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MATE CHOICE FOR NONADDITIVE GENETIC BENEFITS CORRELATE WITH MHC DISSIMILARITY IN THE ROSE BITTERLING (*RHODEUS OCELLATUS*)

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

KEY WORDS: **Additive genetic benefit, female mate choice, genetic compatibility, good genes, mate choice, MHC, nonadditive genetic benefit, olfactory cues, *Rhodeus ocellatus*, sexual selection.**

Females may obtain a fitness benefit under sexual selection by choosing to mate with a male who provides direct, nongenetic, benefits, such as protection from predators or harassment by other males, access to resources or nuptial gifts, or parental care of offspring (Andersson 1994; Qvarnström and Forsgren 1998; Andersson and Simmons 2006). Female mate preference may also be selected through indirect benefits, based on the genetic quality of potential mates (Kirkpatrick 1982; Eberhard 1996). Genetic quality can be considered as the combined effects of good and compatible genes (Neff and Pitcher 2005). In good genes models of mate choice, a male with high genetic quality is predicted to confer a fitness advantage on offspring, with male beneficial alleles acting independently of maternal genotype. Consequently, good genes are predicted to show additive genetic variation (Wedekind et al. 2001; Neff and Pitcher 2005). In populations in which mate choice is driven by good genes, female mating decisions will tend to be congruent, and selection will be directional. Good genes effects are associated with signals of male quality, typically in the form of ornamentation or display (Maynard Smith and Harper 2003). In contrast, compatible genes

are predicted to impart enhanced fitness to offspring only when particular male and female genotypes are combined (Zeh and Zeh 1996, 1997; Penn 2002), thus compatible genes are predicted to show nonadditive variation (Neff and Pitcher 2005; Piálek and Albrecht 2005). In populations in which compatible gene effects operate, females will not necessarily share mate preferences, and selection will not be strongly directional (Tregenza and Wedell 2000; Neff and Pitcher 2005; Ivy 2007). The genetic basis to compatibility is poorly understood, but the process requires an individual to recognize its own genotype as well as those of potential mates, while also allowing for the impact of recombination, asymmetric inheritance patterns, and sex-specific effects (Charlesworth et al. 1987).

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

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

A mate choice system mediated by MHC genes that generates additive genetic benefits could arise through negative frequency-dependent selection (Clarke and Kirby 1966). Here, the prediction is that rare MHC sequence variants confer an advantage on their carrier, although this advantage may eventually be lost through the coevolution of pathogens. An outcome of this model of mate choice is a turnover of advantageous genetic variants in the form of an evolutionary arms race (van Valen 1973). In contrast, a mechanism of mate choice that would confer nonadditive benefits is through heterozygote advantage (Penn 2002). Here, high numbers of MHC sequence variants carried by an individual are predicted to maximize the probability of mounting immune responses against a large number of foreign peptides, and thereby enable an individual to resist a broader range of pathogens. A constraint on maximal MHC diversity may arise through negative T-cell selection during thymic development (Nowak et al. 1992), which would tend to favor an optimum rather than maximum MHC sequence variant diversity (Kalbe et al. 2009). These two models of mate choice evolution are not mutually exclusive.

In the present study, the basis and mechanism of female mate preference was experimentally tested in the rose bitterling (*Rhodeus ocellatus*), a small fish with a resource-based mating system. A sequential blocked mating design was used to test female mate preferences, and a North Carolina Type II breeding design (Lynch and Walsh 1998), utilizing in vitro fertilizations (IVFs) to generate crosses to test for additive and nonadditive genetic benefits of mate choice. The offspring produced by the pairing of preferred and nonpreferred males were reared to maturity and their fitness traits were compared. In addition, rose bitterling MHC genes were analyzed and experimental fish genotyped. Four predictions were tested: (1) Offspring sired by preferred males show enhanced fitness in comparison with nonpreferred males, with the genetic benefits of mate choice either additive or nonadditive. (2) Females show significant mate preferences, which are either congruent among females, suggesting additive genetic effects, or divergent, indicating nonadditive effects. (3) Female mate preferences are based on MHC genotype, with females showing a preference for mating with males that are MHC dissimilar to maximize MHC diversity in the offspring if mate choice is for nonadditive benefits, or a preference for males possessing specific MHC alleles in case of additive benefits. (4) Male offspring that are the products of preferred pairings should have higher mating success than those from nonpreferred pairings in the case of additive benefits, but not if benefits are nonadditive.

Materials and Methods

THE STUDY SYSTEM

Bitterling (Cyprinidae, Acheilognathinae) lay their eggs in the gills of living freshwater unionid mussels, in which their eggs and embryos are incubated for approximately 1 month. During the breeding season, males develop carotenoid-based nuptial coloration, most notably in the iris and on the tail fin, and compete for territories around mussels. Female bitterling develop a long ovipositor that they use to deposit their eggs inside the gills of the mussel through its exhalant siphon. Males vigorously court females and attempt to lead them to mussels in their territories. Both intra- and intersexual selection play a role in the mating system (Kanoh 2000; Candolin and Reynolds 2001; Smith et al. 2002, 2003; Reichard et al. 2005, 2008; Casalini et al. 2009). Female oviposition decisions are based on both male and mussel quality (Smith et al. 2000; Candolin and Reynolds 2001; Smith and

Reichard 2005; Kitamura 2006; Casalini 2007; Kitamura 2007; Casalini et al. 2009). Females spawn in several bouts lasting one day and consisting of approximately 5–10 independent spawnings (Nagata 1985; Smith et al. 2004a). Females deposit one to five (typically three) eggs in the mussel gill chamber. Territorial males release sperm over the inhalant siphon of the mussel so that sperm drawn into the gills fertilizes the eggs. Sneaking behavior, in which a rival male (an adjacent territory holder or a male that does not possess a territory) releases his sperm into a rival's mussel, is common in bitterling (Kanoh 1996, 2000; Smith et al. 2002, 2003; Reichard et al. 2004, 2008, 2009; Smith et al. 2009). Male mating behavior is largely opportunistic and there is no evidence for a morphological or genetic distinction between territorial and sneaking males (Kanoh 2000; Pateman-Jones 2008). Females appear able to undermine male dominance, to some extent at least, by soliciting sneakers, delaying spawning, and performing a spawning action but without depositing eggs (Smith and Reichard 2005; Smith et al. 2007). For further details on bitterling reproductive biology see Smith et al. (2004a).

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

FEMALE MATE CHOICE

Using the isolated sequential mating design of Spence and Smith (2006), four marked males were individually assigned to four aquaria measuring 25 (length) × 40 (width) × 30 (depth) cm in a random order with one *U. pictorum* mussel that did not already contain bitterling eggs. This arrangement was replicated in four independent blocks. This design permits female choice to be measured in the absence of male dominance, which conflicts with female mate choice decisions in rose bitterling (Casalini et al. 2009). Opaque barriers prevented visual contact between adjacent fish. Males were left alone overnight to establish territoriality and the following morning marked females in spawning condition (with a fully extended ovipositor) were randomly paired with each male for 1 h. A mussel-opening device (Kitamura 2005) was used to nondestructively prise the valves of mussels apart and inspect the gills for eggs that had been deposited during pairing. The number of clutches laid was estimated by dividing the total number of eggs by a mean clutch size of three (Nagata 1985; Smith et al. 2004a). Females were moved to a second aquarium within blocks with a different male according to a predetermined randomized order. This process was repeated until each female had been paired once with each of the four males in each of four independent blocks. Thus, a total of 16 males and females was used in 64 independent pairings. The same mussel was used with the same female to remove any effect of oviposition site quality on female spawning preference. Although the presence of eggs in the gills of a mussel can affect female spawning decisions (Smith et al. 2000), the eggs already deposited by a single female were not expected to influence her subsequent spawning decisions, because a mussel can host over 250 eggs in its gills; considerably more than the 30 or so that one female can deposit in a single day (Smith et al. 2001, 2004a; Kitamura 2005, 2007). Female mate preference was measured as the number of eggs laid with individual males within blocks (Spence and Smith 2006); that is, that females showed a preference for particular males by mating more frequently with them, a standard practice for measuring mate choice (Wagner 1998; Smith and Reichard 2005).

After completion of trials the body length of every adult fish was measured from the tip of the snout to the base of tail fin and a small amount of fin tissue removed for MHC typing. The right and left eye of males and red caudal fin spot were photographed under standard light conditions. The red area in the iris of each male eye was analyzed using Photoshop Elements 2.0 following the protocol of Barber et al. (2000). The extent and brightness of the red caudal fin spot were measured using the ordinal scale of Casalini et al. (2009).

IN VITRO FERTILIZATIONS

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

To generate crosses, experimental females were isolated until they ovulated a batch of eggs; obvious from the female's extended ovipositor. The eggs were gently squeezed from the female and divided into approximately four equal groups in separate 70-mm-diameter Petri dishes containing freshwater (mean = 8.0 ± 3.10 SD eggs per group). Sperm was stripped from the four experimental males by gently pressing their abdomens and mixed in 9 mL of teleost saline (Yokoi et al. 2008). A 1-mL subsample of this sperm solution was diluted with a further 9 mL of saline. The concentration of sperm in each diluted suspension was quantified by performing a count of individual spermatozoa. The sample was gently mixed and a subsample pipetted onto a haemocytometer (Neubauer improved, VWR International, Vienna, Austria). A count was made of the number of spermatozoa in the sample using a binocular microscope (Nikon Eclipse E200; Nikon, Tokyo, Japan) with a 40× objective. Counts were made of sperm cells in five 1 × 1 × 0.1 mm squares to obtain an estimate of mean sperm density. Sperm suspensions were pipetted over the eggs and the covered petri dishes were left on the laboratory bench for 30 min. The fertilized eggs were washed with freshwater and incubated at 23°C in an environmentally controlled room until the yolk sac was absorbed and the larvae began exogenous feeding, a period of approximately 30 days. Each family of developing embryos was photographed alongside a scale bar under standard light conditions every day during development using a Canon EOS 300D camera with 60-mm macro lens (Canon, Tokyo, Japan). A daily record was also kept of embryo survival. Egg, embryo and larval sizes were estimated from digital images, as was the rate at which embryos reached the key stage of developing pigmented eyes (Kim and Park 1985).

OFFSPRING FITNESS TRAITS

Free-swimming and exogenously feeding juveniles were retained as separate family groups and raised to maturity in aquaria measuring 35 (length) × 25 (width) × 20 (depth) cm on a recirculating system at 23°C. They were fed twice daily to satiation with a mixture of dried fish flake food and live brine shrimp (*Artemia salina*). A record was kept of the age and size at maturity of all individuals of both sexes. Sexual maturity was attained after a mean of 130 (±20.0 SD) days and at a mean length of 27 (±3.1 SD) mm. In females, maturity was recognized by the first extension of the ovipositor, and in males by the development of red pigmentation in the iris. In addition, male dominance and reproductive success was tested. In bitterling, female body size correlates strongly with lifetime reproductive success (Reichard et al. 2008, 2009), whereas male lifetime reproductive success is determined by dominance and mating success (Reichard et al. 2008, 2009; Casalini et al. 2009). Only nine groups of offspring derived from the same female, but fathered by a preferred and nonpreferred male and containing at least two male offspring, survived to sexual maturity; a total of 71 individuals. To measure male dominance and reproductive success, size-matched male offspring from each of these groups were paired together once both reached sexual maturity. Distinguishing features of each male were noted to enable them to be individually identified and pairs were placed together in isolated experimental aquaria measuring 60 (length) × 40 (width) × 40 (depth) cm. Pairs were provided with a single *U. pictorum* and allowed to settle overnight. The following morning an unrelated female in spawning condition was released in the test aquarium and male behavior (establishment of dominance, rate of aggression, rate of courtship, and rate of leading female to the mussel) (see Smith et al. 2004a for details) was recorded for 20 min. After the female completed spawning, the mussel was dissected and the eggs were incubated in a Petri dish for five days. After this time, the embryos were fixed in ethanol for parentage analysis. A total of 153 embryos were fixed for genotyping. The procedure was repeated with the same pair of males with a second female. After completion of trials, the right and left eye and caudal fin spot of males were photographed under standard light conditions. The red area in the iris of each male eye and the extent and brightness of the caudal fin spot were analyzed using the same methods for the mate choice experiment. A small portion of the tail fin of all experimental fish was removed and fixed in ethanol to assign parentage.

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

MHC TYPING AND ANALYSIS

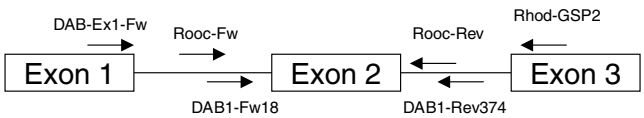
Analysis was focused on MHC Class II, which is known to be associated with mate choice in many vertebrate species. In most cyprinid fish, there is at least one functional gene (named DAB) encoding the MHC class IIβ chain of the protein (Sambrook et al. 2005). This gene can be duplicated, resulting in the expression of

DAB1 and DAB3 genes (e.g., Ottová et al. 2005). We sequenced the complete exon 2 encoding the β1 domain, which is the most polymorphic fragment of MHC Class II molecules responsible for antigen binding. Because of the highly polymorphic nature of MHC genes there is often a problem with null alleles; sequence variants that are not amplified because of substitutions in primer sites. To overcome this problem, for all individuals, the DAB gene was amplified using three combinations of primers (Fig. S1) located in various introns and exons. The primers were designed on the basis of homology with known DAB1 sequences downloaded from GenBank and all combinations amplified the fragment of genomic DNA that included the complete exon 2. Polymerase chain reactions (PCRs) were performed in the following conditions: 3 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM dNTPs, 0.5 U Taq polymerase (Fermentas) in appropriate 1 × PCR buffer and 1 μl of extracted DNA. Deionized water was added to a 10-μl reaction volume. The amplification consisted of an initial denaturation at 94°C (2 min) followed by 35 cycles of denaturation at 94°C (20 s), annealing at 56°C or 59°C (Fig. 1) (30 s), and extension at 72°C (1 min), with a final extension step at 72°C (10 min). The reactions were run on a Mastercycler ep (Eppendorf, Hauppauge, NY).

All PCR products were purified by ExoSAP-IT (USB Affymetrix, Santa Clara, CA) and directly sequenced using the BigDye Terminators Sequencing Kit version 1.1 and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Homozygous sequences had no double peaks in electrophoretograms and 10 of 17 alleles (see Results) were confirmed by independent PCRs using at least two different sets of primers. Heterozygotes showed double peaks in variable sites and had to be cloned to separate individual alleles. Hence, the PCR products were purified using the MinElute PCR purification kit (Qiagen, Valencia, CA), ligated to the vector and transformed to bacteria using the GeneJET™ PCR Cloning Kit (Fermentas, Burlington, ON, Canada) and JM109 Competent Cells (Promega, Madison, WI) according to the manufacturer's protocols. Positive transformants containing inserts of appropriate length were identified by PCR screening using primers on the vector and agarose gel electrophoresis. We randomly chose eight clones, amplified the inserts with specific primers and sequenced the PCR products as described above. The presence of PCR artifacts in cloned sequences (either substitutions or PCR recombinations; Bryja et al. 2005) was checked by comparison with the heterozygous sequence obtained directly from genomic DNA.

To avoid the possibility of analyzing pseudogenes, which can be common in MHC Class II in fish (Sambrook et al. 2005), we compared the genotypes of the DAB gene from six individuals obtained from complementary DNA (cDNA) and genomic DNA (gDNA). Total RNA was extracted from the spleen, stored in RNA later (Qiagen) by RNeasy Plus Mini Kit (Qiagen) and cDNA was prepared by reverse transcription of 5 μg of total RNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers as primers (Roche, Indianapolis, IN). Subsequently, we amplified exon 2 using primer combination III (Fig. 1), sequenced purified PCR products, and compared them with sequences obtained from genomic DNA.

The sequences of exon 2 of the DAB gene derived from *R. ocellatus* were edited and aligned in SeqScape version 2.5 (Applied Biosystems). To determine whether all sequences represented functional classical Class II alleles, the sequences were examined by eye for the presence of insertions or deletions (indels) causing a shift of the reading frame and/or stop codons. The sites encoding the peptide-binding region (PBR) of functional MHC Class II are known to be under positive selection; that is, the number of nonsynonymous substitutions exceeds the number of synonymous substitutions. The presence of selection on specific codons was analyzed separately for each allele



Primer combination	Annealing temperature	Primer name	Primer sequence (5´- 3´)
I.	56°C	Rooc-Fw	CCCATA GTTGACATGATGTCATCTG
		Rooc-Rev	CAT GTGTGACAGGAGGATCAG
II.	59°C	DAB1-Rev374	CAAGAGTTTCCCGTGTGACAG
		DAB1-Fw18	AGTTGACATGATGCCATCTGA
III.	56°C	DAB-Ex1-Fw	CATCYC ATACTGATGCTGTCTGC
		Rhod-GSP2	CCAGTCTCCGTTA GGCATCTCC

Figure 1. Schematic representation of the structure of the DAB1 gene and the positions and names of three combinations of primers used.

using the random effects likelihood (REL) approach (Kosakovsky Pond and Frost 2005). This method involves fitting a distribution of substitution rates across sites with inference of the rate at which individual sites evolve. Inference of selection was made using an empirical Bayes approach. REL analysis implemented in the HyPhy software package (Kosakovsky Pond et al. 2005) and a web-based interface running on a cluster of computers at http://www.datamonkey.org was employed (Kosakovsky Pond and Frost 2005). As recommended by these authors, positively selected sites were considered first with a Bayes factor value of >50. Because this strict identification of positively selected sites revealed only 10 amino acid sites under positive selection (hereafter referred to as strongly positively selected sites), we also used less-stringent conditions for identification of positively selected sites defined as a Bayes factor of >30 (referred to as positively selected sites with a total of 24 amino acid sites under selection) (see also Schwensow et al. 2008).

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

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

STATISTICAL ANALYSIS

All data were tested for normality using a Kolmogorov–Smirnov test and for equality of variance using Bartlett's test. Data that did not meet assumptions of normality and homoscedasticity were transformed. To demonstrate female mate choice the distribution of spawnings among male–female pairings was tested for significant deviation from a Poisson distribution. Congruence in female mate choice decisions was examined using a one-way block analysis of variance (ANOVA) with a significant male effect within blocks indicating congruent female choice. A one-way block ANOVA was also used to test for an effect of order of pairing on the number of eggs spawned by females.

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

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

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

Dissimilarity values were tested between preferred and nonpreferred partners using a *t*-test. An association between parental MHC dissimilarity and offspring survival was tested using a Spearman correlation.

Results

FEMALE MATE CHOICE

Females spawned a total of 233 eggs, distributed among an estimated 78 spawnings (assuming a mean of three eggs per spawning). At least one spawning occurred in 18 of 64 separate pairings. The distribution of spawnings among males deviated significantly from a Poisson distribution (chi-square test, $\chi^2 = 53.2$, df $= 3$, $P < 0.001$); a significantly greater proportion of pairings than expected did not result in a spawning, with a greater than expected proportion of eggs being concentrated on a limited number of males. However, there was no significant male effect on number of eggs received by males within experimental blocks (one-way blocked ANOVA, $F_{12,48} = 1.50$, $P = 0.156$), indicating that female mate preferences were not congruent within blocks. There was no significant effect of the temporal order of pairings within a day on the number of eggs spawned by females (one-way blocked ANOVA, $F_{12,48} = 0.72$, $P = 0.726$).

IN VITRO FERTILIZATIONS

There was no significant correlation between sperm concentration and the fertilization success of IVFs (Spearman correlation, $r_{126} = -0.115$, $P = 0.195$). There was a significant effect of egg size on offspring survival and growth and this variable has been used as a covariate in analyses (Tables 1 and 2). The density of embryos during rearing was not significant for any measured offspring parameters.

There was no significant male effect on offspring survival, growth, or development (Tables 1 and 3); variance attributable to

Table 1. ANCOVAs for fertilization success and offspring performance variables for in vitro fertilizations. Mean and standard deviation of variables are in parentheses.

Source	df	SS	MS	*F*	*P*	Variance	%
Proportion of eggs fertilized (0.98±0.059)							
Egg size	1	0.880	0.880	109.10	<0.001	0.44	91.0
Male (M)	4	0.069	0.017	0.223	0.912	0	0
Female (F)	4	0.557	0.142	1.820	0.288	0	0.4
M×F	4	0.311	0.078	9.641	<0.001	0.03	7.3
Error	64	0.516	0.008	8.162	0.033	0.01	1.7
Proportion of offspring surviving after 72 h (0.90±0.208)							
Egg size	1	849.0	849.0	20.2	<0.001	403.45	67.1
Male (M)	4	264.1	66.0	0.2	0.937	0	0
Female (F)	4	1943.1	485.8	1.3	0.393	3.80	0.6
M×F	4	1456.2	364.1	8.6	<0.001	160.98	26.8
Error	64	2694.0	42.1	4.7	0.040	42.09	7.0
Proportion of offspring surviving after 1 week (0.88±0.211)							
Egg size	1	913.2	913.2	19.2	<0.001	432.82	66.9
Male (M)	4	293.8	73.5	0.1	0.954	0	0
Female (F)	4	2539.6	634.9	1.3	0.407	4.41	0.7
M×F	4	1975.3	493.8	10.4	<0.001	223.14	34.5
Error	64	3042.1	47.5	8.6	0.030	47.53	7.3
Proportion of offspring surviving after 2 weeks (0.82±0.266)							
Egg size	1	13291.1	13291	14.07	<0.001	6173.3	64.2
Male (M)	4	6894.3	1724	0.30	0.865	0	0
Female (F)	4	50853.9	12713	2.21	0.231	217.3	2.3
M×F	4	23035.1	5759	6.10	<0.001	2407.2	25.0
Error	64	60443.8	944	7.38	0.039	944.4	9.8
Proportion of offspring surviving after 4 weeks (0.42±0.369)							
Egg size	1	4679.1	4679	5.70	0.020	1929.0	28.5
Male (M)	4	19710.2	4928	0.56	0.708	0	0
Female (F)	4	32808.4	8202	0.93	0.529	0	0
M×F	4	35406.7	8852	10.78	<0.001	4015.4	59.4
Error	64	52543	821	1.66	0.317	820.98	12.1

Table 2. ANCOVAs for offspring growth and development variables for in vitro fertilizations. Mean and standard deviation of variables are in parentheses.

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

males was typically zero or negative (and hence set to zero). Maternal effects, excepting egg size, were negligible (Tables 1 and 2). However, for all measured offspring variables there was a highly significant male × female interaction. On average, parental interaction effects explained 31% of variance in fertilization success and survival (Table 1), and 37% of growth and development effects, with the biggest impact on survival at 4 weeks (59%) when larval bitterling depart their mussel host and begin exogenous feeding.

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

OFFSPRING FITNESS TRAITS

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

Table 3. **Dissimilarity of amino acids coded by exon 2 of MHC DAB1 between preferred and nonpreferred partners in *R. ocellatus*, measured as phylogenetic and functional distances between amino acids. Distance matrices were derived for strongly positively selected sites (Bayes factor value>50; 10 amino acids), positively selected sites (Bayes factor value >30; 24 amino acids), and all amino acids (92 amino acids). Estimates of effect size (r_{ES}) are given in addition to conventional statistical results. Values of r_{ES}>0.371, 0.243, and 0.100 are considered to represent large, medium, and small effects, respectively.**

	t-value	P	r_{ES}	Preferred			Nonpreferred		
				mean	SD	n	mean	SD	n
Functional distances									
Strongly positively selected sites									
Sum of distances	1.94	0.057	0.262	37.5	20.2	18	26.4	20.8	46
Maximum distances	1.33	0.188	0.197	15.6	4.3	18	13.4	6.6	46
Positively selected sites									
Sum of distances	2.00	**0.049**	0.270	35.8	19.2	18	24.8	19.9	46
Maximum distances	1.44	0.155	0.212	14.9	4.2	18	12.6	6.3	46
All amino acid sites									
Sum of distances	1.77	0.082	0.242	50.8	26.9	18	37.0	28.6	46
Maximum distances	1.20	0.234	0.178	21.8	6.3	18	18.9	9.5	46
Phylogenetic distances									
Strongly positively selected sites									
Sum of distances	1.80	0.076	0.244	2.60	2.62	18	2.60	2.61	46
Maximum distances	1.82	0.074	0.243	2.13	1.27	18	1.51	1.22	46
Positively selected sites									
Sum of distances	1.74	0.087	0.235	0.86	0.51	18	0.61	0.51	46
Maximum distances	1.54	0.129	0.221	0.39	0.13	18	0.32	0.17	46
All amino acid sites									
Sum of distances	1.43	0.158	0.201	0.59	0.31	18	0.45	0.37	46
Maximum distances	1.06	0.295	0.156	0.28	0.10	18	0.24	0.14	46

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

MHC TYPING

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

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

No sequence contained insertions, deletions, or stop codons, suggesting that all MHC alleles were functional. Sequencing of cDNA from six individuals revealed five alleles that are transcribed to mRNA and in all cases the genotype identified from cDNA was identical to that from gDNA, indicating that analyzed loci were fully functional.

MHC DISSIMILARITY AND MATE CHOICE

Females preferred mating with more dissimilar males; that is, with partners whose MHC composition resulted in a higher number of MHC alleles in the offspring, for positively selected sites (two-sample t-test, $t_{62} = 2.00$, $P = 0.0496$) (Table 3). Female preferences approached significance for strongly positively selected sites ($P = 0.057$) and effect sizes were large-medium for both strongly positively selected and positively selected sites

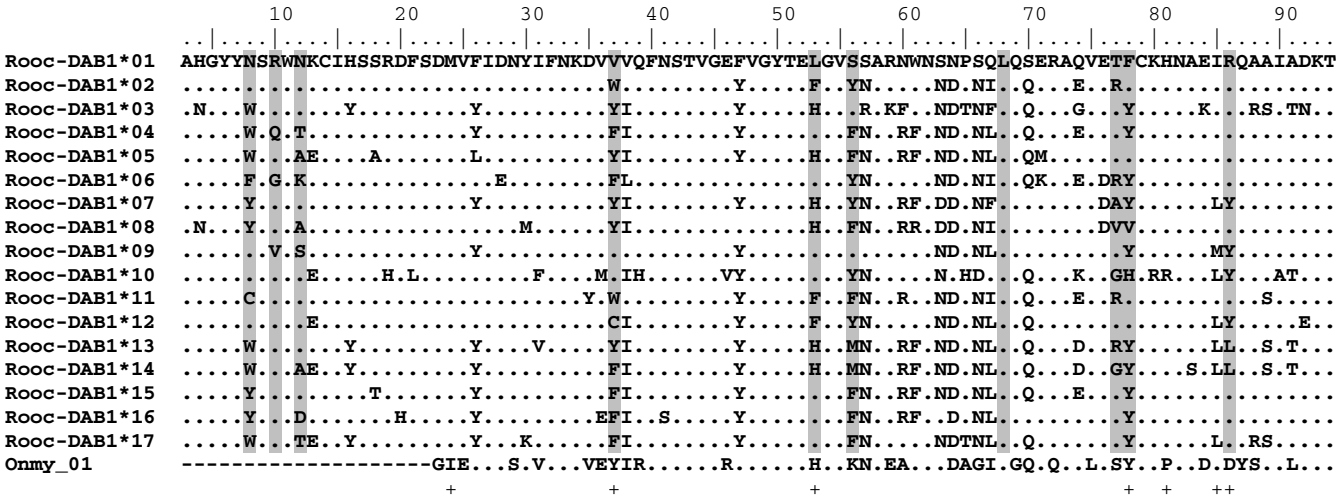


Figure 2. **Amino acid sequence alignment of 17 MHC Class II variants analyzed for codon-specific positive selection. Codons are numbered according to Aguilar and Garza (2007). Positively selected sites based on REL analysis are light- (BF > 50) or dark-shaded (50 > BF > 30). Dots indicate the identity with Rooc-DAB ∗ 01 allele. A "+" marks sites identified to be under positive selection in at least six salmonid species by Aguilar and Garza (2007).**

($r_{ES} = 0.262$ and 0.270 respectively) (Table 3). Functional distances in amino acids explained the pattern of mate choice marginally better (mean $r_{ES} = 0.227$) than phylogenetic distances (mean $r_{ES} = 0.217$). A summation method (mean $r_{ES} = 0.242$) explained the mate choice pattern better than the maximum distance method (mean $r_{ES} = 0.201$) (Table 3). The use of positively selected sites and strongly positively selected sites in the analysis consistently explained more variability than the complete set of amino acid sequences (Table 3).

MHC DISSIMILARITY AND EMBRYO SURVIVAL

Embryo survival was higher for matings between more MHC dissimilar partners (Table 4). The summation method; that is, inclusion of the effect of individual heterozygosity, explained the mate choice pattern better than the maximum distance method (Table 4), and functional dissimilarity at positively selected sites and strongly positively selected sites was the best descriptor of embryo survival among the measures used (Table 4). The effect of MHC dissimilarity on embryo survival was within the range of the effect on mate choice decisions (r between 0.22 and 0.26 for embryo survival, r_{ES} between 0.20 and 0.27 for mate choice using the summation method).

Discussion

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

The fitness of surviving offspring, measured in males as reproductive success in mating trials and in females on the basis of size and age at maturity, did not differ between those sired

Table 4. **Spearman correlation between embryo survival and MHC dissimilarity in *R. ocellatus*, measured as phylogenetic and functional distances between amino acids coded by exon 2 of MHC DAB1. Distance matrices were derived for strongly positively selected sites (Bayes factor value>50; 10 amino acids), positively selected sites (Bayes factor value>30; 24 amino acids), and all amino acids (92 amino acids). Significant results are in bold.**

	Phylogenetic distances		Functional distances	
	r_{62}	P	r_{62}	P
Strongly positively selected sites				
Sum of distances	0.252	**0.044**	0.253	**0.044**
Maximum distance	0.250	**0.046**	0.143	0.261
Positively selected sites				
Sum of distances	0.238	0.058	0.256	**0.041**
Maximum distance	0.227	0.072	0.155	0.221
All amino acid sites				
Sum of distances	0.216	0.087	0.232	0.065
Maximum distance	0.134	0.292	0.087	0.492

by preferred and nonpreferred males. This result supports the conclusion that genetic benefits were nonadditive, because offspring fitness, including the attractiveness of males to females, would not be predicted to improve, whereas in the case of additive genetic benefits the offspring of preferred males would themselves tend to show higher fitness.

Notably, female mate preferences and offspring survival were correlated; females chose males that conferred a nonadditive fitness benefit through enhanced offspring development and survival to independence. Further, both female mate preference and offspring survival to independence were correlated with MHC dissimilarity between paired males and females. Females showed a significantly greater likelihood of choosing MHC dissimilar males (based on functional distances between positively selected amino acids) as mates and the offspring of dissimilar matings had significantly higher survival rates. Mate choice based on MHC dissimilarity is, again, indicative of a nonadditive genetic basis to the rose bitterling mating system. Thus, overall this study shows: (1) Female mate choice is adaptive through an indirect nonadditive genetic fitness benefit. (2) Female mate preferences and offspring survival correlate with the degree of MHC dissimilarity of a mating pair.

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

One hypothesis that has gained ground recently is the proposal that some intermediate, rather than maximal, level of MHC sequence variability will be optimal for offspring viability (Reusch et al. 2001; Milinski et al. 2005; Forsberg et al. 2007; Kalbe et al. 2009). This idea has not been tested in the present study because the number of alleles per individual was relatively low in rose bitterling; an intermediate level of MHC sequence variation may only be applicable in species with duplicated MHC genes. An advantage of intermediate MHC dissimilarity may be important in mating between animals from different populations or races in which outbreeding depression can result from genetic incompatibilities, possibly the result of disruption to co-adapted gene complexes through epistasis, a state recognized in a range of taxa (Turelli and Orr 2000; Barton 2001; Jiggins et al. 2001), including humans (Getahun et al. 2005; Rosenberg et al. 2005; Nystrom et al. 2008).

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

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

Although the present study shows a role for female mate choice in the mating system of the rose bitterling, two other key

features need to be considered; the role of intrasexual competition and spawning site quality in female mating decisions. In the case of the former, there appears to be an intersexual conflict between male aggressive dominance of spawning sites and female mate choice; females do not always prefer dominant males, but dominant males have higher reproductive success than subordinate males (Casalini et al. 2009). Females may be able to circumvent male dominance to some degree by increasing the mating success of subordinates by delaying oviposition and thereby increasing their opportunity to sneak fertilizations (Smith and Reichard 2005; Smith et al. 2007). In respect to the relative significance of mate choice and oviposition choice to female mating decisions, while both appear to play a role in the bitterling mating system, to compare their relative importance it will be necessary to contrast the benefits of each using a common currency related to female reproductive output (reviewed by Kotiaho and Puurtinen 2007). Thus, although female mate choice appears to play a role in the mating system of the rose bitterling, this result still needs to be placed in the context of the other selective forces that contribute to female reproductive success.

A caveat to the results of the present study is the relative weakness of the effect of MHC dissimilarity on mate choice and, to a lesser degree, with offspring survival; no results for MHC dissimilarity were strikingly strong (Tables 3 and 4). Thus, although some studies (this one included) appear to lend credence to the idea that MHC genes may play a role in mate choice, with direct functional significance for offspring fitness, few are entirely convincing. A weakness of these studies is that there is no standard practice for the analysis of MHC data, with the result that multiple variables may be tested by experimenters, though not all may be reported in some studies. We have fully reported all the tests we performed on our own data; the procedures we used were selected on the basis of previous studies of MHC-based mate choice. Note that we chose procedures based on theoretical reasoning and previous evidence from other mating systems, but more measures of MHC dissimilarity can be found in other studies.

An explanation for the relatively weak results for MHC dissimilarity might be because sequencing of rose bitterling MHC Class II genotypes was not be complete; data for DAB3 were missing, though it is probably present in *R. ocellatus* because the gene has been recorded for the related European bitterling, *R. amarus* (M. Vyskocilova, unpubl. data). More complete data including DAB3 may support a stronger link between genetic distance, mate preference, and offspring growth and survival, although the converse may also be the case. It was notable that DAB1 genes were not detected for some individuals, although this is not unusual with MHC genes, for example there is considerable variation in the number of expressed DAB genes in the common carp, *Cyprinus carpio* (van Erp et al. 1996). Those individuals with missing DAB1 genes may have expressed DAB3 genes, and a more comprehensive study of MHC Class II structure in the bitterling would be likely to increase the variability explained.

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

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

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