. Stock aquaria were on a recirculating system with water temperature at 19º C. Each stock aquarium contained a layer of sand substrate, a freshwater mussel and artificial plants as refuges. The fish were kept under a 16h: 8h light: dark regime and fed commercial flake fish food twice each day and a mixture of frozen chironomid larvae and live zooplankton 2–3 times each week. Freshwater mussels used in 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 in Cambridgeshire, UK, stored in 160-L tanks and fed live phytoplankton daily.

Prior to experiments 96 males were haphazardly selected from stock aquaria, assigned by eye to three broad size categories, and individually marked with coloured elastomer tags (Northwest Marine Technology). Tag colours were white, blue and green; red was avoided since this is the hue of male nuptial coloration. Size-sorted males were held in separate stock aquaria and allowed to settle for two days.

Crowding was controlled by varying the number of mussels available for spawning, while density was controlled by varying the number of males. At a high level of crowding the ratio of males to mussels was 2:1 and at a low level 1:2. Experiments were conducted in a large aquarium measuring 180 (length) x148 (width) x100 (depth) cm with a volume of 2400 l. Male density was varied between 2 (low density) and 6 fish (high density). To ensure that the OSR remained unchanged across all treatments the number of females ready to mate was either 1

(low density) or 3 (high density), generating a ratio of males to females ready to mate of 2:1 (Table 2.1). Mussels were arranged in a grid (4 or 12 mussels) or row (3 mussels) equidistant from each other; irrespective of the number of mussels present, the same spacing between adjacent mussels was maintained in each trial. When a single mussel was used it was placed at the centre of the experimental aquarium. Thus, there were four treatment groups: low fish density and low mussel density, high fish density and low mussel density, low fish density and high mussel density, high fish density and high mussel density.

The day before the beginning of each trial males to be tested and females in spawning condition (with a fully extended ovipositor) were haphazardly selected from stock aquaria and placed in the experimental aquarium. Experimental mussels, each in a sand-filled plastic cup covered with a perforated plastic cup to allow inspection of the mussel but not spawning, were placed in the experimental aquarium. Artificial plants were provided as refuges. To replicate the natural size structuring seen in bitterling populations (Smith *et al*. 2000b), males in low-density trials (2 fish) were used from the large and small size classes, and in the high density trials 2 males from each size class (large, medium and small) were used. Body length (mean ± SE) in different size categories was: large 54.3 ± 0.36 mm, medium 47.0 ± 0.31 mm and small 41.3 ± 0.33 mm.

On the morning following stocking the mussels were uncovered and, after 1 h, the behaviour of every male was scored for 20 min using a palm computer with the FIT-system behaviour recording software (Held & Manser 2005). Mussels were again covered for two hours and the procedure repeated in the afternoon, with male behaviour recorded for 20 min. After completion of a trial all mussels and fish were removed and a new group stocked. Thus, one replicate, with all combinations of

crowding and density, was completed over a 4-day period. Treatment order and the order of focal male observations were randomized prior to the beginning of the experiment.

The behaviours scored were: (i) frequency of focal male aggressive defence of a mussel, (ii) frequency of aggression directed at the focal male, (iii) frequency of focal male courtship and (iv) frequency of focal male ejaculation. In addition, a record was made of the mussels defended by focal males and the mussels over which they ejaculated. These data enabled estimation of the proportion of available mussels that were defended, and the proportion into which males ejaculated, an index of the distribution of ejaculates and measure of the frequency of alternative male mating tactics. After completion of a trial, the body length (from the tip of the snout to the base of the tail fin) of every male was measured to the nearest 1 mm and fish were returned to a stock aquarium and were not used again. Experimental mussels were measured to the nearest 1 mm (maximum shell length) and were not used again. A total of 6 independent replicates, each comprising all combinations of crowding and density, were completed using 96 males, 48 females and 120 mussels. Mean male body length did not vary significantly among treatment groups (one-way ANOVA: $F_{3,20} = 1.32$, $P = 0.294$).

3.3.1. Ethical note

Elastomer tags have been widely used to mark fish and have proven to be harmless (Halls & Azim 1998; Malone *et al*. 1999; Imbert *et al*. 2007; Weston & Johnson 2008), which also proved the case in the present study. Aggressive territoriality is a normal feature of male bitterling spawning behaviour, though fish do not inflict physical damage on one another. The high-density experimental treatment in the current experiment consisted of only 6 males in a 2400-l aquarium, within the range

of densities seen under natural conditions (Smith *et al*. 2000a). The test aquarium was furnished with artificial plants as refuges where attacked fish could readily avoid confrontation. Fish were constantly monitored before and during the experiment and none (or any in 15 years of experimental research) showed marks or injuries, or any signs of stress, during territorial disputes.

3.3.2. Data 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 meet these assumptions. Each datum for individual male behaviour referred to the mean of each morning and afternoon, 20-min observation period. A one-way ANOVA was used to test for differences in male size among replicates. A Spearman correlation was used to test the correlation between aggression rate and the mean proportion of mussels defended, between male size and net aggression rate, and between ejaculation rate and the mean proportion of mussels into which sperm was released. A two-way ANOVA was used to test the effect of crowding and fish density on mean male aggression rate, mean male ejaculation rate, mean sneaking rate, mean courtship rate, mean proportion of mussels defended, and mean proportion of mussels into which males ejaculated.

3.4. Results

There was a highly significant correlation between each male net aggression rate (number of aggressions performed minus mean number of aggressions received) and their size (Spearman correlation: $r_{94} = 0.732$, $P < 0.001$; Figure 3.1). Bigger males performed more aggression and smaller males were attacked more frequently.

Mean aggression rate and the mean proportion of mussels defended by males were not correlated (Spearman correlation: $r_{22} = 0.020$, $P = 0.925$). There was no significant effect of either crowding or density or an interaction between variables on mean male aggression rate (rank transformed data, two-way ANOVA, crowding: $F_{1,20} = 3.75$, $P = 0.067$; density: $F_{1,20} = 1.15$, $P = 0.296$; interaction: $F_{1,20} = 0.21$, $P = 0.651$; Figure 3.2), on mean male ejaculation rate (two-way ANOVA, crowding: $F_{1,20} = 0.05$, $P = 0.833$; density: $F_{1,20} = 0.17$, $P = 0.681$; interaction: $F_{1,20} = 2.81$, $P = 0.109$), and on mean male sneaking rate (square root transformed data, two-way ANOVA, crowding: $F_{1,20} = 0.06$, $P = 0.801$; density: $F_{1,20} = 2.03$, $P = 0.170$; interaction: $F_{1,20} = 0.18$, $P = 0.674$). However, there was a significant effect of both variables on the mean proportion of mussels defended by males (two-way ANOVA, crowding: $F_{1,20} = 11.83$, $P = 0.003$; density: $F_{1,20} = 14.89$, $P = 0.001$); a greater proportion of available mussels were defended at high levels of crowding and at low male density. There was no significant interaction between the variables ($F_{1,20} = 0.57$, $P = 0.459$; Figure 3.3).

The proportion of mussels into which males ejaculated was used as an index of the distribution of ejaculates among mussels and, therefore, a measure of the incidence of alternative male mating tactics. Crowding but not density significantly affected the mean proportion of mussels into which males released sperm (two-way ANOVA, crowding: $F_{1,20} = 5.55$, $P = 0.029$; density: $F_{1,20} = 0.66$, $P = 0.425$; Figure 3.4), with the proportion of mussels higher at high crowding irrespective of density. There was no significant interaction between variables ($F_{1,20} = 0.54$, $P = 0.470$). The mean rate of ejaculation and the proportion of mussels into which sperm was released were significantly correlated (Spearman correlation: $r_{22} = 0.619$, $P = 0.001$).

In contrast, density but not crowding significantly influenced mean male courtship rate. Mean courtship rate was lower at high density (two-way ANOVA: $F_{1,20} = 5.79$, $P = 0.026$; Figure 3.5). However, while mean courtship rate was also numerically lower with high crowding, this effect was not significant, though it approached significance ($F_{1,20} = 3.97$, $P = 0.060$). There was no interaction between variables ($F_{1,20} = 0.15$, $P = 0.705$).

3.5. Discussion

The aim of this study was to investigate the effects of two measures of resource competition on components of sexual selection in the rose bitterling, *R. ocellatus*, a fish with a resource-based mating system. The measures of resource competition used were male crowding (*sensu* Lloyd 1967) and density. It was predicted that male aggression would increase at high density and crowding, while courtship rate would decrease, and that the frequency of alternative mating tactics would increase at high density and crowding. A final prediction was that crowding and density would interact.

Surprisingly, neither crowding nor density affected overall levels of male aggression. Aggression among males was shown to be dictated strongly by body length; bigger males tended to perform more aggression and smaller males were attacked more frequently. Body length in the related *R. amarus* also determined male lifetime reproductive success (Reichard *et al*. 2009). This size effect appears to arise through male control of spawning resources and success in sperm competition (Reichard *et al*. 2009). An alternative means of understanding the pattern of male-male interactions with respect to density and crowding would be to utilise social network analysis (Croft *et al*. 2008). To do so, a second observer could identify whether aggressive interactions among males were distributed randomly

among a network of interacting males, or were concentrated on specific individuals, for example among a subset of larger males.

Density, but not crowding, had a significant effect on male courtship rate. At high fish density, courtship rates were lower, possibly the result of a trade-off between defending resources from rivals and attracting mates. This trade-off has been found in previous studies in bitterling (Reichard *et al*. 2004b), as well as in guppies (*Poecilia reticulata*) (Jirotkul 1999), three-spined sticklebacks (*Gasterosteus aculeatus*) (Le Comber *et al*. 2003), and zebrafish (*Danio rerio*) (Spence & Smith 2005). This result suggests that while male courtship rate is sensitive to a change in the number of rivals, access to resources has more trivial significance, though note that the pattern of effect of crowding on courtship rate mirrored that of density and the result approached significance. In addition to the rate of courtship, the intensity of courtship may by modulated by males and has been demonstrated to play an important role in influencing female mate choice, and consequently the strength of sexual selection in several taxa, including bitterling (Reichard *et al*. 2005; Casalini *et al*. 2009), the pacific blue-eye (*Pseudomugil signifier*) (Wong 2004), the guppy (Matthews *et al*. 1997), the bicolor damselfish (*Stegastes partitas*) (Knapp & Kovach 1991) and the katydid (*Requena verticalis*) (Gwynne 1984). Courtship intensity was not measured in the present study, though it might be sensitive to changes in density or crowding. Female willingness to spawn or response to male courtship may have influenced male behaviour but, in the present experiment, the number of spawnings was rare irrespective of mussel crowding or fish density. Also, as females were not marked, it was not possible to test the number of spawning events for each individual female when more than one female was present.

Crowding significantly affected the proportion of mussels defended by males, a measure of male response to rival males adopting alternative mating tactics, with more mussels defended at a high level of crowding. Crowding also affected the success of alternative male mating tactics, measured as the proportion of mussels into which sperm was released by males, with males ejaculating into a higher proportion of mussels at a high level of crowding. Thus, for both these variables an elevated level of crowding had the effect of increasing the intensity of competition among males, either directly for spawning sites or through risk of sperm competition. While the effect of crowding appears predictable with hindsight, this study demonstrates first that, in rose bitterling at least, when resources essential for reproduction are available to males they are used. Second, that crowding and density are two distinct variables, though in the literature density is commonly used as a proxy for crowding.

Male control of spawning sites and distribution of ejaculates are significant determinants of male reproductive success in bitterling (Smith *et al*. 2003; Reichard *et al*. 2004a, 2005; Casalini *et al*. 2009) and other species (Alonzo & Warner 2000; Wedell *et al*. 2002; Spence & Smith 2005; Taborsky 2008). The significant effect of crowding on these variables suggests that crowding around resources critical for reproduction may influence the strength of intra-sexual selection. A consequence of this result is that where male crowding is high, intra-sexual selection will be strong and traits that benefit males in competition for resources will tend to increase in frequency. In the case of bitterling these traits include large body length, red coloration in the eye and on the fins, and aggressive behaviour (Smith *et al*. 2003; Reichard *et al*. 2005; Casalini *et al*. 2009). Conversely, where crowding around resources is low the prediction is for relatively small and drab males that do not

engage in aggressive contests. Under these conditions the mating system would be one in which females would be able to exercise mate choice with little constraint from male dominance and resource monopolisation. Bitterling offer an unusually tractable model for investigating these predictions. Previous work has shown that bitterling occur in lakes in which their population size can vary 90-fold and mussel abundance 500-fold (Smith *et al*. 2000a). Consequently, crowding may vary over several orders of magnitude under natural conditions among bitterling populations, with predictable impacts on mating system evolution among populations.

The key finding of the present study was that male behaviour appeared more sensitive to crowding; the number of conspecifics an individual encounters in competition for a resource, than to density per se. Density, particularly measured simply as the number of individuals per unit area, may fail to capture the degree of competition for resources, with consequent unpredictable responses to density (Kokko & Rankin 2006). Future research should explore the effect of intra-sexual selection on male adaptive responses to variation in crowding in natural populations, with an approach utilising social network analysis one that may be especially rewarding.

Figure 3.1. Net aggression rate (20 min^{-1}), estimated as mean number of aggressions performed minus mean number of aggressions incurred as a function of male body length (mm).

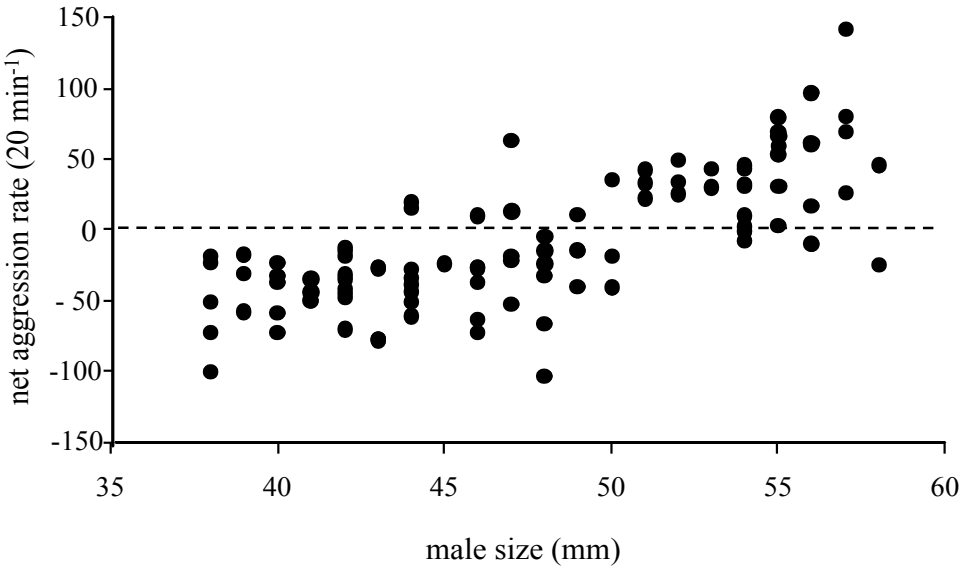


Figure 3.2. Mean ± SE aggression rate (20 min^{-1}) at low and high density and crowding.

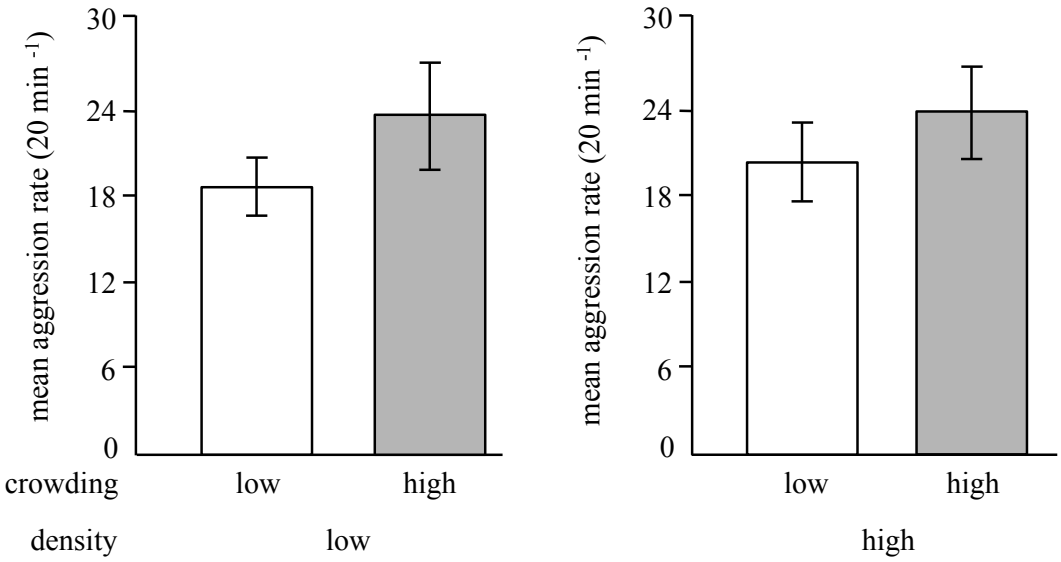


Figure 3.3. Mean ± SE proportion of mussels defended (20 min^{-1}) at low and high density and crowding.

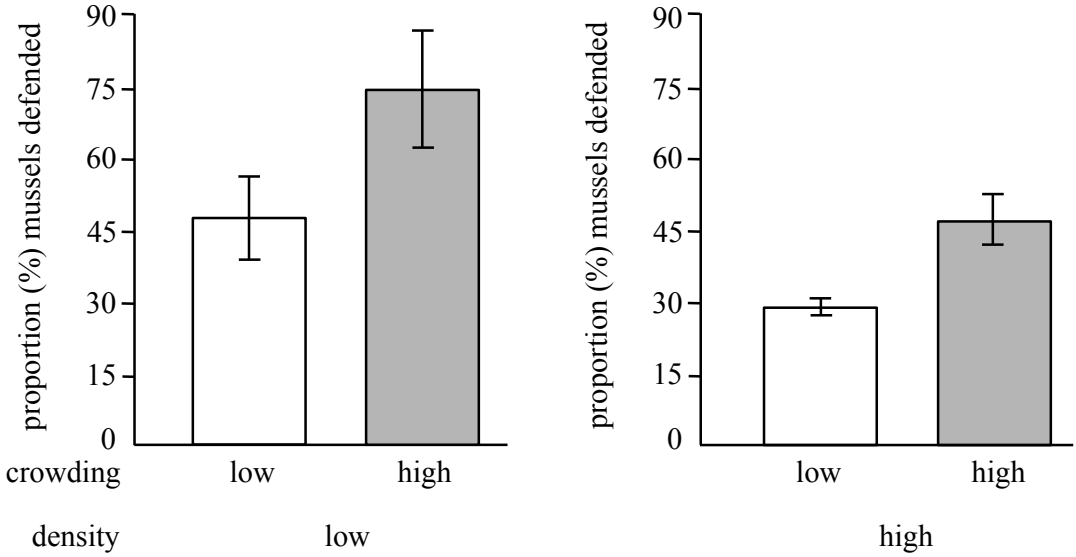


Figure 3.4. Mean ± SE proportion of mussels in which sperm was released at low and high density and crowding.

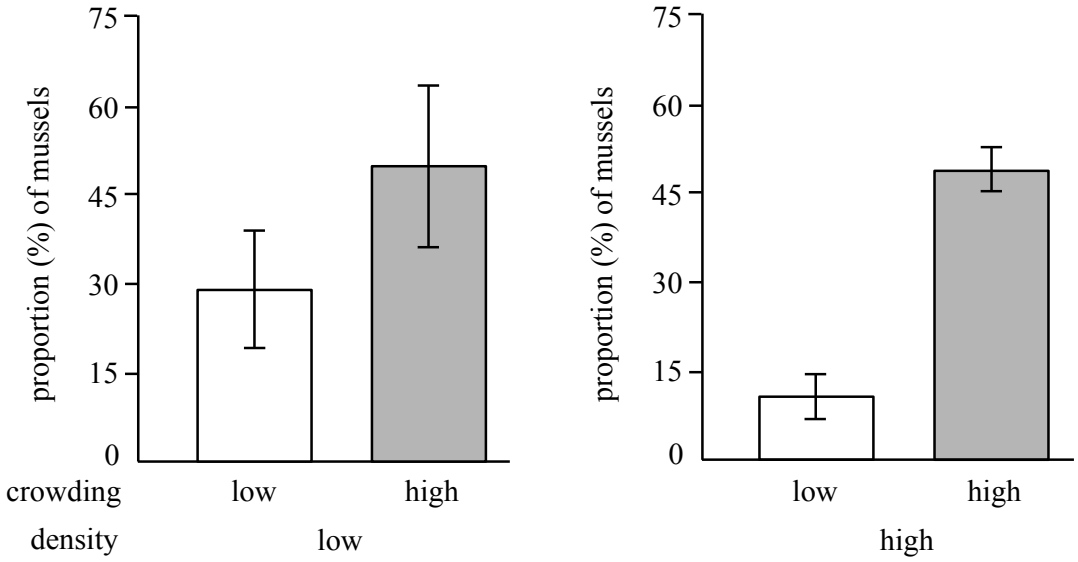


Figure 3.5. Mean ± SE male courtship rate (20 min^{-1}) at low and high density and crowding.

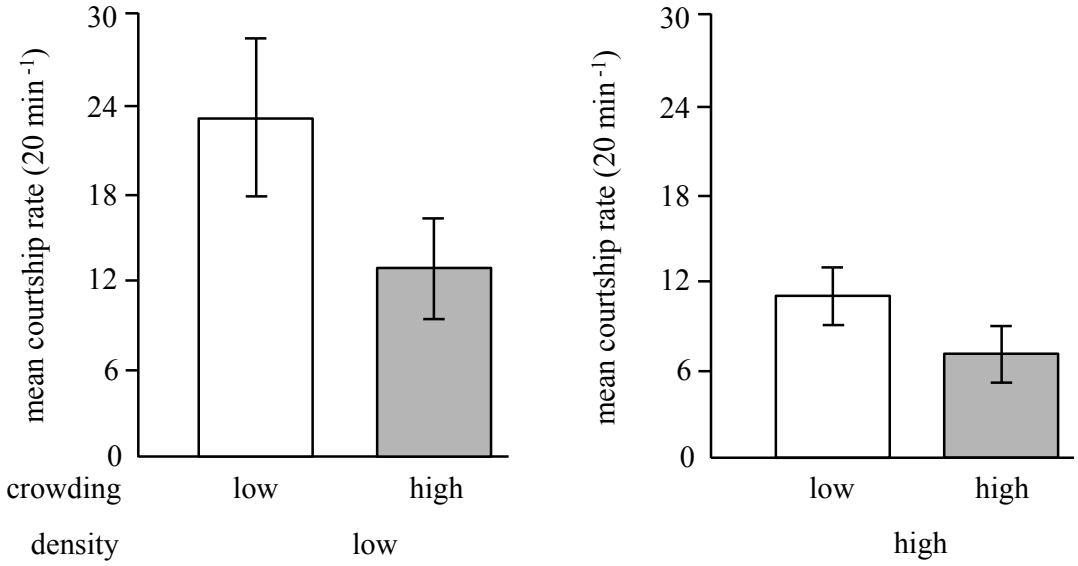


Table 3.1. Experimental design: number of males, females and mussels at different levels of mean crowding and density. Operational Sex Ratio (OSR) was male-biased (2:1) in all treatments.

Crowding	Density	Mussels	Males	Females	Mussels per male	Male density (m^{-2})	OSR
Low	Low	4	2	1	2.0	0.75	2:1
High	Low	1	2	1	0.5	0.75	2:1
Low	High	12	6	3	2.0	2.25	2:1
High	High	3	6	3	0.5	2.25	2:1

Chapter 4. The effect of resource availability and length of allopatric isolation on the mating behaviour of the European bitterling, *Rhodeus amarus*

4.1. Abstract

The availability of mating resources and their spatial and temporal clustering can play an important role in shaping mating behaviours and thus influence strategies and tactics males adopt to increase their reproductive fitness. In populations isolated for longer periods of time differences in mating behaviours can be stronger and even lead to reproductive isolation. Here we investigated the effects of different levels of mating resource availability and length of allopatric isolation on male behaviours in four populations of the European bitterling, *Rhodeus amarus*, a small cyprinid fish with a promiscuous, resource-based mating system. Three experiments were performed; in the first differences in male aggressive behaviour and coloration, and the role of the length of isolation in modulating its strength were tested. In the second, females were paired with males from the same and a different population to assess the role of resource availability on inter-sexual behaviour; hybrids were then obtained with IVF to ascertain whether differences could have led to reproductive isolation in terms of embryo survival. Finally, the effects on male aggressive behaviour of the exposure to different levels of resource availability during ontogeny were tested in juveniles from one of the four populations used in the previous experiments. The combined effect of mussel availability and length of isolation greatly influenced adult aggression rate and coloration, and the longer the isolation the stronger the pressure of selection on male behaviours; conversely, no effect of different resource availability was detected in the behaviour of juveniles. No clear evidence of pre- or postzygotic

isolation emerged from the experiments as no difference was detected either in inter-sexual mating behaviours or in embryo survival rate.

4.2. Introduction

In some species females mate preferentially with dominant males that monopolise high quality resources and adopt energetically expensive courtship or sexual signalling (Andersson 1994). Conversely, females can show no preference for characters connected to male dominance (Qvarnström & Forsgren 1998; Casalini *et al*. 2009), and they can also solicit sneakers to improve their fertilization success (Smith & Reichard 2005). Sneaking is a common alternative tactic adopted by males who fertilize eggs without incurring the costs involved either in establishing and maintaining dominance, or in courtship to attract females or in offspring care (Andersson 1994). Sneaking can undermine the reproductive success of dominant males (Avise *et al*. 2002; Reichard *et al*. 2004a), and is frequently associated with sperm competition (Andersson 1994). Sperm competition, competition between the sperm of two or more males for the fertilization of ova, is an important mechanism of sexual selection that has shaped the evolution of animal mating systems (Andersson 1994; Eberhard 1996; Møller 1998). Because ejaculates are energetically expensive to produce (Wedell *et al*. 2002), in response to the risk of sperm competition males should maximize ejaculate expenditure in competition with a single competitor, but reduce expenditure as the number of rivals exceeds one, since the probability of fertilizing eggs diminishes with the number of competing males (Parker *et al*. 1996).

For dominant males an alternative, or additional, response to competitors is aggression (Petersen & Warner 1998). Aggressive defence of females or sites of reproduction is common in many taxa (Shuster & Wade 2003), and represents a mechanism for avoiding or reducing the risk of sperm competition. However, the conditions that determine whether responses to competition will result in the

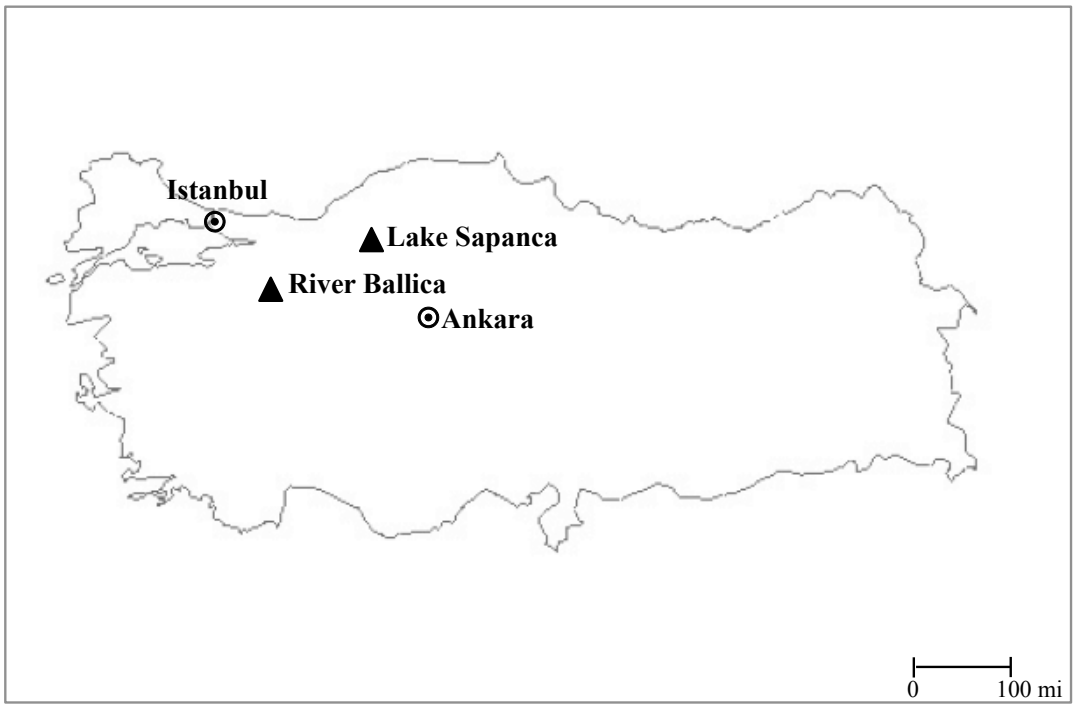
evolution of either aggression or the adoption of alternative tactics are ambiguous. Though not mutually exclusive, each makes different demands on males, which may result in behavioural or physiological trade-offs.

The temporal and spatial distribution of fertilizations shapes male tactics and mating system evolution (Andersson 1994; Shuster & Wade 2003). When fertilizations are spatially clustered or temporally dispersed a small number of males have a greater opportunity to monopolize matings, leading to increased variance in male reproductive success and strong selection on males to control females or the resources they need for reproduction, often through aggressive defence. In contrast, when fertilizations are spatially dispersed and temporally clustered males have less opportunity to compete through controlling sites of reproduction. Under these conditions males may experience stronger selection through female mate discrimination and sperm competition (Shuster & Wade 2003).

Differences in resource availability can affect male mating behaviours and a change in female mate discrimination can ultimately lead to prezygotic reproductive isolation. Even though speciation in allopatric populations has traditionally been ascribed to lack of gene flow due to geographical isolation (Mayr 1942), more recent comparative studies on species richness in dichromatic passerine birds (Barraclough *et al*. 1995; Møller & Cuervo 1998; Seddon *et al*. 2008), and experimental works on female choice for male secondary sexual traits (Boake *et al*. 2000, 2003; Kidd *et al*. 2006) have demonstrated a role for sexual selection in driving reproductive isolation and speciation. When previously allopatric populations come into secondary contact, if the degree of genetic divergence is high, mating may not take place or hybrids may be sterile or not viable; conversely, if it is weak, the two populations may still interbreed (Parker & Partridge 1998).

In the present study a comparison was made of the behaviour and colour of bitterling from populations with historically different availability of spawning sites. The effects of spawning site availability were tested in four allopatric populations of the European bitterling, *Rhodeus amarus*. Two study populations were located in Turkey, in Lake Sapanca (40° 42′ N, 30° 15′ E) and the River Ballica (40° 00′ N, 29° 25′ E), both in western Anatolia.

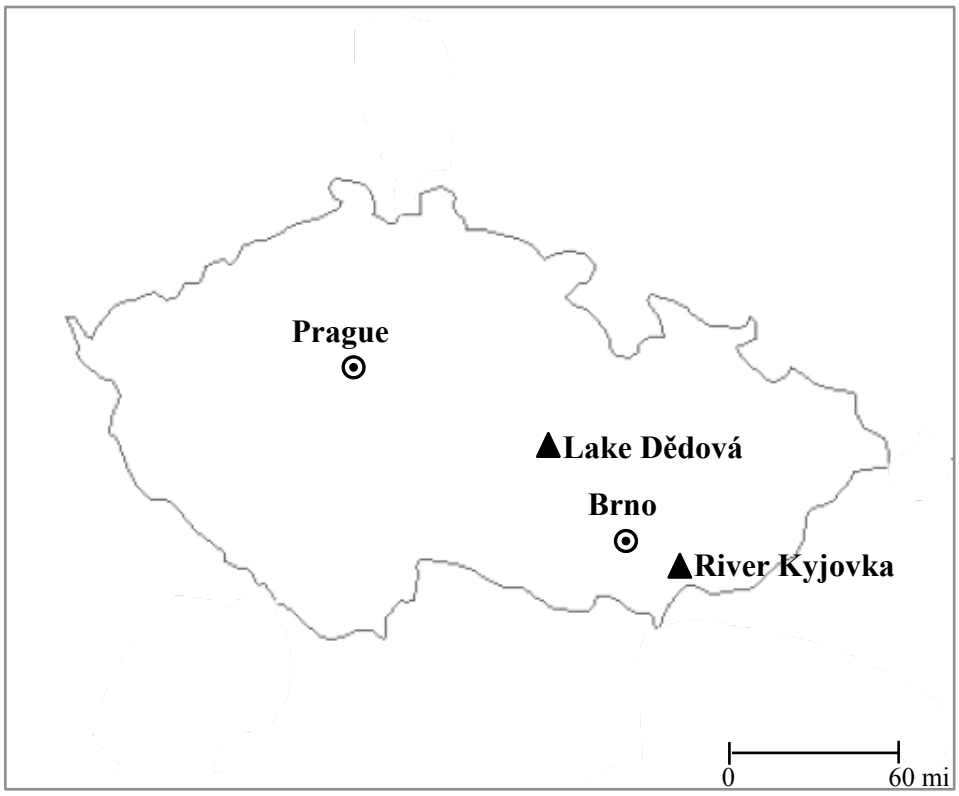
Figure 4.1. Map of Turkey showing the location of the sites where experimental fish were collected. (Adapted from: http://geography.about.com/; approved for inclusion by Matt Rosenberg).



Two further populations were located in the Czech Republic, one in the River Kyjovka (48° 47′ N, 17° 01′ E), a tributary of the River Dyje, the other in Lake

Dědová (49° 45′ N, 15° 59′ E), a gravel pit created in the early 1960s adjacent to the River Dyje that was populated from the river during floods.

Figure 4.2. Map of the Czech Republic showing the location of the sites where experimental fish were collected. (Adapted from: http://geography.about.com/; approved for inclusion by Matt Rosenberg).



The two Turkish populations were genetically distinct, reflecting the difference in their time of divergence (Bryja *et al*. 2010), while the two Czech populations were genetically indistinguishable from each other.

Three separate studies were performed to understand the effects of spawning sites availability on mating system evolution. In the first, differences in male

aggressive behaviour and the role of the length of isolation in modulating its strength were tested in the four populations; in the second, females from one population were paired, and subsequently crossed, with males from the same and a different population to test whether a difference in resource availability can influence inter-sexual mating behaviour and, if so, whether a longer period of isolation can lead to reproductive isolation, measured as embryo survival rate. The third experiment was performed in the Czech Republic with juveniles from one of the populations used in the previous experiments to test for the effect of the exposure to different levels of mating resources during ontogeny on male aggression rate.

In populations with low historical abundances of mussels, crowding (*sensu* Lloyd 1967) was predicted to be high, with a result that spawnings would be spatially clustered enabling a small number of males to monopolise them. In contrast, where crowding was low, the prediction was that spawnings would be spatially dispersed, preventing monopolisation of fertilizations through resource defence. In addition to comparing populations with different levels of clustering, the aim was also to compare populations of different ages of divergence. In older-established populations, the effects of crowding were predicted to be more marked than in more recent populations. Thus, the predictions of the study were that the mating systems of fish from populations with high spatial clustering would be driven by male territorial aggression, with a limited opportunity for female mate choice, whereas in populations with dispersed spawning sites males would be less aggressive, with a more substantial role for female mate choice. These differences in mating system were further predicted to be more divergent for populations of greater antiquity than more recently separated populations. Besides, it was predicted

that there would be a difference in male and female behaviour when females were paired with males from a different population and this would lead to an early stage of reproductive isolation and influence hybrid survival rate, at least in the populations isolated for a longer period of time. As bitterling reach sexual maturity at an early age and well in advance of the beginning of the spawning season, a final prediction was that different levels of resource availability would exert their effect also when experienced during ontogenesis and, therefore, males exposed to an environment poor in mating resources would show a higher level of aggression.

The European bitterling *Rhodeus amarus* is a small freshwater cyprinid fish (subfamily Acheilognathinae) that shares the same reproductive mating system and mating behaviours as the rose bitterling *Rhodeus ocellatus*, the only difference being a shorter length of reproductive season (Chapter 1). For a complete review of bitterling reproductive ecology, see Smith *et al*. (2004).

4.3. Methods

Experiment 1. The experiment was performed in the facilities of Istanbul University in Sapanca, Turkey, and at the Institute of Vertebrate Biology in Brno, Czech Republic during the period of bitterling spawning season. Experimental fish were wild European bitterling *Rhodeus amarus*. Four populations were tested, two in Turkey (one from Lake Sapanca, one from the River Ballica) and two in the Czech Republic (one from the River Kyjovka and one from Lake Dědová).

The fish were captured with traps and transported to the facilities, where they were housed in stock aquaria measuring 70 (length) x 30 (width) x 40 (depth) cm in Turkey and 75 (length) x 40 (width) x 40 (depth) cm in the Czech Republic and fed commercial dried fish flake food twice daily. Stock and experimental aquaria were exposed to a 16: 8 h light: dark regime, and provided with a layer of

sand substrate and artificial plants as refuges. Freshwater mussels used in trials were *Unio pictorum* that commonly co-occur with bitterling in Central Europe and are readily used as spawning sites by *R. amarus* (Reichard *et al.* 2010). Mussels were collected from the lakes and rivers where the fish were captured and, prior to experiments, were stored in an artificial outdoor pond.

To measure the degree of crowding and competition for spawning sites 10 *Anodonta anatina* and 10 *Unio pictorum* mussels were placed in each study site for 3-4 hours so that bitterling could spawn freely in them. The mussels were then dissected and the number of eggs present in each mussel noted down. The mussels had been checked before being placed in the sites to ensure that they did not contain any eggs.

The day before the beginning of the trial a focal male was randomly chosen from the stock aquarium and placed in an aquarium measuring 50 (length) x 40 (width) x 30 (depth) cm in Turkey and 60 (length) x 40 (width) x 30 (depth) cm in the Czech Republic, with a mussel in a sand-filled plastic cup and artificial plants as refuges. Opaque screens between aquaria prevented visual interference with neighbouring fishes. On the following day, in the morning, a female in spawning condition (with an extended ovipositor), constrained in a perforated transparent bottle, was gently placed into the aquarium. When the male started courting the female and inspecting the mussel, so showing that territoriality had been fully established, the female was removed, her body length (from the tip of the snout to the base of the tail fin) was measured to the nearest 1 mm, and the fish was returned to stock tanks and not used again. A tester male, constrained in a perforated transparent bottle, was then placed into the aquarium near the mussel. As soon as the focal male started to approach the constrained male, the behaviours of the focal

male were scored for 10 minutes. The tester male and the mussel were then removed, male body length and maximum shell length of the mussel were measured to the nearest 1 mm and fish and mussel were put back in a stock tank and not used again. To exclude any effect of female, constrained male and mussel on focal male aggressive behaviour, and to test its consistency, the same procedure was repeated after 3 hours with the same focal male but with a different female, tester male and mussel. The behaviours of focal males scored were: (i) head butting, (ii) bouts of side jerking, and (iii) fin spreading. At the end of the trial tester male, female and mussel were to the nearest 1 mm and not used again. The focal male was then lightly anesthetized with a few drops of clove oil and photographed against a scale bar using a Canon EOS 350D with a 60 mm macro lens under standard light condition and his body length and extent of red in the iris were measured from digital photographs using UTHSCSA Image Tool 3.0. The extent of red in the iris and on the tail fin were measured using Photoshop Elements 5.0 following the procedure described in Chapter 2 (page 29). A total of 14 independent replicates were completed for each population.

To exclude any effect of the anaesthetic on the extent of eye redness during the time necessary to photograph the fish, 10 bitterling from L. Sapanca and 10 from R. Ballica, haphazardly chosen from stock aquaria, were photographed against a scale bar 1, 3, 5, 7, 10 minutes after being anesthetized using a Canon EOS 350D with a 60 mm macro lens under standard light condition and the extent of red in the iris was measured using Photoshop Elements 5.0. The fish were then returned to stock aquaria and not used for experimental work.

Experiment 2. The experiment was performed in Turkey and in the Czech Republic, using populations of bitterling from the same sites as the ones used in

Experiment 1. Females collected from R. Ballica (Turkey) and L. Dědová (Czech Republic) were not in spawning condition, thus only females from L. Sapanca (Turkey) and from R. Kyjovka (Czech Republic) were tested. Females from L. Sapanca were paired with males from L. Sapanca and R. Ballica and females from R. Kyjovka with males from R. Kyjovka and L. Dědová. The experimental male was placed in an aquarium measuring 50 (length) x 40 (width) x 30 (depth) cm in Turkey and 60 (length) x 40 (width) x 30 (depth) cm in the Czech Republic with a layer of gravel, a mussel in a sand-filled plastic cup and artificial plants as refuges. The mussels used in the experiment were *Unio pictorum* in Turkey and *Unio tumidus* in the Czech Republic. This mussel species is widespread in sites inhabited by bitterling and is readily used as a spawning site by *R. amarus* (Reichard *et al*. 2010).

When the male was acclimatized, typically after 30 minutes, a female in spawning condition, constrained into a jar, was placed into the aquarium. As soon as the male started to court her, the female was gently released and the behaviours of males and females were scored for 15 minutes. The behaviours scored were: (i) male courtship/leading rate, (ii) male ejaculation rate, (iii) female inspection rate, (iv) female "following" rate (the female follows the male who is courting and leading her to the mussel), and (v) female spawning rate. As males do not interrupt courtship while leading the female to the mussel, data for these two behaviours have been pooled. If the female spawned, the observation was stopped and the time and the number of eggs spawned noted down. At the end of each trial *in vitro* fertilization was performed gently stripping the male and the female. Fish and mussels were then measured to the nearest 1 mm and not used again. The fertilized eggs were put in marked Petri dishes filled with water, covered singly with a black

lid, and kept at room temperature. The water in the Petri dishes was changed every day and embryo survival was checked every day for 4 days in Turkey and 6 days in the Czech Republic. A total of 15 independent replicates were performed for each crossing in Sapanca and 21 in Brno.

Experiment 3. Fish used for experimental work were wild *Rhodeus amarus* collected from the River Kyjovka, Czech Republic, in March, before the beginning of the spawning season. At the time of collection the fish were less than one year old and, prior to experiments, were held for about two months in 24 480L tanks measuring 110 (length) x 110 (width) x 40 (depth) cm provided with a layer of gravel and set outdoors. Four males and four females were housed in each tank; four mussels (*Unio tumidus*), each in a sand-filled plastic cup, were placed in the far corners of twelve tanks and only one mussel was placed in one corner in the remaining tanks.

At the beginning of the experiment the largest male, a haphazardly selected female in spawning condition, and a second haphazardly selected male were collected from one tank. The largest (focal) male together with the female constrained in a glass jar were set in an experimental aquarium measuring 60 (length) x 40 (width) x 40 (depth) cm with a layer of gravel, a mussel in a sand-filled plastic cup, and artificial plants as refuges. When the male started to court the female, the female was removed and the second male, constrained in a glass jar, was placed into the aquarium. As soon as the focal male started to attack the constrained male, the behaviours of the focal male were scored for 10 min. The behaviours scored were: (i) head butting, (ii) side jerking, and (iii) fin spreading.

After completion of the trial the body length of both males and of the female, and the maximum shell length of the experimental mussel were measured to

the nearest 1 mm and fish and mussel returned to stock tanks and not used again. A total of 12 independent replicates were completed for each level of mussel availability.

4.3.1. Data analysis

All data were tested for normality using a Shapiro-Wilk test and for equality of variance using Bartlett's test. Data that did not meet assumptions of normality and homoscedasticity were transformed. Nonparametric tests were used if data did not respond to transformation.

Experiment 1. A Pearson's correlation was used to test for the correlation between aggression rate and male body length, and aggression rate and mean extent of red in the iris. Differences in body length and extent of red in the iris in males from sites with different mussel abundance were tested with one-way ANOVA, and ANCOVA was used to test for the influence of mussel availability and length of time during which the populations have been separated, with male body length as a covariate.

Experiment 2. An unpaired *t*-test was used to test for differences in male and female behaviours when females were paired with males from different populations and to compare embryo survival rate for the populations tested in Brno. Embryo survival rate for the populations tested in Turkey was tested with a Mann-Whitney test.

Experiment 3. Differences in focal male body length between males raised at different levels of mussel availability were tested with an unpaired *t*-test. A Pearson's correlation was used to test for a correlation between focal male aggression rate and their body length and between focal male aggression rate and

the body length of constrained males. ANCOVA was used to test for differences in behaviour between the two treatments, with male body length as a covariate.

4.4. Results

Experiment 1. No difference was detected in the extent of red in the iris of males photographed 1, 3, 5, 7 and 10 minutes after being anesthetized (Table 4.1).

There was a difference in the number of eggs present in the mussels exposed to free spawning by bitterling in the four study sites (Table 4.2); the difference was higher in the study sites in Turkey.

Focal male aggression rate was correlated with their body length (Pearson's correlation, square root transformed data: $r_{54} = 0.622$, $P < 0.001$) and with the extent of red in the iris ($r_{54} = 0.692$, $P < 0.001$), a common finding in *Rhodeus amarus*. There was a significant difference in body length (ANOVA: $F_{1,54} = 77.29$, $P < 0.001$) and in the extent of red in the iris (ANOVA: $F_{1,54} = 28.29$, $P < 0.001$; Figures 4.3 and 4.4) between the males from sites with high or low number of mussels. Males from sites with fewer mussels were bigger and showed a greater extent of red in the iris than the ones from sites with high mussel availability; the differences were higher in the males from the populations tested in Turkey.

Once controlled for focal male body length, mussel availability strongly influenced mean male aggression rate (ANCOVA, square root transformed data: $F_{1,51} = 93.00$, $P < 0.001$). The length of time during which populations have been isolated did not influence mean male aggression rate (ANCOVA, square root transformed data: $F_{1,51} = 0.02$, $P = 0.901$), but the combined effect of mussel availability and length of isolation strongly affected male aggression rate (ANCOVA, square root transformed data: $F_{1,51} = 41.26$, $P < 0.001$; Figure 4.5).

Experiment 2. In the populations tested in Turkey and in the Czech Republic no difference was present either in male courtship rate or in female behaviours when females were paired with males from the same or a different population (Figures 4.6 and 4.7; Table 4.3 and 4.4).

There was a significant difference in the ejaculation rate of the males from the two populations tested in Turkey (unpaired *t*-test, square root transformed data: $t_{28} = 2.72$, $P = 0.011$); the ejaculation rate of the males from R. Ballica (low mussel availability) was higher than the one of the males from L. Sapanca (high mussel availability). Conversely, no difference was detected in the ejaculation rate of the males from the two populations tested in the Czech Republic (unpaired *t*-test, square root transformed data: $t_{40} = 0.84$, $P = 0.406$).

No difference was present in the survival rate of the embryos obtained crossing males from L. Sapanca and R. Ballica with females from L. Sapanca in Turkey and males from R. Kyjovka and from L. Dědová with females from R. Kjyovka in the Czech Republic (percentage of survival on the 4th day in Turkey, Mann-Whitney test: $Z = 0.181$, $P = 0.856$; percentage of survival on the 6th day in the Czech Republic, unpaired *t*-test, $\log_{10+1}$ transformed data: $t_{18} = 1.42$, $P = 0.172$; Figure 4.8).

Experiment 3. Male body length did not vary between treatments (unpaired *t*-test, square root transformed data: $t_{22} = 0.845$, $P = 0.407$), and no correlation was detected between focal male aggression rate and their body length (Pearson's correlation, square root transformed data: $r_{22} = -0.007$, $P = 0.739$). No correlation was detected also between male aggression rate and the body length of constrained males (Pearson's correlation: $r_{22} = 0.104$, $P = 0.626$). No difference was detected in the aggression rate of focal males (ANCOVA: $F_{1, 22} = 0.64$, $P = 0.433$; Figure 4.9).

4.5. Discussion

The aim of the present experiments was to test the effects of mating resource availability and length of allopatric isolation on the mating behaviour of different populations of the European bitterling *Rhodeus amarus*, and whether differences in mating behaviour have led to reproductive isolation. Besides, also the influence of mussel abundance during ontogeny was investigated.

The results of Experiment 1 confirm the predictions: males from the sites with few mussels were larger and more colourful; the ability to control mating resources correlated to larger body length is a common finding in many animal taxa, and, together with a success in sperm competition, has been demonstrated to increase male lifetime reproductive success in *R. amarus* (Reichard *et al*. 2009).

According to predictions male aggression rate was higher in sites with low mussel availability; besides the males from the Turkish sites showed the lowest and highest rate of aggression rate, so confirming that the number of available mating resources and the length of allopatric isolation can exert effects at individual and population level and on the selection for different phenotypic traits.

If resources are scarce and spatially clustered, few males can monopolize them and constrain female choice; the pressure of sexual selection will then favour characters used for intra-sexual competition. Conversely, if resources are abundant and spatially dispersed, females are free to choose, males can devote more time to courtship (Borg *et al*. 2002), and the pressure of sexual selection will favour characters used as inter-sexual signals. Even if the costs individuals incur in sexual displays, resource defence, and risk of sperm depletion can lessen future reproduction and increase their mortality rate (Lindström 2001), the demand for

defence seems to be critical being one of the most important factors determining males' access to mates (Lindström 1988).

The spatial and temporal clustering of resources may affect also the local population density as many males and females gather around few breeding sites (Warner & Hoffman 1980; Reichard *et al*. 2004a,b). In the present study the number of eggs present in the mussels in the study sites (see Methods) was higher in the sites where the number of mussels is low, and the difference was far higher in Turkey. If mating resources are scarce they can erode female choice for mussels (Reichard *et al*. 2010), and mussel defence by males may become more important than the behaviours necessary to attract mates (Warner & Hoffman 1980).

The data collected in the present study confirm the role of mating resources and of the adaptation to the environment; the longer the period of allopatric isolation the stronger the adaptation to the environment and the selective pressure on characters used in intra-sexual competition.

Due to the presence in different habitats of different selective factors that lead to ecological adaptation via natural selection, geographical separation can promote selection also via pleiotropy or hitch-hiking, ultimately impeding gene flow between populations (Rice & Hostert 1993; Ridley 2004). Furthermore, the possibility of a rapid speciation due to sexual isolation and divergence of secondary sexual traits has been confirmed by theoretical works (Lande 1981), and also in laboratory experiments (reviewed by Rice & Hostert 1993).

The overall results of Experiment 2, however, do not support the predictions, as no difference in male and female inspection rate and male courtship rate was present when females were paired with males from a different population. Besides females followed the males leading them to the mussel, that in bitterling

hints at a female willingness to spawn, irrespective of the population males came from. However, there was a difference in the ejaculation rate of the males from the populations tested in Turkey, with a higher rate for males from the site with few mussels, so confirming the results of previous experimental work on the same populations (Reichard *et al*. 2010). If the ratio mussel to males is male skewed, males may have to face the risk of sperm depletion to be able to ensure paternity and increase their reproductive fitness. A longer period of isolation and exposure to a low level of mating resources seems to have enhanced the pressure of sexual selection on male behaviours necessary to defend resources and ensure paternity, but not on male or female inter-sexual behaviour.

The allopatric populations analysed do not seem to be on the verge of a reproductive isolation, as confirmed also by the fact that the compatibility of the embryos obtained with IVF, measured in terms of embryo survival rate, was not statistically different. A possible explanation may be given by the comparatively short period of time during which the populations have been isolated: even if a rapid "ecological speciation" has been demonstrated in some species of fish (Schluter 1996; McKinnon & Rundle 2002), the amount of time necessary for speciation to take place in allopatric populations is generally reputed to exceed by far the approximate period of isolation of the populations tested in the present experiment (Coyne & Orr 1997). Besides, embryo survival rate may not be a definite evidence of hybrid compatibility. In a recent experimental work on guppy, *Poecilia reticulata*, embryo sterility and inviability was documented only in post-F_1 generations, and behavioural sterility only in F_1 guppy obtained with crosses and back crosses from two populations of Trinidadian guppy (Russell & Magurran 2006). Further research on the first and second generation of offspring from the

populations tested in the present experiment is therefore necessary to confirm the lack of reproductive isolation.

The aim of Experiment 3 was to test the influence of different levels of mussel abundance during ontogeny on male aggressive behaviour. For logistic reasons, the experiment was performed only with fish from one of the populations tested in the Czech Republic in Experiment 1 and 2. The results show no difference in male aggression rate between the fish exposed to high or low mussel availability, hinting at a possible role for social learning in shaping behaviours (reviewed by Brown & Laland 2003; Galef & Laland 2005).

When raised in a socially complex environment, fish may learn anti-predator behaviour (Brown & Godin 1999; Utne-Palm 2001), copy other males' mate choice (Schlupp & Ryan 1997), or "eavesdrop" information about other males' fighting ability (Oliveira *et al*. 1998). The fish tested in the present experiment were all juveniles captured before the beginning of the spawning season and, differently from the normal finding in adult bitterling, their aggression rate was not correlated with body size. Smaller bitterling may challenge a bigger one, but they normally adopt alternative mating tactics and rarely engage in fights that would represent a cost in terms of energy expenditure without the benefit of a victory. Juveniles raised with adults may therefore adopt a more submissive behaviour if challenged by dominant males or acquire a more economical aggressive behaviour in defending mating resources (Arnold & Taborsky 2010).

In conclusion, it was demonstrated that the availability of mating resources and their spatial clustering play a role in modulating the aggressive behaviour of male bitterling *R. amarus* and that their effect is stronger in older-established populations, but no clear evidence was found for a role of behaviour in determining

reproductive isolation. Finally, the exposure to different levels of resource availability during ontogeny does not affect male aggression rate.

Figure 4.3. Mean ± SE body length (mm) of males from populations tested in Turkey (A) and in the Czech Republic (B). White bars for males from sites with high mussel availability, grey bars for males from sites with low mussel availability.

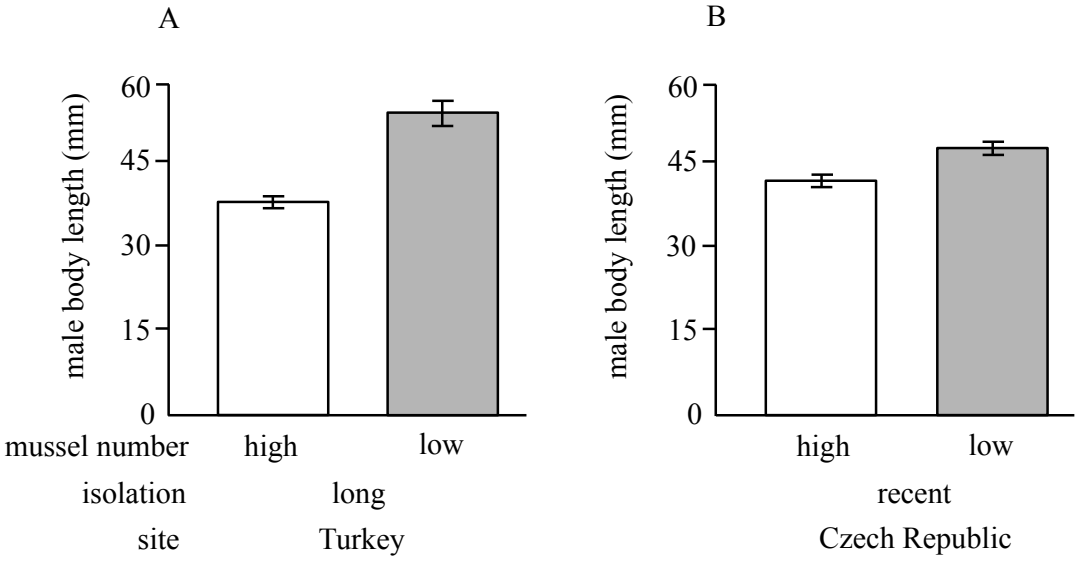


Figure 4.4. Mean ± SE extent of red (proportion) in the iris of males from populations tested in Turkey (A) and the Czech Republic (B). White bars for males from sites with high mussel availability, grey bars for males from sites with low mussel availability.

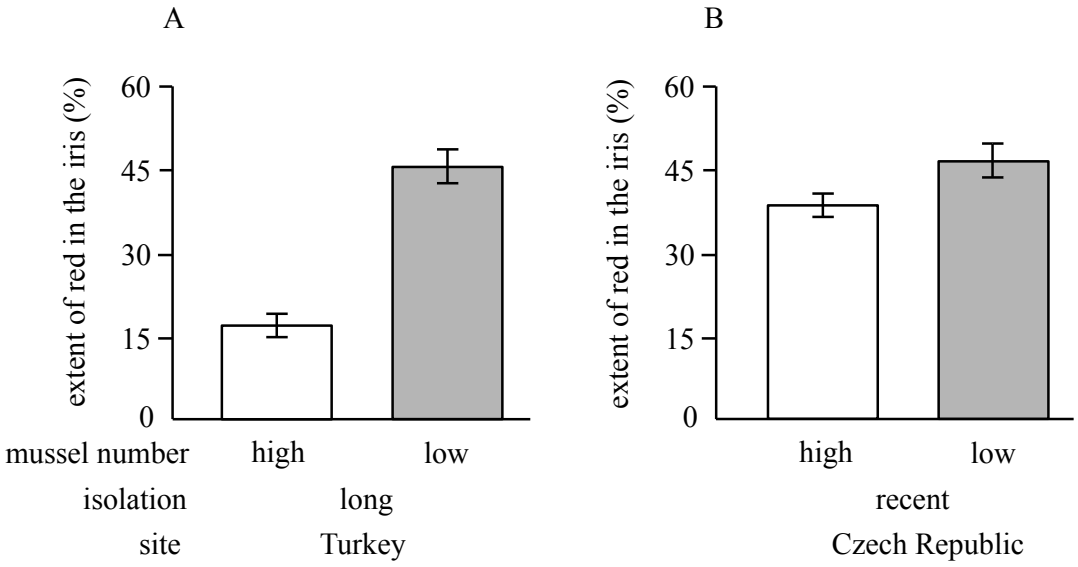


Figure 4.5. Mean ± SE aggression rate (10 min^{-1}) of males from populations tested in Turkey (A) and the Czech Republic (B). White bars for males from sites with high mussel availability, grey bars for males from sites with low mussel availability.

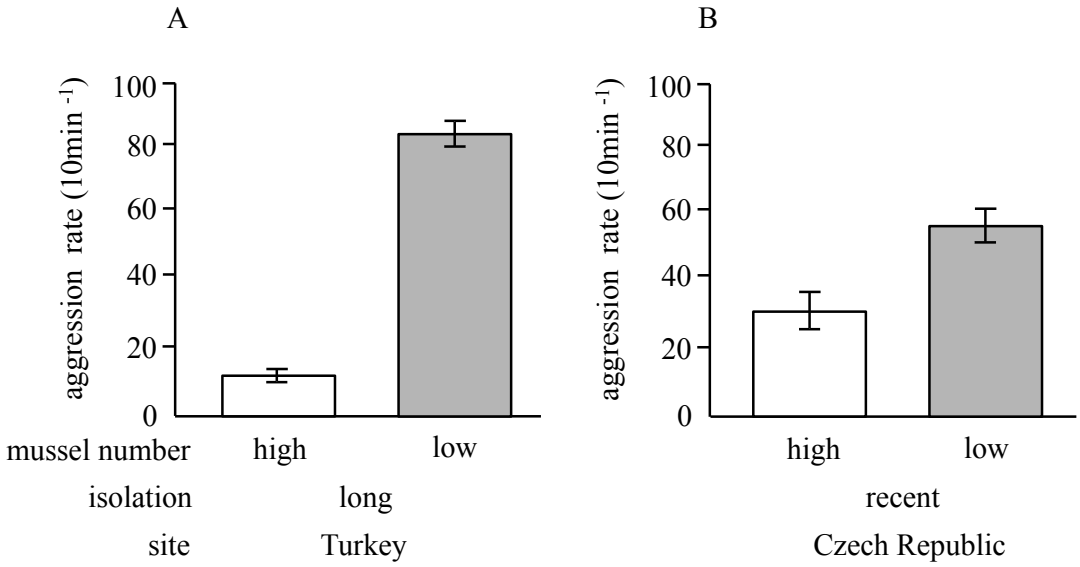


Figure 4.6. Mean ± SE courtship/leading rate (15 min^{-1}) of males from populations tested in Turkey (A) and the Czech Republic (B). White bars for males from sites with high mussel availability, grey bars for males from sites with low mussel availability.

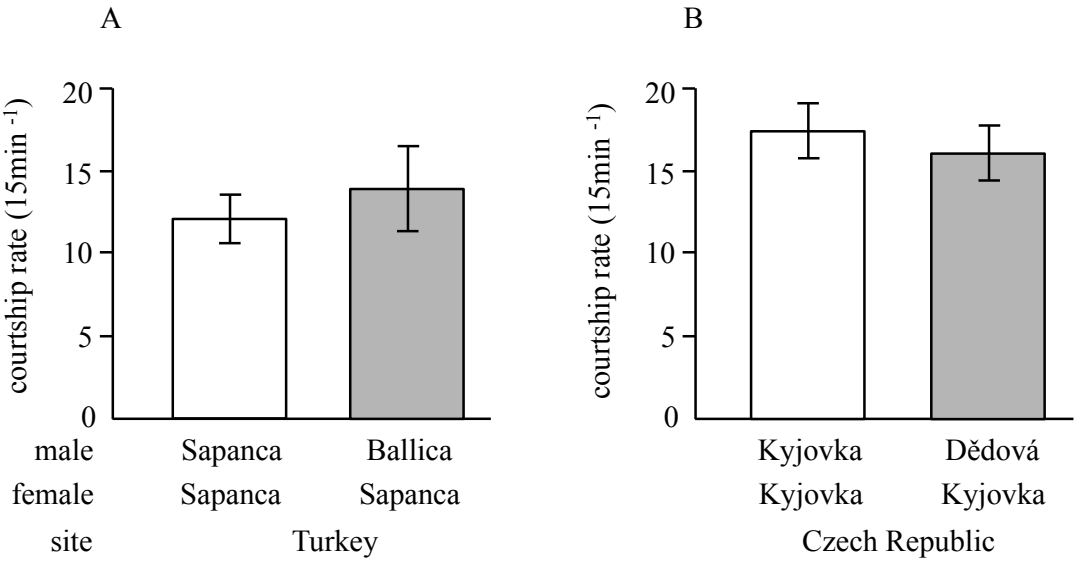


Figure 4.7. Mean ± SE inspection rate (15 min^{-1}) of females from populations tested in Turkey (A) and the Czech Republic (B) when paired with males from sites with high mussel availability (white bars) or with low mussel availability (grey bars).

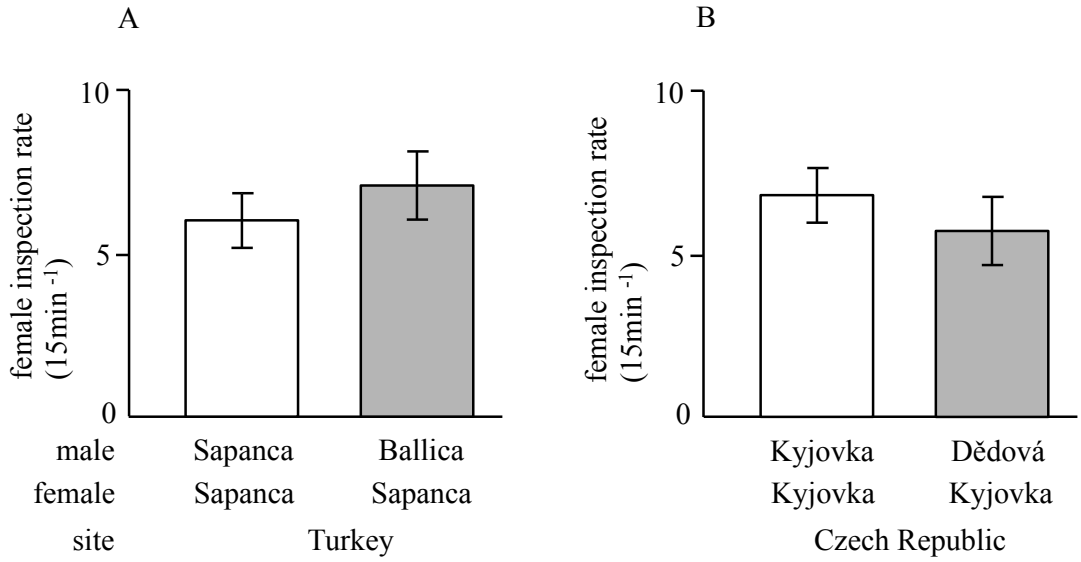


Figure 4.8. Mean ± SE survival rate (proportion) of the embryos obtained by crossing populations from Turkey (A) and the Czech Republic (B). White bars for crosses of males and females from sites with high mussel availability; grey bars for crosses of males from sites with low mussel availability and females from sites with high mussel availability.

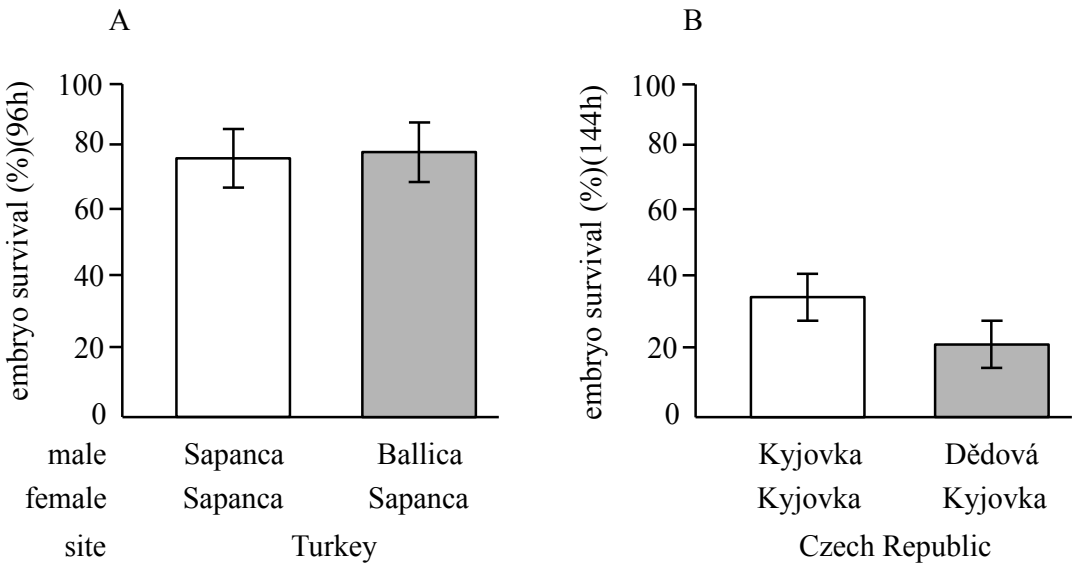


Figure 4.9. Mean ± SE aggression rate (10 min^{-1}) of the juveniles tested in the Czech Republic after being exposed to different levels of mussel availability during ontogeny. High mussel availability (white bars), low mussel availability (grey bars).

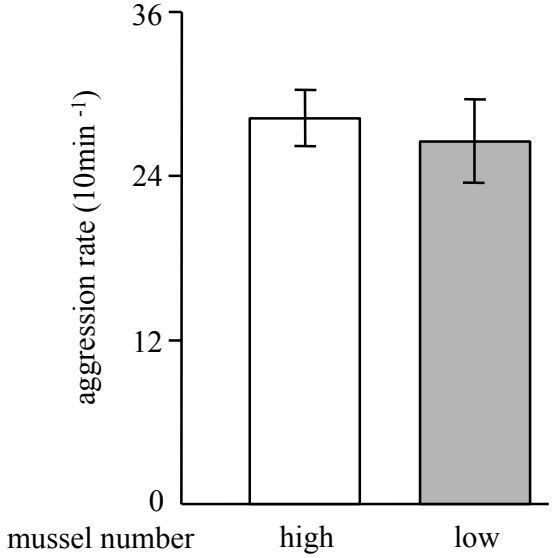


Table 4.1. Mean ± SE extent of red in the iris (proportion) of the 20 males photographed over a 10-minute period after being anesthetized.

Extent of red in the iris (%)		
Time	Mean	SE
1 min	11.07	± 1.65
3 min	11.26	± 1.58
5 min	11.46	± 1.68
7 min	11.26	± 1.70
10 min	11.15	± 1.72

Table 4.2. Mean ± SE egg density (1h) in *Anodonta anatina* and *Unio pictorum* exposed to free spawning by bitterling in the four study sites.

	Turkey		**Czech Republic**	
Mussel availability	High	Low	High	Low
Study site	L. Sapanca	R. Balica	R. Kyjovka	L. Dědová
Mean ± SE number of eggs laid (per 1h of exposure)	0.18 ± 0.10	4.69 ± 0.80	0.31 ± 0.11	0.42 ± 0.13

Table 4.3. Summary of unpaired *t*-test for male and female behaviours (15 min^{-1}) when females from one population were paired with males from the same or a different population. Data for populations tested in Turkey.

Variable	**Mean ± SE (males and females from the same population)**	**Mean ± SE (males and females from different populations)**	**df**	***t-value***	**P**
Male courtship/ leading rate	12.33 ± 1.43	15.67 ± 2.28	28	1.24	0.226
Female inspection rate	5.80 ± 0.78	7.40 ± 0.93	28	1.31	0.198
Female "following" rate	3.67 ± 0.52	5.60 ± 0.88	28	1.88	0.069

Table 4.4. Summary of unpaired *t*-test for male and female behaviours (15 min^{-1}) when females from one population were paired with males from the same or a different population. Data for populations tested in the Czech Republic (log_{10+1} transformed data).

Variable	**Mean ± SE (males and females from the same population)**	**Mean ± SE (males and females from different populations)**	**df**	***t-value***	**P**
Male courtship/ leading rate	16.81 ± 1.29	15.14 ± 1.60	40	1.33	0.191
Female inspection rate	7.00 ± 0.90	5.62 ± 0.79	40	0.79	0.434
Female "following" rate	3.65 ± 0.51	3.14 ± 0.47	40	0.67	0.505

Chapter 5. The role of density during embryogenesis on male aggressive behaviour and phenotypic plasticity in the rose bitterling, *Rhodeus ocellatus*

5.1. Abstract

Environmental conditions experienced during embryogenesis can exert an immediate effect on morphological traits, but their influence can also be expressed at later stages. This phenotypic plasticity, within the limits of genetic constraints, may result in the expression of advantageous traits. Here density during embryogenesis was manipulated and full siblings were raised at high and low densities to test whether this variable can influence male aggressive behaviour, and embryo growth and survival rate. Density experienced during embryogenesis did not influence aggression rate, but embryos raised at high density reached sexual maturity at an earlier age and at a smaller size. Density also influenced mortality rate, with males raised at high density experiencing higher mortality rates. The results are discussed in the context of the evolutionary importance of environmental conditions during development.

5.2. Introduction

A complex interaction of genetic and environmental factors, particularly if experienced during crucial periods of embryonic development, can contribute to the expression of differential morphological and behavioural traits and lead to phenotypic characteristics that will influence future fitness.

Phenotypic plasticity can be defined as the ability of a single genotype to produce alternative forms of morphology, physiological state, and/or behaviour in response to environmental conditions (West-Eberhard 1989); the range of phenotypes that can be expressed by a genotype as a response to different environmental situations is its norm of reaction (Garland & Kelly 2006). Reaction norms can be flexible and can produce different phenotypes in response to environmental change. In some cases, in contrast, once a phenotype has been determined it can be maintained regardless of environmental variation (Stearns 1989).

Phenotypic plasticity can appear as polyphenisms; the expression of two or more discrete phenotypes produced by the same genotype, as a consequence of different environmental signals as, for example, the variation in amount of melanin on the ventral hindwings in the western white butterfly, *Pieris occidentalis*. Melanin plays a role in thermoregulation, and the amount of melanin varies with the season in which broods are produced (Kingsolver & Wiernaz 1991). Another example is the difference, reversible during ontogeny, in morphology and behaviour of treefrog tadpoles, *Hyla versicolor*, in response to the presence of predators (Relyea 2003).

The influence of environmental variables on some fitness traits, such as growth and survival rate, has been demonstrated in experimental studies. In the American rubyspot damselfly, *Hetaerina americana*, a higher availability of food

during larval development affected adult body length, which increased mating opportunities and immune response, while a scarcity of food resulted in delayed larval development (Jiménez-Cortés *et al*. 2012). In the zebrafish, *Danio rerio*, experimentally induced oxygen deficiency in five-day-old larvae affected their subsequent locomotory activity and resulted in an increased mortality rate (Kienle *et al*. 2008).

The environment embryos experience during the early phases of development can also influence subsequent mating behaviour and reproductive fitness. For example, female gerbil (*Meriones unguiculatus*) and house mouse (*Mus musculus*) foetuses located between two males are exposed to elevated levels of androgens and show a masculinised phenotype. These masculinised females are more aggressive towards other females, less attractive to males, and tend to have fewer litters with more male-biased sex ratios (Clark *et al*. 1993; Clark & Galef 1995).

Density is one of the most important environmental variables that can shape the expression of different phenotypes by the same genotype. The effects of density during adulthood have been extensively studied in theoretical and empirical studies (see Chapter 3 and 4 for references). The impact of density during larval development has been investigated in insects and amphibians. For example, in *Drosophila melanogaster* and *D. simulans* the onset of laying and the time to maximum daily fecundity were delayed and the average daily fecundity was shown to be reduced at high density, an effect that was mediated by the impact of density on adult body weight (Barker & Podger 1970). An effect of larval density on mating behaviour was demonstrated in *D. persimilis*, with larvae raised at high density showing reduced male courtship and mating frequency (Spiess & Spiess 1969). In

the marbled salamander, *Ambystoma opacum*, larvae raised at low density had a larger body length at metamorphosis, and once mature, females had greater clutch size. Age at reproduction was also lower in animals raised at low density (Scott 1994). In fish, the permanence of juvenile schooling behaviour has been demonstrated in 100-160 day old African jewel fish, *Hernichrornis bimaculatus*, after 30 and 60 days of exposure to a high density treatment (Coss & Burgess 1981).

The aim of the present study was to test the effect of density during embryo development on adult aggression rate in *Rhodeus ocellatus*, a small freshwater fish with a promiscuous, resource-based mating system. Female lay eggs into the gills of live freshwater mussels and males fertilize them by releasing sperm on the inhalant siphon. Embryos develop inside the gills for about four weeks and then emerge as free swimming larvae able to feed exogenously. When males are sexually mature they aggressively defend a territory that is centred around a mussel or patch of mussels (Smith *et al*. 2009). Male dominance correlates with body size, with smaller males performing a sneaker mating role (Casalini *et al*. 2009). In bitterling alternative mating tactics are not genetically determined and dominant males may behave as sneakers in neighbouring territories defended by other territorial males (Smith *et al*. 2004).

Based on previous studies (Scott 1994; Agbali *et al*. 2011) it was predicted that for embryos raised at a high density: 1) the survival rate would be lower than at low density, 2) growth rate would be lower and they would reach the free swimming stage at a later age, 3) they would become sexually mature at an earlier age and, consequently, at a smaller size, and, 4) they would show a higher aggression rate.

The importance of environmental variables in sex differentiation was not tested as the design of the experiment involved the removal of randomly chosen embryos to maintain an equivalent density for the two experimental treatments after completion of embryo development. In bitterling it is possible to determine sex only at the onset of sexual maturity, when males show a characteristic red carotenoid-based coloration in the iris and on the caudal fin, while females develop a conspicuous ovipositor. Consequently, it was not possible to determine whether the embryos excluded from the study were males or females.

5.3. Methods

Experiments were performed in the aquarium facilities of the University of St Andrews. Parental fish used for experimental work were the second-generation offspring of wild caught *R. ocellatus* from the River Yangtze Basin, China. During the experiment they were 36-40 months old. Prior to experiments fish had been individually marked and were held in stock aquaria measuring 60 (length) x 30 (width) x 35 (depth) cm. Stock and experimental aquaria were maintained at a temperature of 23°C, with a 16:8 h light: dark cycle, and provided with a layer of sand substrate and artificial plants as refuges. Fish were fed a mixture of commercial dried fish flake food twice daily and frozen blood worms (*Chironomus* spp.) once a week.

To conduct *in vitro* fertilizations (IVF), eggs were obtained by gently squeezing the abdomen of a female in spawning condition. The eggs were divided into two differently-sized batches, with a density ratio of 1 (low): 4 (high) (5-7 eggs for the low density treatment; 25-28 eggs for the high density treatment), and placed into separate 50 mm diameter Petri dishes containing 10 ml of fresh water. Sperm was collected from a male by gently squeezing his abdomen; the sperm was diluted

in 5 ml of saline solution (0.05%) and immediately pipetted over the eggs in the two Petri dishes. The Petri dishes were covered and left on the laboratory bench for 30 minutes. The fertilized eggs were rinsed twice with fresh water and the Petri dishes filled with a further 15 ml of fresh water, covered, and incubated at 23ºC. The same procedure was repeated with a series of different males and females.

Figure 5.1. Embryos 3 days after fertilization (low density treatment).

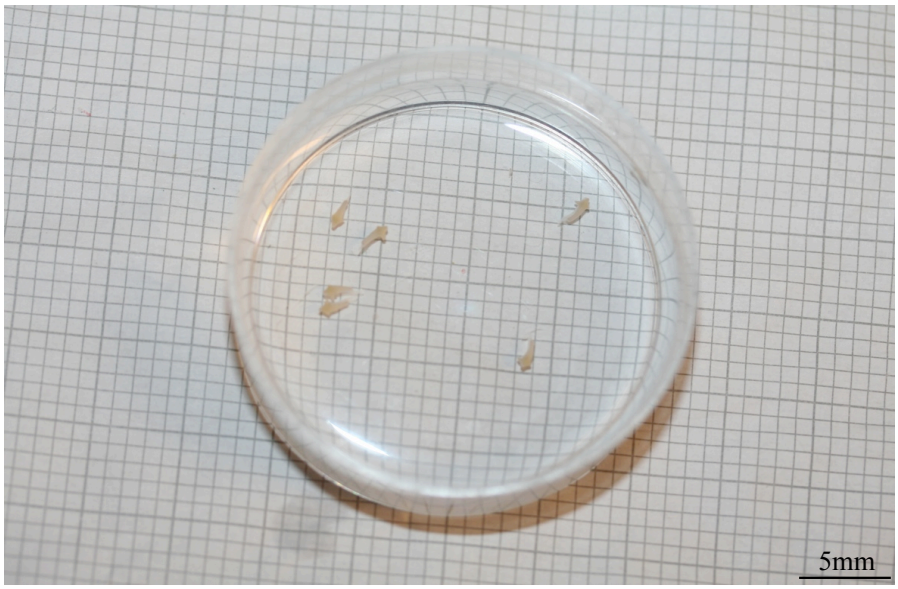


Figure 5.2. Embryos 3 days after fertilization (high density treatment).

The survival of the developing embryos was checked daily and, to keep the density ratio of 1:4 constant, if some died randomly chosen siblings were removed from the corresponding paired Petri dish. If the number of embryos in the low density treatment fell below 3, the replicate was abandoned. The water in Petri dishes was changed daily to maintain water quality. When the embryos were one week old a few drops of diluted Liquifry No.1 (Interpet) were added to Petri dishes every other day. When the embryos had completely absorbed their yolk sac and started to swim, all the low density embryos were retained along with an equivalent, randomly selected, number of high density siblings. They were housed in separate 10 L aquaria measuring 30 (length) x 20 (width) x 20 (height) cm and fed with crushed commercial fish flakes twice each day and live Artemia once each day. Embryos were photographed against a scale bar at 5, 7, 15 days of age and immediately before moving them to aquaria using a Canon EOS 350D with a 60 mm macro lens under standard light condition. Their body length was measured to the nearest 0.1 mm from digital photographs using UTHSCSA Image Tool 3.0. A record was kept of the onset of movement and eye pigmentation.

As soon as juvenile males were sexually mature, obvious by the development of red coloration in the iris and on the tail fin, they were tested for aggression. They were individually placed in experimental tanks measuring 45 (length) x 30 (width) x 30 (depth) cm together with a mussel in a sand-filled plastic cup and an empty perforated transparent bottle. Opaque screens between aquaria prevented visual interference with neighbouring fishes. To initiate male reproductive behaviour, a female in spawning condition was placed in the experimental aquaria. Once the focal male started to court and lead the female to the mussel, the female was removed, and a stimulus male was placed into the

perforated transparent bottle. The aggression rate of the focal male was measured for 15 min. The behaviours recorded were: (i) head butting, (ii) side jerking and (iii) fin spreading (Wiepkema 1961). After completion of each observation the maximum shell length of mussels was measured to the nearest 1 mm and mussels were not used again in trials. The focal male was photographed against a scale bar using a Canon EOS 350D with a 60 mm macro lens under standard light condition and his body length measured to the nearest 0.1 mm from digital photographs using UTHSCSA Image Tool 3.0. After testing, males were returned to the aquaria in which they were raised to maintain a constant density. Fish that had been tested were marked by finclipping to distinguish them from untested fish.

The extent of red in the iris and on the tail fin of test males was scored visually on a scale ranging from 0 to 5, using the criteria listed in Tables 5.1 and 5.2 respectively. Visual scales of this sort have been used reliably in several experimental studies to measure the extent of nuptial coloration in fish (Kodric-Brown & Mazzolini 1992; Kodric-Brown 1996; Östlund Nilsson & Nilsson 2000; Boughman 2001), including in *R. ocellatus*, in which a comparison of visual scoring and more precise estimates of red carotenoid-based coloration on the tail fin were compared without detecting a difference in the methods (Casalini *et al*. 2009).

When females reached sexual maturity they were photographed against a scale bar and their body length measured to the nearest 0.1 mm from digital images. Twenty families of full siblings were used in the study, with a total of 96 males tested for aggression (47 raised at low density and 49 at high density).

5.3.1. Data analysis

All data were tested for normality using a Shapiro-Wilk test and for equality of variance using Bartlett's test. Data that did not meet assumptions of normality and

homoscedasticity were transformed. Nonparametric tests were used if data did not respond to transformation.

An unpaired *t*-test was used to compare embryo survival rate between density treatments at 5 days and 1 week of age, and a Mann-Whitney test for survival at 2 weeks of age and when the larvae were transferred to rearing aquaria. An unpaired *t*-test was used to compare differences between density treatments in the time necessary for embryos to start moving, to develop eye pigmentation, and to reach the free swimming stage. Differences in embryo size between the two treatments at 5 days, 1 week, 2 weeks of age, and when the larvae were moved to aquaria were tested with an unpaired *t*-test. An unpaired *t*-test was used to test for differences in male and female body length and male age at maturity. Female age at maturity was tested with a Mann-Whitney test. The correlation between male aggression rate and body length at maturity, age at maturity, and extent of red in the iris was tested using Spearman correlation. ANCOVA was used to test for the effect of density during embryogenesis on male aggression rate, with male age at maturity and the number of fish in each tank during the larval/ juvenile period as covariates.

5.4. Results

There was a significant effect of density on embryo survival rate during embryogenesis, with a higher mortality rate for embryos raised at high density at 5 days of age (unpaired *t*-test: $t_{38} = 3.11$, $P = 0.003$), at 1 week ($t_{38} = 2.61$, $P = 0.013$), at 2 weeks (Mann-Whitney test: $Z = 3.11$, $P < 0.001$), and at the free swimming stage ($Z = 3.30$, $P = 0.001$; Figure 5.3).

Embryos raised at low density started to move (unpaired *t*-test: $t_{38} = 6.30$, $P < 0.001$) and developed pigmented eyes (unpaired *t*-test: $t_{38} = 7.59$, $P < 0.001$) earlier than those raised at high density (Figure 5.4). There was no difference in the

time for embryos to become free swimming (unpaired *t*-test: $t_{38} = 0.00$, $P = 1.000$), which occurred between 17 and 25 days of age. No effect of density was detected on embryo length at 5 days of age (unpaired *t*-test: $t_{38} = 1.17$, $P = 0.249$), at 1 week ($t_{38} = 1.43$, $P = 0.161$), at 2 weeks ($t_{38} = 1.18$, $P = 0.244$), and at the free swimming stage ($t_{38} = 0.72$, $P = 0.476$; Figure 5.5).

There was a significant effect of density on both male age (unpaired *t*-test: $t_{94} = 2.68$, $P = 0.008$) and body length ($t_{94} = 9.38$, $P < 0.001$) at maturity; males raised at high density reached sexual maturity at an earlier age and at a smaller size (Figure 5.6). Females reached sexual maturity at an older age and at a bigger size than males, a common finding in bitterling, but with no significant effect of density either on age (Mann-Whitney test: $Z = 0.82$, $P = 0.410$) or on body length (unpaired *t*-test: $t_{52} = 1.78$, $P = 0.079$).

Male age and body length at maturity were highly correlated (Spearman correlation: $r_{94} = 0.605$, $P < 0.001$). Male aggression rate was negatively correlated with age at maturity ($r_{94} = -0.257$, $P = 0.011$); however, there was no correlation between male aggression rate and body length at maturity ($r_{94} = -0.063$, $P = 0.539$), and between male aggression rate and the extent of red in the iris ($r_{94} = 0.040$, $P = 0.693$).

Once controlled for male age at maturity and fish density in the tanks during the larval/juvenile period, density during embryogenesis did not influence male aggressive behaviour (Figure 5.7; Table 5.3).

5.5. Discussion

The aim of this experiment was to test the influence of different levels of density during embryogenesis on morphological and behavioural traits in *R. ocellatus*.

The results confirm the first prediction: the survival rate of embryos raised at high density was lower than the one of embryos raised at low density, which is consistent with a study on spotted salamanders, *Ambystoma maculatum*, in which mortality rate was higher for embryos raised at high density (Davies & Maerz 2009). In an experiment performed with the same bitterling species, on the contrary, no difference was detected in survival rate between embryos raised at different levels of densities (Agbali & Smith 2012). A likely explanation for this discrepancy is that the Petri dishes used to raise embryos in the present experiment, and, consequently, the quantity of oxygen available, were smaller than the ones used in the previous experiment.

The data collected do not confirm the second prediction: no difference was detected either in the body length of embryos raised at high or low density during the period of development up to 3-4 weeks of age or in the time necessary to reach the free swimming stage.

Bitterling embryos, to be able to survive in an environment protected from predators but poor in oxygen or even hypoxic when the mussel closes its valves, hatch very quickly (about 38 hours after spawning) (Kim & Park 1985), and develop an extended cutaneous vascularisation in the yolk sac. Embryos begin to move approximately 4 days after hatching and, after about 4 days, develop pigmented eyes (Kim & Park 1985; Smith *et al*. 2004). The number of eggs present in the mussel gills is highly variable, from a few up to 250 (Smith *et al*. 2004), therefore embryos must have developed the ability to adapt to different environments. In the present study, the effect of density affected the onset of movement and the development of eye pigmentation in the very early phases of development, but the time for embryos to become free swimming was comparable

and no effect was present on growth rate. A possible explanation could be that only the fittest embryos were able to survive and develop in an environment very poor in oxygen. Alternatively, the lack of difference in growth rate and time to reach the free swimming stage between the two density treatments could indicate either that density does not impinge on growth, or that the experimental densities were insufficiently high to elicit an effect on these variables.

Males raised at high density reached sexual maturity at an earlier age and a smaller size so confirming the third prediction. In rose bitterling, male dominance is correlated with body size (Casalini *et al*. 2009). Alternative male mating tactics are not genetically determined and the environment experienced by the fish influences behavioural plasticity. Males that reach sexual maturity at an earlier age can increase their overall reproductive fitness by sneaking fertilization while still small and adopt a different strategy later in the spawning season when, having increased their size, they can successfully defend a territory. As demonstrated by Reichard *et al*. (2004a,b) in *Rhodeus amarus*, territorial male reproductive success varied with male density: at a low density dominant males were, on average, 17 times more successful than sneakers in fertilizing eggs, but at a high density territorial males and sneakers achieved the same reproductive success. Female bitterling do not prefer to mate with larger, dominant males (Casalini *et al*. 2009; Agbali *et al*. 2010) and solicit sneakers to improve their fertilization success (Smith & Reichard 2005), so conferring a further advantage in reproductive fitness to males that reach sexual maturity at a smaller size.

Embryos raised at high and low density showed no difference in their aggression rate, contradicting the fourth prediction. The exposure to different levels of mussel abundance during ontogeny (Chapter 4), and in the present experiment to

different levels of density during embryogenesis did not affect male aggressive behaviour. These two environmental variables, so important in shaping older males' behaviours, do not seem to exert the same influence when experienced early in life.

In adult bitterling, aggression rate is normally correlated with body size, and larger males perform more aggressions while smaller males are attacked more frequently and withdraw without escalating fights (Chapter 3). Conversely, juvenile aggression rate both in the present experiment and in research performed with *R. amarus* (Chapter 4) was not correlated with body length.

As already underlined in Chapter 4, some experimental studies have demonstrated the importance of learning in moulding many behaviours that are essential to survival, such as predator avoidance and foraging, or to increase reproductive fitness, such as mate recognition, mate choice, or aggressive behaviour. Juvenile bitterling, raised without the presence of other fish, may fail to develop some behaviours that are shaped by social interactions; alternatively, the full expression of phenotypic behavioural plasticity may be delayed to a later period of bitterling life history.

In conclusion, it was demonstrated that a high density experienced during the period of early embryonic development affected survival, the onset of movement, and eye pigmentation but not growth rate. Embryos raised at high density reached sexual maturity at an earlier age and at a smaller size, but their aggression rate was comparable to those raised at low embryo density.

Figure 5.3. Mean ± SE survival rate (proportion) of embryos raised at high density (white bars) and low density (grey bars) at five days, one week, two weeks of age and when they were able to swim freely.

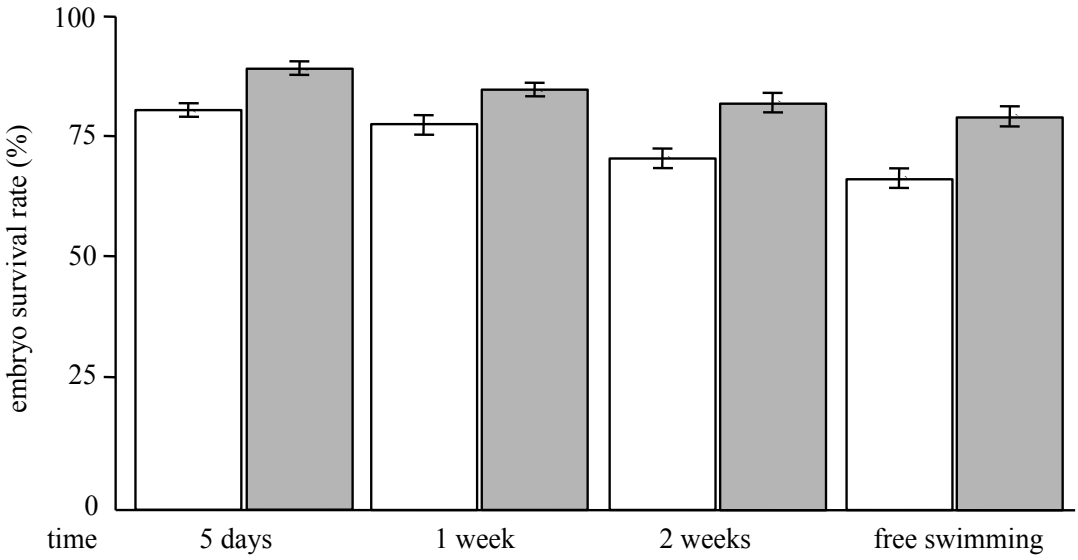


Figure 5.4. Mean ± SE time (d) from fertilization to the onset of movement (A) and eye pigmentation (B). White bars show data for embryos raised at high density and grey bars for embryos raised at low density.

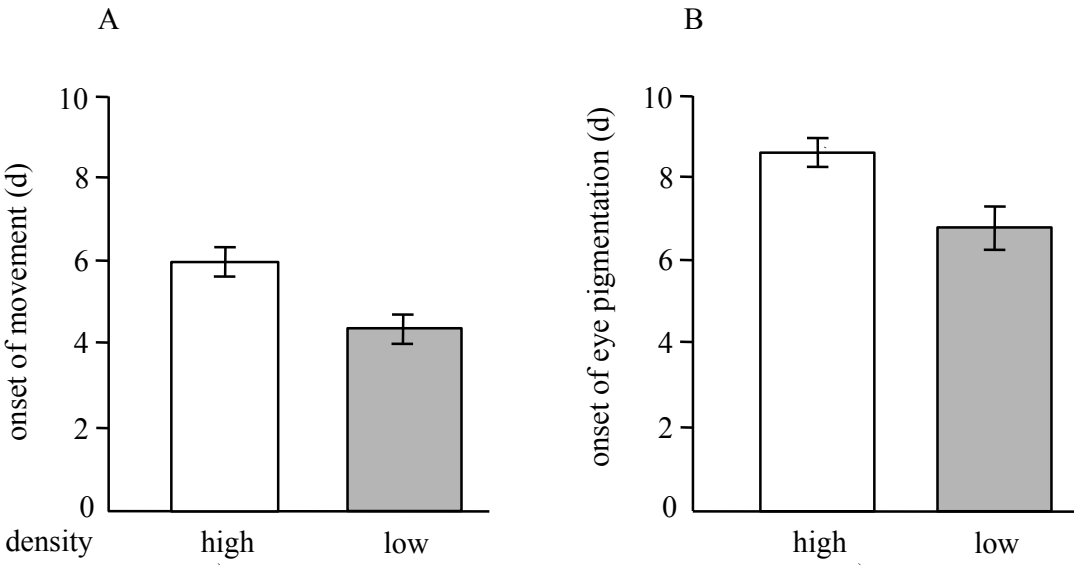


Figure 5.5. Mean ± SE body length (mm) of embryos raised at high density (white bars) and low density (grey bars) at five days, one week, two weeks of age and when they were able to swim freely.

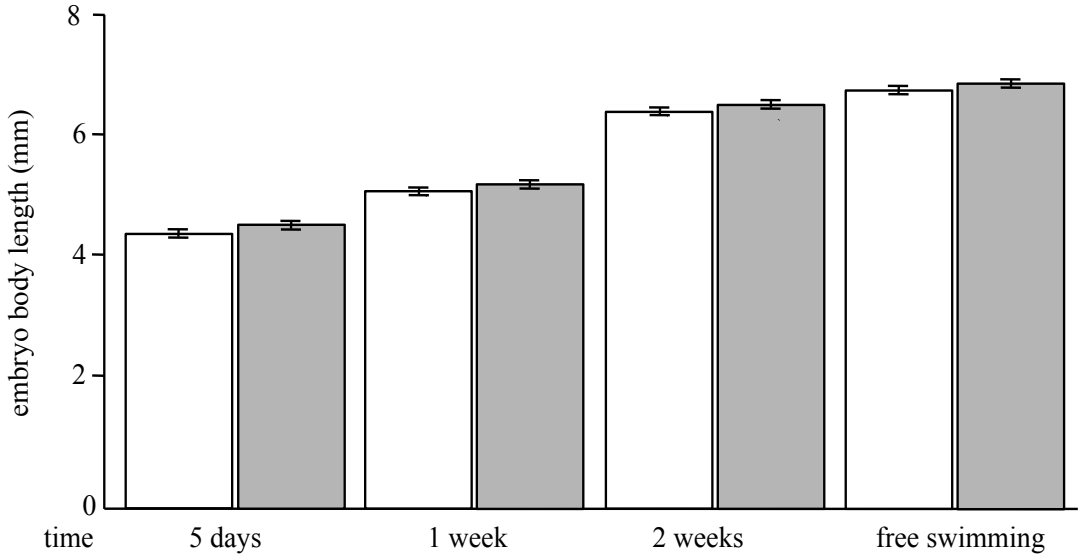


Figure 5.6. Mean ± SE male age (d) (A) and body length (mm) (B) at maturity. White bars for embryos raised at high density, grey bars for embryos raised at low density.

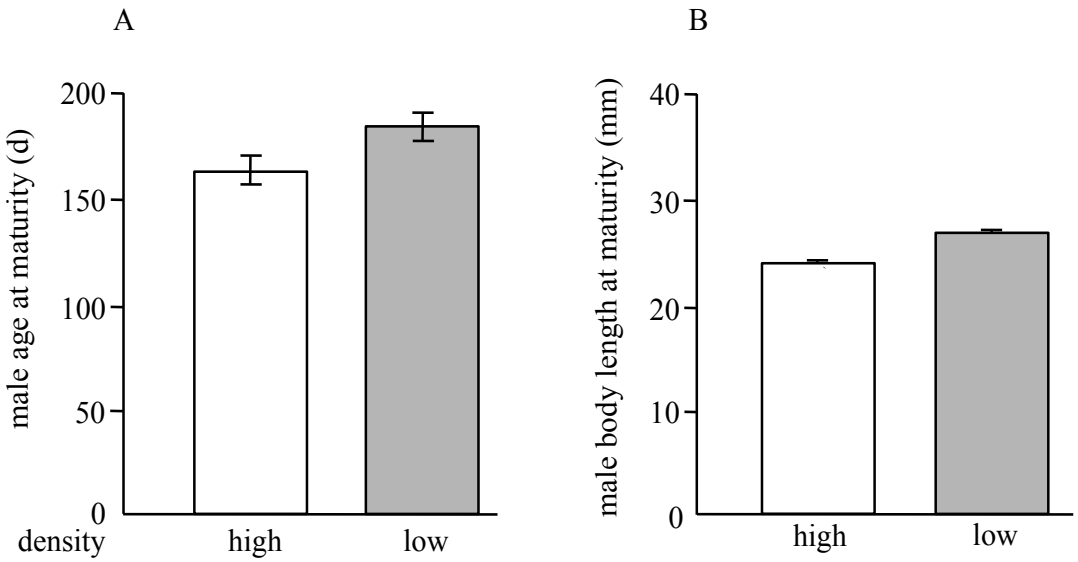


Figure 5.7. Mean ± SE male aggression rate (15 min^{-1}) of embryos raised at high density (white bars) and at low density (grey bars).

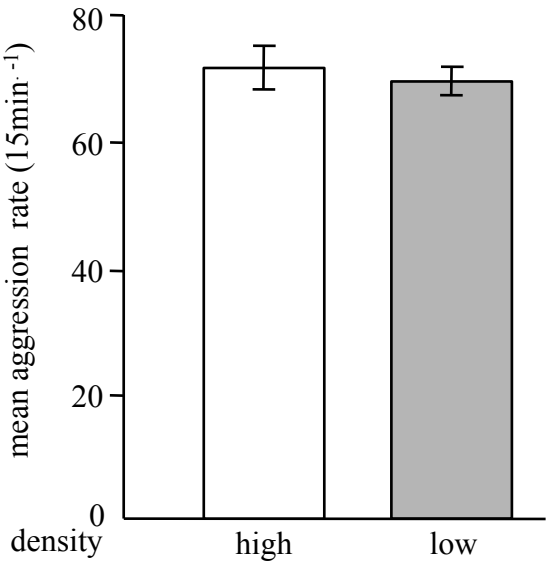


Table 5.1. Ordinal scale to score the extent of male tail colour.

Ordinal rank	Descriptor
5	An area of brilliant red extending from the body to the hind edge of the tail fin.
4	An area of brilliant red extending from the body for ¾ of the length of the tail fin.
3	An area of brilliant red extending from the body to ¼ - ½ of the length of the tail fin.
2	An area of pale red extending from the body to ¾ of the length of the tail fin.
1	An area of pale red extending from the body to ¼ - ½ of the length of the tail fin.
0	No distinct red coloration.

Table 5.2. Ordinal scale to score the extent of male eye colour.

Ordinal rank	Descriptor
5	An area of red covering > 45% of the area of the iris.
4	An area of red covering between 36-45% of the iris.
3	An area of red covering 27-35% of the iris.
2	An area of red covering 18-26% of the iris.
1	An area of red covering 9-17% of the iris.
0	An area of red covering < 9% of the iris.

Table 5.3. ANCOVA for the effect of different levels of densities during embryogenesis on male aggression rate (square root transformed data). Mean and standard error of male aggression rate are in parentheses.

Source	df	SS	MS	F	P
Mean male aggression rate (70.5 ±1.39)					
Focal male age at maturity	4	2.84	0.71	1.09	0.364
Number of fish in rearing aquaria	4	4.64	1.16	1.78	0.139
Density during embryogenesis	1	0.01	0.01	0.03	0.868

Chapter 6. Variation in colour signals in the rose bitterling *Rhodeus ocellatus*

6.1. Abstract

In many animal taxa, colour patterns function either as camouflage or as a visual signal. Carotenoid-based coloration is used in social contexts and, if costly, it can signal dominant status to opponents or fitness status to potential mates. In some taxa, nuptial coloration can undergo rapid changes in its extent and intensity. Here the variation in the extent of red coloration as an inter- or intra-sexual signal was tested in male *Rhodeus ocellatus*, a small cyprinid fish with a red carotenoid-based nuptial coloration in the iris and on the tail fin. The results show that variation in male colour is influenced by the presence of both potential rivals and mates, and that the extent of colour can increase rapidly, while decreasing relatively slowly. Variation in the pigmentation of the tail fin, but not of the iris, is influenced by the presence of a mating resource. The results are discussed in the context of sexual selection.

6.2. Introduction

Male secondary sexual traits are under strong sexual selection as they can be used to signal a superior status to opponents and, being related to higher male fitness, females are supposed to choose superior males that can provide them either with direct benefits as food, mating sites, and protection from other males' harassment, or indirect benefits by increasing offspring fitness (Andersson 1994). Traits related to male dominance as body length, songs and coloration may have evolved primarily as armaments used in male-male competition and subsequently undergoing selection as ornaments through inter-sexual selection (Berglund *et al*. 1996).

Coloration is a widespread feature in many animal taxa and can be used to serve different functions: as species recognition (Detto *et al*. 2006; Genner *et al*. 2007), to appear inconspicuous and so avoid predation (Magurran 2005; Vallin *et al*. 2006; Cooper *et al*. 2008), as intraspecific mimicry (Dominey 1981), as an aposematic signal to advertise toxicity (Blount *et al*. 2009), or for intra- and inter-specific communication in male-male context and mate choice (Darwin 1871; Andersson 1994; Houde 1997).

Colour patterns may be permanent, seasonal or ephemeral. Permanent colour patterns develop at maturity, seasonal colorations are present during the breeding season, while ephemeral colours consist of quick change in colour used as signals in social communications (Kodric-Brown 1998; Price *et al*. 2008).

Conspicuous red or orange carotenoid-based colorations are present, more often in males, in many species of arthropods, fish, reptiles and birds (reviewed in Svensson & Wong 2011), and become more conspicuous during the reproductive season. To be honest signals must be costly and therefore only the fittest individuals

are thought to be able to express them (Zahavi 1975). Carotenoid-based ornaments are costly as being conspicuous increases predation risk (Endler 1980, 1983; Godin & McDonough 2003), and may increase parasite load (Hamilton & Zuk 1982). Fishes cannot produce carotenoids endogenously but must obtain them from food; so carotenoid-based colours may also signal males' ability to forage (Kodric-Brown 1998). Furthermore, carotenoids enhance immune function (Blount *et al*. 2003; McGraw & Ardia 2003; Dijkstra *et al*. 2007), and exert a role as antioxidants, in parasite resistance, and growth (Olson & Owens 1998; Chew & Park 2004); therefore males must trade off between using carotenoids as a signal and lowering their immune defence (Lozano 1994; Svensson & Wong 2011).

Androgen levels are modulated by male social status and social interactions (Oliveira *et al*. 1996, 2001; Dijsktra *et al*. 2007), and a relationship between levels of circulating androgens and carotenoid- based coloration has been demonstrated in fish (Borg & Mayer 1995; Oliveira & Almada 1998), reptiles (Salvador *et al*. 1996), and birds (Mougeot *et al*. 2009). The hormone 11-ketostestosterone (KT) is the androgen present in the highest concentrations in males during the breeding season and it regulates male mating behaviour and secondary sexual traits (Borg & Mayer 1995). Testosterone supplementation has been demonstrated to enhance nuptial coloration in lizards (Salvador *et al*. 1996), to increase aggression and bill colour in birds (Ardia *et al*. 2010) and, in bitterling fishes, injections of 11-KT or methyltestosterone cause female *Rhodeus ocellatus* to develop male nuptial coloration with a male-like distribution of chromatophores in treated females (Tajima *et al*. 2008; Kobayashi *et al*. 2009).

The aim of this study was to test variation in male carotenoid-based coloration as an inter- or intra-sexual signal in the rose bitterling, *Rhodeus*

ocellatus. To do so, males were paired either with a female in spawning condition, or with a male or with both. To exclude the effect of the presence of a conspecific on male signalling, males were also paired with a non-spawning female. In addition, to test whether the presence of a mating resource (a mussel) influenced the variation in male coloration, the same study was performed with and without a mussel. Three predictions were tested: 1) males would not increase coloration in the presence of a female not ready to spawn; 2) males would increase the extent of red in the iris and on the tail fin both as a signal to rival males and to females ready to spawn; and, 3) the variation in the extent of red would be greater when males defend a territory around a mating resource. In fish an increase in colour is well documented in males performing mating behaviours (Kodric-Brown 1998), but its plasticity has yet to be tested in bitterling.

Bitterling are small freshwater fishes from East Asia, with a single species-complex present in Europe (Zaki *et al*. 2008). When sexually mature, male bitterling develop a red carotenoid-based coloration in the iris and on the tail fin that appears to intensify while performing courtship and territorial behaviours. Females maintain a uniform silvery colour and, when ready to spawn, develop an extended ovipositor that they use to lay their eggs in the gills of fresh water mussels (Duyvené de Wit 1955). Males fertilize the eggs by releasing sperm over the inhalant siphon of the mussel (Wiepkema 1961). Dominant males aggressively defend territories against rival males and vigorously court females and lead them to the mussel (Smith *et al*. 2004; Casalini *et al*. 2009). Bitterling have a promiscuous mating system (Smith & Reichard 2005) and alternative mating tactics are not genetically determined; dominant males often sneak fertilization in the territory of neighbouring territorial males. There is no morphological distinction between

territorial males and sneakers (Kanoh 2000), though the former are generally larger (Smith *et al*. 2004; Reichard *et al*. 2004b, 2005). For a review of bitterling reproductive ecology see Smith *et al*. (2004).

6.3. Methods

Experiments were performed in the facilities of the University of St Andrews. Fish used for experimental work were the second-generation offspring of wild caught *R. ocellatus* from the River Yangtze Basin, China. During the experiment they were 36 - 40 months old. Prior to experiments fish were held in stock aquaria measuring 60 (length) x 30 (width) x 35 (depth) cm. Stock and experimental aquaria were kept at 23°C, exposed to 16:8 h light: dark cycle, and provided with a layer of sand substrate and artificial plants as refuges. Fish were fed a mixture of commercial dried fish flake food twice daily and frozen chironomid larvae once a week. Freshwater mussels used in trials were *Unio pictorum* that are readily used as a spawning site by *R. ocellatus* (Casalini 2007). Mussels were collected from the Grand Union canal in Leicestershire, UK, and prior to experiments were stored in an outdoor pond.

The design of the experiment consisted of four different treatments:

1. focal male paired with a free female not ready to spawn
2. focal male paired with a male constrained in a perforated transparent bottle
3. focal male paired with a free female in spawning condition
4. focal male paired with a free female in spawning condition and with a male constrained in a perforated transparent bottle.

The day before the beginning of trials, four focal males were haphazardly selected from stock aquaria, randomly assigned to one of the four treatments, individually placed in experimental aquaria measuring 45 (length) x 30 (width) x 30

(depth) cm together with a mussel in a sand-filled plastic cup and an empty perforated transparent bottle, and allowed to acclimatize overnight. Opaque screens between aquaria prevented visual interference with neighbouring fishes.

The following morning, at the start of the trial, the extent of red in the iris and on the tail fin of focal males were measured visually; the extent of red was checked three times: at the start of the trial, and twice more at 5 minute intervals. The mussels were covered with a perforated plastic cup to allow inspection but not spawning, and stimulus males and females, haphazardly chosen from stock aquaria, were gently placed in the experimental aquaria according to the different treatments.

The extent of red in the iris and on the tail fin of focal males was scored visually 1, 2, 3, 4, 5, 10, 15, and 20 minutes after exposure to stimulus using the ordinal scale described in Chapter 5 (see Tables 5.1 and 5.2, page 103). Stimulus males and females were then removed and the extent of red in the iris and on the tail fin of focal males were measured again after 1, 2, 3, 4, 5,10,15, 20, 30, 40, and 50 minutes.

The length of observations before, during and after imposing experimental treatments was defined on the basis of the results of pilot trials. A total of 15 independent replicates were completed for each treatment. Once complete, the same procedure was repeated without placing a mussel in the experimental aquaria, with a 15 further independent replicates completed for each treatment using different focal males, stimulus males and females. After completion of trials the body length of focal males, stimulus males and females, and the maximum shell length of mussels were measured to the nearest 1 mm and fish and mussels were returned to stock tanks and not used again.

6.3.1. Data analysis

Data were tested for normality using a Shapiro-Wilk test and for equality of variance using Bartlett's test. Nonparametric tests were used if data did not respond to transformation. A Spearman test was used to test the correlation between the extent of red in the iris and on the tail fin of focal males at the beginning of the trials and when the stimulus was removed, and between focal male body length and increase in the extent of red (difference between the initial extent at the start of the trials and maximum extent when the stimulus was removed) in the iris and on the tail fin. ANCOVA was used to test for the effect of stimulus males, females, and mussels on the variation in the extent of red in the iris and on the tail fin, with focal male body length as a covariate.

6.4. Results

No significant difference was present in male body length among treatments (Table 6.1). There was a significant correlation between the extent of red in the iris and on the tail fin of focal males at the start of the trials (Spearman correlation: r_{118} = 0.546, $P < 0.001$) and the maximum extent of red in the iris and on the tail fin (Spearman correlation: $r_{118} = 0.801$, $P < 0.001$). Male body length did not correlate with the increase in the extent of red (difference between the initial extent at the start of the trials and maximum extent when the stimulus was removed) in the iris (Spearman correlation: $r_{118} = 0.075, P = 0.414$) and on the tail fin ($r_{118} = 0.159$, P = 0.081). There was a significant difference in the time from exposure to stimulus to the expression of colour change and from exposure to stimulus to the expression of maximum colour change as compared to the time from maximum colour change to the beginning of decrease in the extent or red and to the original colour state after removal of the stimulus (Figures 6.1 and 6.2; Tables 6.2 and 6.3). At the end of the

trials all males but 3 showed the same extent of red in the iris measured initially, and all males but 4 the same extent of red on the tail fin. Males exposed to a female not ready to spawn did not increase the extent of red in the iris (mean ± SE difference between the initial extent of red and the maximum increase 0.20 ± 0.14) or on the tail fin (mean ± SE difference between the initial extent of red and the maximum increase 0.07 ± 0.07). Conversely the change in the extent of red in the iris and on the tail fin was affected by the presence either of a rival male, a female in spawning condition or a male and a female in spawning condition, though there was no difference in the effect of different stimuli (Figures 6.3, 6.4, 6.5, and 6.6; Tables 6.4 and 6.5). The presence of a mussel did not influence the variation in the extent of red in the iris (Table 6.4), but an effect was detected for the variation of the extent of red on the tail fin (Table 6.5).

6.5. Discussion

The aim of this experiment was to test whether male *R. ocellatus* increased the extent of red colour signals in the iris and on the tail fin as an inter- or intra-sexual signal or both. The aim was also to examine whether the presence of a key mating resource (a mussel) influenced variation in colour signals, and to test the plasticity of the increase and decrease of the extent of red coloration.

The results confirm the first prediction: notably, the control stimulus (a female not ready to spawn) did not influence the change in colour in the iris or on the tail fin. The presence of a conspecific that is not a potential mate or a potential opponent does not elicit an intensification of the signal as confirmed in a study where male pupfish, *Cyprinodon pecosensis*, were paired with juveniles (Kodric-Brown 1996).

The data collected confirm also the second prediction: the increase in the extent of red was influenced both by agonistic behaviour in the presence of a rival male or by courtship when males were paired with a female ready to spawn.

The function of male coloration as an inter- and intra-sexual signal in mating contexts has been demonstrated in fish (Kodric-Brown 1996; Cubillos & Guderley 2000) and birds (Griggio *et al*. 2007). Red coloration appears to signal fighting ability to rivals in male red- collared widowbirds, *Euplectes ardens*, and red-shouldered widowbirds, *Euplectes axillaris* (Pryke *et al*. 2001; Pryke & Andersson 2003), and to be a general indicator of male dominance in the European bitterling, *R. amarus* (Smith *et al*. 2002; Reichard *et al*. 2005). In contrast, a correlation between the extent of red and dominance is less clear-cut in *R. ocellatus* (Casalini *et al*. 2009).

The influence of this visual signal on female choice is still controversial. Some experimental work has demonstrated the role of male carotenoid-based coloration in determining female choice (Kodric-Brown 1985; Houde 1987; Bakker 1993, Pilastro *et al*. 2004). Conversely, female choice for more colourful males may be limited (Braithwaite & Barber 2000), or weak (Östlund Nilsson & Nilsson 2000), or females may base their choice on multiple cues (Cubillos & Guderley 2000; Candolin & Reynolds 2001; Candolin 2003). Female bitterling do not appear to choose more colourful males as mates (Casalini *et al*. 2009); their choice is strongly influenced by male courtship (Reichard *et al*. 2005; Casalini *et al*. 2009), and they mate preferentially with genetically compatible MHC-dissimilar males (Agbali *et al*. 2010).

In the present experiment, colour change took place when males were paired with a female ready to spawn or in the presence of a rival male; as red carotenoid-

based coloration is costly, its increase may be used by males to signal their superior fitness both to potential rivals and mates as functional and physiological parameters can determine, among others, morphology and design of sexual signals (Irschick *et al.* 2007). A larger extent of carotenoid- based coloration used as a badge of status (Andersson 1994), can allow males to win more contests (Evans & Norris 1996) or to spend less effort in territorial maintenance and agonistic interactions towards less coloured intruders (Pryke *et al*. 2001). In bitterling, intra- and inter-sexual mating behaviours take place simultaneously: the presence of a female ready to spawn and inspecting a mussel attracts competitors and the territorial male, in a few seconds, switches from courtship, that influences female willingness to spawn, to aggression to prevent rivals from releasing sperm into the mussel he defends. Given the time necessary to increase and decrease the extent of red in the iris and on the tail fin, it would be impossible for males to modulate the expression of colour according to different but concomitant mating behaviours. The signal may then have evolved and be maintained by males in a mating context, irrespective of female mate choice. Further investigation is necessary to test whether a greater extent of red in the iris or on the tail fin of *R. ocellatus* males can influence the willingness of rival males to fight.

The results of the experiment only partly support the prediction that colour variation in the iris and on the tail fin of males was influenced by the presence of a mussel; the presence of a mating resource affected the variation on the fin tail, but not in the iris. This result needs further investigation, as the mechanism is unclear.

Previous experiences at winning a contest or a fight can determine a higher probability of a future success either by altering the winner's own perception of his fighting ability or influencing the propensity of opponents to start a fight (Hsu *et al*.

2006; Oliveira *et al*. 2009). This mechanism provides a likely explanation for the lack of influence of the presence of a mussel on male colour change in the iris: if winning a fight may result in future success or in a dominant position in a social group (Dugatkin & Druen 2004), it can be worth fighting even in the absence of a mating resource; the behaviour can then become fixed at population level and be adopted in any mating context. The design of the present experiment did not allow a physical contact between focal males and potential opponents but, prior to the experiment, fish were housed together and can have experienced direct fights with rival males. Alternatively, as levels of circulating androgens and nuptial coloration are often correlated, the presence of a potential rival or of a female ready to spawn may trigger the increase in circulating androgens and, consequently, in the extent of red even in the absence of a mussel.

The extent and intensity of ephemeral colours may increase and decrease rapidly in a mating context (Kodric-Brown 1998). In the present experiment only the variation in the extent of red was measured and the rapid increase in colour expression after exposure of a male to a stimulus contrasted with the slow loss of colour after the stimulus was removed. This result suggests a strong effect of mating context on the mobilization of carotenoids into nuptial coloration, probably linked to the effect of a concurrent rise in circulating androgens. The different rate of response may relate to the difference in the time for androgens to have an effect once the male is stimulated, compared with the time taken for circulating androgens to be removed once the stimulus disappears.

In conclusion, the results of this experiment show that the variation in the extent of red in the iris and on the tail fin of *R. ocellatus* males may be used both as an inter- and an intra-sexual signal, while the presence of a mating resource

influences only the variation in colour on the tail fin. The extent of red increased rapidly in response to the appearance of a potential mate or rival, and decreased relatively slowly when rivals or mates were no longer present. Further research is necessary to clarify the physiological mechanisms underlying this variation and its plasticity.

Figure 6.1. Mean ± SE colour change in the iris of focal males before, during, and after treatments. Vertical bars mark the beginning of treatments. Dotted black line for replicates with mussels in the aquaria.

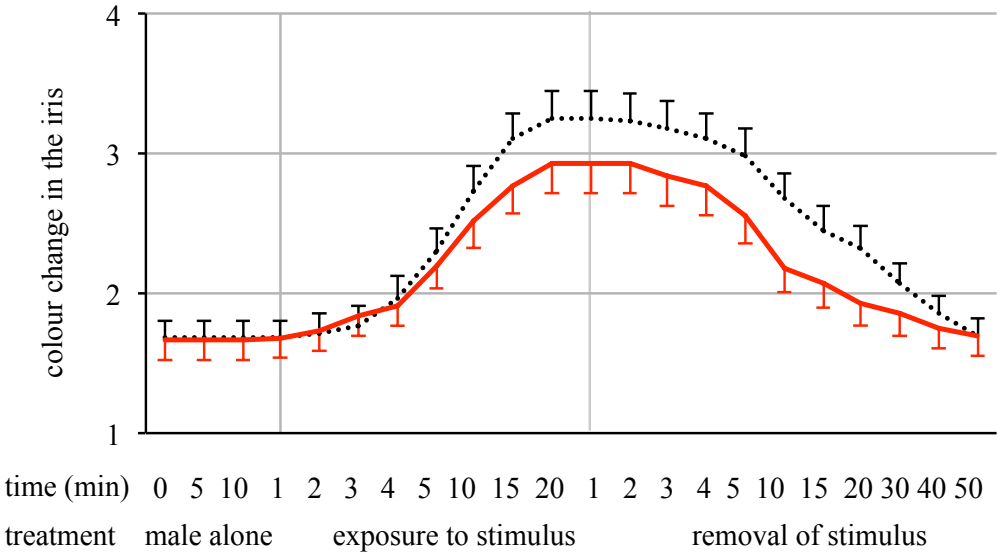


Figure 6.2. Mean ± SE colour change on the tail fin of focal males before, during, and after treatments. Vertical bars mark the beginning of treatments. Dotted black line for replicates with mussels in the aquaria.

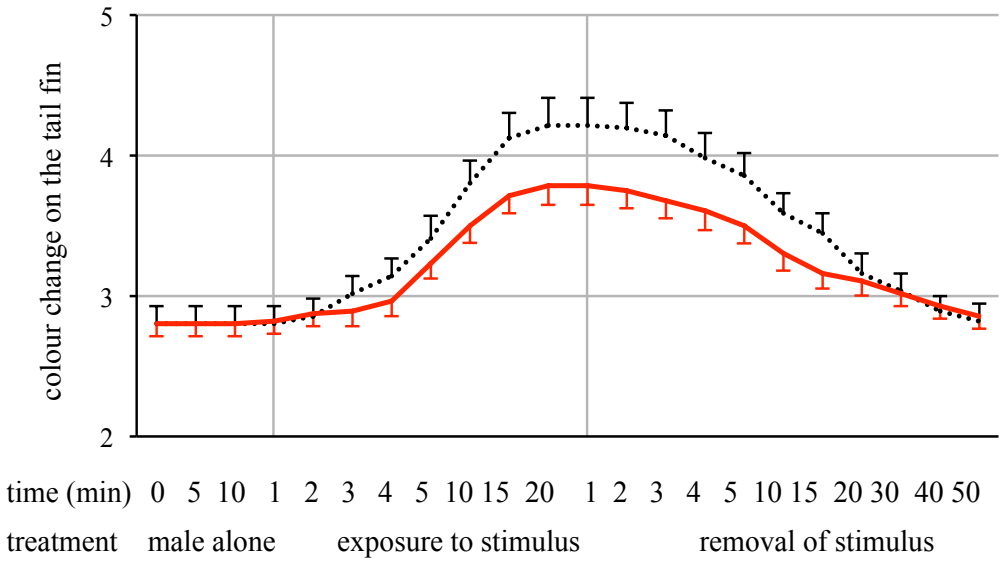


Figure 6.3. Mean ± SE colour change in the iris of focal males paired with a free female not ready to spawn (treatment 1), with a constrained male (treatment 2), with a free female in spawning condition (treatment 3), and with a free female in spawning condition and a constrained male (treatment 4). Replicates with mussels in the aquaria.

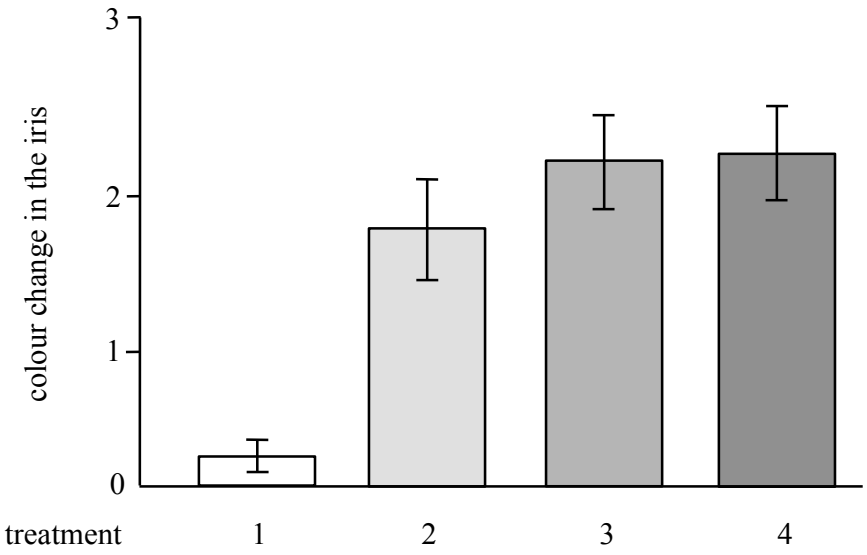


Figure 6.4. Mean ± SE colour change in the iris of focal males paired with a free female not ready to spawn (treatment 1), with a constrained male (treatment 2), with a free female in spawning condition (treatment 3), and with a free female in spawning condition and a constrained male (treatment 4). Replicates without mussels in the aquaria.

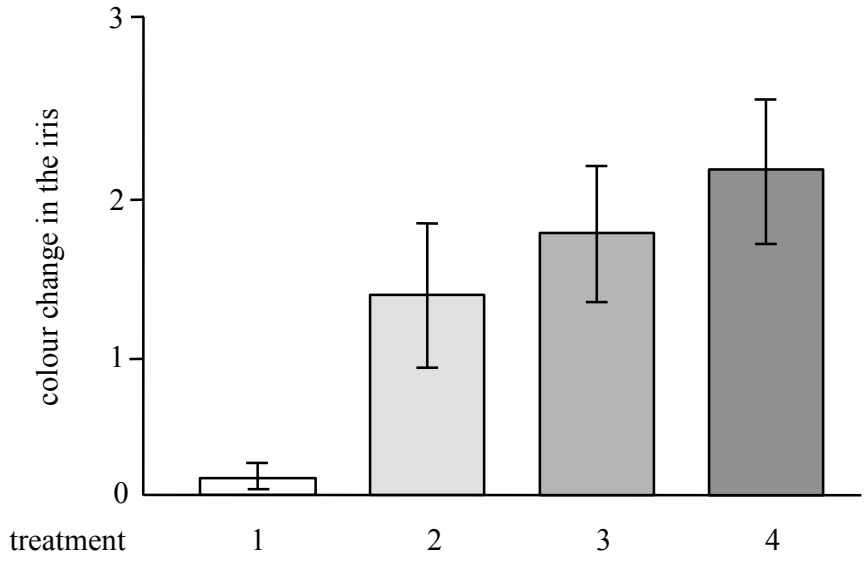


Figure 6.5. Mean ± SE colour change on the tail fin of focal males paired with a free female not ready to spawn (treatment 1), with a constrained male (treatment 2), with a free female in spawning condition (treatment 3), and with a free female in spawning condition and a constrained male (treatment 4). Replicates with mussels in the aquaria.

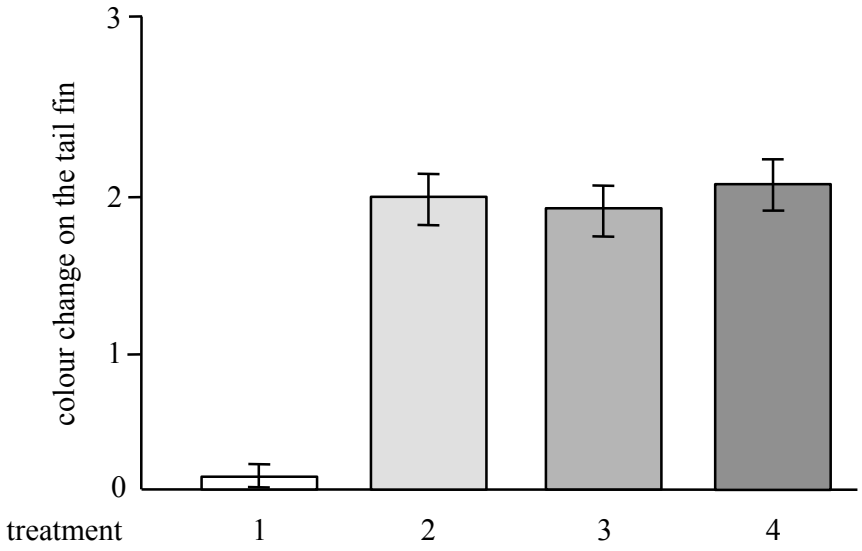


Figure 6.6. Mean ± SE colour change on the tail fin of focal males paired with a free female not ready to spawn (treatment 1), with a constrained male (treatment 2), with a free female in spawning condition (treatment 3), and with a free female in spawning condition and a constrained male (treatment 4). Replicates without mussels in the aquaria.

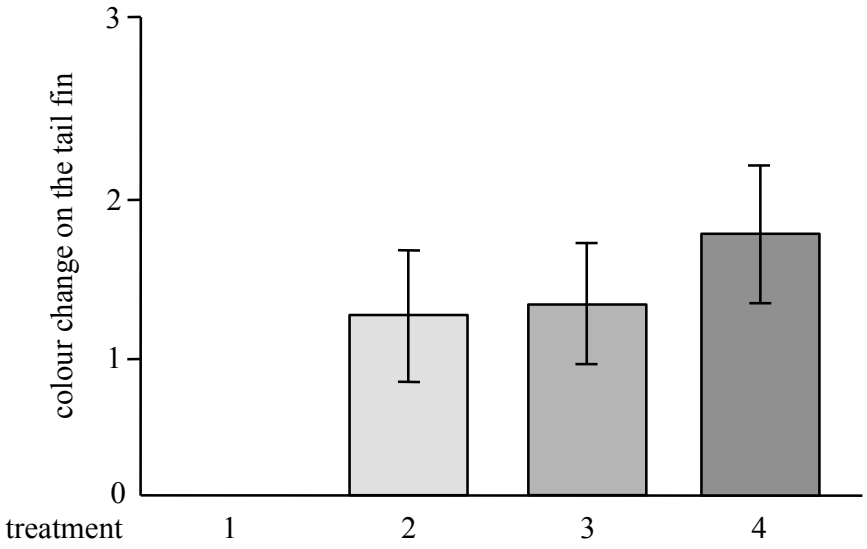


Table 6.1. Mean ± SE body length (mm) of focal males assigned to the four treatments. Pooled data for replicates with and without mussels in the aquaria.

	Mean male body length (mm)	
Treatment	**Mean**	**SE**
Focal males paired with a female not ready to spawn	53.07	± 1.50
Focal males paired with a constrained male	54.13	± 1.32
Focal males paired with a free female in spawning condition	54.34	± 1.64
Focal males paired with a free female in spawning condition and with a constrained male	53.63	± 1.16

Table 6.2. Mean ± SE time (min) from the exposure to stimulus to the expression of eye and tail colour change and to the expression of maximum colour change. Pooled data for replicates with and without mussels in the aquaria.

	Eye		Tail	
	Mean	**SE**	**Mean**	**SE**
Time (min) from exposure to stimulus to the expression of colour change	6.82	± 0.46	6.44	± 4.43
Time (min) from exposure to stimulus to the expression of maximum colour change	12.55	± 0.64	11.89	± 0.60

Table 6.3. Mean ± SE time (min) from eye and tail maximum colour change to the beginning of decrease in the extent of red and to the original colour state after removal of the stimulus. Pooled data for replicates with and without mussels in the aquaria.

	Eye		Tail	
	Mean	**SE**	**Mean**	**SE**
Time (min) from the maximum colour change to the beginning of decrease in the extent of red after removal of stimulus	12.37	± 1.26	11.30	± 1.13
Time (min) from the maximum colour change to the original colour state after removal of stimulus	26.52	± 1.78	25.06	± 1.76

Table 6.4. ANCOVA for colour change in the iris of focal males exposed to stimulus males and females, with focal male body length as covariate.

Source	df	SS	MS	F	P
Focal male body length	1	0.27	0.27	0.23	0.633
Constrained male (treatment 2)	1	20.08	20.08	16.92	< 0.001
Female in spawning condition (treatment 3)	1	52.25	52.25	44.01	< 0.001
Constrained male and female in spawning condition (treatment 4)	1	5.13	5.13	4.32	0.039
Mussel	1	1.90	1.90	1.60	0.207

Table 6.5. ANCOVA for colour change on the tail fin of focal males exposed to stimulus males and females, with focal male body length as covariate.

Source	df	SS	MS	F	P
Focal male body length	1	1.17	1.17	1.41	0.237
Constrained male (treatment 2)	1	25.35	25.35	30.51	< 0.001
Female in spawning condition (treatment 3)	1	20.28	20.28	24.41	< 0.001
Constrained male and female in spawning condition (treatment 4)	1	10.13	10.13	12.19	< 0.001
Mussel	1	3.68	3.68	4.43	0.037

Chapter 7. Male choice for mates and mating resources in the rose bitterling, *Rhodeus ocellatus*

7.1. Abstract

The traditional view of sexual selection acting through male-male competition and female choice has been challenged, in recent years, by an increasing body of experimental work that has demonstrated the role of male choosiness influenced by cues such as body length that correlate with female fecundity, or for good quality mating resources that are preferentially chosen by females. Here male response to differently sized females and male choice for mating resources were tested in the rose bitterling, *Rhodeus ocellatus*. Male mating behaviours did not vary with female size. The lack of male response, which contrasts with other species, can be explained through weak selection pressure on males for choosiness since neither the number or size of eggs spawned by females correlated with female size. Male choice for different mating resources was not consistent: when presented with two different mussel species and a constrained female, males did not show any preference for either mussel. When paired with free females, however, males complied with female choice. The results underline the relevance of female choice for resources in influencing male mating behaviour.

7.2. Introduction

Sexual selection operates through male-male competition and female mate choice (Darwin 1871; Andersson & Simmons 2006). The conventional view is that, to maximize their reproductive fitness, males should mate with the greatest possible number of females and choose resources females prefer. Conversely females, given their higher cost of gamete production and lower potential reproductive rate, should be choosy over the males with which they mate and the territories or the resources males defend (Bateman 1948; Trivers 1972). In consequence, mate choice research has focused primarily on females, attempting to understand the relative importance of direct and indirect benefits for their reproductive fitness conferred by males (Andersson 1982; Thornhill 1983; Eberhard 1996), and whether females may base their choice more on the quality of territory or resources than on male characteristics (Warner 1987; Candolin & Reynolds 2001).

However, an increasing body of experimental work has investigated and demonstrated the role of male choice for females and for resources that can attract females in different animal taxa (fishes: Sargent *et al*. 1986; Bakker & Rowland 1995; Werner & Lotem 2003; birds: Hill 1993; reptiles: Olsson 1993; anurans: Liao & Liu 2009; and invertebrates: Zahradnik *et al.* 2008).

Males are predicted to mate preferentially with larger females when female body length is correlated with fecundity or offspring survival, as reported in teleost fishes (Wootton 1990), in chameleons, *Chamaeleo chameleon* (Cuadrado 1998), in the Japanese beetle, *Popillia japonica* (Saeki *et al*. 2005), and in the European pond turtle, *Emys orbicularis* (Poschadel *et al*. 2006).

Male choice is expected to evolve if mating with successive females leads to sperm depletion (Andersson 1994; Smith *et al*. 2009), if males can detect a

difference in quality among receptive females, and, in general, if the cost of choice in terms of probability of finding alternative mates and assessing their quality does not exceed the benefits resulting from variation in quality (Bonduriansky 2001).

Male territoriality and aggressive defence of mating resources is a widespread behaviour in many animal taxa and can be crucial to increase reproductive fitness if territory or mating resource quality are among the cues on which females base their mate choice. In oviparous species the choice of good quality oviposition sites by females can be as important as mate choice to increase their reproductive fitness, in terms of greater offspring likelihood to survive, and has been demonstrated in anurans (Roithmair 1994), birds (Alatalo *et al*. 1986; Temeless & Kress 2010), and fish (Warner 1987; Bisazza *et al*. 1989; Svensson & Kvarnemo 2005). The possession of better quality spawning sites can even determine female choice to mate preferentially with heterospecific males (Dijkstra *et al*. 2008). Thus, female choice for resource quality rather than mates can limit male reproduction and lead to male choice for resources; given the choice, males should allocate more time and energy expenditure on behaviours that allow them to possess and then attract females to their preferred mating resources (Wong & Jennions 2003).

Being choosy, however, may carry a cost in terms of time and energy expenditure (Parker 1983), greater risk of predation (Forsgren 1992; Houde 1997; Herdman *et al*. 2004), and loss of potential future matings. Thus males must trade-off the benefits of increased reproductive fitness in seeking larger females or better mating resources against the costs associated with choosiness.

Here male mate choice was investigated in the rose bitterling, *Rhodeus ocellatus*, to test whether different sized males invested more energy in courting

females and in attacking a rival male when presented with larger or smaller females (Experiment 1), and whether larger female size correlated with egg volume and clutch size (Experiment 2). Besides, male choice for mating resources was investigated to test whether males inspected and tried to attract females preferentially to mussels of one of the two species provided, and if they were consistent in their behaviour when presented with a constrained or a free female (Experiment 3). Four predictions were tested: 1) males would invest more energy in courtship and aggression of larger females; 2) female size would correlate with egg volume, 3) larger females would lay a higher number of eggs per spawning event, and, given the known choosiness of female bitterling for mating resources, 4) males would be influenced in their choice for mating resource by female behaviour.

Rose bitterling *R. ocellatus* are small freshwater fishes from East Asia. During the spawning season female bitterling develop a long ovipositor that they use to insert their eggs into the gill cavity of live unionid mussels through the mussel exhalant siphon (Duyvené de Wit 1955). Females spawn in bouts lasting one-two days and the eggs are fertilized by males releasing sperm over the inhalant siphon of the mussel (Wiepkema 1961). The embryos develop inside the mussel gills before emerging, after 3-4 weeks as free swimming larvae. Both males and females carefully inspect mussels, probably to ascertain their quality that is essential to embryo survival and development. Dominant males are usually bigger, aggressively defend territories to monopolize mussels, court females and, while courting, try to lead them to the mating resource (Wiepkema 1961; Smith *et al*. 2004; Casalini *et al*. 2009).

7.3. Methods

Experiment 1. Experiments were conducted in the aquarium facility of the Department of Biology at the University of Leicester. Fish used for experimental work were first generation *R. ocellatus* derived from wild caught fish from the River Yangtze Basin, China. During the experiment they were 24-30 months old. Prior to experiments fish were held in stock aquaria measuring 120 (length) x 40 (width) x 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 layer of sand substrate. Fish were fed a mixture of commercial dried fish flake food twice daily and bloodworm (*Chironomus* spp.) and live zooplankton (*Daphnia* spp.) three times each week. Freshwater mussels used in trials were *Unio pictorum* collected from the River Cam, kept in 100 L outdoor tanks and fed with live phytoplankton daily.

Prior to experiments 42 males and 42 females were haphazardly selected from stock aquaria and assigned by eye to three broad size categories (males) and two broad size categories (females). Differently sized males were used to exclude size-related differences in males' behaviour and to mirror the actual composition of a natural population. Size-sorted males and females were held in separate stock aquaria and allowed to settle for two days.

At the start of the experiment, two size-matched males were haphazardly selected and placed in an experimental aquarium measuring 60 (length) x 40 (width) x 40 (depth) cm with a mussel in a sand-filled plastic cup and artificial plants as refuges. When the males were acclimatized and dominance was fully established, the aggression rate of the focal male (the one who had established dominance over the second size-sorted male) was scored for 15 minutes using a

palm computer with the FIT-system behaviour recording software (Held & Manser 2005). After covering the mussel with a perforated plastic cup to allow mussel inspection but not spawning, either a large or a small female in spawning condition, randomly selected from the stock aquaria, was gently released into the experimental aquarium and allowed to settle for one hour. The mussel was then uncovered and the behaviours of the focal male were scored with the FIT-system behaviour recording software for 15 min. The behaviours scored were: (i) aggression rate, (ii) duration of courtship/leading, and (iii) pre- and post-oviposition ejaculation rate. After completion of the trial female body length was measured to the nearest 1 mm and the female was not used again.

The same procedure was repeated with the same males and mussel but with a haphazardly selected female from the alternate size class to the first tested. In each paired replicate the between-female difference in body length was ≥ 20%. The order of different sized males and females to be tested was randomized prior to the beginning of the experiment. After completion of the trial the body length of both males and of the second female, and the maximum shell length of the experimental mussel were measured to the nearest 1 mm and all fish were returned to stock aquaria and not used again. A total of 21 independent paired replicates were completed.

Experiment 2. Experiments were conducted in the aquarium facility of the Department of Biology at the University of Leicester. Fish used for experimental work were first generation *R. ocellatus* derived from wild caught fish from the River Yangtze Basin, China. During the experiment they were 24-30 months old. Prior to the start of the experiment 32 males were haphazardly selected from stock aquaria and placed in an aquarium measuring 60 (length) x 40 (width) x 40

(depth) together with 16 wild or first generation females. Females were assigned by eye to two size classes (large and small). Haphazardly selected pairs of males were placed in a smaller aquarium measuring 25 (length) x 40 (width) x 30 (depth) cm with a mussel in a sand-filled plastic cup and a female in spawning condition randomly chosen between the two size classes. Two males were used to maximise the likelihood that females would spawn (Smith & Reichard 2005). After one hour the mussel was inspected by gently opening a 1-cm gap between the valves using a mussel-opening device (Kitamura 2005), which allows checking the presence of eggs inside the mussel without harming it. If eggs were present, the mussel was measured and dissected and the eggs counted, assigned to one or multiple spawnings and photographed against a scale bar using a Canon EOS 350D with a 60 mm macro lens under standard light condition. If eggs were not present the mussel was put back into the tank and examined at hourly intervals until spawning had occurred. Female body length and maximum shell length of the mussel were measured to the nearest 1 mm and the fish and the mussel were not used again. Female *R. ocellatus* spawn eggs in batches into mussel gills making it easy to ascertain the number of eggs released during each spawning event. However, in two different replicates, some of the eggs were in batches adjacent to each other making identification of the exact number of eggs per spawning uncertain. The replicates were discarded and repeated using males and females, size-matched with the ones of the discarded replicates, haphazardly chosen from stock aquaria.

The diameter of the major and minor axis of all eggs was measured to the nearest 0.1 mm from digital photographs using UTHSCSA Image Tool 3.0. Egg volume was calculated using the function:

$$\text{Egg volume (mm}^3\text{)} = 1/6\ \pi\ ab^2$$

where *a* and *b* are the lengths of the major and minor axes respectively (Coleman 1991). A total of 16 independent replicates were completed.

Experiment 3. Experiments were performed in the facilities of the School of Biology, St Andrews. Experimental fish were the second-generation offspring of wild caught *R. ocellatus* collected from the River Yangtze Basin, China, in 2005. The fish were raised in captivity and were 30-36 months old when experiments were conducted. Fish were fed a mixture of commercial dried fish flake food twice daily and exposed to 16:8 h light: dark cycle. The two mussel species used in the experiment, *Unio pictorum* and *Anodonta anatina*, were collected from the Grand Union canal in Leicestershire, UK, and, prior to experiments were stored in an outdoor pond. These mussel species occur across Eurasia and are readily used as a spawning site by *R. ocellatus* males, which are generalists (Reichard *et al*. 2007).

At the start of the experiment a male, haphazardly chosen from stock aquaria, was placed in an aquarium measuring 60 (length) x 30 (width) x 30 (depth) cm with a layer of gravel and artificial plants as refuges. Two mussels, one *A. anatina* and one *U. pictorum*, each in a sand-filled plastic cup, were placed in a randomly selected position at the same distance from the aquarium walls. Once the male was acclimatized and had established territoriality, a female confined in a perforated transparent bottle was placed into the aquarium and when the male started to court her, his behaviours were tested for 10 minutes. Male behaviours scored were: (i) mussel inspection rate, (ii) courtship/leading rate, and (iii) ejaculation rate. The female was then gently freed into the aquarium and male and female behaviours were tested for 10 further minutes. Female behaviours scored were: (i) mussel inspection rate, (ii) skimming rate, and (iii) spawning rate. At the end of each trial male and female body length and mussel maximum length were

measured to the nearest 1 mm and fish and mussels were not used again. Sixteen independent replicates were performed.

7.3.1. Data analysis

All data were tested for normality using a Shapiro-Wilk test and for equality of variance using Bartlett's test. Data that did not meet assumptions of normality and homoscedasticity were transformed to meet these assumptions.

Experiment 1. An unpaired *t*-test was used to test the difference in body length between the two female size classes. A paired *t*-test was used to test for differences in the aggression rate of focal males before and after they were presented with a female, and for differences in aggression rate, courtship/leading duration and ejaculation rate (mean pre- and post-ejaculation) when males were presented with differently sized females.

Experiment 2. An unpaired *t*-test was used to test the difference in body length between the two female size classes; the correlation between female size and mean number and volume of eggs per spawning was tested with a Pearson's correlation.

Experiment 3. Male inspection rate and courtship/leading rate were tested with a paired *t*-test to ascertain male choice between the two mussel species when females were constrained. A paired *t*-test was used to test for differences in inspection rate and courtship/leading rate with both mussel species when males were paired with free females. Differences in female inspection rate in *U. pictorum* and *A. anatina* were tested with a paired *t*-test. A Pearson's correlation was used to test the correlation between female inspection rate and male inspection rate and between female inspection rate and male courtship/leading rate.

7.4. Results

Experiment 1. Male body length (mean ± SE) varied between the size categories: large (58 ± 0.79 mm), medium (50.57 ± 0.37 mm), and small (44.14 ± 0.46 mm). There was a significant difference in body length between the two female size classes (unpaired *t*-test, square root transformed data: $t_{40} = 18.06$, $P < 0.001$). When males were presented with differently sized females no difference was detected in male aggression rate (paired *t*-test: $t_{20} = 1.54$, $P = 0.138$; Figure 7.1), intensity of courtship (paired *t*-test, square root transformed data: $t_{20} = 0.43$, $P = 0.670$), or mean ejaculation rate (paired *t*-test, $\log_{10+1}$ transformed data: $t_{20} = 0.56$, $P = 0.585$; Figure 7.2). Conversely, there was a significant difference between male aggression rate before and after they were presented with a female; male aggression rate increased when they were presented with a female irrespective of female size (paired *t*-test, square root transformed data: $t_{20} = 5.18$, $P < 0.001$, small females; large females: $t_{20} = 4.57$, $P < 0.001$).

Experiment 2. There was a significant difference in body length between the two female size classes (unpaired *t*-test: $t_{14} = -5.81$, $P < 0.001$). There was no significant correlation between either female size and mean number of eggs laid per spawning event (Pearson's correlation: $r_{14} = 0.127$, $P = 0.637$; Figure 7.3), or between female size and mean egg volume ($r_{14} = 0.202$, $P = 0.451$; Figure 7.4).

Experiment 3. When females were constrained there was no difference in male inspection rate and courtship rate near *A. anatina* and *U. pictorum* (paired *t*-test; inspection rate: $t_{15} = 1.92$, $P = 0.073$; courtship/leading rate: $t_{15} = 0.64$, $P = 0.527$). Conversely, male inspection rate and courtship/leading rate in the two mussel species were significantly different when males were paired with constrained or free

females (Figures 7.5 and 7.6; Table 7.1). Male ejaculation rate in both mussel species was low, both with free or constrained females.

Female inspection rate was significantly different between the two mussel species (paired *t*-test, square root transformed data: $t_{15} = 22.68$, $P < 0.001$); females inspected *U. pictorum* much more frequently than *A. anatina*. Male behaviours changed accordingly and there was a significant correlation between female inspection rate and both male inspection rate and courtship/leading rate in the two mussel species (Figure 7.7; Table 7.2).

7.5. Discussion

The aim of this study was to investigate male mating behaviour when presented with differently sized females and male choice for different mussel species.

The results do not confirm the first three predictions: differently sized males behaved consistently and the rate of aggression, the intensity of courtship and mean pre- and post- ejaculation rate did not vary when males were presented with larger or smaller females. In addition, female size was not correlated either with egg volume or with the number of eggs laid per spawning. Similar results were obtained also in a pilot test with *R. ocellatus* (12 males and 12 females) and *R. amarus* (10 males and 10 females); neither male nor female size affected the number of eggs laid per spawning event (Reichard, unpublished data).

A strong pressure of selection on males to choose larger females can be expected in taxa where females produce large and variable number of eggs, and if this can lead to an increase in the number and survival of offspring (Bonduriansky 2001). In oviparous species the quantity of yolk nutrients represents one of the most important means of survival for developing embryos; a greater egg volume can be correlated with a greater supply of yolk and, consequently, with a comparatively

higher chance of survival. However, a lack of male choice for large females is not uncommon and has been demonstrated, for example, in the frog, *Rana dalmatina*, even if fecundity was positively correlated with female size (Hettyey *et al*. 2005). Besides, in a study on *Rhodeus ocellatus kurumeus* Kitamura (2005) found that egg volume was positively correlated to female size and condition but only in some periods of the spawning season; female size remained unchanged over time but their somatic condition declined, so hinting at a paramount effect of female condition on egg size and volume.

A possible explanation for the lack of male choice for larger females may be the loss of potential fertilization success by choosy males due to the choosiness of *R*. *ocellatus* females (Casalini 2007; Casalini *et al*. 2009). Actually, as the spawning season of *R*. *ocellatus* extends for a comparatively long period of time (from April to late September) (Asahina *et al*. 1980), and females mate with several males and spawn repeatedly also on the same day laying 1-5 eggs per spawning, male choosiness over mates can result in a decrease of their overall reproductive fitness. A low variation in female fecundity may limit the evolution of male choosiness based only on female size (Pélabon *et al*. 2003) as males can achieve the same reproductive fitness by mating randomly with a greater number of small females (Itzkowitz *et al*. 1998).

The results of the third experiment support the prediction: mussel species did not influence male behaviour. Males inspected, courted and tried to lead females to both mussels when females were constrained. When females were free and showed a preference for *Unio pictorum*, males altered their behaviour to match female decisions. This result is in agreement with a previous study where male *R. amarus* leading rate was found to correspond to female mussel choice (Smith *et al.*

2002).

Female can base their choice both on male and mating resource quality as both can increase their reproductive fitness (Wells 1977). Thus, a strong selective pressure is expected to affect male choice and aggressive defence of good quality territories and mating resources. For example, male mottled triplefins, *Forsterygion varium*, defend territories with a better spawning substratum where females spawn preferentially (Thompson 1986), and the presence of vegetation and the temperature of the water influence female bullfrog, *Rana catesbeiana,* choice of a spawning site and, consequently, male territoriality (Howard 1978).

Both male and female bitterling inspect the mating resource before releasing sperm or spawning. Experimental studies (Smith *et al*. 2001, Mills & Reynolds 2002; Reichard *et al*. 2007) have demonstrated that, by inspecting a mussel, females are able to detect its quality in terms of oxygen content that can be influenced either by the number of embryos already present or by the change in oxygen concentration between inhalant and exhalant siphon typical of the mussel species. Bitterling females are extremely choosy and, given the chance, they avoid spawning in poor quality mussels, so ensuring a better reproductive fitness in terms of embryo survival.

The cues males use to determine mussel quality seem less clear. The presence of previously released sperm in a mating resource is one of the cues that can influence male behaviour and ejaculation expenditure is predicted to increase in the presence of sperm competition with another male (Parker *et al.* 1996), but to decrease when the number of competitors increases (Smith *et al*. 2002). In bitterling pre-oviposition ejaculation is a common finding, either to ensure paternity in successive spawnings (Smith *et al*. 2004) or as a sneaking tactic to get a higher

fertilization success (Kanoh 1996). Smith *et al*. (2002, 2003) demonstrated that male *R. amarus* seem to be able to detect the presence of rival males' sperm in a mussel: when testis solution of other males was released into the mussels or after observing another male releasing sperm they increased their inspection rate, though keeping leading and ejaculation rate unaltered. Conversely, male *R. ocellatus* did not increase their inspection and ejaculation rate when sperm of different males was released into the mussels (Agbali 2011). However, male *R. amarus* are probably able to assess one of the aspects of mussel quality as they decreased their leading rate to mussels that already contained a high number of eggs or embryos (Smith *et al*. 2003).

The mussels used in the present experiment did not contain eggs or sperm, and males did not have to compete with rival males, which can explain the overall scarcity of pre-oviposition ejaculation and the lack of male mussel choice when females were constrained. When females were free, their choice for a mussel species clearly influenced male decisions: as inspecting and courting are time and energy consuming, males can lose a mating opportunity by trying to lead females to a mussel they are not willing to use as a spawning site.

In conclusion, this study has demonstrated that male *R. ocellatus* do not invest more in courtship or aggressive behaviour when presented with larger females, that female size is not correlated to the number and size of eggs laid per spawning event, and that female choice for a mating resource influences male mating behaviours. Further research is necessary to investigate whether male *R. ocellatus* discriminate among females basing their choice on cues different from female size, and whether male bitterling are able to detect mussel quality.

Figure 7.1. Mean ± SE focal male aggressive defence of mussels when presented with small females (white bar) and large females (grey bar) (15 min^{-1}). In each paired replicate the between-female difference in size was ≥ 20%.

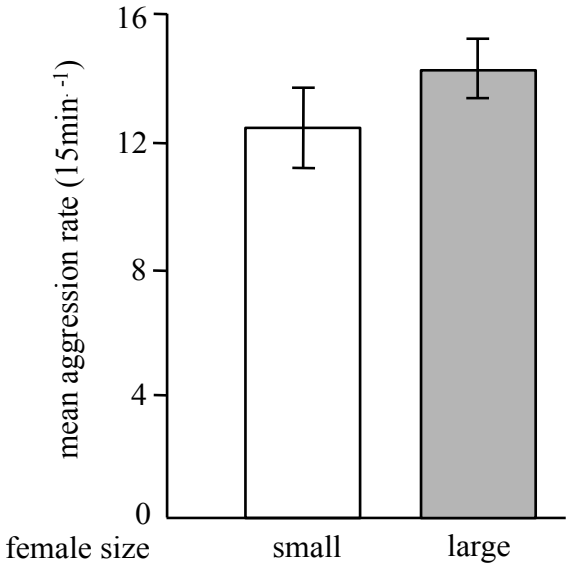


Figure 7.2. Mean ± SE courtship length (s) (15 min^{-1}) (A), and ejaculation rate (pre- and post-oviposition ejaculation) (15 min^{-1}) (B) of focal males when presented with small females (white bars) and large females (grey bars).

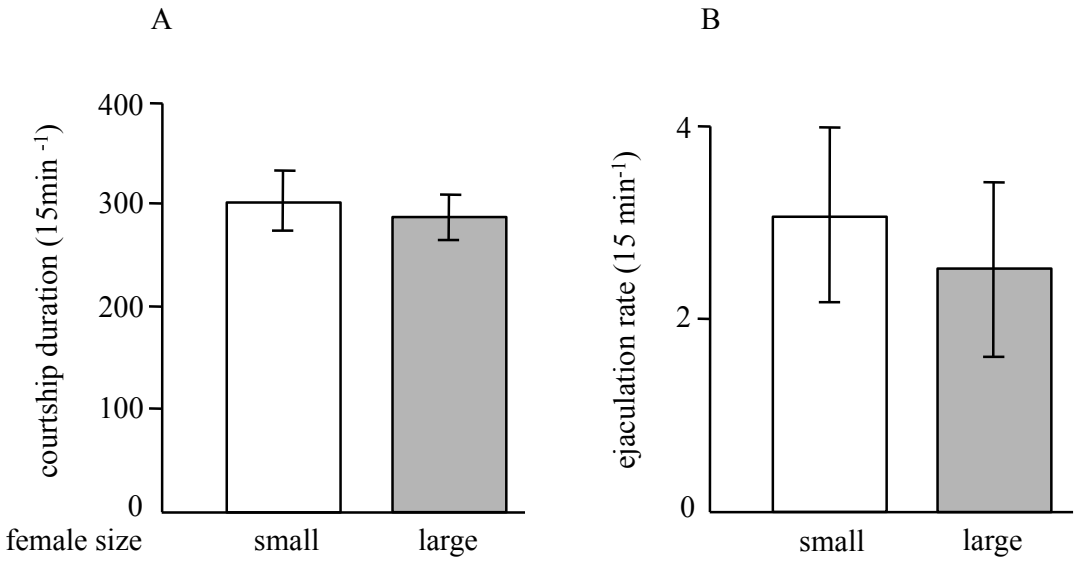


Figure 7.3. Correlation between female body length (mm) and mean number of eggs per spawning event.

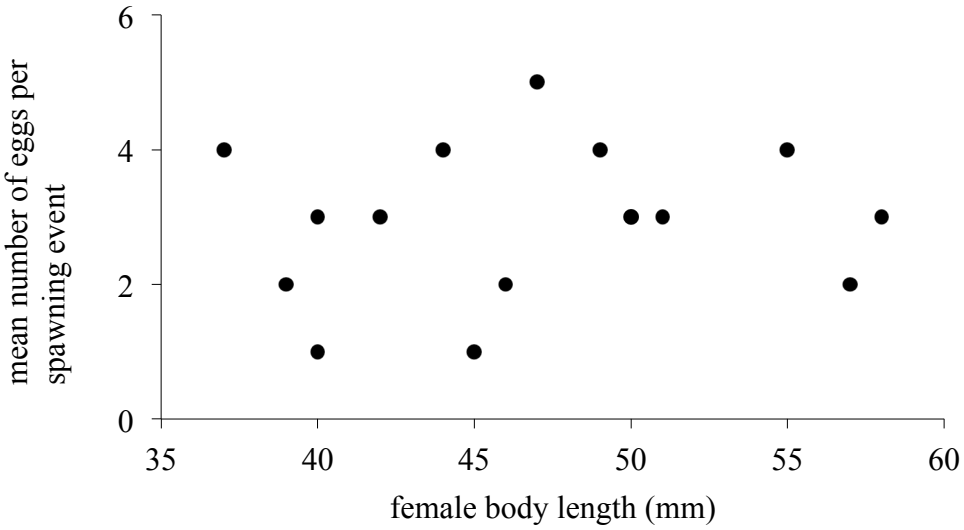


Figure 7.4. Correlation between female body length (mm) and mean volume (mm³) of eggs.

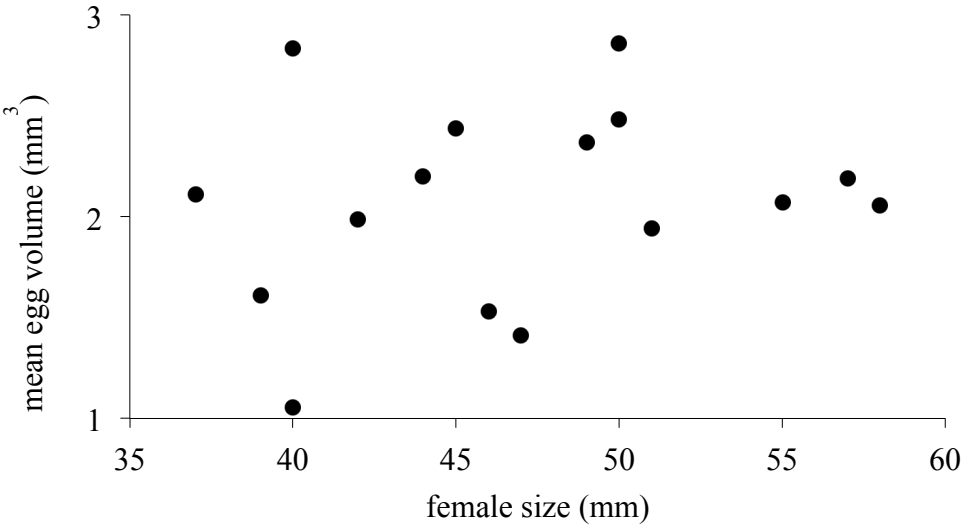


Figure 7.5. Mean ± SE male inspection rate (10 min^{-1}) in *Anodonta anatina* (white bars) and *Unio pictorum* (grey bars) when paired with constrained females (A) and free females (B).

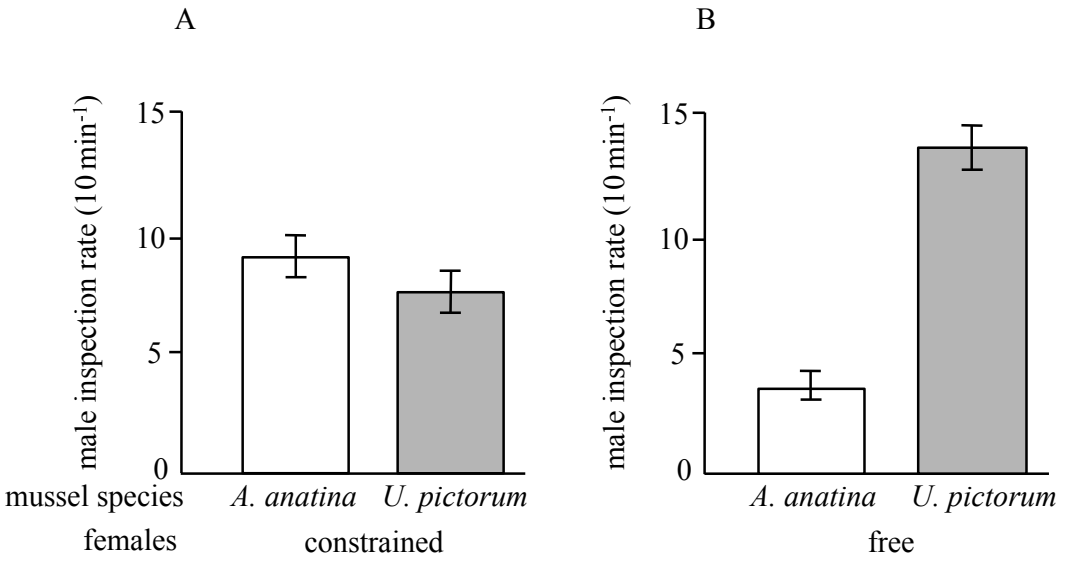


Figure 7.6. Mean ± SE male courtship/leading rate (10 min^{-1}) near *Anodonta anatina* (white bars) and *Unio pictorum* (grey bars) when paired with constrained females (A) and free females (B).

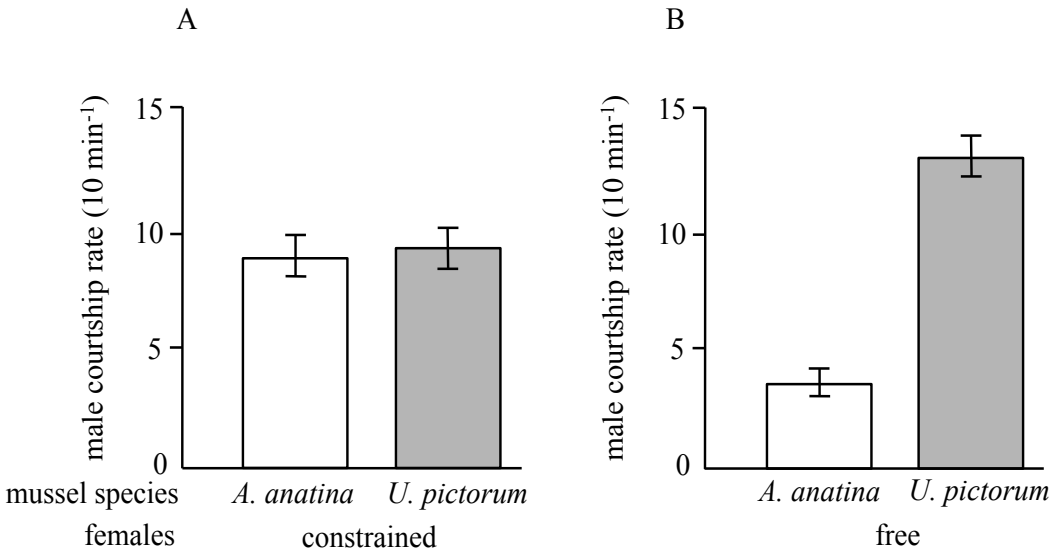


Figure 7.7. Correlation between male courtship/leading rate and female inspection rate of *Unio pictorum* (10 min^{-1}).

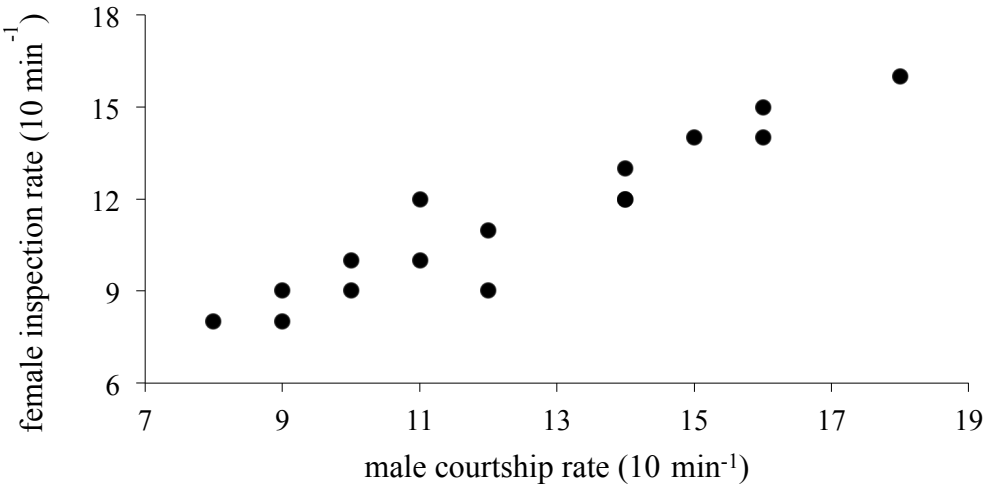


Table 7.1. Summary of paired *t*-test for male inspection and courtship/leading rate (10 min^{-1}) in *Anodonta anatina* and *Unio pictorum* with constrained or free females (square root transformed data for inspection and courtship rate in *Anodonta anatina*).

Variable	**Mean ± SE** **(constrained females)**	**Mean ± SE** **(free females)**	**Df**	***t*-value**	**P**
Male inspection rate in *Anodonta anatina*	7.86 ± 0.64	3.07 ± 0.31	15	12.59	< 0.001
Male inspection rate in *Unio pictorum*	6.71 ± 0.58	11.57 ± 0.57	15	7.56	< 0.001
Male courtship/ leading rate in *Anodonta anatina*	7.86 ± 0.58	3.14 ± 0.26	15	11.63	< 0.001
Male courtship/ leading rate in *Unio pictorum*	7.86 ± 0.59	12.50 ± 0.74	15	8.49	< 0.001

Table 7.2. Summary of Pearson's correlation between female inspection rate and male inspection and courtship/leading rate (10 min^{-1}) in *Anodonta anatina* and *Unio pictorum*.

Variable	Mean ± SE (female)	Mean ± SE (male)	df	*r*	P
Female and male inspection rate in *Anodonta anatina*	2.43 ± 0.28	3.07 ± 0.31	14	0.669	< 0.001
Female and male inspection rate in *Unio pictorum*	11.43 ± 0.63	11.57 ± 0.57	14	0.906	< 0.001
Female inspection rate and male courtship/leading rate in *Anodonta anatina*	2.43 ± 0.28	3.14 ± 0.26	14	0.856	< 0.001
Female inspection rate and male courtship/leading rate in *Unio pictorum*	11.43 ± 0.63	12.50 ± 0.74	14	0.944	< 0.001

Chapter 8. Male mating tactics in the rose bitterling (*Rhodeus ocellatus)* and in the European bitterling (Rhodeus amarus)

8.1. Synthesis

The aim of this thesis was to obtain a deeper understanding of the mechanisms that shape male mating decisions and their consequences for male reproductive fitness. The study investigated:

1. The heritability of male aggressive behaviour and sexually selected traits
2. The role of crowding and density on male mating tactics
3. The effect of resource availability on male mating behaviour
4. The effect of density during embryogenesis on male aggressive behaviour
5. The role of male colour signals
6. Male preference for resource quality
7. Male mate choice.

The genetic basis to behaviour and sexually selected traits has been investigated in fish (Rosenau & McPhail 1987; Magurran 1990), birds (Dingemanse *et al*. 2002), and insects (Majerus *et al*. 1982, Iyengar *et al*. 2002). The genetic component to aggressive behaviour, in particular, has received considerable attention since it was demonstrated in the 1960s in lines of mice artificially selected for high or low levels of aggression (Lagerspetz 1964). Using a North Carolina II design, *in vitro* fertilization, and behavioural assays I demonstrated that aggressive behaviour in male *Rhodeus ocellatus* has a genetic component, with an additive maternal effect (**Chapter 2**). A significant non-additive effect was detected on the extent of red in the iris of males. In addition, embryo and juvenile fitness traits at

sexual maturity were influenced by additive paternal and maternal effects, but with a substantial environmental component. Aggression and sexually selected traits are therefore influenced by an interplay between genetic and environmental factors.

The spatial distribution of resources, mates and potential rivals can play a role in influencing male mating decisions. I did not find evidence for a role of the crowding of fish around mussels, or of the density of individuals on the overall level of male aggression (**Chapter 3**); density and crowding, however, did affect male mating behaviours. At high density male courtship rate decreased, while at high crowding a higher proportion of mussels were controlled by males, and they released sperm into a higher proportion of mussels. The crowding of males around resources can therefore influence the adoption of alternative mating tactics, and elevate the strength of sexual selection on traits used in intra-sexual competition to control spawning sites. A potential outcome is that female mate and mussel choice will be constrained, perhaps resulting in a sexual conflict. Conversely, if crowding is low, male defence of resources will diminish and female choice will play a greater role.

I tested these hypotheses in a further study with populations of the European bitterling, *Rhodeus amarus*, historically separated and exposed to a different level of mussel availability for a different length of time (**Chapter 4**). Males from sites with low mussel availability were larger, more colourful and more aggressive than those from sites with high mussel availability and these differences were far higher in the populations with a long history of isolation. However, no reproductive isolation was detected in the populations tested: male and female mating behaviours did not change when they were paired with a mate from the same or from a different population. Furthermore, no difference was present in the survival rate of

embryos obtained by crossing males and females from different populations using *in vitro* fertilizations. Thus, resource availability plays a role in shaping adaptive intra-sexual behaviours, and the longer the period of isolation the stronger the pressure on characters used in male competition for mating resources.

Bitterling reach sexual maturity at 3-6 months of age, well in advance of the beginning of the spawning season. Thus, I tested whether a difference in mussel availability during ontogeny could modify male territoriality and aggressive behaviour (**Chapter 4**). The aggression rate of one-year old males captured before the start of the spawning season and exposed to different levels of mussel availability was comparable. The aggression rate of the juveniles tested in this study was not correlated with body size, which suggested a role for social learning. During the spawning season juveniles can copy adult fish mating behaviours and adopt a more economical defence of mating resources thereafter.

The environment embryos experience during development can exert an immediate effect on morphological traits, but can also influence mating behaviours and reproductive fitness later in life (Kienle *et al*. 2008; Jiménez- Cortés *et al*. 2012). I tested the role of density during embryogenesis on male mating behaviour in the rose bitterling, *Rhodeus ocellatus* (**Chapter 5**). Full siblings obtained with *in vitro* fertilizations were exposed to a high or low density treatment up to the free swimming stage and juveniles were tested for aggressive behaviour once they reached sexual maturity. At high densities, embryo survival rate was lower and movement and eye pigmentation appeared at a later date than in embryos raised at low densities. Conversely, growth rate and the time taken to reach the free swimming stage were comparable in both treatments. Embryos raised at high density, however, reached sexual maturity at an earlier age and, consequently, at a

smaller size but no difference was observed in male aggression rate. Reaching sexual maturity at an earlier age can confer a higher reproductive fitness to juveniles who can be able to fertilize eggs early in the spawning season by adopting alternative mating tactics. The effects of a high rearing density on morphological and physiological traits are felt, even if partially, up to sexual maturity but do not seem to play a role in influencing juvenile aggressive behaviour. The lack of correlation between aggression rate and body size is consistent with the results of the study performed with *Rhodeus amarus* juveniles (**Chapter 4**) and hints at a possible role of social learning in influencing male behaviour.

The role of red carotenoid-based coloration as an inter- or intra-sexual signal, its plasticity, and its relationship with circulating androgens is well documented in many animal taxa but the exact role of this signal and its plasticity have never been tested in bitterling. *R. ocellatus* males did not intensify red coloration in the presence of a female not ready to spawn, so excluding the effect of a conspecific on male signalling, but the extent of red increased in the presence of a potential rival or a potential mate (**Chapter 6**). The colour change both in the iris and on the tail fin was rapid after exposure to the stimulus, but it took longer to return to the original colour state after the stimuli were removed. In bitterling, female mate choice does not appear to be strongly influenced by male extent of red; thus, the signal may have evolved to be used in intra-sexual communication to threaten rivals and avoid overt contests. It may be maintained in spite of the lack of female preference for colour because courtship and aggression take place simultaneously and males are not able to modulate the change in a short period of time. Surprisingly, the presence of a mussel affected only the colour change on the

tail fin; the physiological mechanisms underlining this effect need further investigation.

In recent years experimental studies have demonstrated that males do choose mates or resources that females prefer. However, while female choice for mate and resource quality has been well documented in bitterling fishes, evidence for male choice is still scanty. I tested male mate choice between differently sized females and between different mussel species (**Chapter 7**). Focal males did not invest more energy in aggression towards rival males, in ejaculates, mussel inspection, or courtship when paired with larger females. In this study larger females did not spawn more or larger eggs, which can explain the lack of male choice (**Chapter 7**). The dependence of bitterling on mussels as spawning sites and for embryo development makes the possession and assessment of good quality resources essential for their reproductive fitness. *Rhodeus ocellatus* are generalists in mussel use (Reichard *et al*. 2007), but if mussel quality is comparable, females avoid spawning in *Anodonta anatina* and prefer to spawn in *Unio pictorum* (Casalini 2007). I tested male choice between *A. anatina* and *U. pictorum* first in the presence of constrained females, and then when the same females were free to inspect the mussels and interact with the males (**Chapter 7**). When females were constrained male inspection, courtship and leading rate were the same irrespective of mussel species; when females were free they showed a clear preference for *U. pictorum* and males showed concordant choice. Thus, female choice for spawning sites determines male choice and could lead to a higher aggression rate of males to defend territories around the mussels females prefer.

The general findings of my study on the mating system of male *Rhodeus ocellatus* and *Rhodeus amarus* can be summarised as follows:

- Aggressive behaviour has a large heritable component with a significant maternal additive effect.
- The availability of mating resources (mussels) plays a dominant role on adult male aggressive behaviour. The length of exposure to environments with different levels of resources influences the adaptive evolution of behaviour.
- The crowding of males around resources and the density of potential rivals increase male alternative tactics and the trade off between courtship and aggression.
- The exposure to a high number of mating resources during ontogenesis and to a high density of individuals during embryogenesis do not affect male aggressive behaviour.
- Male red carotenoid-based coloration is plastic and is used in intra- and inter-sexual contexts.
- Male do not choose larger females as mates and their choice for a mating resource is driven by female choice.

8.2. Future research

The genetic basis to male and female behaviours has been demonstrated in many animal taxa and aggressive behaviour, in particular, has been extensively studied in animals and men. In my research I demonstrated that, even if female bitterling are not overtly aggressive, male aggressiveness has a large genetic component with a significant maternal additive effect. Therefore research on the relevance of an additive maternal effect on the genetic component of male behaviours in different taxonomic groups would be especially rewarding.

A still unanswered question is the role of red carotenoid-based coloration as an intra- or inter-sexual signal. In this study I demonstrated that males increase the

extent of red in the iris and on the tail fin both in the presence of a potential mate and of a potential rival, and that the presence of a mating resource influences only the variation in colour on the tail fin. From previous research there is no evidence that female mate choice is influenced by the extent of red coloration, but the exact role of the extent of redness as an intra-sexual signal is still unclear. In some species of birds and fish coloration can be used by males to deter rivals from overt aggression and, therefore, to avoid the costs involved in escalating fights. Male bitterling extent of red ranges from no distinct coloration to an area of brilliant red covering a large portion of the iris and of the tail fin. Thus, further research could investigate whether the increase in this secondary sexual trait is modulated according to the perception of a rival's strength, and whether different physiological mechanisms play a role in the mobilization of carotenoids into eye or tail colour change.

A further question concerns male choice of mating resources. Females can base their mate choice on the quality of the territory or the resources males possess; consequently, the possession of good quality resources can increase male reproductive fitness. In this study I found evidence that females show a preference for specific mussel species and that males readily adapt their behaviour to comply with female choice. However, bitterling males invest time and energy in frequent inspections of the mussels present in their territory even when no rivals or females are present. Therefore, further studies should be addressed to test which aspects, if any, of mussel quality males are able to assess and whether they tune their aggressive territorial defence according to the perceived higher quality of the mating resource.

In my research performed on the European bitterling, *Rhodeus amarus*, I

have found evidence that allopatric populations isolated for long periods may evolve different mating behaviours as an adaptation to the environment. However, the same variation in aggressive behaviour, though with a lesser degree of differentiation, has been found also in populations isolated for a short period of time. In recent years the distribution of European bitterling has expanded, and they are now present in England and northern Italy, where they have been exposed to different environments for about fifty and twenty years respectively. Thus, further research might investigate behavioural plasticity within recently introduced populations.

Finally, as the presence of bitterling eggs and developing embryos on the gills of mussels significantly reduces mussel growth rate (Reichard *et al*. 2006), further research could investigate the potential threat to local fauna posed by bitterling expansion in new territories and, in particular, the potential fitness cost to the mussels bitterling use as oviposition sites.

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