

'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*)

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According to the theory of mate choice based on heterozygosity, mates should choose each other in order to increase the heterozygosity of their offspring. In this study, we tested the 'good genes as heterozygosity' hypothesis of mate choice by documenting the mating patterns of wild Atlantic salmon (*Salmo salar*) using both major histocompatibility complex (MHC) and microsatellite loci. Specifically, we tested the null hypotheses that mate choice in Atlantic salmon is not dependent on the relatedness between potential partners or on the MHC similarity between mates. Three parameters were assessed: (i) the number of shared alleles between partners (x and y) at the MHC (M_{xy}), (ii) the MHC amino-acid genotypic distance between mates' genotypes (AA_{xy}), and (iii) genetic relatedness between mates (r_{xy}). We found that Atlantic salmon choose their mates in order to increase the heterozygosity of their offspring at the MHC and, more specifically, at the peptide-binding region, presumably in order to provide them with better defence against parasites and pathogens. This was supported by a significant difference between the observed and expected AA_{xy} ($p = 0.0486$). Furthermore, mate choice was not a mechanism of overall inbreeding avoidance as genetic relatedness supported a random mating scheme ($p = 0.445$). This study provides the first evidence that MHC genes influence mate choice in fish.

Keywords: mate choice; good genes; heterozygosity; major histocompatibility complex; Atlantic salmon

1. INTRODUCTION

The theory of sexual selection proposes that males are selected to enhance their reproductive success by mating with as many females as possible, whereas females are selected to maximize their reproductive success by choosing mates that will provide the best resources, genes or both (Bateman 1948; Trivers 1972). In species where males provide no direct material benefits, females should prefer males that will increase the genetic quality of their progeny (reviewed in Searcy 1982). Males can offer two types of genetic benefits to females. First, they can provide 'good genes' to offspring and, thus, increase their relative fitness. Second, males can be chosen in order to increase the genetic diversity within clutches (Loman *et al.* 1988; Yasui 1998). These two hypotheses are not mutually exclusive, since good genes do not necessarily correspond to the relative status of males but may be the genes that will allow females to increase the heterozygosity of their offspring (Brown 1997). Accordingly, in order to produce more viable progeny, a female need not only assess the quality of males' genes, but must also take into account how well they complement her own genes in order to ensure heterozygous offspring (Qvarnström & Forsgren 1998). Empirical support for the 'good genes as heterozygosity' hypothesis mainly stems from evidence of inbreeding avoidance (Keane 1990; Simmons 1991). However, as pointed out by Brown (1997), the nature of the genetic quality, in either terms of good genes or heterozygosity, has rarely been defined (but see Weatherhead *et al.* 1999).

Among the potential candidates for the genetic basis of mate choice in vertebrates are the genes of the major

histocompatibility complex (MHC) (Von Schantz *et al.* 1996; Ober *et al.* 1997; Paterson & Pemberton 1997; Edwards & Hedrick 1998; Jordan & Bruford 1998). Several adaptive hypotheses for MHC-dependent mating preference have recently been proposed. First, since these genes are very polymorphic, they may be used as a cue for kin discrimination and, accordingly, MHC mate preference might be an effective way of avoiding inbreeding and enhancing offspring variability (Penn & Potts 1999). Second, since heterozygous offspring can protect themselves against a wider range of parasites than homozygous ones, MHC negative assortative mating might provide progeny with an enhanced immunological surveillance (Doherty & Zinkernagel 1975; Wakeland *et al.* 1990; Carrington *et al.* 1999). Experimental evidence has been obtained for both hypotheses but evidence from natural populations is very scarce and has mostly been limited to mammals (Tregenza & Wedell 2000).

Salmonid fishes are well suited as model organisms for investigating these hypotheses. Given the importance of olfactory cues in other important behavioural mechanisms, such as homing and kin recognition (Nordeng 1977; Brown & Brown 1992; Dittman & Quinn 1996), the allelic composition at MHC genes could be an important factor potentially determining mate choice (see Olsén *et al.* 1998). Furthermore, the MHC polymorphism described in salmon suggests that they are evolving in the same way as their mammalian counterparts and that it seems to be an important factor for individual fitness (e.g. Miller & Withler 1996; Langefors *et al.* 2001). In addition, it is unlikely that female Atlantic salmon should gain any material benefits from mate preference, since there is no post-spawning parental care in this species (Fleming 1996) and a single male is potentially capable of fertilizing all the eggs a female produces (Gjerde 1984). Thus, mate choice in

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Atlantic salmon should theoretically be mainly driven by potential genetic benefits.

In this study, we tested the good genes as heterozygosity hypothesis of mate choice by documenting the mating patterns of wild Atlantic salmon using both the MHC and microsatellite loci. More specifically, we tested the null hypotheses that mate choice in Atlantic salmon is neither dependent on the relatedness between potential partners nor on the MHC similarity between mates. These hypotheses would be rejected by evidence for mating patterns departing from random mating expectation. In such a case, the alternative hypotheses would be that salmon choose mates in order to either specifically enhance their offspring's MHC heterozygosity or to avoid inbreeding by enhancing the overall heterozygosity of their progeny. Challenging these two hypotheses can in turn allow us to discriminate between the two proposed roles of the MHC in mate choice.

2. MATERIAL AND METHODS

(a) *Sampling and polymorphism analysis*

A total of 41 adult male salmon and 35 adult female salmon were caught at the fish ladder located on the north-eastern branch of the Sainte-Marguerite River, Québec, Canada (48°20' N, 70°00' W). These were measured, sexed and their adipose fins clipped and collected. All spawners were then relocated in an upstream part of the same river branch. This river stretch, which had not been previously occupied by Atlantic salmon, was 19 km long and isolated by two impassable waterfalls. We chose a stretch of river previously devoid of Atlantic salmon for exclusively sampling the offspring of introduced spawners. Electrofishing was conducted from 27 August to 6 September 1996 throughout accessible spawning and nursery areas in order to sample as many young salmon as possible. A total of 650 fry were randomly sampled over a four-day period, as detailed in Garant *et al.* (2001). Whole fry (young of the year) and adult adipose fins were preserved for genetic analysis in 95% ethanol.

Total DNA extraction was performed from *ca.* 30 mg of tissue according to Bernatchez *et al.* (1992). Microsatellite polymorphism was analysed at five loci used in a previous study detailed in Garant *et al.* (2001). A 254-bp fragment of the exon 2 of an MHC class II β gene was obtained for MHC polymorphism analysis using the primers CL007 (5'-GATCTGTAT-TATGTTTTTCCTTCAG-3') and AL1002 (5'-CACCTGTCT-TGTCCAGTATG-3') (Olsén *et al.* 1998). Alleles were first identified using radioactive single-strand conformation polymorphism (SSCP) as this method provides a rapid and sensitive screening of mutations and polymorphism of haplotype (Thorpe *et al.* 1999). The primer CL007 was end labelled with [γ -³³P] and a polymerase chain reaction (PCR) was carried out in a total volume of 10 μ l containing 20–40 ng of genomic DNA, 0.25 mM dNTPs, 1 μ l reaction buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 0.1% Triton X-100 and 50 mM KCl), 1 unit of Taq polymerase, 1 pmol of [γ -³³P]-CL007, 4 pmol of CLMHC and 5 pmol of AL1002. Five microlitres of a denaturing loading buffer were added to the PCR products and 6 μ l of the mix were loaded onto a non-denaturing acrylamide gel (10% 49:1 acrylamide:bis-acrylamide, 5% glycerol and 0.5X TBE) for a 15-h and 20-W migration at 4 °C. The dried gels were exposed on an X-ray film for 24–48 h. Since both strands of the PCR product had a different electrophoretic mobility, two gels out of approxi-

mately ten were run by labelling both strands in order to avoid false variant identification or homoplasy. The following PCR profile produced one or two bands for each of the 76 spawners analysed: 95 °C for 3 min, 32 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s followed by 72 °C for 10 min. Allelic segregation was also tested in the progeny of two families. One allele was from the female and the other one from the male in all genotypes (unpublished data). This further supported the existence of a single MHC class II β locus in Atlantic salmon, as recently proposed (Langefors *et al.* 1998, 2000; Landry & Bernatchez 2001).

The reliability of the SSCP gel scoring was confirmed by sequencing analysis of identified SSCP haplotypes. Molecular cloning was performed using an Invitrogen Topo TA cloning kit (Invitrogen, Carlsbad, Foster City, CA, USA). Insertions were sequenced using the BigDye Terminator cycle-sequencing kit on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Several (two to eight) clones were sequenced for many individual representatives of different allelic variants in order to avoid erroneous allelic identification due to reading errors or recombinant sequences generated by the PCR (Ennis *et al.* 1990; Bradley & Hillis 1997).

(b) *Statistical analyses*

We used the maximum likelihood-based method detailed in Sancristobal & Chevalet (1997) and Bernatchez & Duchesne (2000) for inferring parentage from microsatellite multiloci genotypes from putative parents and all offspring caught. The results of these analyses are detailed in Garant *et al.* (2001). Briefly, the likelihood of each offspring genotype was quantified for each pair of parental genotypes. An accurate estimation of the identity and number of mates for each adult salmon was then obtained by identifying all successful pairs of spawners.

In order to determine whether mate choice occurred so as to specifically increase the variability or heterozygosity of MHC genes in the progeny, three parameters were quantified: (i) the number of MHC class II β shared alleles between partners, (ii) the MHC amino-acid genotypic distance between mates' genotypes, and (iii) genetic relatedness between mates.

(c) *Number of shared allele at the MHC locus*

Traditionally, models of MHC mating preference have considered the number of alleles shared between mates without considering the amino-acid composition of the different alleles (Hedrick 1992; Paterson & Pemberton 1997). This assumes that an individual preferentially mates with partners that differ genetically from itself, without any regard to the nature and quantity of the difference. This should result in more frequent mating between dissimilar genotypes according to the number of alleles they share than expected by chance alone. We tested this by quantifying the basic similarity statistic M_{xy} (Blouin *et al.* 1996) based on the numbers of shared MHC alleles (none, one or two) between males and females involved in every mating event.

(d) *The sum of pairwise amino-acid differences*

MHC alleles could be different at the molecular level, but functionally similar in terms of immune defence. For instance, these could differ slightly in sequence, but the protein they encode would still bind the same peptides as their binding specificities depend on only a few characteristics of the molecule (Rammensee 1995; Sidney *et al.* 1995). In addition, since heterozygotes can respond to any antigen recognized by either

parental MHC haplotype, an offspring could be better protected with an increasing heterozygosity or complexity of its MHC genotype (Doherty & Zinkernagel 1975; Potts & Wakeland 1990; Carrington *et al.* 1999). In this context, MHC mate preference could imply not only avoidance of mates that share alleles but also a preference for partners that will maximize the complexity of their offspring's genotypes. This was tested by computing a genotypic distance (AA_{xy}) value for each realized mating pair, which corresponds to the sum of all the pairwise amino-acid differences (D) of the four possible alleles (A , a , B and b) carried by mates ($D_{AB} + D_{Ab} + D_{aB} + D_{ab}$), thus reflecting the genotypic complexity of the offspring. Since odours may be influenced by the MHC genotype, either indirectly via the parasitic load and binding characteristic of the molecules (reviewed in Singh 1998) or directly by whole MHC proteins in the body fluids (Singh *et al.* 1987; Yamazaki *et al.* 1990), two distances were used. First, all the amino-acid differences were considered and, second, only the putative sites involved in the peptide-binding region, which is known to be the basis of peptide recognition, were examined.

(e) Relatedness

The genetic relatedness between mating partners was estimated using the r_{xy} -statistic of Queller & Goodnight (1989), which is an unbiased estimate of the true relatedness between individuals x and y . Computations of the r_{xy} -statistic using microsatellite data allowed us to first determine the degree of genetic similarity among spawners and then verify whether more related individuals tended to avoid each other in mating.

The association of each of the above three parameters with mate choice was statistically investigated by estimating the likelihood of the values of each observed statistic under random mating using Monte Carlo simulations. Mating pairs established from parentage analysis (Garant *et al.* 2001) were used as a template for performing the simulations. Specifically, all observed mate pairs were disassociated and reassembled at random 5000 times in order to create a distribution of the AA_{xy} -, M_{xy} - or r_{xy} -values under random mating conditions. In doing so, every individual 'mated' with the same observed number of partners, which was therefore respected in each simulation. Finally, the mean observed value of each parameter was compared with the random distribution.

3. RESULTS

(a) Genetic polymorphism

A description of the genetic polymorphism of the microsatellite and MHC loci is summarized in table 1 and detailed in Garant *et al.* (2001) and Landry & Bernatchez (2001). Briefly, we observed a mean of 14 alleles per locus for the five microsatellites and 16 alleles at the MHC with an expected heterozygosity of 0.85 for both markers. The MHC alleles differed by one to 17 amino acids. The putative peptide-binding region was established according to W. C. Jordan, K. A. Foley, H. J. Hall and M. W. Bruford (unpublished data) following Brown *et al.* (1993) and Dixon *et al.* (1996).

(b) Mating patterns

A total of 262 mating events were resolved from the analysis of the 650 offspring (detailed in Garant *et al.* 2001). However, the largest individuals of both sexes (one male and nine females) had reduced reproductive success

Table 1. Number of alleles and expected (H_e) and observed (H_o) heterozygosities in the 76 spawning Atlantic salmon at five microsatellite loci and an MHC locus

(GenBank accession numbers for the MHC class II β alleles (AF373692, AF373693, AF373695, AF373699, AF373701–AF373709).)

loci	number of alleles	H_e	H_o
Ssa171	22	0.92	0.95
Ssa197	14	0.84	0.95
SSOSL85	13	0.89	0.89
Ssa202	13	0.87	0.84
MST-3	8	0.75	0.79
MHC class II β	16	0.85	0.88

and a small number of mates in proportion to expectations relative to their size (Garant *et al.* 2001). These were all putative repeat spawners that were not undergoing their first reproductive event and, thus, they might have been less effective in the spawning grounds relative to other fish than their size would suggest (Garant *et al.* 2001; G. Chaput, personal communication). As this could also confound the process of mate choice, these ten individuals were left out the analyses, resulting in subsequent analysis of 221 mating events.

The results of the randomization procedure for each statistic are presented in figure 1. These did not support the hypothesis that mates discriminate between or choose each other on the basis of their number of MHC shared alleles. Thus, there was a slight but non-significant trend for the mean observed numbers of shared alleles at the MHC class II β locus to differ from random mating expectations (mean observed $M_{xy} = 0.423$ and $p = 0.224$) (figure 1a). In contrast, the analysis of MHC genotypic distances supported the hypothesis that mates tend to choose each other according to the degree of dissimilarity of their functional MHC class II β proteins. When only considering the changes of amino-acids at the peptide-binding region, a significantly higher AA_{xy} -value than expected under random mating (mean observed $AA_{xy} = 24.5$ and $p = 0.049$) was observed (figure 1d). However, the null hypothesis of random mating could not be rejected at $\alpha = 0.05$ when considering all amino-acid differences between the genotypes of mating partners (mean observed overall $AA_{xy} = 36.5$ and $p = 0.094$) (figure 1b), although the trend was marginally significant. Finally, there was no evidence of inbreeding avoidance related to the degree of relatedness between mates as quantified from r_{xy} (mean observed $r_{xy} = 0.024$ and $p = 0.445$) (figure 1c). Most of the mating events occurred between unrelated individuals, as suggested by the weak variance in the r_{xy} -values (0.032).

4. DISCUSSION

The main objective of this study was to test the two competing hypotheses that mate choice in Atlantic salmon operates so as to (i) specifically increase the variability of MHC genes in their progeny, and (ii) enhance the global heterozygosity of their offspring by choosing unrelated mates (inbreeding avoidance). As such, to the best of the authors' knowledge, this study represents the first investigation of the potential roles of MHC genes in

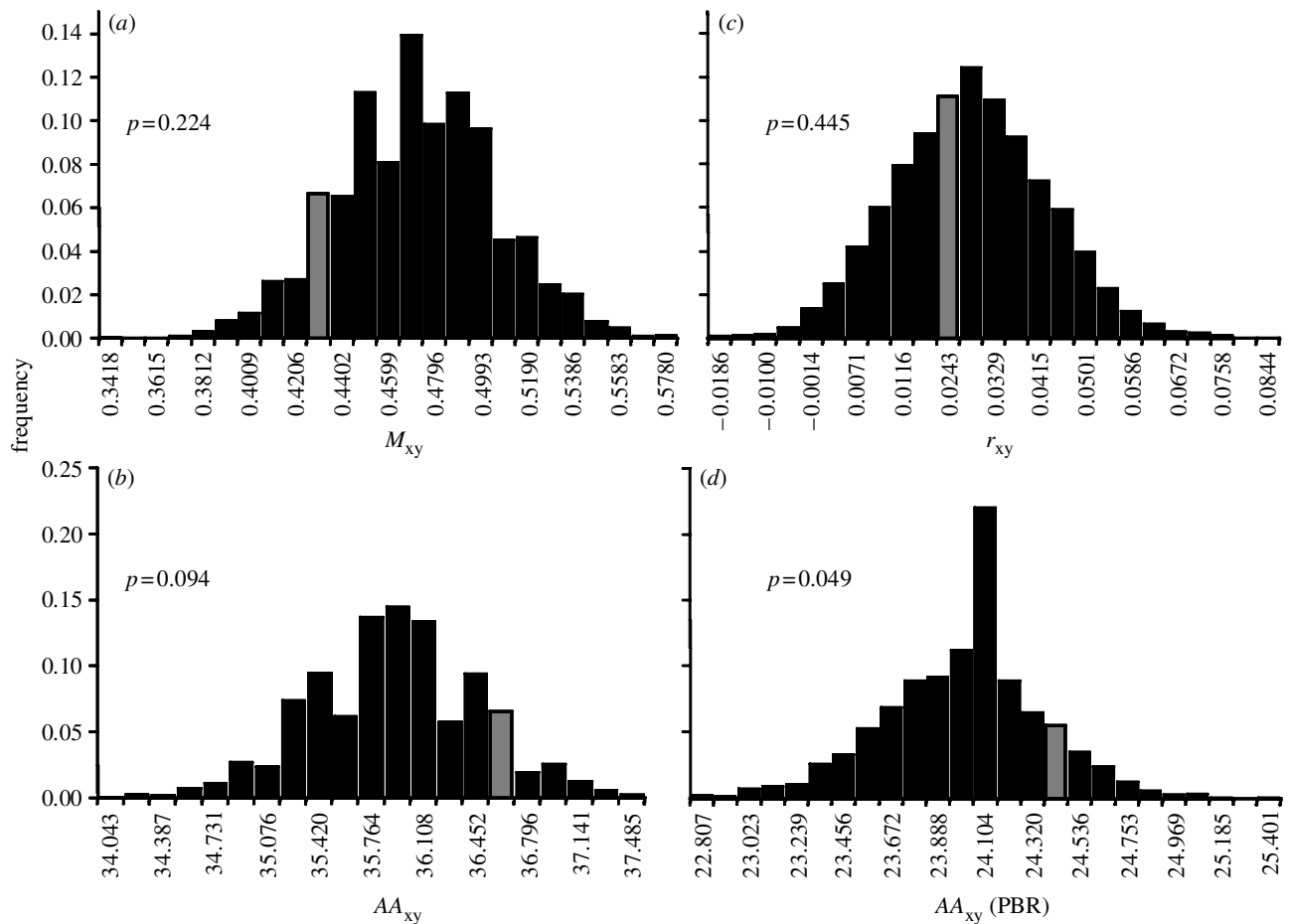


Figure 1. Frequency distributions of the mean values of each reproductive event, as established by 5000 simulated dissociations and associations of the 221 observed mates. The range of randomized values containing the observed values is marked and the corresponding p -value is indicated. (a) Mean number of MHC shared alleles (M_{xy}), (b) mean over all amino-acid pairwise differences (AA_{xy}), (c) overall genetic relatedness (mean r_{xy}) and (d) AA_{xy} at the peptide-binding region.

mate choice in fish. Furthermore, by documenting mating patterns in wild Atlantic salmon using MHC and microsatellite loci, this study provides one of the only tests of the good genes as heterozygosity hypothesis of mate choice proposed by Brown (1997). Overall, the results supported the first hypothesis by indicating that mates choose each other in order to increase the genotypic complexity (in terms of pairwise amino-acid differences) of their offspring at the peptide-binding region and not in a way to enhance the heterozygosity over the genome (inbreeding avoidance), as revealed by the microsatellites.

The statistical method employed in this study allowed a more precise assessment of non-random mating patterns than those usually achieved, namely in that the relatedness and MHC similarity between individual genotypes were directly assessed instead of simply relying on departure from Hardy–Weinberg equilibrium for inferring disassortative mating (Potts *et al.* 1991; Ober *et al.* 1997). Conclusions based on such criteria may potentially be biased by the effect of other population characteristics such as selective mortality, abortion and a small effective population size (Robertson 1965; Hedrick 1994). Furthermore, the combined analyses of multilocus, microsatellite and MHC loci allowed us to discriminate between the two types of information that the MHC signal might communicate, namely relatedness and immune functional

similarities. This also circumvents the possible confusion that may create the overall genetic similarity between individuals by causing seemingly MHC-correlated behaviours, related individuals being more likely to share alleles than unrelated individuals, and vice versa (Manning *et al.* 1992).

(a) *MHC and inbreeding avoidance in salmonids*

Jordan & Bruford (1998) proposed that species with a high probability of inbreeding, such as highly philopatric fishes, might benefit from a genetically based kin recognition system. This would particularly be the case in many anadromous salmonids, such as Atlantic salmon, which exhibit homing behaviour on a small geographic scale (Jordan *et al.* 1992; Garant *et al.* 2000) and are consequently structured in a large number of populations with relatively small effective population sizes (Stabell 1984). The capacity of salmonids for discriminating the odours of urine from siblings and non-siblings has previously been documented (Moore *et al.* 1994). However, the genetic basis of this ability remains largely unknown. The only evidence that kin recognition is based on the genetic similarity between fish comes from Olsén *et al.* (1998) who recently documented that the MHC had a significant influence on the odours used for kin recognition in the Arctic charr (*Salvelinus alpinus*). These authors

also hypothesized that, if active, the role of the MHC in mate choice should be related to inbreeding avoidance in this species. The results of our study do not support this latter hypothesis, as we found no evidence for inbreeding avoidance but rather a random mating pattern with some mating events occurring between relatives (maximum $r_{xy} = 0.56$).

Many studies of MHC-based mate choice have relied on quantification of the number of shared alleles between mate genotypes (Potts *et al.* 1991; Hedrick 1992; Paterson & Pemberton 1997). Although we observed a trend for the observed mean number of shared MHC alleles between partners to be lower than expected by chance, this pattern was not statistically significant. This tendency is nevertheless consistent with the excess of heterozygous offspring on spawning sites that we have reported in another study (Landry & Bernatchez 2001). The limited statistical support could be partly explained by the high variability in the MHC genes observed, which render all mating types quite rare and particularly those sharing two alleles. In such a case it is therefore difficult to demonstrate negative assortative mating due to lack of power (Hedrick & Black 1997).

The most salient result of this study was the statistically significant support for non-random reproduction according to the differential composition of the amino acids involved in the putative peptide-binding region of mates. This reproductive pattern is concordant with the good genes as heterozygosity hypothesis that predicts, in the case of MHC genes, that mate choice should operate so as to enhance offspring fitness by providing them with the best immune defence. In this context, the best immune defence would be a heterozygote MHC genotype (Doherty & Zinkernagel 1975; Brown 1997; Carrington *et al.* 1999) and an enhanced divergence between alleles, as proposed by Wakeland *et al.* (1990). This may reflect a tendency for individuals to choose mates with different genotypes. Moreover, it may suggest that this preference might be extended to mates exhibiting the most 'divergent' genotypes. Not considering the amino-acid differences between alleles implicitly assumes that many of them are functionally different whereas they could be playing a similar role if they diverge at only few sites (Rammensee 1995; Sidney *et al.* 1995). From this perspective, the information added by the genotypic distance may partially explain the lack of significance in the M_{xy} analysis and still provide evidence for disassortative mating. Choosing mates with a more different MHC genotype could also be adaptive in salmon if more complex genotypes provide better defence against parasites, as reported in humans (Carrington *et al.* 1999). The observation that the olfactory acuity of mice enables them to discriminate MHC class I molecules differing by only three amino acids suggests that salmon could also discriminate odours produced by slightly different genotypes (Yamazaki *et al.* 1983).

Other explanations, such as differential fertilization success depending on MHC genotype (Wedekind *et al.* 1996) or higher fitness in more heterozygous offspring, could also potentially account for the significant association between mate choice and genotypic complexity. However, a differential fertilization success would imply that MHC molecules are expressed on the surface of

spermatozooids. This has been shown in humans (e.g. Paradisi *et al.* 2000) but has never been looked for in fish. Similarly, better survival of more heterozygous offspring at this stage is unlikely since it is not clear whether fry are able to mount a specific immune response (Bakke & Harris 1998). Finally, it is also possible that the distance value we quantified not only reflects the difference between specific exons (exon 2 of the β chain) but also that of a larger linked chromosomal region. For instance, class II α and β genes (but not classes I and II) apparently co-segregate in salmon (Stet *et al.* 1997). However, the finding of a stronger tendency for disassortative mating when considering only sites at the peptide-binding region rather than all differences is not supportive of such a process.

The MHC genes of teleosts appear to have evolved and to act like those of their mammalian counterparts, the polymorphism of which is believed to be maintained both by parasite-driven selection and MHC-based mating preference (reviewed by Stet & Egberts 1991; Ono *et al.* 1993; Van Muiswinkel *et al.* 1999). A high level of polymorphism at the MHC class II β gene has recently been documented in several populations of Atlantic and Pacific salmon (Miller & Withler 1996; Langefors *et al.* 1998, 2000; Landry & Bernatchez 2001). The overall high level of diversity and the pattern of nucleotide substitutions (ratio of synonymous and non-synonymous substitutions) reported in these studies indicate that this gene is under balancing selection, as is its mammalian counterparts. Our results indicate that, along with natural selection, disassortative mating at this MHC locus may contribute to maintaining a high level of polymorphism within a population. They also provide the first empirical evidence for the role of MHC genes in the behavioural ecology of wild salmonids.

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