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Costly major histocompatibility complex signals produced only by reproductively active males, but not females, must be validated by a 'maleness signal' in three-spined sticklebacks

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Olfactory information about individual major histocompatibility complex (MHC) immune genotypes is important for mate choice in several species. For example, during the mate choice decisions of three-spined sticklebacks, females assess males on the basis of odour cues that convey information about their MHC diversity. Here, we show that an additional 'maleness' signal is needed to validate the MHC signal. Furthermore, using interaction between natural odour of sticklebacks and synthetic MHC-ligand peptides, we show that MHC signals are conditional on the reproductive state in males. By contrast, we find that gravid females do not produce such signals. Since MHC olfactory signals relevant to mate choice decisions are conditional upon gender and reproductive state, we suggest that their manufacture is likely to be costly to senders, and therefore, potentially conditional on the health/parasitization status of the sender. We hypothesize that shedding of peptide–MHC complexes compromises immune function, selecting against unconditional use of these signals.

Keywords: major histocompatibility complex; mating decision; olfaction; stickleback

1. INTRODUCTION

Sexual selection has been proposed as one mechanism to maintain the extraordinarily high degree of sequence polymorphism at immune genes such as the major histocompatibility complex (MHC) (Penn & Potts 1999; Bernatchez & Landry 2003; Mays & Hill 2004; Milinski 2006). Besides their immune function (Janeway *et al.* 2005), MHC genes are known to influence both body odour and reproductive behaviour (Penn & Potts 1998; Milinski 2006). MHC-based sexual-selection strategies are known to involve olfactory mechanisms in fish (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Milinski *et al.* 2005), lizards (Olsson *et al.* 2003), mice (Yamazaki *et al.* 1976; Singh *et al.* 1987; Penn & Potts 1999) and humans (Wedekind *et al.* 1995; Jacob *et al.* 2002; Thornhill *et al.* 2003). Recent studies have suggested a role for short peptides with general features of MHC ligands as part of the natural odour signals in mice and fish (Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005; Slev *et al.* 2006).

Male three-spined sticklebacks (*Gasterosteus aculeatus* L.) build nests into which they try to attract as many gravid females as possible during the few days before they start guarding the eggs (Wootton 1976). Since, under natural conditions, a male's reproductive success increases with the number of egg clutches he receives (Kraak *et al.* 1999), it is not surprising that males are not selective when approached by a single gravid female, which seems to be the normal situation (Rowland 1982). When presented simultaneously with two gravid female stickleback dummies that differ in fecundity signals, males prefer the more fecund females (e.g. Rowland 1982; Bakker & Rowland 1995; Kraak & Bakker 1998 used real females). Furthermore, males invest in costly signals to be chosen as mates (Milinski & Bakker 1990, 1992). Because of their usually lower potential reproductive rates female three-spined sticklebacks are, as predicted (Clutton-Brock & Parker 1992; Wootton *et al.* 1995; Johnstone *et al.* 1996), highly selective and, during mate choice, evaluate a number of olfactory (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; McLennan 2003; Milinski *et al.* 2005; Rafferty & Boughman 2006; Sommerfeld *et al.* 2008) and costly visual (McLennan & McPhail 1990; Milinski & Bakker 1990, 1992; Bakker *et al.* 1999) male cues. For instance, they assess male MHC-dependent olfactory cues in order to achieve an optimal degree of MHC diversity in their offspring (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Milinski *et al.* 2005) that equips them with maximal resistance towards pathogens (Wegner *et al.* 2003, 2008) and maximizes their lifetime reproductive success (Kalbe

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et al. 2009). This optimal MHC diversity has been predicted to occur as a consequence of a trade-off between a high probability for presentation of pathogen-derived peptides and a high probability that resulting peptide–MHC complexes are recognized by T-cells (Nowak *et al.* 1992; Borghans *et al.* 2003; Woelfing *et al.* 2009). An optimal individual MHC diversity has subsequently been demonstrated in various species (Wegner *et al.* 2003; Bonneaud *et al.* 2004; Madsen & Ujvari 2006; Forsberg *et al.* 2007).

Here, we ask whether the elaboration of the MHC odour cue is costly to sticklebacks. One way to approach this problem is to assess whether or not the MHC-associated signal is produced constitutively or only when needed (conditional production). In the latter case, sticklebacks can accrue a benefit despite associated cost. Under this scenario, two predictions can be made. First, males should only send the MHC signal when they are reproductively receptive, i.e. actively maintain and 'glue' (Wootton 1976) a nest. Second, females should have no incentive to pay the cost of producing it, because male sticklebacks are usually not choosy. On the other hand, evaluating the mixture of her and his MHC signal might enable the female to test directly for the optimal complement potentially provided by the male. So she might send the signal because it helps her to select the right male. Here, we use a previously developed experimental interaction analysis (Milinski *et al.* 2005) to examine these predictions.

During mate choice, gravid female sticklebacks evaluate MHC diversity of prospective mates via an olfactory mechanism. The quality of the signal associated with MHC diversity (henceforth referred to as 'MHC signal') can be predictably modified by synthetic MHC peptide ligands, suggesting that peptides form at least part of the natural MHC signal (Milinski *et al.* 2005): For a mating pair with suboptimal numbers of MHC alleles, adding synthetic peptides and thereby simulating the possession of additional MHC alleles increases the attractiveness of male water for the choosing female, whereas for a mating pair with optimal or super-optimal numbers, attractiveness is decreased. Thus, females avoid males who provide either a sub- or a super-optimal complement to the female's MHC diversity.

In the present study we used males who already provide the optimal MHC complement for the choosing female. Adding synthetic peptides should mimic a super-optimal complement and thus decrease the male's attractiveness, but only if the male sends his MHC signal. However, if the male does not send an MHC signal, adding peptides to his odour should increase his attractiveness, because the peptide signal should be more attractive than no MHC signal. Thus, a negative or positive response of the choosing female to added peptides allows discrimination between two possibilities: whether the male's odour contains the MHC signal or not. This type of interaction analysis was exploited here as a test to examine the presence or absence of the MHC signal under various conditions.

2. MATERIAL AND METHODS

(a) *Animals*

Adult three-spined sticklebacks were caught in the Grosse Plöner See in January 2005 and kept in the laboratory, first under spring (12°C, 12 h light/12 h dark) and then summer (18°C, 16 h light/8 h dark) temperature and light conditions, the latter for a maximum of 6 weeks before use in the experiments. Under summer conditions, fish were fed a rich diet of live food, i.e. *Tubifex*, chironomids, *Chaoborus, Daphnia*. MHC class IIB alleles were determined for all individual fish as described previously (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Wegner *et al.* 2003). All stimulus males had developed a bright breeding coloration and had built a nest. Every day each male was shown ('stimulated' with) a gravid female in a separate tank (same size as in Milinski & Bakker 1990) positioned in front of a male's tank for 5 min. We observed whether the male glued during these 5 min. All males had previously been reproductive, at least for a while, and had maintained a nest. We hypothesized that when males stop maintaining their nest it would make sense to stop producing the MHC signal if it is costly, or, alternatively, to give up maintaining the nest, when they can no longer afford to produce the costly signal. Therefore, we predicted that while the signal would still be present within two days of the male giving up maintenance of the nest, males who had not maintained their nests for two weeks would have stopped producing the signal.

(b) *Mate choice tests*

Female sticklebacks that were ripe for spawning were placed in a flow chamber that was fed by two water columns, to each of which stimulus water (1 l during 600 s) was continuously added, under conditions of laminar flow as previously described (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Milinski *et al.* 2005). Fish were able to freely investigate the composition of water in the two halves of the chamber for two periods of 300 s each, with spatial reversal of water sources at halftime. Their choice in the chamber was video-recorded from above. There were lines drawn on the screen of the monitor, by which it was divided either in front and back quarters. We measured the time the tip of the female's nose spent in a each front quarter. If the two sources were equally attractive, the fish should spend an equal period of time (i.e. about 150 s) with each source of stimulus. Odour preference, as determined in the flow channel set-up, reliably predicts mate choice (electronic supplementary information of Milinski *et al.* 2005).

(i) *First experiment*

Single females were tested in different treatments during one day. Stimulus water samples were taken just prior to the experiment. Stimulus water was taken from (i) the tank at about 2 cm above the nest of a single male, which had not maintained (i.e. glued, see below) its nest on the day of testing, but either 2 days before/within 2 days after or two weeks before the test; (ii) the tank of a gravid female; (iii) the tap supplying the flow tanks. In each treatment the stimulus water was used both as peptide-supplemented stimulus water (after addition of synthetic peptides in solvent) continuously added to one half, and control water (after addition of solvent only) added continuously to the other half of the flow channel as described (Milinski *et al.* 2005). The concentrations of solvent and the volume of supplement added to the water columns were identical on both sides in all treatments (i–iii). The code for peptide/solvent tubes was broken only after the data had been analysed. Test females were selected such that their genotypes were close to the optimal combined number of different MHC class IIB alleles

of 10.4 alleles (Aeschlimann *et al.* 2003; Milinski 2003) with both the stimulus male (9.75 ± 0.18 alleles, mean $\pm$ s.e.) in (i) and the stimulus female (9.77 ± 0.18 alleles) in (ii). The sequence of treatments (i), (ii) and (iii) was randomized among test females. For the last treatment (iv), the test female was presented only with un-spiked (no peptides supplemented) stimulus water from the tank of the stimulus male from (i) on both sides of the flow tank. All test females were used for only one set of treatments (i)–(iv) and all stimulus sources were used with only one test female. According to our study protocol, three such sets could be repeated (once the females were gravid again) on days when no new sets of previously untested fish were possible; data of replicated sets were entered as mean values to avoid pseudo-replication (Milinski 1997). Retesting and averaging the results increases the reliability of data from single test fish.

(ii) *Second experiment*

Single females were tested in three different treatments during one day. (i) Stimulus water was taken from the tank of a single male, which had maintained (i.e. glued) its nest on the day of testing, and from the tank of a gravid female, each to be added to one arm of the flow tank, respectively. Animals producing the stimulus specimens were selected such that the males (8.06 ± 0.30 alleles) were below and the females (9.44 ± 0.66 alleles) were closer to the optimal combined number of different MHC class IIB alleles of 10.4 alleles (Aeschlimann *et al.* 2003). (ii) Water taken from the tank of the stimulus male was mixed either with an equal volume of tap water or water taken from the tank of the stimulus female. All triplets had been selected such that the three fish together were closer (11.06 ± 0.35 alleles) to the optimal combined number of different MHC class IIB alleles of 10.4 alleles (Aeschlimann *et al.* 2003) than the test female with only the stimulus male (8.06 ± 0.30 alleles). The sequence of treatments (i) and (ii) was randomized among test females. All test females were used for only one set of (i)–(ii) and all stimulus specimens were used with only one test female. The bottles with stimulus water were prepared and coded such that the experimenter was blind with respect to the side of the flow tank to which each type was added. In both experiments bottles had been cleaned with hot tap water.

(c) *Derivation of peptides*

We used a mixture of four peptides SYIPSAEKI, SFVDTRTLL, ASNENMETM and AAPDNRETF as before (Milinski *et al.* 2005). Peptides were chemically synthesized, purified, verified by mass spectroscopy (MALDI-TOF) and dissolved in phosphate-buffered saline (PBS).

(d) *Statistical analysis*

Statistical analysis was performed with StatViewTMSE, SAS Institute Inc. 1998. We used very simple statistics, e.g. paired or unpaired *t*-tests, when conditions were met. We used two-tailed tests throughout.

3. RESULTS

First, we examined whether the MHC signal depends on the reproductive state of male sticklebacks. To this end, water from a tank of males that had not glued nests for at least 14 days, i.e. that were in a non-reproductive state, was used. We then tested whether females preferred plain male water or male water spiked by the addition of a mixture of four synthetic peptides (as in Milinski *et al.* 2005). The number of different alleles for paired females and males was chosen close to the previously determined optimum number of alleles (Aeschlimann *et al.* 2003). Under these conditions, our previous results (Milinski *et al.* 2005) predict that, in the presence of an MHC signal in the water from non-reproductive males of the present study, the female should prefer the plain water source, because the additional peptides serve to increase the apparent near optimal MHC diversity to super-optimal levels, which would be avoided. Surprisingly, however, females preferred the spiked water source ($t = 5.149$, $n = 7$ pairs, $p = 0.0021$, using a two-tailed paired *t*-test; figure 1 left), suggesting the absence of an MHC signal in water from a non-reproductive male. In the absence of an MHC signal, the spiked side mimics a male with four MHC alleles, which is closer to the optimum than an apparent 0 alleles at the un-spiked side.

When other females were offered the same choice (spiked versus un-spiked) but with stimulus water from males that had stopped maintaining (i.e. glueing) their nests 2 days before the test, or did not begin to glue their nests until at least 2 days after the test, females tended to prefer the un-spiked water ($t = -2.493$, $n = 6$ pairs, $p = 0.0549$, using a two-tailed paired *t*-test; figure 1 middle). The preference for the un-spiked water source, i.e. time in 'spiked quarter'/time in 'un-spiked quarter', of these males was significantly different from the preference for the un-spiked water of males who had not maintained a nest for at least two weeks (figure 1 left, middle; $t = 3.800$, $n_1 = 7$ pairs, $n_2 = 6$ pairs, $p = 0.0029$, using a two-tailed unpaired *t*-test), showing that the water from the males' tanks now sometimes contained an MHC signal. Our previous results (right half of fig. 3*a* in Milinski *et al.* 2005), where stimulus males were actively maintaining their nests on the day of the test are shown for comparison in figure 1 right; here, females also preferred the un-spiked water ($t = -3.646$, $n = 8$ pairs, $p = 0.0082$, using a two-tailed paired *t*-test). In all previous studies (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Milinski *et al.* 2005) we have measured the female's preference for the spiked versus un-spiked 'half' of the tank. We have re-analysed all video records for 'time in front quarters' as requested by one reviewer; however, for comparison with previous data, all significant differences shown in figures 1 and 2 are also significant when analysed for 'halves'. The preferences reported in figure 1 middle and right are not statistically distinguishable ($t = 0.505$, $n_1 = 8$ pairs, $n_2 = 6$ pairs, $p = 0.6225$, using a two-tailed unpaired *t*-test). Collectively, these data indicate that the presence of an MHC signal coincides with maintenance of a nest, strongly suggesting that the MHC signal may be conditional upon the reproductive state of males.

Second, we asked whether gravid females also send an MHC signal. Using the test applied above, we found that test females did not significantly distinguish between the spiked and the un-spiked front quarter when female stimulus water was used ($t = -0.440$, $n = 13$ pairs, $p = 0.67$, using a two-tailed paired *t*-test; figure 2 middle). This result suggests that gravid female sticklebacks ignored supplemental peptides that were added to water from a tank of a gravid female stickleback.

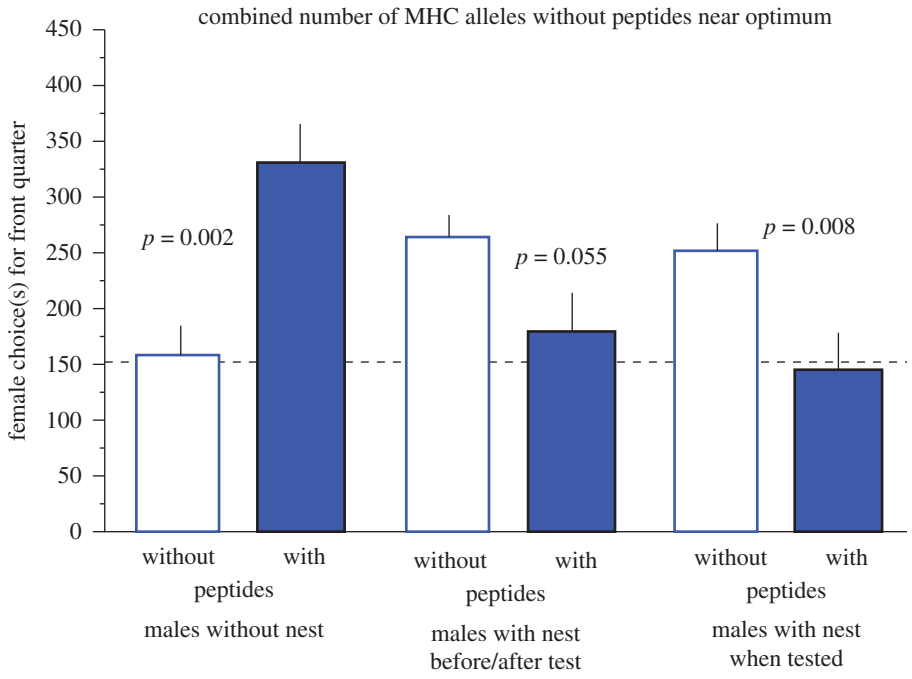

Figure 1. Male MHC signals are conditional upon reproductive state. Mean (+s.e.) time (of a total of 600 s) females spent in each of the two front quarters of a flow tank to which water from the tank of a male providing about optimal complementation of MHC class IIB alleles was added as plain water (open) on one side and spiked with four different 9-mer MHC ligand peptides (blue) on the other side. (Left) Non-reproductive males that had not maintained (glued) their nests for at least 14 days; (middle) non-reproductive males that had stopped maintaining their nests two days before the test, or did not begin to glue nests until two days after the test; (right) reproductive males that maintained their nests on the test day (data (right) taken from Milinski *et al.* 2005). Stippled line depicts expectation for no preference, i.e. random choice (150 s) of each front quarter of the tank (see text for statistics).

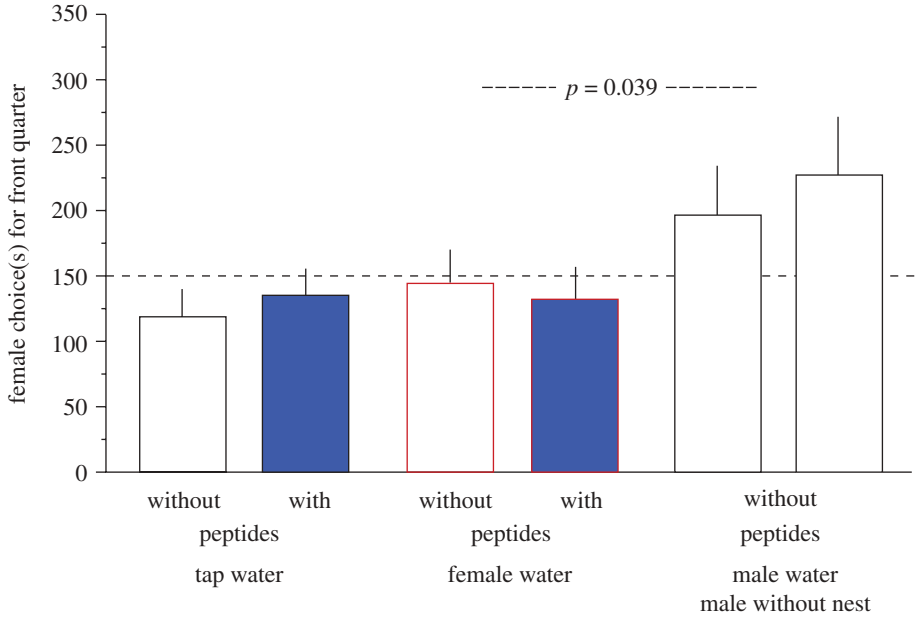

Figure 2. Which kind of signal do gravid females produce? Mean (+s.e.) time (of a total of 600 s) females spent in the front quarters of a flow tank to which (left) tap water was added plain (white) on one side and spiked with four different 9-mer MHC ligand peptides (blue fill) on the other side; (middle) water from the tank of a gravid female providing about optimal complementation of MHC class IIB alleles was added as plain water (red) on one side and spiked with four different 9-mer MHC ligand peptides (blue fill) on the other side; (right) water from the tank of a non-reproductive male providing about optimal complementation of MHC class IIB alleles was added as plain water (blue) on both sides, left and right side shown; stippled line depicts expectation for no preference, i.e. random choice (150 s) of each front quarter of the tank (see text for statistics).

Likewise, no significant preference was found when gravid females were offered the choice between spiked and un-spiked tap water ($t = 0.637$, $n = 13$, $p = 0.54$, using a two-tailed paired t-test; figure 2 left). Furthermore, the female's presence in the two front quarters of the tank in these two experiments was close to random expectations (stippled line in figure 2), suggesting that both plain tap water and spiked tap water were

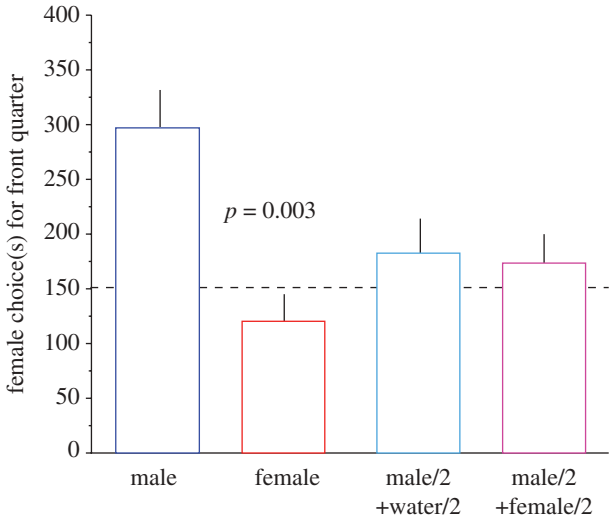

Figure 3. Interaction of potential male and female MHC signals. Mean (+s.e.) time (of a total of 600 s) females spent in each front quarter of a flow tank to which water from the tank (left) of a reproductive male providing suboptimal complementation of MHC class IIB alleles (blue) was added on one side and water from the tank of a gravid female providing almost optimal complementation of MHC class IIB alleles (red) was added on the other side; (right) of a reproductive male providing suboptimal complementation of MHC class IIB alleles diluted with equal volumes of either tap water (light blue) on one side or female water (pink) on the other side. The genotypes of the fish were chosen such that male plus choosing female were suboptimal, whereas the triple combination (i.e. plus the contribution of the second gravid female) was always closer to the optimum. Stippled line depicts expectation for no preference, i.e. random choice (150 s) of each half of the tank (see text for statistics).

treated as being neutral. Although these results give no hint that gravid females send MHC signals, it is also possible that female water contains the MHC signals but lacks a second signal that in male water validates MHC signals. We postulate the existence of a 'maleness' signal that validates the peptide signal of male water; this signal would be missing in both spiked tap water and potentially in water taken from the tank of a gravid female. We would expect that she sends the MHC signal only if she needs to evaluate the mix of her MHC signal with that of the male's.

In order to examine whether male water indeed contains such a presumptive MHC-independent attractive factor, a 'maleness' signal, females were presented with water from the tank of males who had not maintained their nest for at least two weeks, for which we had found evidence that they lack the MHC signal. The two stimulus water sources in channels of the flow tank were not spiked with peptides. In this situation, females spent significantly more time in the front quarters of the tank (figure 2 right), indicating that this water was more attractive than female water ($t = -2.24$, $n_1 = 13$, $n_2 = 6$, $p = 0.039$, using a two-tailed unpaired *t*-test).

Hence, the possibility remains that females may send the MHC peptide signal but not an additional signal validating it. To test for such cryptic MHC signals in female water, we used mixed female and male water sources, to supply female water with the presumptive 'maleness' signal. To this end, we first analysed a preference test between water of a MHC suboptimal male who maintained his nest on the test day (and thus should send both the MHC and the maleness signal) and the water of an almost optimal gravid female. Test females preferred the male water in the flow tank (figure 3 left; $t = 4.111$, $n = 9$, $p = 0.0034$, using a two-tailed paired *t*-test) extending our previous findings (figure 2 middle, right). In the next analysis, the suboptimal male's water was diluted with equal volumes of either tap water or female water. With respect to MHC diversity, the contributions of the male and that of the choosing female are invariant in this experiment; the difference lies in the presence or absence of female water in one arm of the flow channel. If female water lacks the MHC signal it should have the same effect as tap water and the two sides would have the same attractiveness. The genotypes of the fish were chosen such that male plus choosing female were sub-optimal (as in figure 3 left), whereas the triple combination (i.e. plus the potential contribution of the stimulus female) was closer to the optimum. If the female water contains the MHC signal, the combination of male and female water should be preferred to the combination of male plus tap water. Under these conditions, there was no significant preference for either side (figure 3 right) ($t = 0.150$, $n = 9$, $p = 0.8847$, using a two-tailed paired *t*-test). This result suggests that the odour of the gravid female indeed lacks an MHC signal.

4. DISCUSSION

Collectively, our results suggest that male and female sticklebacks do not constitutively produce MHC signals. Rather, its production by males is conditional upon their reproductive state, whereas reproductive females seem to lack the signal. Our previous experiments showed that MHC class II diversity makes a major contribution to the MHC signal assessed during mate choice (Milinski *et al.* 2005). Since peptide ligands, rather than the complete MHC–peptide complexes are the likely MHC signals as indicated by the interaction analyses (Milinski *et al.* 2005) and the results obtained with mice (Leinders-Zufall *et al.* 2004), peptides must be liberated from MHC complexes situated at the cell membrane to become available for assessment. Therefore, proteolytic shedding of MHC class II molecules induced as a consequence of the physiological changes during sexual maturation would explain the conditional presence of MHC signals in tank water of reproductive male sticklebacks. If this were true, one would expect to see defined degradation products of MHC class II molecules in bodily fluids (i.e. urine) as a result of this inducible shedding mechanism. Indeed, such MHC fragments have been found in the urine of rodents (Singh *et al.* 1987). According to the results reported above, such products should appear in the urine only of reproductive stickleback males, but not in that of non-reproductive males or reproductive females.

Our behavioural data provide compelling evidence that MHC-dependent odour signals are conditionally produced by male three-spined sticklebacks only when they are in a reproductive state. Males that have not recently glued or are not close to maintaining and gluing nests do not send such signals. This is in general agreement with earlier observations that both stickleback (Häberli & Aeschlimann 2004) and round goby (Gammon *et al.*

2005) females exert an olfactory preference for reproductive over non-reproductive males, although the role of the MHC was not investigated in these earlier studies. To the best of our knowledge, our present results are the first demonstration that the production of olfactory MHC signals relevant to mate choice decisions is conditional implying that they are costly to senders. We thus speculate that less healthy or parasitized males can send only a weak or no MHC signal before they give up maintaining their nests.

The MHC signal, i.e. MHC-ligand peptides, is not recognized if not combined with a 'maleness' signal validating it. If the olfactory MHC signalling system is indeed conserved in all jawed vertebrates (Boehm 2006), it can be neither species-specific nor individualized: a female stickleback tracking an MHC signal in a lake cannot tell whether the signal had been sent by a single pike or jointly by a pike and a stickleback and might approach a pike instead of a stickleback. It is therefore conceivable that the MHC signal is combined with an odour cue signalling at least the species' identity of the sender. It has been shown that female three-spined sticklebacks discriminate in favour of their own males when asked to choose between conspecific and heterospecific odours (McLennan 2003). The nature of the maleness signal is, however, elusive.

Our results strongly suggest that reproductive females do not send an MHC signal: reproductive females ignored MHC ligand peptides added to female water as they did with MHC ligand peptides added to tap water. However, they might send the MHC signal without a validating factor. In this case female water should affect the MHC signal from males that send both the MHC signal and the signal validating it. However, the MHC signal of a reproductive male offering a suboptimal complement for the choosing female could not be 'spiked' successfully with female water. This latter result suggests that reproductive females do not send an MHC signal. Because we assume that the validating male signal is species-specific, males themselves should have problems distinguishing between conspecific and heterospecific females approaching them. A recent study (Kozak *et al.* 2009) found that male three-spined sticklebacks did not prefer conspecific females, which corroborates our results.

It is interesting to note that pregnancy failure was not induced by the presentation of a MHC ligand peptide that did not belong to the male that initiated the pregnancy, i.e. the 'Bruce-effect', when presented alone to the pregnant female; however, pregnancy failure was induced readily when this 'unfamiliar male MHC peptide' was presented together with the urine of her mate (Leinders-Zufall *et al.* 2004). The urine of the familiar male alone does not induce pregnancy failure. This shows that in order to induce pregnancy failure in a mouse, the MHC-peptide signal has to be validated by an additional male olfactory signal, which is contained in his urine. Thus, both in sticklebacks and in mice, the MHC odour signal needs an additional odour signal to be validated to the receiver.

Because of our failure to find a MHC-signal sent by reproductive females, we have to reject the hypothesis that evaluating the mixture of her and his MHC signal might enable the female to test directly for the optimal complement potentially provided by the male. Thus, the method the female uses to compare her and his MHC genetics remains elusive. Would a male profit from evaluating a female MHC signal? In a situation where two gravid females simultaneously approaching a male, i.e. a situation where males have been shown to be choosy (e.g. Rowland 1982), the male could only smell the combined MHC signals of the two females if they were to send those signals. Since a male has nothing to gain from smelling out the MHC mix of two females, males would not profit from evaluating MHC signals here. This is corroborated by Rowland (1994): 'In a pilot study, I presented territorial male three-spine stickleback with two visually identical dummies, each fitted with a fine plastic tube that permitted water to flow slowly from the ventral opening. When water from a jar containing a receptive, gravid female was allowed to flow from one dummy, males showed no obvious difference in response to this dummy compared with response to the dummy through which plain tank water flowed.' If there is just one gravid female, he is not expected to be choosy anyway (Kraak *et al.* 1999).

How might MHC signals incur fitness costs to senders? It has been known for a long time that the immune system essentially depends on MHC class II expression for proper immune surveillance and function (McHeyzer-Williams & McHeyzer-Williams 2005). MHC class II deficiency renders vertebrates vulnerable to infections by various pathogens and parasites (Nekrep *et al.* 2003). In view of this, shedding MHC class II complexes into the excretory system can be expected to cause localized MHC class II deficiency. We propose that male sticklebacks minimize the ensuing risks by sending MHC signals only when they are ready to reproduce. In this way, they would maximize the benefit/cost ratio of reproduction. This would agree with assumptions of the 'immunocompetence handicap hypothesis' (Folstad & Karter 1992; Kurtz *et al.* 2007), which suggests a trade-off between costly sexually selected ornaments and the immune system. Males, whose immune system is activated by an infection may not be able to produce excess MHC for signalling purposes and may thus stop maintaining their nest. An intermediate state may exist when infected males maintain a nest but send only weak MHC signals. On the other hand, the MHC signal is appreciated by selective females not (only) because it is costly but also because it transmits information about the sender's MHC diversity, which might or might not complement the female's. If MHC signals cannot be produced cost-free, we should expect conditional MHC signalling to occur also in other vertebrates, including humans.

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