A COMPARISON OF THE IMPACT OF DIRECT AND INDIRECT BENEFITS OF MATING DECISIONS IN THE ROSE BITTERLING (*RHODEUS OCELLATUS*)

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ABSTRACT

Research on sexual selection has tended to focus on indirect benefits of female mating decisions, and few attempts have been made to quantify the relative effect of direct and indirect selection simultaneously. Here we compared direct and indirect selection on female mating decisions in the rose bitterling (*Rhodeus ocellatus*), a fish with a resource-based mating system, using experimental treatments with equivalent consequences for female reproductive success. Direct selection was varied by manipulating the quality of sites available to females for oviposition, and indirect selection by presenting females with males of known genetic compatibility. Manipulating the strength of direct and indirect selection had specific, quantified consequences for embryo survival during incubation. There was a significant effect of both direct and indirect selection on female mating decisions, though direct selection accounted for more variance in female oviposition rate compared with indirect. No interaction between direct and indirect selection was detected. Although effects on female reproductive success were additive, selection for direct mate choice benefits appears to be stronger than for indirect benefits. A possible explanation for weaker selection on indirect benefits in the study species is because females are constrained in making mate choice decisions through alternative male mating tactics, which generate a sexual conflict.

Keywords: bitterling, direct selection, indirect selection, mate choice, oviposition, *Rhodeus ocellatus*, sexual selection

INTRODUCTION

Sexual selection is a powerful and pervasive mechanism of adaptive evolution. It arises through differences in the strength of selection acting on the sexes, either as a result of competition between members of the same sex, usually among males, or as a consequence of (usually) female mate choice. Sexual selection through female mating decisions can be attributed to what have been termed "direct" benefits. Direct benefits

of mate choice are those that promote female welfare or reproductive productivity. Examples of direct benefits include better access to food, assistance with parental care, access to oviposition sites, and protection from harassment or predation. In addition, and more controversially, selection may act on female preferences for "indirect" benefits, based on the genetic quality of potential mates (Kirkpatrick, 1982; Eberhard, 1996). "Good genes" (more correctly "good allele") models predict additive genetic benefits of female choice, while "compatible genes" ("compatible allele") models predict that genetic benefits are non-additive and only a particular combination of male and female haplotypes can generate fitness benefits in the offspring (Neff and Pitcher, 2005; Kempenaers, 2007). Male genetic quality can be considered as the total effects of good and compatible alleles.

Both direct and indirect benefits potentially shape mate choice decisions, and thereby mating system evolution (Neff and Pitcher, 2005). The direct benefits of mate choice to females are often taxon- or mating-system-specific. For example, in species with paternal or biparental care, selection on female mate choice decisions will be generated through variation in male parental investment (Kempenaers, 2007). In contrast, indirect benefits are wholly genetic and female mate choice decisions are selected through enhanced offspring viability and reproductive success (Andersson and Simmons, 2006). Because indirect selection presents the greatest theoretical challenges, it has been the focus of much research on sexual selection (Kotiaho and Puurtinen, 2007). However, while direct selection on mate choice has attracted less attention, its importance is no less significant. Indeed, there is surprisingly little compelling evidence for a significant role of indirect sexual selection on female fitness in many mating systems (Kotiaho and Puurtinen, 2007). Where indirect benefits have been properly quantified their magnitude has proven limited (for example, Qvarnström et al., 2006), and theoretical models suggest that the strength of indirect sexual selection on mate choice may be minor (Kirkpatrick and Barton, 1997).

Direct and indirect selection on female mate preference alleles are not mutually exclusive. If both operate simultaneously, then it is important to assess their relative magnitudes. A further uncertainty is whether the extent of their contribution to selection varies consistently among mating systems. Despite this uncertainty, few studies have compared the magnitude of direct and indirect selection, though a number have examined how direct and indirect benefits of mate choice are traded off against each other (Brooks and Endler, 2001; Calsbeek and Sinervo, 2003, Hadfield et al., 2006). The only systematic experimental study is that of Maklakov and Arnqvist (2009), who performed experimental manipulations on female seed beetles (*Callosobruchus chinensis*) to vary the extent to which they could resist male mating attempts. Their results showed that female mating preferences are maintained predominantly through direct rather than indirect selection, though the generality of these results is unclear. In this study, we measured the relative significance of direct and indirect selection on female mating preferences by experimentally manipulating the outcome of direct and indirect selection to have identical fitness consequences for females, then examined the relative strength

of female mating preferences. To do this we used the rose bitterling (*Rhodeus ocellatus*), a freshwater fish with a resource-based mating system.

Bitterling fishes use living freshwater mussels for oviposition (Wiepkema, 1961). Male *R. ocellatus* compete for territories around mussels and court females (Kanoh, 2000). Females have long ovipositors that they use to place one to eight eggs in the gill chamber of a mussel in one spawning act, and can spawn up to five clutches each day (Nagata, 1985). Males release sperm over the inhalant siphon of the mussel and fertilized eggs develop inside the mussel gill, where they are protected and ventilated. Bitterling emerge from mussels as independent larvae after approximately one month (Smith et al., 2004). The mortality rate of developing eggs and embryos in mussel gills varies with density, with eggs suffocating at high densities (Smith et al., 2000). Female oviposition decisions represent a key mating decision with direct fitness consequences for the female (Smith et al., 2000, 2001). Female bitterling are choosy about which mussels they use for oviposition and prefer mussels that do not already host eggs and embryos, where egg survival is higher (Smith et al., 2000). Dissolved oxygen concentration in the exhalant current is the probable cue used by females to assess mussel quality (Smith et al., 2001).

Female bitterling are also choosy about mates. Mate choice decisions are based on olfactory cues that identify male genetic compatibility (sensu Neff and Pitcher, 2005), such that females spawn preferentially with males with alleles complementary to their own. Female mate preferences are adaptive, with significant differences in embryo survival rates between embryos fathered by genetically compatible and incompatible males (Casalini et al., 2009, Agbali, 2011; Reichard et al., 2012). Agbali et al. (2010) used an NCII breeding design (Lynch and Walsh, 1998) to measure additive and non-additive genetic effects in *R. ocellatus*. Their results showed a significant interaction of male and female genotype on offspring survival during the period of incubation in mussels, with females demonstrating significant mate preferences corresponding with male genetic compatibility, which correlated with the degree of functional dissimilarity of the major histocompatibility complex (MHC) between males and females (Agbali et al., 2010). A further study that experimentally paired females of known genotype with MHC-dissimilar (compatible) and MHC-similar (incompatible) males confirmed this correlational result (Reichard et al., 2012), showing a significant female mating preference for genetically compatible males.

In bitterling, then, female oviposition decisions are acting on offspring survival under both direct and indirect selection, through mussel quality and male genetic compatibility, respectively. The aim of this study was to contrast the relative role of direct and indirect selection on female oviposition decisions, measured using a common currency: offspring survival to independence. Direct benefits were manipulated using oviposition sites of known quality as sites for incubation, while indirect benefits were manipulated using males of known genetic compatibility with experimental females. We manipulated direct and indirect benefits to yield identical fitness consequences for females (measured by offspring survival to independence).

METHODS

EXPERIMENTAL CONDITIONS

Experimental *R. ocellatus* were the first generation of a large outbred lab population. Experimental aquaria were maintained on a recirculating system at 23 ºC and exposed to 16:8 h light/dark regime. Fish were fed twice daily a mixture of commercial flake food and bloodworm (*Chironomus* spp.) supplemented with live zooplankton. Freshwater mussels used in trials were *Unio pictorum*. This mussel occurs across Eurasia and is readily used by *R. ocellatus* as a spawning site (Casalini, 2007). Mussels were collected from the River Cam and kept in a 100 L stock tank, and fed live phytoplankton.

QUANTIFYING DIRECT AND INDIRECT BENEFITS OF FEMALE MATING DECISIONS

Direct and indirect selection on female mating decisions was varied at two levels; high and low. Indirect selection was controlled by presenting females with males of known genetic compatibility, previously estimated in a study by Agbali et al. (2010). Individually marked females were paired with two males with which they had previously been crossed, and for which offspring survival rates were known. Of the pair of males, one was genetically compatible and known to be preferred by the female. Crosses with preferred males were associated with a mean ± s.e. offspring mortality for the period of incubation of 28 ± 3.9%. The other male was genetically incompatible and generated a mean ± s.e. offspring mortality rate of 74 ± 5.7%.

Direct selection was manipulated by presenting females with mussel spawning sites that differed in quality, again with predictable effects on egg survival during incubation. Mussel quality was varied by controlling the number of eggs laid in mussels to generate mortalities equivalent to those of mating with compatible and incompatible males. Smith et al. (2000) derived a function for the mortality rate of bitterling embryos in different species of mussel. For *U. pictorum*, the function takes the form: $Z = 0.035n - 0.049$, where Z is the instantaneous daily mortality rate and n is $\log_e$ embryo density. Embryo mortalities in *R. ocellatus* occur up to development stage 4 (Kitamura, 2005), corresponding to an age of two weeks at 23 °C (Suzuki and Jeon, 1988). On the basis of this mortality relationship, eggs deposited in a mussel containing 80 eggs were predicted to suffer a mortality rate of 76% over 2 weeks, generating selection on females approximately equivalent to mating with a genetically incompatible male.

A high-quality direct benefit for a female was predicted to come from using a mussel that had previously received no eggs. Thus, the instantaneous mortality rate predicted for eggs laid in an unused mussel was that for a single egg clutch only. The mean ± s.e. clutch size for *R. ocellatus* is 4.4 ± 1.3 eggs per spawning, with a range from 1–8 eggs (Reichard et al., 2007a). Therefore we assumed the maximum mortality rate for a female spawning in a mussel without eggs would match the rate generated by a single clutch of maximum size (8 eggs). Over a two-week period 8 eggs were predicted, on the basis of the egg mortality function of Smith et al. (2000), to suffer a 28% mortality rate, identical to that of mating with a compatible male.

To manipulate mussel quality, experimental mussels were haphazardly selected from

the stock tank and randomly assigned to either low- or high-quality treatment groups. Those assigned to the low-quality treatment were exposed to groups of *R. ocellatus* that were allowed to use the mussels for spawning until the mussels had accumulated approximately 80 eggs. In some cases slightly more than 80 eggs were deposited in mussels, though this had only a marginal effect on mortality rate (100 eggs would generate a mortality rate of 78%, rather than 76% with 80 eggs). Under natural conditions mussels can receive over 250 bitterling eggs (Smith et al., 2004), so the low- and high-quality experimental treatments were within the natural range for mussels and bitterling. Egg numbers were confirmed by using a mussel-opening device (Kitamura, 2005) to gently open the valves of the mussel so that the eggs could be counted. Mussels assigned to the high-quality mussel group received no spawnings, but were housed in aquaria with identical water quality and received the same degree of handling as those in the low-quality treatment.

Thus, using data derived from a study of embryo mortality rates at both different densities during incubation, from Smith et al. (2000), and with genetically compatible and incompatible mates, from Agbali et al. (2011), it was possible to predict a priori that weak direct selection (high mussel quality) would result in an offspring mortality rate of 28% and strong direct selection (low mussel quality), of 76%. Weak indirect selection (male genetic compatibility) was predicted to impose an offspring mortality rate of 28%, and strong indirect selection (male incompatibility), a rate of 74%. There was no significant difference between strong direct and indirect selection ($t_{10} = 0.52$, $p = 0.614$) or weak direct and indirect selection ($t_{10} = 0.22$, $p = 0.830$). In contrast, there were highly significant differences between estimated strong and weak direct ($t_{10} = 6.39$, $p < 0.001$) and indirect selection ($t_{10} = 8.11$, $p < 0.001$).

MEASURING FEMALE MATING DECISIONS

Female mating decisions were tested using an isolated sequential mating design. Two males were randomly assigned to each female; one genetically compatible and the other incompatible. Allocation of compatible and incompatible males to females was randomized, with no fish used more than once in trials. Males were housed singly in aquaria measuring 25 (length) × 40 (width) × 30 (depth) cm with a sand substrate and two artificial plants. Opaque barriers prevented visual contact between adjacent fish. High- or low-quality mussels were randomly assigned to the two males, which were able to inspect the mussel and establish territoriality around it. The female was paired with each male in random order for one hour and allowed to spawn. A mussel-opening device was used to gently open the mussel, and any eggs that had been spawned were counted. Recently deposited eggs could be distinguished from older eggs by their stage of development. After one hour mussels were exchanged, with high-quality mussels substituted for low-quality mussels and vice versa. The mate choice trial was repeated so that each female was paired with a compatible and incompatible male with a high and low-quality mussel. After completion of a trial, mussel length was measured. There was no difference in mussel length between high and low mussel quality treatments (paired t-test $t_{10} = 1.40$, $p = 0.190$).

DATA ANALYSIS

A total of 44 trials in 11 independent replicates were completed, using a total of 11 fe-males, 22 males, and 22 mussels. A generalized linear model (GLM) with quasi-Poisson distribution for two within-subject factors was used to compare the number of eggs laid by females with respect to direct and indirect selection and their interaction. A logistic regression was also used to test the probability of a female spawning in response to direct and indirect selection.

RESULTS

There was no significant two-way direct × indirect selection effect on the number of eggs laid by females (Table 1). However, there was a highly significant effect of direct selection on oviposition (Table 1), with females spawning significantly more eggs in high-than low-quality mussels (Fig. 1). There was also a significant effect of indirect selection (Table 1); females spawned more eggs with genetically compatible than incompatible males (Fig. 1). A logistic regression model of the probability of a female spawning with a male returned a significant term for direct effects only ($\chi^2 = 6.01$, d.f. $= 1$, $p = 0.014$).

DISCUSSION

Explicit empirical comparisons of direct and indirect selection are rare, but essential for understanding the relative role of each in mating system evolution (Kotiaho and Puurtinen, 2007; Maklakov and Arnqvist, 2009). Our results showed a significant effect of both variables, though direct selection explained almost three times more variance in oviposition rates than indirect selection. Notably, no interaction between direct and indirect selection was detected. This result implies that direct and indirect selection acted independently on female oviposition decisions (additive effects). Thus, female assessment of male quality, for example, is predicted to be unaffected by the quality of the resources that a male controls. These findings support Kotiaho and Puurtinen (2007)

Table 1

Analysis of variance with two within-subject factors (direct and indirect selection) on the oviposition rate of female rose bitterling, *Rhodeus ocellatus*

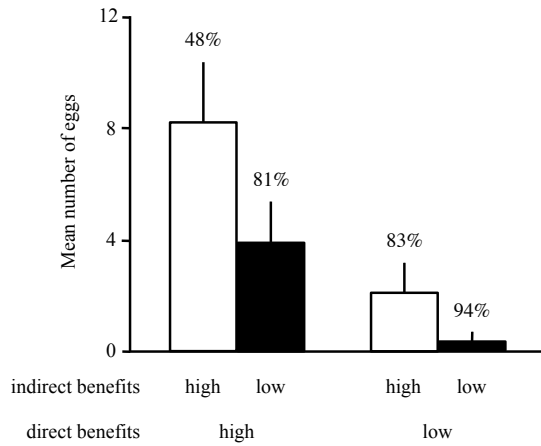| Source of variation	SS	d.f.	MS	F	p
subjects	27.50	10			
within subjects	27.34	3			
direct benefits	19.91	1	19.91	14.42	<0.001
indirect benefits	7.02	1	7.02	5.09	0.032
direct × indirect	0.41	1	0.41	0.30	0.589
residual	41.42	30	1.38		

Fig. 1. Effect of direct and indirect selection on female oviposition decisions in the rose bitterling, Rhodeus ocellatus. Error bars are one standard error. Numbers above bars are estimated embryo mortality rates for each treatment.

who argued that direct selection is at least as significant as indirect selection, and corroborates the few empirical (Maklakov and Arnqvist, 2009) and theoretical (Kirkpatrick and Barton, 1997; Cameron et al., 2003) studies that have simultaneously compared direct and indirect selection on female mating decisions.

Although the experimental design used here enabled a comparison between direct and indirect selection on female oviposition decisions that affected offspring mortality, other aspects of offspring fitness may also play a role in female mating decisions. Conditions during sensitive periods of embryo development can have a pronounced effect on the adult owing to epigenetic effects, either by irreversible disruption to development or through inducing alternative adult phenotypes (Gluckman et al., 2005). Another outcome is "coping" whereby challenges to development are compensated in the short-term, but with a longer-term fitness cost in the adult (Gluckman and Hanson, 2004). In bitterling, embryos that develop at high densities do so at a significantly slower rate than those at low density, though whether this effect has implications for offspring fitness is unclear (Agbali, 2011).

For offspring sired by incompatible males, there appear to be no costs in addition to the increased offspring mortality rate associated with mating with such males. Agbali et al. (2010) raised the surviving offspring of compatible and incompatible males to maturity. The reproductive success of males from each group was compared in pairwise mating contests, while females were compared in body size, a reliable predictor of female lifetime reproductive success (Reichard et al., 2009). In neither sex were the surviving offspring of compatible mates superior to those of incompatible (Agbali et al., 2010). Thus, in bitterling at least, indirect selection had no long-term fitness consequences for

female oviposition decisions once young achieved independence.

An assumption of the study is that female bitterling can discriminate proximate cues associated with direct and indirect selection with equal precision. For neither mussel nor male quality have the cues used by females been unequivocally identified, though in the former case it appears to be the dissolved oxygen concentration of water flowing from the mussel gill (Smith et al., 2001). In the latter, genetic compatibility appears to be linked to genetic distance between parents, possibly linked to variability in the MHC gene complex (Agbali et al., 2010; Reichard et al., 2012). The relatively weaker effect of indirect selection on oviposition choice in the present study may hinge on the accuracy with which females are able to discriminate compatible and incompatible males. The cues used by females in mate choice are probably olfactory (Casalini et al., 2009; Agbali, 2011). Limitations on females in their ability to discriminate these olfactory cues may constrain the strength of indirect selection, though the evidence suggests that female discrimination of males is consistent and accurately reflects offspring survival (Agbali et al., 2010).

In comparing direct and indirect selection, an implicit assumption is that females possess the capacity to exercise choice over each to an equivalent extent. This assumption may not always be met, and could alter the relative strength of selection. In bitterling, choice of a mussel for oviposition is not susceptible to interference; if a female chooses to oviposit in a mussel she will not be prevented from doing so. In contrast, the high frequency of sneaky matings in bitterling (Kanoh, 2000; Casalini et al., 2009) can mean that female choice of compatible males can be usurped by sneaking incompatible males, which carries a substantial fitness cost for the female. The frequency of sneaking rates are not trivial, and in over half of all natural spawnings in a population of European bitterling (*Rhodeus amarus*) more than one male was observed to participate (Smith et al., 2009). Sneaking rates in *R. ocellatus* appear comparable (Kanoh, 2000). Moreover, males tend to monopolize high-quality oviposition sites and may limit female ability to express choice for direct and indirect benefits simultaneously (Reichard et al., 2005; Casalini et al., 2009). Thus, although in this study the outcome of direct and indirect selection on female oviposition decisions were equivalent, there is good reason to believe that the strength of indirect selection for female preference alleles would be eroded in nature due to male mating behavior undermining female mate choice decisions (Reichard et al., 2007b).

In summary, this study demonstrates a primary role for direct selection, in the form of choice of oviposition site, in female oviposition decisions. Indirect selection through mate choice had a significant effect, but accounted for less variance in female oviposition rate under the experimental design we used. The strength of indirect selection may be constrained through a sexual conflict between female mate choice decisions and male alternative mating tactics.

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