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Male mate choice relies on major histocompatibility complex class I in a sex-role-reversed pipefish

O. ROTH*, J. SUNDIN†, A. BERGLUND†, G. ROSENQVIST‡ & K. M. WEGNER§

*Evolutionary Ecology of Marine Fishes, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
†Department of Animal Ecology, Evolutionary Biology Centre (EBC), Uppsala University, Uppsala, Sweden
‡Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway
§Coastal Ecology, Alfred Wegener Institute-Helmholtz Centre for Polar and Marine Research, List, Germany

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Abstract

Mate choice for compatible genes is often based on genes of the major histocompatibility complex (MHC). Although MHC-based mate choice is commonly observed in female choice, male mate choice remains elusive. In particular, if males have intense paternal care and are thus the choosing sex, male choice for females with dissimilar MHC can be expected. Here, we investigated whether male mate choice relies on MHC class I genes in the sex-role reversed pipefish *Syngnathus typhle*. In a mate choice experiment, we determined the relative importance of visual and olfactory cues by manipulating visibility and olfaction. We found that pipefish males chose females that maximize sequence-based amino acid distance between MHC class I genotypes in the offspring when olfactory cues were present. Under visual cues, large females were chosen, but in the absence of visual cues, the choice pattern was reversed. The use of sex-role reversed species thus revealed that sexual selection can lead to the evolution of male mate choice for MHC class I genes.

Introduction

Mate choice evolution is driven by the differential investment of parents into reproduction and offspring (Trivers, 1972; Halliday, 1983; Kokko *et al.*, 2003). The sex with the highest investment into offspring spends relatively more time in a sexually unreceptive stage and is thus generally assumed to be more discriminating during mate choice (Owens & Thompson, 1994; Deutsch & Reynolds, 1995). Hence, in particular in species with biparental investment into offspring, not only females but also males may exhibit mate choice (Sargent *et al.*, 1986; Rosenqvist, 1990) as the reciprocal choice of both male and female attributes (mutual choice) will result in better quality offspring (Real, 1991; Sandvik *et al.*, 2000).

Aside from direct benefits over the choice of good quality mates, two evolutionary functions are associated with mate choice: choice for good genes and choice for compatible genes. Both enable the transfer of attractive attributes onto the offspring as honest signals, which ultimately couple fitness to secondary signalling traits. In particular for immune traits, rather the choice of compatible genes (nonadditive effects) that are complementary to the own genes, results in better-adapted offspring (Tregenza & Wedell, 2000). Whereas the choice of good genes or direct benefits may provide a general fitness advantage (additive genetic effects), the choice of compatible genes depends on the interaction between the genotype of the choosing and the selected individual (nonadditive genetic effects) (Neff & Pitcher, 2005).

Prime examples for compatibility choice are immune traits associated with highly polymorphic classical genes of the major histocompatibility complex (MHC). MHC class I and II genes play a crucial role for mounting an efficient adaptive immune response against infections by parasites but are also involved in mate choice (Penn & Potts, 1999; Piertney & Oliver, 2006). The role of presenting self- and non-self-peptides to T cells makes

Correspondence: Olivia Roth, GEOMAR Helmholtz Centre for Ocean Research Kiel, Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, D-24105 Kiel, Germany. Tel.: +49 431 600 4557; fax: +49 431 600 4553; e-mail: oroth@geomar.de

MHC genes one of the key innovations of the vertebrate immune system (Klein, 1986). Due to strong pathogen-mediated selection and several events of gene duplication (Klein *et al.*, 2007), MHC genes display the highest polymorphism of all coding loci found among vertebrates (Apanius *et al.*, 1997). Importantly, chemical cues associated with individual MHC genotypes influence mate choice decisions in many vertebrate species, allowing a direct link of the genetic basis of a trait, here immunity, to male or female choice (Yamazaki *et al.*, 1979; Potts *et al.*, 1993; Wedekind *et al.*, 1995; Reusch *et al.*, 2001; Shohet & Watt, 2004; Milinski *et al.*, 2005; Bonneaud *et al.*, 2006; Milinski, 2006; Forsberg *et al.*, 2007; Neff *et al.*, 2008).

According to the diversity-advantage hypothesis, mate choice should aim at maximizing individual MHC diversity (Doherty & Zinkernagel, 1975), which in turn positively affects fitness because of an enhanced immune-competence and increased pathogenic scope due to a broader recognition-spectrum of pathogen-derived antigens (Janeway *et al.*, 2001). Interestingly, mate choice strategies in some of the species examined aim for an intermediate (optimal) MHC diversity in offspring. Experiments and also theoretical considerations suggest that the damaging effects of MHC-mediated autoimmunity (Milinski *et al.*, 2005) and/or deselection of autoreactive T cells with maximal MHC diversity are responsible for such a strategy (Woelfing *et al.*, 2009). Choice for maximal or optimal MHC diversity and the positive correlation with successful combatting of infectious disease has been found in various species, ranging from fish, over birds and mice to humans, (e.g. Wegner *et al.*, 2003; Kurtz *et al.*, 2004; Martin *et al.*, 1989; Penn *et al.*, 2002; Carrington *et al.*, 1999; Arkush *et al.*, 2002).

To date, the main research focus has been MHC-based female choice, as under conventional sex roles, which comprise most of the species on the planet, females are the choosing sex. Only few studies addressed male mate choice alone or with the female component and revealed inconsistent results. Either no influence of MHC genes on male mate choice (Forsberg *et al.*, 2007; Neff *et al.*, 2008; Bahr *et al.*, 2012) was found, or male mate choice for MHC dissimilarity was detected (Gillingham *et al.*, 2009). In humans, no difference between sexes was found for smell preference of potential mating partners (Wedekind & Füri, 1997), indicating that males may also prefer females with dissimilar MHC, provided a sensorial requirements to perform mate choice, that is, olfactory receptors.

Here, we use a particular group of teleost fishes, the seahorses and pipefishes (Syngnathids), in order to test long-standing ideas of the prevalence of olfactory mate choice based on MHC immune genes. In particular, if sex roles are reversed to intensified paternal care, the evolution of MHC-dependent male mate choice could be expected to be highly adaptive due to strong selection for provisioning of offspring with efficient immune defence (Roth *et al.*, 2012b).

Due to the evolution of male pregnancy, pipefish and seahorses, as members of the family of Syngnathidae, represent a prominent example for extreme paternal care justifying stringent male mate choice. Our study species, the deep-snouted pipefish *Syngnathus typhle,* has a functional absence of MHC class II (Haase *et al.*, 2013) and thus relies exclusively on MHC class I diversity.

Using an experimental mate choice setting, we applied Roche 454 FLX amplicon sequencing to perform MHC class I genotyping (Babik *et al.*, 2009) and could therefore directly determine allelic diversity on the sequence level. This allowed us to detect signatures of selection (nonsynonymous to synonymous amino acid substitution ratio – dN/dS) and use this information to define compatibility on the fundamental, functional level of amino acid divergence. To account for the importance of MHC class I diversity for immune defence dynamics, we further measured baseline activity of the pipefish immune system in a natural habitat determining both activity of innate and adaptive immunity. We then correlated baseline activities with the MHC class I genetic diversity to provide a functional link between choice and immunity.

Taking together, we combined several understudied aspects of olfactory-mediated mate choice and tested the following hypotheses: (1) in a sex-role-reversed species with strong male investment into offspring, we find MHC-based male mate choice; (2) in a species lacking the MHC class II system, mate choice is based on the polymorphism of MHC class I genes; (3) MHC-based mate choice is only important when olfactory cues are present; (4) MHC choice decisions directly affect immune traits in the offspring.

Materials and methods

Experiment

The pipefish *Syngnathus typhle* is a prominent model system for the investigation of mate choice and sexual selection. Behavioural experiments have shown that visual signals, such as female size and parasite load affect male mate choice (Berglund *et al.*, 1986; Sandvik *et al.*, 2000; Silva *et al.*, 2006; Widemo, 2006). As yet, the importance of olfactory cues for mate choice remains elusive (Sundin *et al.*, 2010) as opposed to the gulf pipefish *Syngnathus scovelli*, where males use olfactory cues for mate choice (Ratterman *et al.*, 2009). The typical choice for larger body size (Berglund *et al.*, 1986) was, however, not observed in the absence of visual cues, suggesting that olfactory and visual signals are not redundant and may evolve independently (Ratterman *et al.*, 2009). To investigate whether olfactory MHC cues are correlated with visual indicators such as

female size, we now analysed the MHC genotype-based mate choice of a behavioural experiment that was performed by Sundin *et al.* (2010). To do so, we genotyped the MHC class I, and 10 microsatellite loci of pipefish used in that experiment. For detailed experimental description, refer to Sundin *et al.* (2010).

The mate choice experiment was conducted at Sven Lovén Centre for Marine Sciences, Kristineberg, in Sweden, using *Syngnathus typhle* collected in the Gullmar Fjord (58°15′N, 11°28′E). In mate choice trials, a male could choose between a large and a small female since body size is a visual mate choice cue in this species (Berglund *et al.*, 1986). Mate choice was assessed, such that the male and the two females were each placed in their own compartments. The large female was placed in the right or left compartment in alternating order to avoid side effects. Three levels of visual contact were tested (full vision, impaired vision, no vision), and two olfactory levels (smell, i.e. connection of water from the females to the male, and no smell, i.e. no connection of water between males and females), thus resulting in five treatments (full vision/smell; impaired vision/smell; no vision/smell; full vision/no smell; impaired vision/no smell). Each trial started with an acclimation period, after which the position of the male (before the large female, before the small female, in the no choice zone) was recorded. Choice was defined as when the male was observed within 15 cm from the female compartment. Physical proximity has earlier been shown to correlate well with actual mating propensity (Berglund, 1993; Berglund *et al.*, 2005). Triplets where males were not observed in front of both the large and small female at least once were excluded to ensure that the male had seen the two females and hence could choose between them. This resulted in 20 replicates in the full vision/no smell and full vision/smell treatments, 21 in the impaired vision/smell treatment, 19 in the impaired vision/no smell treatment, and 17 in the no vision/smell treatment.

A fin clip was taken of each individual after the mate choice trials and stored in ethanol (ethical permission Dnr 118-2008).

MHC genotyping

DNA from all fin clips was extracted using DNeasy 96 Tissue kit (Qiagen, Hilden, Germany) and stored at −20 °C until use. Primers for genotyping the MHC class I antigen alpha 1 Exon 2 were designed from an alignment of MHC class I sequences from a pipefish EST library (Haase *et al.*, 2013) (Appendix S1). Primers used were as follows:

Primer Pipe_MHCI_forward: CCTGYCATTCACACGC TCAATTATTTCWG
Primer Pipe_MHCI_reverse: CTCCAGTTTGGTTGAAG CGCTTTTTA

Since the last two basepairs of the forward primer could potentially lead to mismatch, we omitted them from further analysis and suggest to use a primer shortened by 2 bp on the 3′ end. We verified the performance of our genotyping assay by cloning and sequencing. Alleles identified from 20 individuals via cloning can also be found in Supplement 1. For large-scale genotyping, we used amplicon sequencing via 454 next generation sequencing technologies (Babik *et al.*, 2009; Wegner, 2009). Each individual was coded by a unique combination of one of 40 different multiplex identifier (MID) tags attached to the forward primer and one of the same 40 MID tags attached to the reverse primers resulting in 1600 unique primer combinations (Binladen *et al.*, 2007; Wegner *et al.*, 2012). The amplified sequence is 211 base pairs long. To assist downstream artefact detection, we used two independent replicates per individual by coding each individual with two distinct primer combinations. For each individual, a nested PCR was performed. The first PCR consisted of 12 cycles and was run in a 10-μL reaction using Dream Taq polymerase 0.3 U (Thermo Scientific, Dreieich, Germany), 100 μM dNTP, 0.5 μM each primer without MID tags and 10–100 ng DNA. PCR Program: 94 °C 30 s, 58 °C 30 s, 72 °C 60 s, final extension: 72 °C 3 min. The PCR product was diluted 1 : 5 in HPLC water. For the second nested PCR, primers with the MID tags were used and 20 cycles of PCR were performed using 0.65 U Dream Taq polymerase, 100 μM dNTP, 0.5 μM each primer with individual tag and 2 μL of the diluted PCR product.

The resulting single PCR products were then purified using QIAquick 96 PCR purification kit (Qiagen) after standard protocols, 2% Agarose gels were run to determine product presence. The concentration of each PCR product was determined photometrically (Nanodrop, PEQLAB Biotechnology GmbH, Erlangen, Germany), and each product was diluted to 30 ng/μL. Ultimately, 100 ng per individual were pooled and sent for Roche 454 FLX sequencing to GATC Konstanz, Germany.

Genotyping of neutral markers (microsatellites)

MHC genotype may serve as a marker for individual relatedness and choice for MHC dissimilar mates could therefore just represent a mechanism of inbreeding avoidance. Hence, we investigated whether mate choice decisions are influenced by relatedness among focal individuals. To determine genome-wide genetic similarity, we genotyped 10 microsatellite loci in all pipefish used for the mate choice experiments. Multiplex-polymerase chain reactions were performed for all 10 microsatellites as described under GenBank Accession Numbers JQ598279–JQ598290 (Roth *et al.*, 2012a).

MHC diversity and immunity

A total of 29 fish were examined for both MHC diversity and their baseline immunological activity in the field (i.e. immunocompetence) (Roth *et al.*, 2011). Analogous to MHC genetic distance between pairs of fish, we calculated intra-individual genetic distance by summing up pairwise protein distances between all alleles of an individual. This way we accounted for variation in functional diversity in terms of amino acid composition. Intra-individual genetic distance was then correlated with the proportion of proliferating head kidney lymphocytes (lymphocytes in S phase) as a measure of activity of the adaptive arm of the immune system and to a respiratory burst assay that indicated the phagocytosis activity of monocytes, that is, the innate arm of the immune system [methods are described in detail in (Roth *et al.*, 2011)].

Data analysis

Assignment of reads to individual PCR reactions was done using modified python scripts from the cogent package. Each raw read was searched for both primers ensuring that a complete PCR product was sequenced. Afterwards, we identified individual PCRs by the specific combination of MID tags allowing for a maximum hemming distance (allowed mismatch between a pair of sequences) of one in each MID tag. All reads assigned to one PCR were trimmed of primer, adaptor and MID sequences and written into a single FASTA file. Within each file we sorted the unique sequences by their abundance and eliminated each sequence that occurred less than three times in total, or less than 3% of the total number of reads found for the respective PCR. The co-occurrence of sequences between the replicate PCRs for each fish was then checked for pairs of fasta files, and only those sequences that occurred in both replicate PCRs were retained for genotype calls. Individuals with low coverage naturally displayed lower allelic diversity and we set the lower coverage cut-off level to 100 reads per individual PCR, because the number of alleles called did not depend on the coverage anymore when only individuals were included that fulfilled this criterion. After this rigorous quality control and successful allele calling, we ended up with a total of 61 triplets for further statistical analysis (impaired vision/smell: 14; full vision/smell: 11; impaired vision/no smell: 13; full vision/no smell: 10; no vision/smell: 13). The remaining 36 triplets had to be excluded due to incomplete genotype calls.

To account for the paired experimental design, we expressed male mate choice as the number of observations in the choice zone adjacent to the large female minus the number of observations at small female (Δ_{choice}) [behaviour of fish was observed every 5 min over 2 h as described in (Sundin *et al.*, 2010)]. The overall time a male spent in the choice zone can be regarded as a measure of choosiness and was added as a weighing variable to statistical analyses. The genetic distance of MHC class I was based on the Jones–Taylor–Thornton matrix for calculating pairwise distances between all alleles as implemented in the *protdist* routine in PHYLIP v3.69 (Felsenstein, 2000). As a second approach, we calculated genetic distance of MHC class I for positively selected sites. To test whether full allele distance matrices correlated with distance matrices on only positively selected sites, we used a Mantel test based on 999 random permutations. The distance between two genotypes within a triplet was then calculated as the mean of the pairwise distances between all alleles of the male and the respective female. Analogous to Δ_{choice}, we used the difference of the genetic distance between the male and the large female minus the genetic distance between the male and the small female ($\Delta_{Genetic\ Distance}$). We then investigated with two ANOVAS whether $\Delta_{Genetic\ Distance}$ (covariate) and the different levels of vision (fixed factor) affected male mate choice behaviour for large vs. small female (Δ_{choice} as response variable), in the smell treatment vs. the no smell treatment. These analyses were run for both genetic distance calculated from full sequences and for only positively selected sites. We had to perform separate tests for smell and no smell treatments, because the factor vision under smell conditions had three levels (no vision, impaired vision, full vision) but only two levels under no smell conditions (impaired vision, full vision) and the calculation of interaction terms was therefore not possible. As a measure of the choosiness of each male, we used the number of times a male was observed in either of the choice zones and entered this as a weight into the statistical analysis. Statistical analyses were performed in R (R Development Core Team, 2012).

To calculate dN/dS ratios along the sequence, we used omegaMap v0.5 (Wilson & McVean, 2006) and ran two independent chains of $2*10^5$ iterations with a thinning interval of 100 and three random start orders each. We used default parameters and set block size to single codons. Sites experiencing positive selection were identified by > 95% of samples from the posterior distribution exceeding a value of dN/dS > 1.

To test whether MHC-based mate choice corresponded to genome-wide similarity, we further tested whether preferred females shared fewer microsatellite alleles. Analogous to the MHC-based $\Delta_{Genetic\ Distance}$, we calculated the difference of microsatellite alleles the male shared with the large female minus the number of alleles he shared with the small female $\Delta_{Allele\ Share}$ and analysed its influence on Δ_{Choice} with the same ANOVA model as for $\Delta_{Genetic\ Distance}$ above.

To assess the influence of MHC class I diversity on parameters of innate and adaptive immune-competence, we used a ANOVA with individual number of

MHC class I alleles and proportion of proliferating lymphocytes. As an approximation for strength of innate immunity, individual number of MHC I diversity or intra-individual MHC genetic distance (the distance of the different MHC alleles found in an individual) was correlated with the phagocytosis activity (area under curve in a respiratory burst assay) and to the proportion of proliferating lymphocytes as a approximation for the activity of the adaptive immune system (Roth *et al.*, 2011).

Results

We could assign 171'207 reads to individual PCRs used in this study resulting in 214 individuals with sufficient coverage above our lower cut-off of 100 reads per individual. Within these, we found 183 fish stemming from 61 complete choice triplets. In these fish, we found a total of 37 MHC class I partial alleles. None of these contained a stop codon suggesting that these alleles are at least potentially functional. We cannot exclude that the primers used did not amplify all possible alleles in the individuals, which would indicate that we underestimate the MHC allelic diversity and that some alleles could be lacking in our dataset. However, this underestimation would concern both MHC genotypes alike – the choosing males but also the chosen females. The pairwise protein distances between alleles ranged from 0.001 to 0.494 (mean 0.259 ± 0.136) taking the whole protein sequence and increased substantially when only sites under positive selection were included in the analysis (range: 0.01–0.993, mean: 0.360 ± 0.169). On average, individual fish displayed 1.75 alleles (range 1–4) suggesting that sequences from at least two loci were amplified (contigs and alleles displayed in S2). The mean number of alleles was further in accordance with the number of alleles identified via cloning and Sanger sequencing of 20 individuals [2.3 alleles (range 1–5)]. The average coverage per replicate PCR was 403 ± 14 and 796 ± 23 for each individual leading to a coverage of approximately 100 reads per individual allele, which should offer sufficient sequencing depth for reliable genotype calls, although amplification efficiency might vary among alleles. Sequences could be unambiguously aligned and showed a significant signature of positive selection (dN/dS = 1.94, $t_{(\text{d.f.} = 69)} = 2.687$, $P = 0.009$) owing to 14 positively selected codons corresponding to 20% of all codons (Fig. 1). In a first approach, we used the entire sequence to calculate amino acid based genetic distance between partners in mate choice triplets. This procedure is conservative because average distance over the whole sequence will be lower than for positively selected sites only and covers the whole information contained in the sequence. Inference of sites involved in antigen binding is usually based on homology to crystallized human or mouse MHC proteins, but can be misleading for distantly related species like pipefish in which probably also the functional properties of the protein changed. The use of the whole sequence therefore seemed to be an unbiased estimator of amino acid based genetic distance. In a second approach, we used only positively selected sites to calculate amino acid based genetic distance between partners in mate choice triplets. Distance matrices that were based on positively selected sites correlated well with distance matrices based on the whole sequence (Mantel test, $R = 0.598$, $P < 0.001$) showing that a major proportion of genetic distance was caused by these sites.

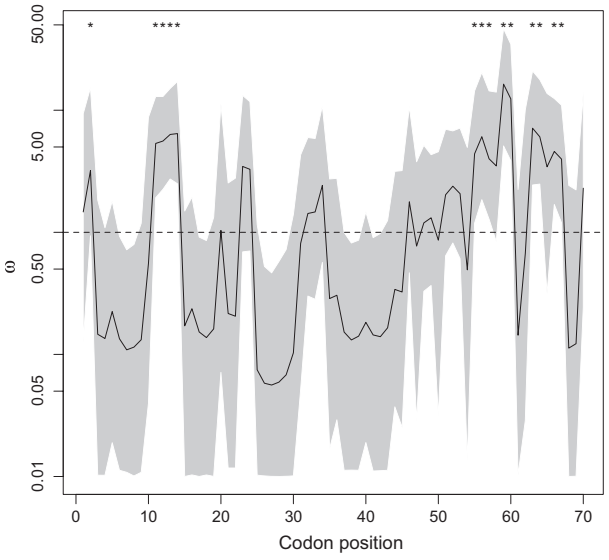

Fig. 1 Signature of positive selection (w = dN/dS) along the sequence of *S. typhle* MHC class I genes. The black line represents the highest posterior density with shaded areas showing the 95% confidence intervals around this estimate. The dashed line shows the null expectation of neutral evolution ($\omega = 1$) and sites experiencing positive selection are marked by *.

As predicted, when females were visible a preference for large females was evident in both smell treatments. As described in Sundin *et al.* (2010), this preference disappeared when vision condition deteriorated and was even reversed in the smell treatment when no visual contact was allowed (Fig. 2, Table 1A). As expected, the MHC class I genetic distance did not have an influence on the mate choice decision in the absence of smell. However, under smell conditions, males preferred the female with a more distant, different or unrelated MHC class I genotype in terms of genetic distance (Fig. 3; Table 1). The effect of MHC genotype was independent of female size because it could be observed with and without visual contact (Table 1), whereas genetic distance between MHC genotypes $\Delta_{\text{Genetic Distance}}$ was not significantly biased towards larger females (t-test, $t_{57} = 1.22$, $P = 0.227$). This suggests an odour preference of male pipefish for females with an MHC class I geno-

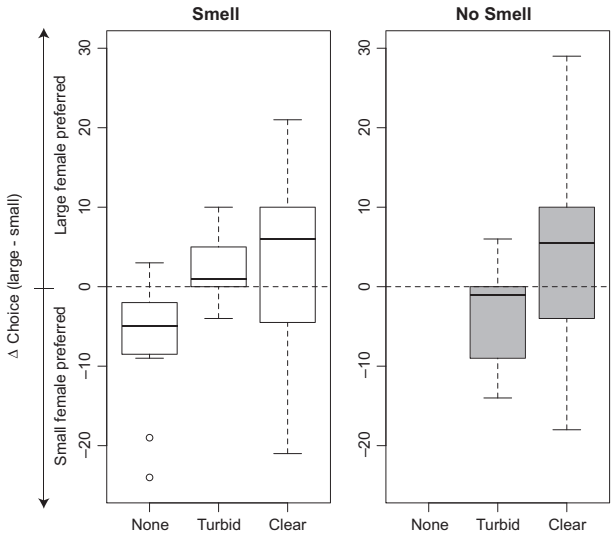

Fig. 2 The choice differential (Δ_{choice}) of the focal male (number of choice measurements at the large minus number of choice measurements at the small female) is displayed either under smell (left graph, white) or no smell (right graph, grey) conditions for the different vision treatments applied (none = no vision, turbid = impaired vision, clear = full vision). Positive values indicate choice for large females, negative choice for small females. Boxes show 75% quantiles with medians as lines. Whiskers comprise 95% quantiles and single dots show outlier values. Arrows indicate either choice for large females (positive Δ_{choice}) or choice for small females (negative Δ_{choice}).

Table 1 ANOVA tables for the effect of the fixed factors genetic distance of major histocompatibility complex (MHC) and vision on the choice differential (Δ_{choice}, number of choice observations at the large female minus number of choice observations at the small female), under smell or no smell conditions either for full sequence (A) or for only sites under selection (B).

	d.f.	SumSq	*F* value	*P*
(A) MHC full sequence				
Smell				
Genetic distance	1	474.88	6.5	0.016*
Vision	2	661.82	4.53	0.019*
Genetic distance x vision	2	112.33	0.77	0.473
Residuals	29	2118.86		
No smell				
Genetic distance	1	18.46	0.2	0.662
Vision	1	476.04	5.1	0.036*
Genetic distance x vision	1	115.63	1.24	0.28
Residuals	19	1773.87		
(B) MHC selected sites only				
Smell				
Genetic distance	1	444.42	10.03	0.004*
Vision	2	450.77	5.09	0.013*
Genetic distance x vision	2	20.92	0.24	0.791
Residuals	29	1285		
No smell				
Genetic distance	1	7.17	0.50	0.703
Vision	1	535.23	11.19	0.003*
Genetic distance x vision	1	89.87	1.88	0.187
Residuals	19	909.13		

*Indicates significance *P* value ($P < 0.05$).

type that is more different in terms of amino acid sequence than their own genotype. Twice the amount of variation in choice could be explained when only selected sites were considered (Table 1B), indicating that these sites comprise the major proportion of relevant functional variation. The reversal of size preference in no vision conditions of the smell treatment (Fig. 2) suggests that the olfactory signal may be enhanced in smaller females and can sometimes override the obvious benefit of size.

We see a positive correlation of intra-individual MHC distance with the activity of the adaptive immune system (proliferating lymphocytes) (*t*-test: $t_{27} = 5.28$, $P = 0.030$), however, no correlation with phagocytosis activity could be identified (*t*-test: $t_{25} = 1.93$, $P = 0.177$) (Appendix S2).

Male mate choice was unaffected by genome-wide allele sharing. When using the sharing of microsatellite alleles to assess male mate choice, no significant effect could be found (Table 2).

Discussion

Our study provides evidence for pronounced male olfactory mate choice based on the genotype of the MHC class I loci in a species with sex-role reversal lacking MHC class II. MHC-based choice complements phenotypically based selection for female size which probably provides a direct benefit. Importantly, both cues represent nonredundant signals. When visual cues were absent, we observed a reversal of size preference (Fig. 2). This use of multiple cues for mate choice can be less costly as fewer mates need to be inspected (Candolin, 2007). In addition, the observed choice mechanism will increase immunogenetic diversity in the offspring, and will therefore boost the adaptive immune system (Fig. 3), which may in turn help offspring to cope better with a diverse set of parasites (Apanius *et al.*, 1997).

Since *Syngnathus typhle* is a sex-role-reversed species, with males displaying extreme paternal care, they invest much more into the offspring compared with species with conventional sex roles. Due to the evolution of male pregnancy, males rather than females have longer and fewer reproductive events. This suggests a reversed Bateman Gradient (relationship between mating success and fertility characterized by the sexual selection gradient) (Andersson & Iwasa 1996) in *S. typhle* (Jones *et al.*, 2000), which makes evolution of MHC-mediated mate choice very likely. Bateman's principle also applies to sexual immune dimorphism. Here, male *S. typhle* display a stronger immuno-

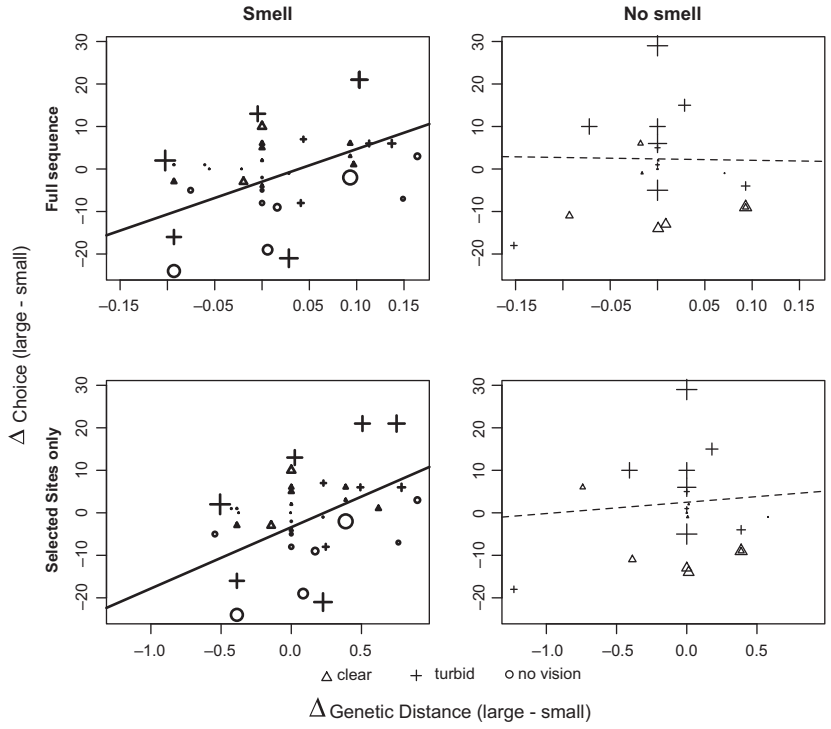

Fig. 3 A weighed regression for the choice differential (Δ_{choice}) of the focal male (number of choice measurements at the large minus number of choice measurements at the small female) on the *y*-axis and the genetic distance differential ($\Delta_{Genetic\ Distance}$) on the *x*-axis using either full sequence (upper panel) or only selected sites (lower panel) (genetic distance of focal male and large females minus genetic distance of focal male and small female). The left panel shows animals that were permitted to use olfactory cues (smell), animals displayed on the right panel did not have access to olfactory cues in mate choice (no smell). The symbols show different vision treatments (Δ: clear; +: turbid; o: no vision) and the size of the symbols corresponds to the proportion of observations a male spent in the choice zone, which was added as a weighing variable representing overall choosiness of each male.

Table 2 ANOVA tables for the effect of the fixed factors microsatellite allele sharing and vision on the choice differential (Δ_{choice}) (number of choice measurements at the large female minus number of choice measurements at the small female), under smell (A) or no smell conditions (B).

	d.f.	SumSq	*F* value	*P*
ANOVA microsats				
(A) Smell				
Allele share	1	1.83	0.03	0.86
Vision	2	464.01	4.01	0.029*
Allele share × vision	2	58.43	0.51	0.609
Residuals	29	1676.83		
(B) No smell				
Allele share	1	108.63	2.24	0.151
Vision	1	457.93	9.45	0.006*
Allele share × vision	1	54.42	1.12	0.302
Residuals	19	920.42		

*Indicates significance *P* value ($P < 0.05$).

competence than females (Roth *et al.*, 2011). In addition, both males and females enhance the immune response of their offspring via biparental immune priming (Roth *et al.*, 2012b). This suggests that the evolution of intense paternal care is associated with additional traits that enhance offspring immunity (Roth *et al.*, 2012b; Keightley *et al.*, 2013). As in many other species, MHC-based male mate choice is likely to be adaptive in pipefish as it serves a variety of potential benefits in parasite resistance in offspring and thus enhances fitness (Consuegra & de Leaniz, 2008). Even though we lack a comprehensive dataset to support the existence of a negative correlation of MHC diversity with parasite prevalence and disease resistance in pipefish, our data suggest that the baseline activity of the adaptive immune system correlates positively with the individual MHC diversity. High parasite loads often negatively affect growth in fish (Wegner *et al.*, 2003) making a correlation of MHC genotype and size conceivable. In the present study, genetic distances between MHC genotypes were not correlated with size differences among adult females, suggesting two largely independent signals. Whereas larger females may provide immediate benefits via larger eggs, MHC-mediated mate choice will only become effective in the next generation by boosting the offspring immune response.

Alternative explanations including inbreeding avoidance (Pusey & Wolf, 1996) seem to be much less likely because MHC-based mate choice did not select for more distantly related individuals and can therefore not be considered as a marker for genome-wide relatedness (Table 2).

The species most closely related to *S. typhle* from which data on MHC-mediated mate choice is available in the potbellied seahorse *Hippocampus abdominalis*. As opposed to *S. typhle* that lacks MHC class II, *H. abdominalis* was recently suggested to have a single functional MHC class II locus that, however, displays high sequence divergence compared with other teleost species (Bahr & Wilson, 2011). In the seahorse, only MHC class II-based female choice was identified, whereas males showed a preference for direct benefits (size of the mating partner). This discrepancy may have evolved due to the differences in the genomic architecture with MHC class I and class II both being present in seahorses, whereas only MHC class I is functional in pipefish (Haase *et al.*, 2013). On the other hand, our model pipefish *Syngnathus typhle* has a polygamous mating system, whereas most seahorses live monogamously. This implies that even though males brood the eggs, seahorses are not male limited and thus have conventional sex roles (Wilson *et al.*, 2003; Wilson & Martin-Smith, 2007). Sex role reversal in combination with polygamy will intensify sexual selection on females (Jones *et al.*, 2000) and can furthermore induce strong selection for male mate choice mechanisms, such as the reported MHC-mediated mate choice in *S. typhle*.

Our experimental set-up also included visual cues for direct benefits and, possibly, good genes [i.e. female size (Sundin *et al.*, 2010)], since mate choice was performed under different degrees of visibility simulating deterioration of visibility in the natural habitat (Orth *et al.*, 2006; Wennhage & Pihl, 2007; Sundin *et al.*, 2011, 2013). Whereas size of partners mattered under full visibility conditions, this trait decreased in importance under impaired visibility (Sundin *et al.*, 2010). However, choice for MHC sequence dissimilarity persisted as long as olfactory cues were present. Hence, in line with our initial hypothesis, not only partner size is important for mate choice decisions (reviewed in Rosenqvist & Berglund (2011)), but that the decisions also depend on a complex combination of direct benefits (body size) and genotype-dependent benefits of compatible genes (MHC). We may not be able to finally resolve the relative importance of both mechanisms for Darwinian fitness with this study, but our data clearly indicates that when vision is obstructed, choice for a large animal is absent, whereas choice for distinct MHC class I genotypes persists.

In conclusion, we found support that selection for male mate choice in sex-role-reversed species can be sufficiently strong to utilize MHC class I-mediated choice, optimize offspring immunity and trade off additive effects of good genes against condition-dependent effects of compatible (i.e. divergent) MHC genotypes. Ultimately, choice for optimal offspring immunity via MHC-dependent mating may even outweigh a pure choice for size, in particular if parasite prevalence is high.

Data accessibility

See Appendix S1 for sequences used for primer design and all MHC class I alleles of *S. typhle*. Microsatellites genotyping primers: GenBank Accession Numbers JQ598279–JQ598290.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Alignment of all sequences used for the design of primers for MHC class I (contigs, transcriptome_contigs out of Haase *et al*., 2013), cloned sequences (cloning) for 20 individuals, and all alleles identified in the experimental animals (St_MH classI).

Appendix S2 A correlation between the intra-individual MHC genetic distance (*x*-axis) with proliferating lymphocytes (proportion of cells in S phase) (left *y*-axis, black o symbols, dashed line) and with the phagocytosis activity (area under curve in a respiratory burst assay) (right *y*-axis, grey Δ symbols, solid line).

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