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Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism

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The origin and maintenance of polymorphism in major histocompatibility complex (MHC) genes in natural populations is still unresolved¹. Sexual selection, frequency-dependent selection by parasites and pathogens, and heterozygote advantage have been suggested to explain the maintenance of high allele diversity at MHC genes^{2–4}. Here we argue that there are two (non-exclusive) strategies for MHC-related sexual selection, representing solutions to two different problems: inbreeding avoidance and parasite resistance. In species prone to inadvertent inbreeding, partners should prefer dissimilar MHC genotypes to similar ones. But if the goal is to maximize the resistance of offspring towards potential infections, the choosing sex should prefer mates with a higher diversity of MHC alleles. This latter strategy should apply when there are several MHC loci, as is the case in most vertebrates^{5,6}. We tested the relative importance of an ‘allele count-

ing’ strategy compared to a disassortative mating strategy using wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) from an interconnected system of lakes. Here we show that gravid female fish preferred the odour of males with a large number of MHC class-IIB alleles to that of males with fewer alleles. Females did not prefer male genotypes dissimilar to their own.

Sexual selection, the preference of certain mating partners over others, is ubiquitous among animals^{6,7}. The choosing sex may be able to increase the attractiveness of offspring, or gain direct benefits such as parental care⁸. Another function of mate choice is to increase the fitness of offspring by either choosing ‘good genes’, or avoiding incompatible ‘bad genes’—for example in matings with close kin. Particularly suited for testing the idea of choosiness with respect to genes is the MHC, a multigene family that is important in controlling the vertebrate immune system by presentation of self and foreign peptides to T cells². MHC alleles confer specific resistance against pathogens and parasites. Therefore, mate choice should increase the fitness of offspring by maximizing the heterozygosity at MHC loci^{1,4,9,10}, allowing a wider spectrum of pathogens to be recognized during early infection.

The focus of previous studies was disassortative mating, that is, the preference of dissimilar males or females as a mating partner^{11–13}, as a means of inbreeding avoidance, or in order to increase the heterozygosity at MHC loci^{3,14}. In the context of sexual selection, it has not been taken into account that most vertebrates possess several MHC loci⁵. As a result, there are many possible combinations of alleles at different loci². The chances of choosing a partner with identical MHC haplotypes become very unlikely because the combination of alleles at multiple loci renders the expected likelihood of existing MHC genotypes very small, even under linkage disequilibrium. A mechanism of sexual selection focusing on the distinction between similar and dissimilar MHC genotypes becomes inefficient when increasing parasite resistance is important. Females should rather choose partners that maximize the number of different MHC alleles in their offspring³.

We studied populations of the three-spined stickleback (*Gasterosteus aculeatus*) where we identified high MHC diversity for partial sequences of MHC class-IIB loci, coding for the peptide-binding region. At an estimated six loci¹⁵, we identified 24 distinct sequences in only eight fish from a system of interconnected populations, and many more alleles were identified on the basis of single-strand conformation polymorphisms (SSCP) in a total of 144 fish. We also observed marked differences in the number of different alleles per individual fish, varying between two and eight detectable alleles across all loci (Fig. 1). For one location (Schöhsee) we calculated that the chance a female has of mating with an MHC-identical male is only 1% if she mates at random. But the probability of choosing a mate with fewer alleles is 46% under random expectations (see Methods). Therefore, we predicted that females

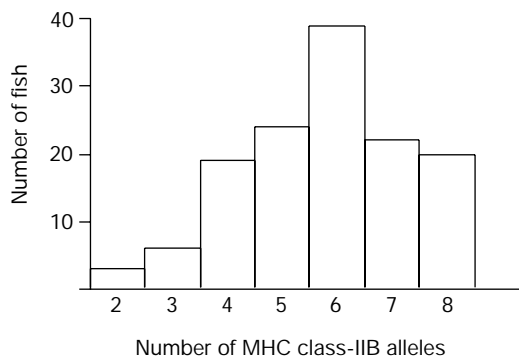


Figure 1 Frequency distribution of the number of MHC class-IIB alleles (peptide-binding region) detectable by SSCP in 144 fish from one population (Schöhsee). The mean number of MHC alleles \pm s.e. was 5.8 ± 0.13 .

would choose according to the number of different MHC alleles in males rather than according to similarity/dissimilarity of genotype.

MHC genes influence individual body odour^{16–19}, which is used as a cue for dissimilarity in the mate choice of mice¹¹ and humans¹³. Therefore, we assessed MHC-related mating preference through odour-related behaviour of gravid fish in a flow chamber.

We tested whether females preferred the odour of males with many different MHC class-IIB alleles to the odour of males with few MHC alleles. We presented gravid females with water for the tanks of two males that differed in the number of MHC class-IIB alleles. Males with ‘few’ alleles had 3–6 alleles, whereas those with ‘many’ displayed 6–8 alleles. In experimental combinations, male allele number differed by an average of 2.3. We found that choosing females preferred males with a higher number of alleles over those males with few alleles (paired *t*-test: *t* = 2.39, *n* = 29, *P* = 0.023; Fig. 2a). Since inbreeding avoidance can be ruled out as a mechanism of mate choice (see below), the results support a parasite-driven mechanism¹⁴ that maintains the observed diversity of MHC alleles.

In a second experiment, we tested for disassortative mating. Accordingly, we examined whether females prefer the odour of males that differ in their MHC alleles from the female to that of males that share all alleles with the female, with males having the same number of MHC class-IIB alleles as the female. Female sticklebacks did not prefer the odour of MHC class-IIB dissimilar males (sharing as few alleles as possible with the female) to the odour of MHC class-IIB identical males (*t* = 0.34, *n* = 21, *P* = 0.738, statistical power = 0.802, effect size taken from previous experiment; Fig. 2b). These results indicate that disassortative mating is less important than the strategy of female allele counting in a natural population of sticklebacks.

We can exclude the possibility that preferred males were less related to the females than unpreferred males. Over both experiments, we calculated pairwise relatedness coefficients among all pairs of experimental fish on the basis of microsatellite polymorphism. There was no significant correlation among preference time and individual pairwise relatedness (*n* = 46, correlation coefficient, *r*² = 0.04, *P* = 0.19).

Modelling studies^{3,20} indicate that there is an optimal number of MHC alleles, since too many different alleles increase the risk that self-peptides will mount harmful autoimmune responses. Accordingly, we find that females with 7 and 8 alleles tend to be less choosy of males with more alleles than are females with 3–6 MHC class-IIB alleles (analysis of variance, ANOVA, among all categories of female

fish possessing 3–8 different alleles, response variable ‘time spent on side of male with more alleles’, *P* = 0.037; subsequent contrast 3–6 alleles versus 7 and 8 alleles, *F*_{1,27} = 3.2, *P* = 0.08).

Choosing many rather than few alleles may be a strategy to maximize the general genomic heterozygosity of offspring and not the diversity specifically at MHC loci. However, the number of detectable MHC class-IIB sequences per individual was not correlated with genome diversity, measured at seven microsatellite loci (multi-locus heterozygosity: *r*² = 0.0004, *P* = 0.8; mean *d*², a measure of microsatellite heterozygosity²¹: *r*² = 0.016, *P* = 0.3; both *n* = 124).

The experimental set-up enabled us to test odour preference, as a precursor of mate choice^{13,16,17,19}. There are strong indications that female sticklebacks were indeed choosing mating partners in the experimental runs. Most females spawned spontaneously immediately after the experiments, even in the absence of a male.

The observed MHC-related mating patterns may be direct or indirect. An alternative, but less likely, interpretation of indirect MHC-correlated preference is that fish with high overall MHC class-IIB heterozygosity were less infected with parasites, and that this can be smelled by females. However, experimental males were not infected with parasites that are known to have a pronounced impact on their fitness²¹. Furthermore, screening of 263 wild-caught fish revealed no simple correlation between infection status and MHC class-IIB allele number (K. M. Wegner, T.B.H.R. and M. Kalbe, unpublished work).

Which of the two proposed mate-choice strategies that are associated with MHC polymorphism is more important will depend on the natural population structure of the species studied. In sticklebacks, the chances of mature fish mating accidentally with close kin are low²². On the other hand, in species (mouse, rat, human) where avoidance of similar MHC genotypes (that is, disassortative mating) has been found, the social genetic structure introduces the danger of encountering close kin as a mating partner^{11,12}. Inbreeding avoidance through MHC-related choice is facilitated in species with extended parental care, such as mice, since progeny can learn the MHC odour of its kin by familial imprinting²³. In salmon (*Salmo salar*) adults return to their natal spawning grounds and may locally meet close kin. Nevertheless, there is no indication of inbreeding avoidance associated with MHC alleles²⁴. Salmon possess only one MHC class-IIB locus, so no preference for allele number can be expected. Instead, mating patterns are nonrandom with respect to the distance of MHC alleles in terms of their amino-acid sequence. The inclusion of more species with different population structures will allow us to examine the relative proportion of both MHC-correlated mate choice strategies: disassortative mating and allele counting.

We have thus shown that in natural populations of fish, females will increase individual heterozygosity and hence the population-wide polymorphism at MHC loci, by choosing males with many alleles. We suggest that such a strategy is ubiquitous in vertebrates with several MHC class-IIB loci. □

Methods

MHC characterization

In order to characterize the MHC class-IIB genes of *G. aculeatus*, 96 sequences, spanning the complete exon 2 (coding for the peptide-binding region, PBR) were obtained from two populations of three-spined sticklebacks (Schöhsee, *n* = 6 individuals; Ascheberg, *n* = 2). On the basis of intron 3-length variation, we and others¹⁵ estimate that there are up to six MHC class-IIB loci in *G. aculeatus*. For cloning, we used all necessary precautions to avoid erroneous overestimations of allele diversity (that is, proof-read polymerase, plasmid preparation as sequencing template). The forward primer was: AAC TCC ACT GAG CTG AAG GAC ATC (ref. 15). The antisense primer (GCA GTA CAA GTA CCA GTC ATG TAC) was a consensus primer placed in intron 3 on the basis of published sequences¹⁵ and sequences from local populations. Sequences, which were obtained at least twice independently, were translated to amino-acid sequences and revealed a large homology to published MHC class-IIB sequences, but nevertheless represented new alleles in all cases. Amino-acid sequence divergences among alleles were between 2 and 29%. Sequences were deposited in GenBank under accession numbers AF395709–730.

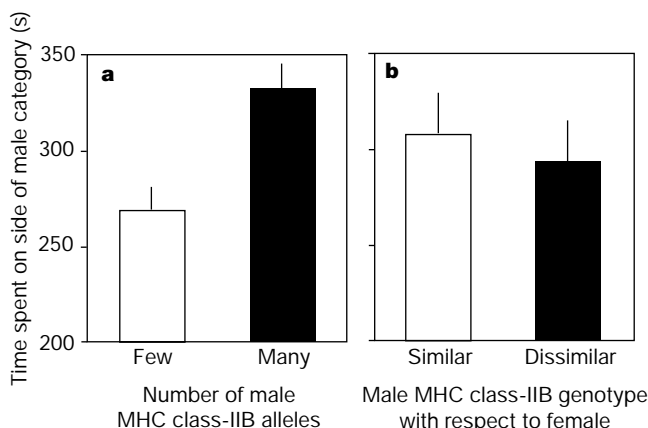


Figure 2 Odour preference of gravid female three-spined stickleback for males in a flow tank. **a**, Mean (+ standard error, s.e.) duration (in seconds) spent on side of male category with either few (2–6) or many (6–8) MHC class-IIB alleles (‘many’ always having more alleles than ‘few’); sample size *n* = 29. **b**, Mean duration (+s.e.) spent on side of male category sharing either all MHC class-IIB alleles (identical to female) or as little as possible (different from female); sample size *n* = 21.

SSCP genotyping

SSCP genotyping was performed using polymerase chain reaction (PCR) with exon 2 internal primers, amplifying a 120-base pair portion of the PBR, and subsequent capillary electrophoresis on an ABI310 automated sequeencer²⁵ with modifications. These were the inclusion of a second different reverse primer (GTT GTG CAG ACA GTA AAC CTC CTT C) to increase the number of alleles detectable with SSCP. Both primer pairs together amplified 17/24 (thus 74%) of the PBR that was identified by sequencing in the experimental fish. Isolated sequences cloned into plasmids were subjected to SSCP in order to standardize the fluorescent signals with sequence information. In three cases, complete PBR residues that differed only in 2–4 amino acids cannot be resolved by SSCP, such that our method underestimated the true MHC class-IIb diversity. In all cases, SSCP signals obtained from PCR of plasmids were also present in genotypes based on fish DNA.

Microsatellite genotyping, heterozygosity and relatedness

All fish of the first experimental block were genotyped for seven polymorphic microsatellites (GenBank accession numbers: AJ010352, -54, -55, -57, -58, -60; ref. 26), representing a total of 72 alleles. Individual heterozygosity was calculated as the number of different alleles per microsatellite locus, and as mean d^2 taking the length difference of alleles into account²⁷. We calculated a correlation between allele number of MHC class-IIb loci and individual heterozygosity. Relatedness coefficients R (ref. 28) were calculated for all pairs of fish using a program called Relatedness 5.0 (K. F. Goodnight, available from <http://gsoft.smu.edu/gsoft.html>). R coefficients were tested for correlation with female mating behaviour using the time difference of females among preferred/unpreferred male as variable for the female preference.

Permutation procedure

The risk that females will choose an MHC-identical male, or a male with fewer alleles, when mating completely at random was assessed in a permutation procedure. We combined 46 male and 46 female genotypes from Schöhsee 100 times at random, each time counting genotypic similarity and difference in allele number.

Sexual selection experiments

Adult three-spined sticklebacks were seine-netted from an interconnected natural system of large lakes near Plön, Germany, in spring. A bit of a dorsal spine of each fish was cut for MHC class-IIb and microsatellite genotyping (see above). Thereafter fish were housed in individual tanks (10 litres, continuous exchange of 1 litre per hour, 18°C, 18 h of light). Males (every second tank) were offered artificial nesting material. Only bright red males with completed nests were used for experiments.

Gravid females were tested in a flow channel²². In the test compartment (25 × 20 cm long, 10 cm water level) each female was offered the choice between the two sides of the current (1.4 cm s⁻¹), which was video-recorded from above. The inlet compartment, which was separated upstream by a net from the test compartment, was divided laterally into halves. A peristaltic precision pump (ISMATEC MV, 100 W, 50 Hz) supplied water (2 × 65 ml min⁻¹) taken from the tanks of each of two males with defined MHC II properties through silicon tubes to the halves of the inlet compartment in the sequence: 2 min neutral water, 5 min 'male' water, 2 min neutral water, 5 min 'male' water after switching sides. The bottles with the stimulus water had been coded by a third person. Each of 50 females was tested only once. Of the 57 males in both experiments, there were three combinations in the disassortative experiment which were tested twice.

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Illusory perceptions of space and time preserve cross-saccadic perceptual continuity

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When voluntary saccadic eye movements are made to a silently ticking clock, observers sometimes think that the second hand takes longer than normal to move to its next position¹. For a short period, the clock appears to have stopped (chronostasis). Here we show that the illusion occurs because the brain extends the percept of the saccadic target backwards in time to just before the onset of the saccade. This occurs every time we move the eyes but it is only perceived when an external time reference alerts us to the phenomenon. The illusion does not seem to depend on the shift of spatial attention that accompanies the saccade. However, if the target is moved unpredictably during the saccade, breaking perception of the target's spatial continuity, then the illusion disappears. We suggest that temporal extension of the target's percept is one of the mechanisms that 'fill in' the perceptual 'gap' during saccadic suppression. The effect is critically linked to perceptual mechanisms that identify a target's spatial stability.

Although most observers have experienced the 'stopped clock' illusion, previous psychophysical experiments that have tested when subjects perceive the time of transient external events relative to saccadic eye movements have yielded contradictory results^{2,3}. A