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Sperm is a sexual ornament in rose bitterling

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Abstract

In many taxa, odour cues mediate mating decisions. A key question is what these odours comprise, where they are produced, and what they signal. Using rose bitterling, fish that spawn in the gills of freshwater mussels, we investigated the role of sperm cues on female oviposition decisions using individuals of known MHC genotype. Male bitterling frequently released sperm prior to female oviposition and females responded with an increased probability of oviposition and released a greater number of eggs, particularly if males had a dissimilar MHC genotype. These mating preferences by females were shown to be adaptive, with MHC dissimilarity of males and females correlated positively with embryo survival. These results support a role for indirect benefits to rose bitterling mate choice, and we propose that sperm acts as a releaser pheromone in bitterling, functioning as a sexual ornament signalling male quality as a mate.

Introduction

Many taxa use chemical signals as components of communication in the context of mating, functioning as attractants to the opposite sex, signalling an individual's dominance, health status, mating status, receptivity, genetic 'quality' and parasite burden (Penn & Potts, 1998; Wyatt, 2003). In fish, olfactory signals are involved in a wide range of functions, such as antipredator responses, migration, kin recognition and mating decisions (Milinski, 2014; Wootton & Smith, 2015). Pheromones, which are chemical signals that have evolved to elicit a specific reaction in a conspecific, play a key role in the courtship and mating behaviour of fishes (Liley, 1982; Stacey *et al.*, 2003), although many aspects of olfactory signalling in fishes, including signalling behaviour and signal structure, are poorly understood (Rosenthal & Lobel, 2006).

Whereas terrestrial animals typically release pheromones from specialized exocrine glands onto a substrate and rely on airborne diffusion transmission to disseminate odour, fish release odour cues directly into water where the rate of transmission is substantially slower than in air. Thus, while terrestrial pheromones tend to belong to a limited family of volatile chemicals, those of fish typically comprise a wide range of unspecialized water-soluble compounds (Stacey *et al.*, 1986). A result is the evolution of highly flexible chemical communication systems in fish with a diverse range of chemicals potentially serving as pheromones, either priming physiological responses in conspecifics or acting as releasers, inducing intrinsic adaptive responses (Stacey *et al.*, 1986; Sorensen, 2015).

One mechanism by which odour cues may mediate mating preferences in vertebrates is through the influence of an individual's major histocompatibility complex (MHC) genotype. The MHC is a family of highly polymorphic genes that play a key role in resistance to infectious disease in vertebrates. MHC genes encode a set of trans-membrane proteins that function in distinguishing between self- and nonself-antigen, presenting foreign peptides to immune-surveillance cells, such as T lymphocytes. Individuals with a wide range of antigen-binding molecules are able to recognize and eradicate a wider range of pathogens and tend to have a fitness advantage over individuals with a more limited MHC profile (Doherty & Zinkernagel, 1975; Penn & Potts, 1998; Boehm & Zufall, 2006). It has also been demonstrated that an optimal rather than maximal individual MHC diversity can confer enhanced resistance to

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pathogens through negative T-cell selection during thymic development (Nowak *et al.*, 1992; Kalbe *et al.*, 2009). Because MHC-dissimilar parents are more likely to produce offspring with a diverse MHC genotype, MHC genes have received attention as possible targets of sexual selection through mate choice (Firman *et al.*, 2017).

An assumption is that MHC polymorphism generates a specific odour signature, which is perceived by the olfactory system of a potential mate and results in mating if the odour cues indicate MHC compatibility (Penn & Potts, 1998; Milinski *et al.*, 2005) or new or rare MHC alleles that have a selective advantage through frequency-dependent selection (Van Valen, 1973; Hamilton, 1980). A key question is what these odour signatures comprise and where they reside. Urine and body odour have been implicated as the primary source of compounds linked to mate choice and individual recognition in terrestrial vertebrates (Santos *et al.*, 2016; Leclaire *et al.*, 2017; Ferkin, 2018), but given the flexible chemical communication systems in fish, other sources of MHC-specific odours may operate.

We investigated the role of odour cues in the mate choice decisions of a fish, the rose bitterling, *Rhodeus ocellatus* (Kner, 1866). Rose bitterling, in common with all other bitterling fishes, lay their eggs in the gills of living freshwater mussels. Males release sperm over a mussel, and after fertilization, the eggs complete development inside the mussel gill, which typically lasts 3–4 weeks (Smith *et al.*, 2004). Female bitterling are choosy over which mussels they will use for oviposition. Decision-making is primarily based on olfactory cues in the exhalant flow from a mussel's gill and include mussel odour and dissolved oxygen concentration (Phillips *et al.*, 2017).

Mate choice by female *R. ocellatus* is at least partly based on genetic compatibility. Female mate preferences are strong, but incongruent among individual females, and positively correlated with offspring survival and growth rate (Agbali *et al.*, 2010). There is good evidence that male and female MHC dissimilarities affect offspring fitness, and female mate preferences correlate with MHC similarity, with females depositing more eggs with MHC-dissimilar mates (Reichard *et al.*, 2012). However, how female bitterling recognize MHC compatibility in potential mates is not known.

Male bitterling guard mussels and attempt to lead females to mussels in their territory to spawn (Smith *et al.*, 2004). Male bitterling perform regular pre-oviposition ejaculations over mussels in their territory, and there appears to be an association between the likelihood of a female spawning in a mussel and the frequency of pre-oviposition ejaculations in bitterling (Smith & Reichard, 2013; Smith *et al.*, 2014), although this has yet to be formally tested. If the case, an implication is that pre-oviposition ejaculation may provide females with odour cues regarding the likelihood of fertilization of her eggs and, alternatively or additionally, on mate compatibility.

We tested the role of sperm cues on female oviposition decisions in *R. ocellatus* with an experimental approach using fish of known MHC genotype. We conducted three experiments. The first was to identify the role of sperm on female oviposition decisions (response to sperm cues), with the prediction that sperm release by males prior to oviposition influenced female reproductive investment. In the second experiment, the aim was to test the role of male genetic compatibility in female mating decisions and understand the way that sperm release mediated female spawning decisions (response to MHC compatibility). Here, the prediction was that sperm carried cues that females used to measure male mate compatibility, with the predicted outcome that females would prefer MHC-dissimilar males and that mating decisions were associated with ejaculation frequency. In this experiment, we additionally examined whether females responded to a single male or groups of three, with either similar or dissimilar MHC genotypes. The aim in performing this comparison was to examine whether MHC genotype, or genomewide variability in the case of groups of three males, contributed to female mating decisions. The prediction in this case was that if only MHC genotype influenced female mating decisions, there would be no difference in female response between single males and groups of three, but that cues associated with genomewide variability would result in a preference for three males. Finally, we examined whether MHC genotype influenced egg survival (embryo survival), with the prediction that pairings between males and females with dissimilar MHC genotypes would result in greater offspring developmental success.

Materials and methods

Experiment 1: Response to sperm cues

Rose bitterling used in the experiment were the second generation of a large outbred population of *R. ocellatus* originally imported from the River Yangtze basin, China. A sexually mature male was selected from a stock aquarium and housed in an experimental aquarium measuring 250 (length) × 400 (width) × 300 (depth) mm with a single *Unio pictorum* mussel in a 57-mm-diameter ceramic flower pot and left alone overnight to establish a territory. A length of 3-mm-diameter silicon tubing was suspended directly over the inhalant siphon of the mussel, 50 mm from the mussel inhalant siphon and connected to a 20-mL plastic syringe. On the following day, a female with ovulated eggs (recognizable by extension of her ovipositor) was gently removed from a stock aquarium and transferred to the experimental aquarium containing the male and allowed at least 15 min to settle. During this time, the

mussel was covered with a perforated plastic cup that allowed the fish to see and smell the mussel but prevented spawning. Once the female started approaching the mussel, it was uncovered, and experimental treatments imposed.

Each experimental pair of fish was randomly assigned to a sperm or control treatment. In the case of the control treatment, a 20 mL solution of water from an aquarium housing six male *R. ocellatus* was drawn into a plastic syringe, attached to the silicon tubing suspended over the experimental mussel and slowly released over the inhalant siphon of the mussel. In the case of the sperm treatment, a 20 mL sperm solution was released in the same way. The sperm treatment was obtained from six male *R. ocellatus* randomly selected from stock aquaria and kept together for 1 day in an aquarium measuring 1200 (length) × 400 (width) × 450 (depth) mm with a female in spawning condition and a *U. pictorum* mussel. The mussel was covered with a perforated plastic cup to allow inspection of the mussel but not spawning. On the following day, sperm was stripped from each male in 5 mL of water by gently pressing their abdomens. A 3 mL subsample of the sperm solution from each male was combined and mixed with 2 mL of freshwater to make a 20 mL sperm solution. Sperm solutions were made up within 5 min of each experimental test; bitterling spermatozoa remains viable for up to 14 min after ejaculation (Smith *et al.*, 2004).

During exposure to the mussel and imposition of treatments, fish behaviour was videoed for 5 min and male and female behaviour subsequently scored. Behaviours recorded were frequency of female mussel inspection (the female positions its snout close to the exhalant siphon of the mussel) and male ejaculation frequency (the male skims smoothly over the inhalant siphon of the mussel and releases sperm). In the case that spawning occurred, the valves of the mussel were gently opened, and the number of eggs deposited in the gills were counted. The mussel was subsequently covered and the pair left for 1 h. After this period, the mussel was uncovered and the process repeated using the alternative treatment. After completion of a paired trial, fish and mussels were measured and none, including sperm donor males, were used again in the experiment.

Experiment 2: Response to MHC compatibility

A total of 65 males and 28 females were haphazardly selected from stock aquaria, individually marked using coloured visible implant elastomer tags (VIE, Northwest Marine Technology company) and genotyped for MHC Class II, which is known to be associated with mate choice in several vertebrate taxa, including the rose bitterling (Agbali *et al.*, 2010; Reichard *et al.*, 2012). Individual MHC profiles were identified for each male and female from DAB1 and DAB3 genes, using a fin clip (for details on genotyping methods see below). Females were randomly allocated to one of four treatment groups: single male MHC similar, single male MHC dissimilar, three males MHC similar, three males MHC dissimilar. Males were assigned from the pool of genotyped males to a treatment group based on their MHC profile and its relationship to a corresponding female MHC profile. MHC similarity/dissimilarity was maximized in terms of the number of DAB1 and DAB3 alleles shared between the partners, analogous to the summation method of Landry & Bernatchez (2001) and Eizaguirre *et al.* (2009). In *R. ocellatus*, the summation method provided a stronger contrast than an alternative method based on functional differences (allele divergence method) and the two measures were strongly correlated (Reichard *et al.*, 2012).

We aimed to maximize contrasts between similar and dissimilar males by allocating the most similar and the most dissimilar males to particular females, given the constraints of our set of genotyped fish. For the similar genotype treatment, we attempted to pair partners with identical MHC genotypes, and in nine replicates, this was achieved (F01, F02, F06, F07, F09, F11, F14, F15, F22). In five replicates, an identical match between male and female MHC genotypes was not possible (F03, F10, F18, F20, F25). In two replicates, a female was paired with a similar male that possessed either an identical DAB1 or DAB3 allele but had an additional DAB1 or DAB3 allele that was lacking in the male (F10 and F25). In another replicate, the reverse was the case (F03). In two further replicates, a male had an additional DAB1 (F20) and DAB3 (F18) allele not found in the female. These deviations from an identical match between females and males in the MHC similar treatment still represented a major contrast with the dissimilar treatment, with a median of 6 different DAB alleles (range 3–6) for three males and median of 2 (range 1–3) different alleles for a single male (Table 1). A double-blind approach was employed for MHC testing; genotyping and treatment assignment were performed in Brno (Czech Republic), whereas behavioural tests were conducted blind to MHC similarity in St Andrews (UK). Three females (F02, F16, F26) repeatedly failed to ovulate, resulting in a final sample size of 25 experimental females, paired with 51 males (Table 1).

Experimental fish were housed in single-sex groups in ten 60 L aquaria containing a sand substrate and artificial plants. Mean (± SE) water temperature was 23.1 (± 1.3) °C. Lighting was maintained on a 12 : 12-h light: dark cycle. Fish were fed once daily with a mixture of frozen bloodworm and flake food. Female reproductive status was monitored each morning, and those with ovulated eggs were gently transferred to a separate experimental aquarium measuring 600 (length) × 300 (width) × 300 (depth) mm. The single or group of three males assigned to the female were also

Table 1 Experimental crosses, MHC genotypes of females and males and experimental outcomes. Females that failed to ovulate (shaded) were excluded from data analyses.

Female			Male 1			Male 2			Male 3			MHC	
ID	DAB1	DAB3	ID	DAB1	DAB3	ID	DAB1	DAB3	ID	DAB1	DAB3	Similarity	Outcome
F01	Rooc*06	None	M05	Rooc*06	None	M10	Rooc*06	None	M50	Rooc*06	None	Similar	Spawned
F02	Rooc*03	None	M03	Rooc*03	None	–	–	–	–	–	–	Similar	Failed to ovulate
F03	Rooc*06	None	M18	Rooc*06	Rooc*05	–	–	–	–	–	–	Similar	Failed to spawn
F04	Rooc*03/27	None	M30	Rooc*14	Rooc*02	–	–	–	–	–	–	Dissimilar	Failed to spawn
F05	Rooc*02/31	None	M12	Rooc*01/18	None	M35	Rooc*18/25	None	M48	Rooc*06/18	None	Dissimilar	Spawned
F06	Rooc*02/31	None	M32	Rooc*02/31	None	–	–	–	–	–	–	Similar	Spawned
F07	Rooc*03	None	M25	Rooc*03	None	M27	Rooc*03	None	M49	Rooc*03	None	Similar	Failed to spawn
F08	None	Rooc*02	M29	None	Rooc*04	–	–	–	–	–	–	Dissimilar	Failed to spawn
F09	Rooc*03	None	M07	Rooc*03	None	–	–	–	–	–	–	Similar	Failed to spawn
F10	Rooc*25	Rooc*02	M08	Rooc*25	None	M33	Rooc*25	None	M43	None	Rooc*02	Similar	Spawned
F11	Rooc*01	None	M55	Rooc*01	None	–	–	–	–	–	–	Similar	Spawned
F12	Rooc*02/31	None	M54	Rooc*01/21	None	–	–	–	–	–	–	Dissimilar	Spawned
F13	Rooc*03	None	M21	Rooc*05	None	M23	Rooc*20	None	M53	Rooc*24	None	Dissimilar	Spawned
F14	Rooc*03/21	None	M22	Rooc*03/21	None	–	–	–	–	–	–	Similar	Failed to spawn
F15	Rooc*04	None	M16	Rooc*04	None	M42	Rooc*04	None	M46	Rooc*04	None	Similar	Failed to spawn
F16	Rooc*19	Rooc*01	M17	Rooc*03/26	None	M19	Rooc*06/29	None	M37	Rooc*14/21	None	Dissimilar	Failed to ovulate
F17	Rooc*02/30	None	M14	Rooc*01/09	None	M31	Rooc*03/26	None	M52	Rooc*03/26	None	Dissimilar	Spawned
F18	Rooc*06	Rooc*02	M24	Rooc*06	Rooc*02/03	M34	Rooc*06	Rooc*02	M45	Rooc*06	Rooc*02	Similar	Failed to spawn
F19	Rooc*22	None	M02	Rooc*20	None	M20	Rooc*03/04	None	M41	Rooc*14	Rooc*02	Dissimilar	Spawned
F20	Rooc*18	None	M01	Rooc*18	None	M06	Rooc*18	None	M09	Rooc*18/19	None	Similar	Failed to spawn
F21	Rooc*19	None	M39	Rooc*02	None	–	–	–	–	–	–	Dissimilar	Spawned
F22	Rooc*01	None	M04	Rooc*01	None	M15	Rooc*01	None	M38	Rooc*01	None	Similar	Failed to spawn
F23	None	Rooc*03	M26	Rooc*18/32	None	M28	Rooc*20	Rooc*05	M44	Rooc*06/33	None	Dissimilar	Spawned
F24	Rooc*25	Rooc*04	M11	Rooc*03/23	None	–	–	–	–	–	–	Dissimilar	Failed to spawn
F25	Rooc*01	Rooc*01	M56	Rooc*01	None	–	–	–	–	–	–	Similar	Failed to spawn
F26	Rooc*03/21	Rooc*02	M51	Rooc*06/19	None	–	–	–	–	–	–	Dissimilar	Failed to ovulate
F27	Rooc*03/19	Rooc*04	M13	Rooc*02/06	None	M36	Rooc*06/25	None	M47	Rooc*14/23	None	Dissimilar	Spawned
F28	Rooc*19	Rooc*06	M40	Rooc*03/21	None	–	–	–	–	–	–	Dissimilar	Spawned

caught from their respective holding aquaria and released in the experimental aquarium. Experimental aquaria had a layer of sand as a substrate and a single *U. pictorum* mussel in a ceramic flower pot for spawning. The fish were left in the aquarium for at least 1 h to settle with the mussel covered, after which it was uncovered. Once courtship and spawning behaviour started, the behaviour of the fish was recorded for 10 min. Behaviours recorded were male ejaculation frequency and courtship frequency (male undulates body at high frequency and low amplitude and swims towards the mussel, Smith *et al.*, 2004) and female oviposition.

Experiment 3: Embryo survival

The survival of embryos fathered by males with MHC similar and dissimilar genotypes was measured using fertilized eggs from Experiment 2. Thus, 1 h after the completion of each replicate in Experiment 2, the fish and mussel were removed and measured. The valves of the mussel were gently opened, and the number of eggs laid in the mussel gill were counted. The remaining ovulated eggs were stripped from the female by gently pressing her abdomen and placed in aquarium water in a 70-mm-diameter Petri dish. Sperm was stripped from the paired male, in the cases where females were exposed to three males the sperm from just one randomly selected male was collected. Egg fertilization followed an established protocol described in Agbali *et al.* (2010). Embryos were scored for development to the neurula stage (Nagata & Miyabe, 1978), indicating successful onset of development (Kimmel *et al.*, 1995). Fish and mussels were not used again.

MHC analysis

For MHC analysis, we used the same protocol as Reichard *et al.* (2012). In brief, genotyping focused on MHC Class II, known to be associated with mate choice in rose bitterling. A gene encoding the MHC class IIβ chain of the protein (Sambrook *et al.*, 2005) (named DAB) can be duplicated in cyprinids, resulting in the expression of DAB1 and DAB3 genes, but there is no evidence of a further gene duplication at either DAB1 or DAB3 (Šimková *et al.*, 2006). We sequenced the complete (DAB1) or partial (DAB3) exon 2 encoding the β1 domain; the most polymorphic fragment of MHC Class II molecules that are responsible for antigen binding. To minimize problems with null alleles, we used a combination of three primers located in introns and exon for DAB1 alleles (Fig. S1) and two primer sets for DAB3 alleles (Fig. S2). DAB3 gene was only present in some individuals. DNA sequences were translated into amino acid sequences, and those were used in all subsequent analyses.

A total of 23 DAB1 alleles (92 amino acids long, Fig. S3) and 6 DAB3 alleles (43 amino acids long, Fig. S4) were detected. Heterozygote deficiency was observed, indicating the absence of the DAB1 and DAB3 loci on some chromosomes in our study population. Heterozygote deficiency resulted from copy number variation rather than resulting from the existence of null alleles; see Reichard *et al.* (2012) for full details. To avoid the possibility of analysing pseudogenes, we compared the genotypes of the DAB1 gene from six individuals obtained from complementary DNA (cDNA) and genomic DNA (gDNA) following RNA extraction from the spleen and reverse transcription. In all cases, the sequences of exon 2 obtained from RNA and DNA were identical. Additionally, the exon 2 sequences of all DAB alleles were aligned in SeqScape v2.5 (Applied Biosystems, Foster City, CA, USA) and examined for the presence of stop codons and/or insertions or deletions ('indels') causing a shift of the reading frame. None showed these types of mutation.

Data analysis

Female response to the sperm treatment was modelled with a Poisson GLMM, which took the form:

$$\mathrm{Eggs}_{ij} \sim \mathrm{Poisson}(\mu_{ij})$$

$$E(\mathrm{Eggs}_{ij}) = \mu_{ij}$$

$$\log(\mu_{ij}) = \eta_{ij}$$

$$\eta_{ij} = \mathrm{treatment}_{ij} + \mathrm{ejaculation}_{ij} + \mathrm{fsl}_{ij} + \mathrm{female}_{j}$$

$$\mathrm{female}_{j} \sim N\left(0, \sigma^2_{\mathrm{female}}\right)$$

where the number of eggs, denoted by Eggs_{ij}, spawned by female j was assumed to follow a Poisson distribution with mean and variance μ_{ij}. A log link function was used to model the expected number of eggs spawned as a function of the covariates. The covariate $\mathrm{treatment}_{ij}$ was a categorical covariate with two levels, corresponding with experimental treatment, water control or sperm solution. The model also contained a linear effect for experimental male ejaculation frequency ($\mathrm{ejaculation}_{ij}$) and female standard length (fsl_{ij}). The random intercept female_j was included to introduce a correlation structure between observations for the same experimental female with variance σ^2, distributed normally and equal to 0.

In the case of female response to MHC compatibility, because females frequently failed to spawn with the males with which they were paired (44% of cases), the data contained a large number of zeros. Consequently, these data were modelled with a zero-altered Poisson (ZAP) GLM. A ZAP (hurdle) model is partitioned into two parts, with a binary process modelling zeros and positive counts, and a second process modelling only positive counts using a zero-truncated model (Hilbe, 2014). This modelling approach permitted us to separately identify the variables that elicited spawning

(binary part), and number of eggs laid when spawning occurred (zero-truncated part) (Zuur *et al.*, 2009). The model took the form:

$$\text{Eggs}_i \sim \text{ZAP}(\mu_i, \pi_i)$$

$$E(\text{Eggs}_i) = \frac{1-\pi_i}{1-e^{-\mu_i}} \times \mu_i$$

$$\text{var}(\text{Eggs}_i) = \frac{1-\pi_i}{1-e^{-\mu_i}} \times \left(\mu_i + {\mu_i}^2\right) - \left(\frac{1-\pi_i}{1-e^{-\mu_i}} \times \mu_i\right)^2$$

$$\text{logit}(\pi_i) = \text{mhc}_i \times \text{ejaculation}_i$$

$$\log(\mu_i) = \text{males}_i + \text{courtship}_i + \text{ejaculation}_i$$

A log link function was used to model the expected number of eggs spawned as a function of the covariates for the zero-truncated part of the model, and a logit function for the binomial part, to ensure the fitted probability of spawning lay between 0 and 1. The covariate males_i was a categorical covariate with two levels, corresponding with females exposed to either a single male or three males, whereas mhc_i was a second categorical variable, corresponding with female exposure to males with a similar or dissimilar MHC genotype. The model contained linear effects for experimental male ejaculation frequency (ejaculation_i) and courtship frequency (courtship_i).

The best-fit ZAP model was selected based on second-order Akaike's information criterion (AICc), by removing predictor variables from the full model until the model with the lowest AICc value was identified. To assess model robustness, we simulated 1000 datasets from the best-fitting model and compared these with observed data, using the procedure of Zuur & Ieno (2016) for hurdle models.

Embryo survival data were modelled using a binomial GLM assuming egg survival for i replicates followed a binomial distribution with probability π_i. Thus:

$$\text{Survived}_i \sim \text{Bin}(\pi_i, \text{Eggs}_i)$$

$$E(\text{Survived}_i) = \text{Eggs}_i \times \pi_i$$

$$\text{var}(\text{Survived}_i) = \text{Eggs}_i \times \pi_i \times (1-\pi_i)$$

$$\text{logit}(\pi_i) = \eta_i$$

$$\eta_i = \text{mhc}_i + \text{fsl}_i$$

The variable Survived_i was the number of eggs that survived to the neurula stage and Eggs_i was the initial number of eggs incubated. The covariates mhc_i and fsl_i correspond with definitions above.

All models were implemented using Bayesian inference with Integrated Nested Laplace Approximation (INLA; Rue *et al.*, 2009) in the R statistical environment, ver. 3.4.3 (R Development Core Team, 2017), with diffuse or noninformative priors put on all parameters. The advantage of using Bayesian inference is that is provides probability distributions for parameters of interest, so that probability statements about the magnitude of model parameters can be made with confidence. This approach avoids reliance on hypothesis testing and *P*-values, which are increasingly recognized as unreliable statistical tools for any but the simplest models (Burnham & Anderson, 2014; Nuzzo, 2014; Wasserstein & Lazar, 2016).

Results

Experiment 1: Response to sperm cues

Females spawned more eggs in the gills of mussels into which a sperm solution was released than those receiving a water control, with zero falling outside the upper and lower credible intervals of the posterior mean (Table 2). There was also a statistically important positive effect of male ejaculation frequency on the number of eggs spawned, though no effect of female size (Table 2; Fig. 1).

Experiment 2: Response to MHC compatibility

For the binomial part of the best-fitting ZAP model, the probability that females spawned was greater with males with a dissimilar MHC genotype (Table 3). A greater ejaculation frequency by males also increased the probability of spawning (Table 3; Fig. 2). For the zero-truncated part of the model, the number of eggs spawned was greater with a single male than with three males. There were also statistically important positive effects of courtship frequency and ejaculation frequency on the number of eggs spawned (Table 3; Fig. 2).

Experiment 3: Embryo survival

The probability of embryos surviving to the neurula stage was greater for those fathered by males with MHC genotypes that were dissimilar to the MHC

Table 2 Posterior mean estimates for number of eggs spawned by female *R. ocellatus* as a function of sperm treatment, ejaculation frequency and female standard length (mm), modelled using a Poisson GLMM with individual females fitted as random intercepts. CrI is the 95% Bayesian credible interval. Credible intervals that do not contain zero in bold to indicate statistical importance.

Model parameter	Posterior mean	Lower CrI	Upper CrI
Fixed intercept	−0.36	−7.79	4.08
$\text{Treatment}_{(\text{control})}$	**−1.53**	**−2.34**	**−0.88**
Ejaculation	**0.30**	**0.15**	**0.54**
Female length	0.02	−0.08	0.19

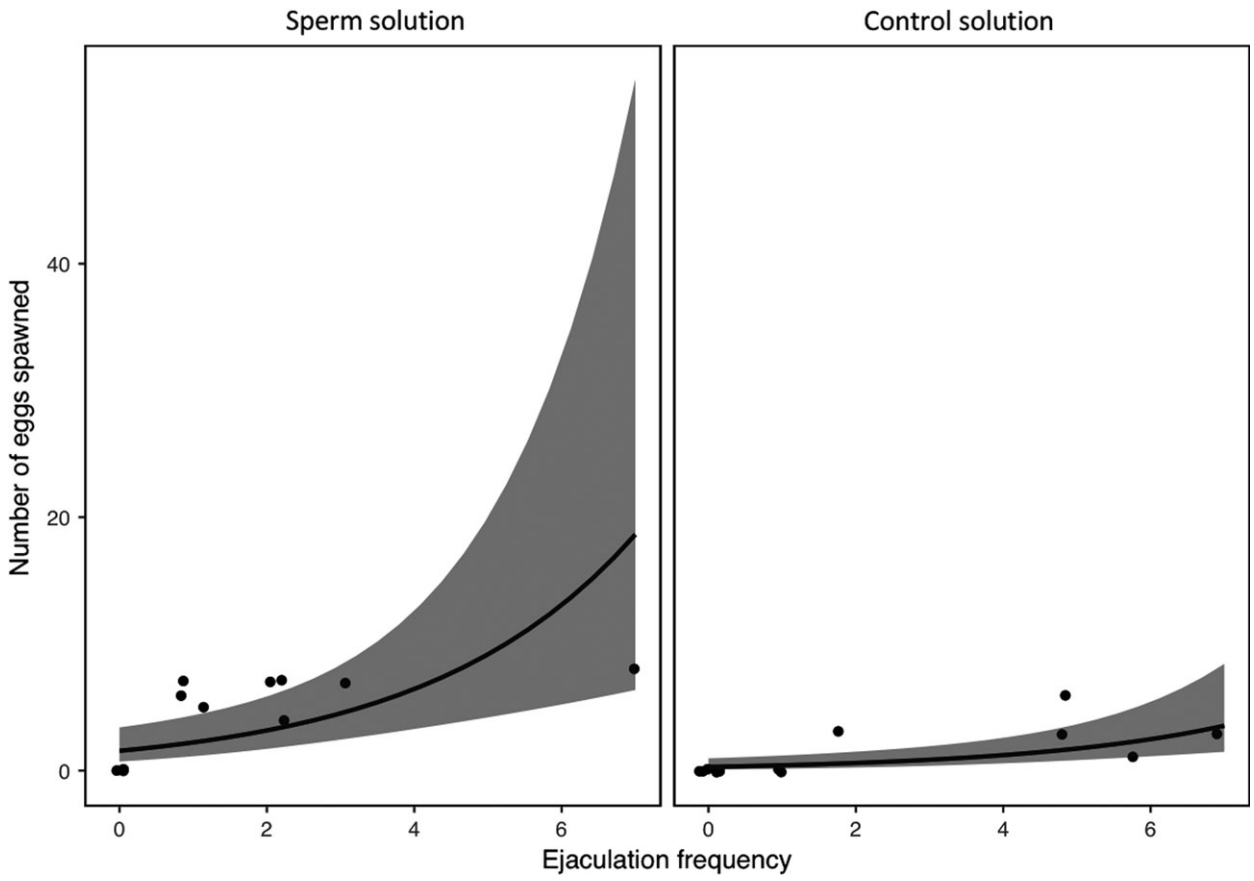

Fig. 1 Posterior mean fitted number of eggs spawned by female *R. ocellatus* as a function of male ejaculation frequency with 95% credible intervals (shaded area) exposed to an experimental sperm solution and control solution. Data were modelled with a Poisson GLMM with individual females fitted as random intercepts. Black circles are observed data.

Table 3 Posterior mean estimates for number of eggs spawned by female *R. ocellatus* as a function of MHC similarity, number of males, male courtship frequency and ejaculation frequency modelled using a zero-altered Poisson GLM. CrI is the 95% Bayesian credible interval. Credible intervals that do not contain zero in bold to indicate statistical importance.

	Occurrence model			Frequency model		
Model parameter	Posterior mean	Lower CrI	Upper CrI	Posterior mean	Lower CrI	Upper CrI
Fixed intercept	−0.88	−3.36	1.29	**2.27**	**1.64**	**2.89**
$\text{Similarity}_{(similar)}$	**−4.09**	**−8.14**	**−1.02**	–	–	–
$\text{Males}_{(three)}$	–	–	–	**−0.38**	**−0.73**	**−0.03**
Courtship	–	–	–	**0.04**	**0.01**	**0.07**
Ejaculation	**1.54**	**0.48**	**2.97**	**0.07**	**0.01**	**0.13**
Similarity × Ejaculation	**−29.4**	**−52.0**	**−14.8**	–	–	–

genotype of the female. There was also a positive relationship between embryo survival and female size (Table 4; Fig. 3).

Discussion

Our results provide evidence that sperm release functions as a releaser pheromone in *R. ocellatus*, driving an adaptive, innate spawning response in females. Adding a sperm solution from multiple males enhanced the attractiveness of a mussel to females (Fig. 1), although multiple ejaculations by a guardian male, particularly those with dissimilar MHC genotypes, increased the probability of female oviposition and simultaneously amplifying the number of eggs spawned (Table 2; Fig. 2). MHC dissimilarity also correlated with mate

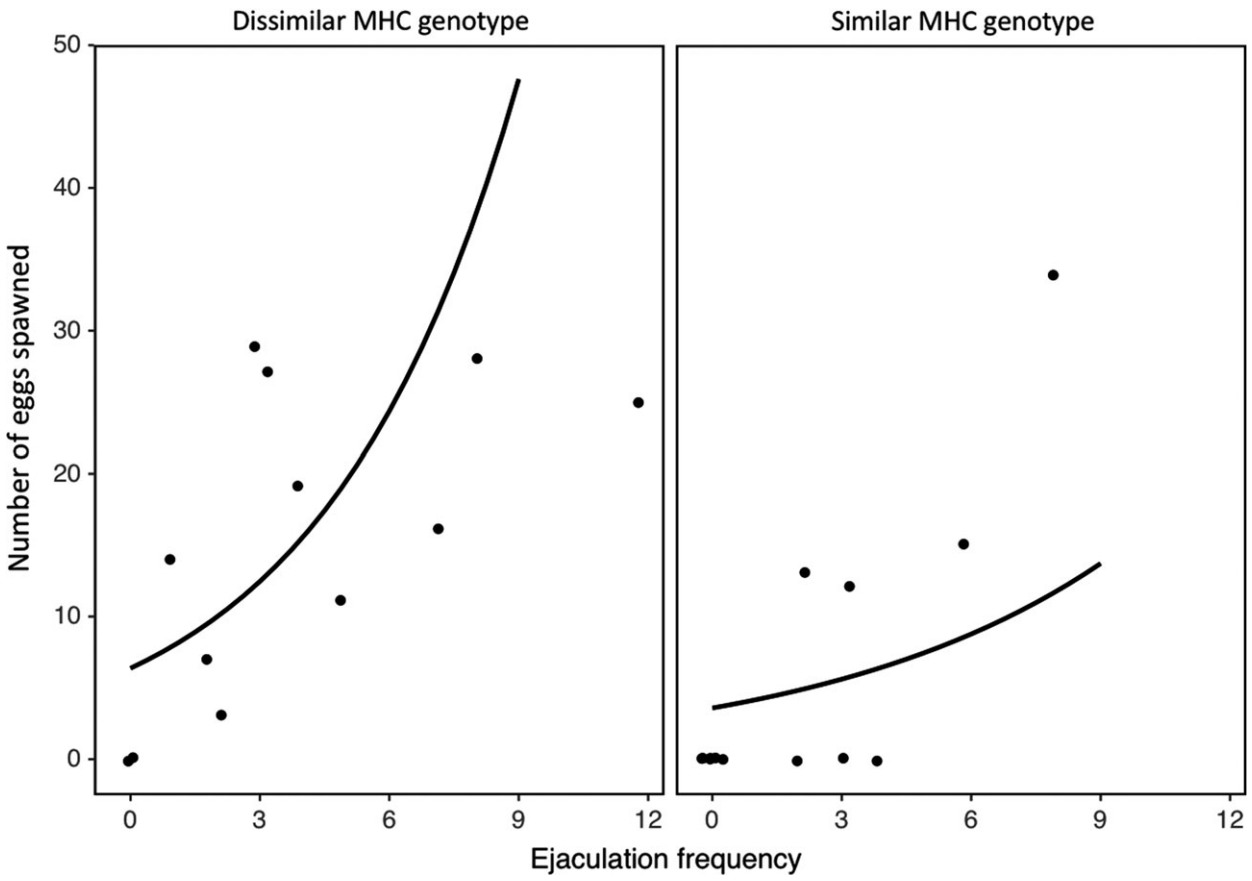

Fig. 2 Posterior mean fitted number of eggs spawned by female *R. ocellatus* as a function of male ejaculation frequency with males with a similar or dissimilar MHC genotype. Data were modelled with a zero-altered Poisson GLM. Black circles are observed data.

Table 4 Posterior mean estimates for number of *R. ocellatus* eggs surviving to the neurula stage as a function of MHC similarity and female standard length (mm), modelled using a binomial GLM. CrI is the 95% Bayesian credible interval. Credible intervals that do not contain zero in bold to indicate statistical importance.

Model parameter	Posterior mean	Lower CrI	Upper CrI
Fixed intercept	−1.43	−4.76	1.88
$\text{Similarity}_{(\text{similar})}$	**−1.15**	**−1.70**	**−0.64**
Female length	**0.08**	**0.01**	**0.14**

choice, and these mate preferences were adaptive; embryo survival was greater with MHC-dissimilar parents (Fig. 3). Taken together, these findings offer two conclusions. The first is that odour cues produced by the male signal MHC compatibility and elicit spawning by the female, and the presence of sperm also serves to elicit spawning, but independently of MHC-related odour cues. The second, more parsimonious, conclusion is that MHC-related odour cues reside in the ejaculate and function as releaser pheromones that females use in making adaptive oviposition decisions. In this scenario, the ejaculate has a dual function, as a medium for delivering spermatozoa to the egg to accomplish fertilization and as an ornament signalling male quality as a prospective mate.

The proximate mechanism by which individuals judge MHC dissimilarity in mating partners has been persuasively demonstrated to be through olfactory cues in a range of vertebrates (Eggert *et al.*, 1998; Penn, 2002; Ziegler *et al.*, 2005), even including taxa, such as birds, with relatively poorly-developed olfaction (e.g. Rymešová *et al.*, 2017). The functional benefits of selecting a mate with dissimilar MHC variants are recognized as coming through increased MHC diversity and elevated heterozygosity in the offspring, as well as from an enhanced performance accruing from specific haplotype combinations (Tregenza & Wedell, 2000). However, a conceptual difficulty arises with the evolution of a mate choice system based on a preference for MHC dissimilarity because it demands an unusually complex set of traits, with an individual required to reference-specific components of their own genotype as well as those of potential mates in making mate choice decisions (Puurtinen *et al.*, 2009). Elucidating the

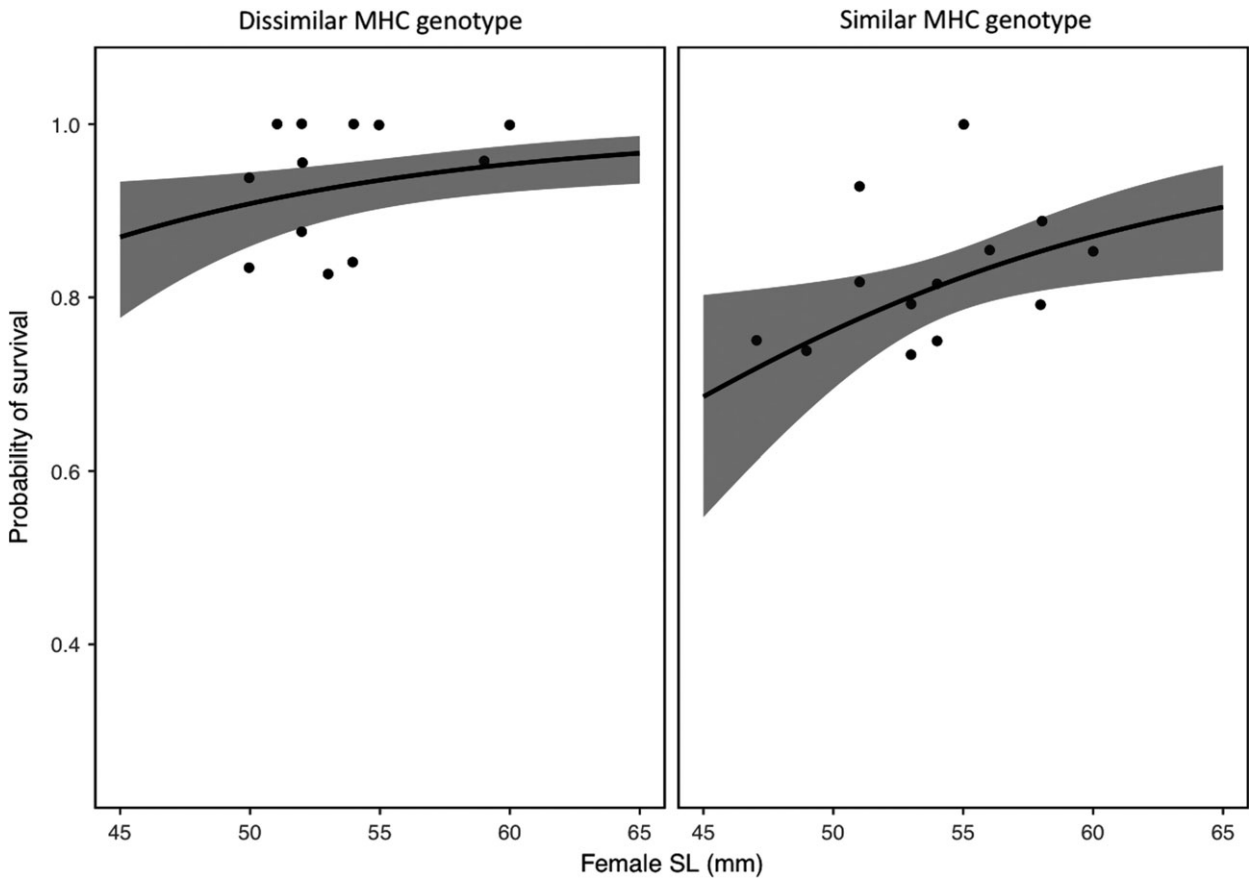

Fig. 3 Posterior mean probability of survival to the neurula stage of *R. ocellatus* embryos produced by *in vitro* fertilization as a function of female standard length (mm) with 95% credible intervals (shaded area) for parents with a similar or dissimilar MHC genotype. Data were modelled with a Binomial GLM. Black circles are observed data.

mechanisms by which genetic compatibility functions in mate choice remains a significant challenge.

The association between female mate preference, MHC dissimilarity and embryo survival in *R. ocellatus* reinforces previous findings for a nonadditive genetic basis to the rose bitterling mating system (Agbali *et al.*, 2010; Reichard *et al.*, 2012). The present study further provides circumstantial evidence that the proximate cue for mate choice is associated with olfactory cues associated with sperm release. The chief components of seminal fluid in teleost fishes are lipids, proteins, free amino acids and monosaccharides. Seminal fluid also exhibits phosphatase, β-glucuronidase and protease activity (Wootton & Smith, 2015). An additional component of seminal fluid in some species, including bitterling (Pateman-Jones *et al.*, 2011), is a sialoglycoprotein-rich fluid termed mucin, which functions in slowly releasing active spermatozoa over an extended period after ejaculation (Marconato *et al.*, 1996; Scaggiante *et al.*, 1999). Thus, seminal fluid comprises a range of constituents that potentially carry MHC-dependent olfactory cues, although these have yet to be identified.

Chemical signals, or pheromones, are widespread in nature (Wyatt, 2003), including in fishes (Sorensen, 2015), potentially performing the function of sexual ornaments comparable to colouration, morphological traits or display behaviour (Corkum & Cogliati, 2015). Female pheromones are recognized in initiating male reproductive behaviour in fishes (Stacey *et al.*, 2003; Wootton & Smith, 2015), but pheromones are also produced by males and serve to attract females and promote spawning synchrony. Male pheromones derive from a variety of sources, including the urine (Maruska & Fernald, 2012; Keller-Costa *et al.*, 2014), mesorchial glands (Gammon *et al.*, 2005) anal glands (Serrano *et al.*, 2008), seminal vesicles (Lambert & Resink, 1991) and testes (van den Hurk & Resink, 1992; Arbuckle *et al.*, 2005). In the Pacific herring (*Clupea pallasii*), a releaser pheromone is associated with sperm (Stacey & Hourston, 1982) and functions in initiating group spawning behaviour (Carolsfeld *et al.*, 1997). For

pheromones to function as ornaments, they must stimulate the receiver's sensory system, be innate and not learned, carry a cost in their production and show variation among individuals, such that they serve as a measure of individual identity in mate choice (Sorensen, 2015). In the case of bitterling, cues associated with sperm release appear to satisfy all these criteria. Sperm is evidently detectable by females (Fig. 1), with female responses apparently innate; responses are seen in females that have not spawned previously and are shared by related taxa (Phillips, 2018). Sperm production is recognized as costly in fishes (Wootton & Smith, 2015), and demonstrably so in bitterling (Smith *et al.*, 2009). Finally, we present evidence that the strength of female response to sperm release is conditional on male MHC genotype (Fig. 2).

A striking feature of the reproductive behaviour of male bitterling is the frequency with which males ejaculate over mussels during reproduction (Smith *et al.*, 2004). Male bitterling repeatedly inspect the exhalant siphon of the mussels they guard, ejaculating over them up to 250 times over the course of a day of matings under natural conditions (Smith *et al.*, 2009). Notably, males engage in pre-oviposition ejaculations, releasing sperm over mussels as part of courtship, and even in the absence of a female. The function of pre-oviposition ejaculations is opaque. It may function in obtaining precedence in fertilization when a female subsequently spawns; alternative mating tactics are common in bitterling, and sperm competition between guarder and sneaker males inside the mussel gill appears common (Reichard *et al.*, 2004a). Males may also keep mussel gills 'topped up' with their sperm (*sensu* Parker, 1998), and thereby ensure fertilization of eggs should a female deposit eggs in a mussel in the male's absence, as water filtration by the mussel depletes sperm in the mussel gill (Smith & Reichard, 2013). The present results suggest that an additional explanation for pre-oviposition ejaculation may be in signalling male traits to prospective mates, including MHC compatibility, with sperm thereby functioning as an ornament.

We showed that the number of males with which females were paired had a statistically important effect on the number of eggs spawned by females in the zero-truncated part of the ZAP model (Table 3), with females depositing more eggs with single males rather than groups of three, irrespective of male MHC genotype. This outcome may result from an artefact of our experimental design, as groups of males tended to disrupt spawning by females in attempting to ejaculate over the mussel during oviposition, which can significantly constrain oviposition rate at the population level (Reichard *et al.*, 2004b). Our predicted outcome for this treatment was that, in the case that genome-wide variability contributed to female mating decisions rather than MHC genotype alone, groups of three males would present females with greater variability in olfactory cues than single males. However, this proved not to be the case and it appeared to be MHC dissimilarity specifically that influenced female mate choice, though with the caveat that we failed to adequately control male–male and male–female interference in our design.

Females spawned a greater number of eggs with males that performed courtship displays more frequently (Table 2). The courtship behaviour of male bitterling is striking, involving the male undulating his fins and body in front of the female at high frequency and interspersed with sperm releases over a mussel (Wiepkema, 1961; Smith *et al.*, 2004). Male bitterling are brightly coloured, and a possible function of courtship is to display these nuptial colours to the female, which may signal direct or indirect mate choice benefits to females (Smith *et al.*, 2004). Vigorous courtship movements may also function in directing sperm and associated odour cues to the female. The release of olfactory signals by fish is often associated with fin or body movements performed during courtship displays (Passos *et al.*, 2015) possibly because the diffusion of compounds in water is relatively slow (Atema, 1996). In the swordtail *Xiphophorus birchmani*, males release urine-borne chemical cues upstream of females, so that odours are carried to the female (Rosenthal *et al.*, 2011). Thus, the positive effect of male courtship frequency on female mating decisions may reflect the role of this behaviour in displaying visual or olfactory ornaments to females, or both in the case that multiple cues operate in the rose bitterling mating system.

Larger females produced more viable eggs, indicating significant maternal effects in embryo survival (Table 3). Across a wide range of teleost species, egg size correlates positively with female body size (Wootton, 1998), and female age and size are recognized as predictors of egg and embryo 'quality' (Wootton & Smith, 2015). Egg size was not measured in the present study, although Agbali *et al.* (2010) did measure egg size in their investigation of *R. ocellatus* and demonstrated that additive maternal effects were largely explained by female size and egg size, and the same is assumed to be the case in the present study.

In summary, female rose bitterling responded positively to the presence of sperm released over mussels during spawning. Multiple ejaculations by males, particularly those with dissimilar MHC genotypes, increased the probability of oviposition, as well as increasing the number of eggs that females spawned. These mating preferences by females were adaptive, with MHC dissimilarity correlated with improved embryo survival. We propose that sperm has a dual function in rose bitterling, transporting the spermatozoa to the egg and as a sexual ornament by acting as a releaser pheromone.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Schematic representation of the structure of the DAB1 gene and the positions and names of three combinations of primers used.

Figure S2 Schematic representation of the structure of the DAB3 gene and the positions and names of three combinations of primers used.

Figure S3 Amino acid sequence alignment of 23 MHC Class II DAB1 variants. Codons are numbered according to Aguilar & Garza (2007). Dots indicate the identity with the Rooc-DAB1*01 allele.

Figure S4 Amino acid sequence alignment of 23 MHC Class II DAB3 variants. Codons are numbered according to Aguilar & Garza (2007). Dots indicate the identity with the Rooc-DAB3*01 allele.

Data deposited at Dryad: https://doi.org/10.5061/dryad.0rr6pm3.

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