

# MHC-linked susceptibility to a bacterial infection, but no MHC-linked cryptic female choice in whitefish

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## Keywords:

*Coregonus*;  
cryptic female choice;  
egg disease;  
fish;  
gamete fusion;  
MHC class II;  
*Pseudomonas fluorescens*;  
second meiotic division;  
sperm-egg interaction;  
within-family comparison.

## Abstract

Non-random gamete fusion is one of several potential cryptic female choice mechanisms that have been postulated and that may enhance the survival probability of the offspring. Previous studies have found that gamete fusion in mice is influenced by genes of the major histocompatibility complex (MHC) region. Here we test (i) whether there is MHC-dependent gamete fusion in whitefish (*Coregonus* sp.) and (ii) whether there is a link between the MHC and embryo susceptibility to an infection by the bacterium *Pseudomonas fluorescens*. We experimentally bred whitefish and reared sibships in several batches that either experienced or did not experience strong selection by *P. fluorescens*. We then determined the MHC class II B1 genotype of 1016 surviving larvae of several full sibships. We found no evidence for MHC-linked gamete fusion. However, in one of seven sibships we found a strong connection between the MHC class II genotype and embryo susceptibility to *P. fluorescens*. This connection was still significant after correcting for multiple testing. Hence, the MHC class II genotype can considerably influence embryo survival in whitefish, but gamete fusion seems to be random with respect to the MHC.

## Introduction

One evolutionary explanation for the success of sexual reproduction assumes that sex is an advantage in the co-evolutionary arms race between pathogens and hosts. Accordingly, an important criterion in mate choice and cryptic female choice could be the allelic specificity on polymorphic loci involved in host–parasite interactions (reviews in Westneat & Birkhead, 1998; Møller & Alatalo, 1999; Møller *et al.*, 1999; von Schantz *et al.*, 1999; Jennions & Petrie, 2000).

The major histocompatibility complex (MHC) is currently the best-studied example of genes that are important in both, host–pathogen co-evolution and sexual selection (Apanius *et al.*, 1997; Edwards & Hedrick, 1998; Penn & Potts, 1999). Products of the MHC present antigens to lymphocytes. A link between the MHC and the susceptibility to an infectious organism is therefore strongly expected. Indeed, many studies report evidence for the existence of such a link (reviews in Tiwari & Terasaki, 1985; Apanius *et al.*, 1997; Hedrick, 2002; see recent examples in the Discussion). There is also accumulating evidence in a number of species that the MHC influences mate choice. The MHC somehow influences body odours and body odour preferences in, for example, mice (Yamazaki *et al.*, 1976, 1994; Singer *et al.*, 1997; Carroll *et al.*, 2002), humans (Wedekind *et al.*, 1995; Wedekind & Füre, 1997; Montag *et al.*, 2001), and fish (Reusch *et al.*, 2001). Probably because of its link to odours, the MHC has been found several times to

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influence sexual selection (Egid & Brown, 1989; Potts *et al.*, 1991; Ober *et al.*, 1997; Landry *et al.*, 2001). The effect of MHC-linked mate choice on offspring survival is, however, still unclear.

Sexual selection includes cryptic female choice. There exist a number of potential cryptic female choice mechanisms in species with internal fertilization (Birkhead & Møller, 1993; Wedekind, 1994; Eberhard, 1996; Birkhead & Pizzari, 2002) and there is much evidence for cryptic female choice in various taxa (reviews in Eberhard, 1996; Fernandez *et al.*, 1999; Tregenza & Wedell, 2000; Olsson & Madsen, 2001; recent examples include Dorak *et al.*, 2002; Tregenza & Wedell, 2002). However, the exact kind of selection is often unclear in species with internal fertilization. In fish with external fertilization there are typically three potential possibilities for cryptic female choice that may eventually result in non-random gamete fusion:

(1) Sperm could somehow signal their haploid specificity at certain loci and eggs could selectively accept sperms on the basis of these signals. There are contradictory reports about whether or not sperm bear MHC-antigens on their cell surface that reveal parts of their haploid genome. On the one hand, gene transcription during spermatogenesis actually seems to occur in some mammals after the last meiotic division, i.e. from the haploid genome, and protein synthesis continues some times afterwards (Hecht, 1990; Eddy *et al.*, 1993). This could explain the finding of some authors that MHC-antigens on sperm of mice and humans seem to be haploid expressed (Fellous & Dausset, 1970; Halim & Festenstein, 1975; Arnaiz-Villena & Festenstein, 1976; Halim *et al.*, 1982). On the other hand, however, several groups could not find any MHC-antigens on human sperm (Haas & Nahhas, 1986; Kuhlmann *et al.*, 1986) and others found MHC-antigens on human sperm of only few individuals of a sample (Kurpisz *et al.*, 1987), or found only some HLA-A and -B antigens but not others (Rodríguez-Córdoba & Arnaiz-Villena, 1985). Wedekind *et al.* (1996) suggested as a possible explanation of these controversial findings that haploid expression of MHC on sperm could be condition-dependent, e.g. dependent on the infection status of the male.

(2) Selection could also occur later, i.e. after the fusion of the gametes and during the formation of the second polar body. This is possible, as in many vertebrates (including many fish), the second maturational division is completed only after the sperm has penetrated the egg (Wolgemuth, 1983). The significance of this suspension is not yet clear. It could, however, be an important prerequisite allowing eggs to optimally complement the sperm's haplotype with their own haplotype on loci like the MHC (Wedekind, 1994; Wedekind *et al.*, 1996).

(3) Ovarian fluids could have a selective effect on sperm (Turner & Montgomerie, 2002).

First evidence for nonrandom gamete fusion was found in a tunicate where eggs resist fertilization by sperm with the same allele on the fusibility locus for a longer period than sperm with a different allele (Scofield *et al.*, 1982). In another tunicate, the self-discrimination could be shown to be controlled by the products of overlying follicle cells (de Santis & Pinto, 1991; Marino *et al.*, 1999). Within vertebrates, several authors tested whether the MHC segregates randomly with respect to the MHC in rats or mice. They found nonrandom segregation in most tests (Palm, 1969, 1970; Hings & Billingham, 1981, 1983, 1985; Potts *et al.*, 1991). A recent study on over 400 newborn human babies found that nonrandom segregation of the MHC interacts with gender: male embryos seem to be under higher selection than female embryos (Dorak *et al.*, 2002). A series of *in vitro* fertilization experiments in MHC-congenic inbred mice revealed that MHC-dependent gamete fusion contributes to this nonrandom segregation (Wedekind *et al.*, 1996). These first experimental *in vitro* results were later confirmed by Rüllicke *et al.* (1998). The physiology behind such nonrandom gamete fusion in vertebrates is still unknown. Ziegler *et al.* (2002) suggest that testis-expressed MHC genes and MHC-linked olfactory receptor genes may be functionally connected and play a role in sperm selection.

In the present study we test whether there is nonrandom gamete fusion with respect to the MHC in a fish. This is, to our knowledge, the first study on possible MHC-dependent gamete fusion in a fish. We then ask whether MHC-linked gamete fusion would matter with respect to offspring survival. We therefore test whether there is an association between the MHC and embryo susceptibility to a virulent infectious organism. In order to control for potentially disturbing effects of non-MHC linked background genes or different maternal reproductive strategies (Arkush *et al.*, 2002; Lohm *et al.*, 2002), we only do comparisons within full sibship, i.e. we compare full-sibs of different MHC-types.

As a model species, we chose the Alpine whitefish (*Coregonus* sp., see Douglas *et al.*, 1999, for a discussion of the taxonomy). Females of this species produce thousands of eggs that can be experimentally fertilized *in vitro* under controlled conditions. The eggs can then be reared until hatching in batches of about 100–200 eggs (Wedekind *et al.*, 2001a). This, and the fact that some of the polymorphism on the MHC class I and class II loci has recently been described (Binz *et al.*, 2001a), makes the whitefish a convenient model for studying selection on the MHC. Moreover, Wedekind *et al.* (2001b) found significant paternal effects on offspring susceptibility to *Pseudomonas fluorescens*, a virulent fish disease (Schäperclaus, 1990; Wedekind, 2002). Here, we specifically ask whether some of these genetic effects are because of different MHC class II haplotypes and whether eggs can get these 'good genes' via nonrandom fertilization.

## Methods

### Sibship breeding and infection

We individually stripped the gametes of wild-caught whitefish into Petri dishes and used these gametes to produce 100 different sibships. Therefore, we put about 500–1000 freshly stripped eggs of one female each into a dry Petri dish (diameter = 9 cm) and added 10  $\mu\text{L}$  of milt of only one male each. The eggs were still in ovarian fluids when the sperm was added. We then half filled the Petri dish with lake water and shook it gently for about 5 s. After transporting the freshly fertilized eggs to the laboratory, we exchanged the water and distributed the eggs from each Petri dish into five new Petri dishes (diameter = 9 cm, water level = about 1 cm, no cover). From then on we kept the eggs in a climate chamber at 8 °C. One randomly chosen Petri dish per sibship was separated for another study. Care was taken to randomize the position of the remaining 400 batches of eggs in the climate chamber (randomized block design with respect to parental origin and to shelf in the climate chamber).

During regular checks at least three times per week, we removed dead, badly developed and obviously infected eggs with a pipette and recorded their numbers with marks at the respective Petri dish. Water was exchanged every 2 weeks during the first 30 days, and once per week from then on by emptying the Petri dish over a stiff piece of nylon net (1000  $\mu\text{m}$ ) and immediately adding sand filtered lake water that had been stored for at least 1 day in an aerated aquarium in the climate chamber.

As we expected the eggs to take about 60 days to hatch (Ventling-Schwank & Müller, 1991), we decided *a priori* to record the cumulative mortality during the first 30 days as 'early mortality' (including all possibly unfertilized eggs) and the remaining mortality as 'late mortality' (=number of dead eggs between day 30 and hatching/number of live eggs on day 30). Regular checks under the microscope indicated that the early mortality was mainly caused by developmental abnormalities of the embryos. From about day 35 on, a marked increase in mortality could be observed that was connected to an uncontrolled epidemics by *P. fluorescens*. We did not attempt to treat the infection but instead recorded the mortality in all Petri dishes (described in Wedekind *et al.*, 2001b).

For the present study we selected seven sibships that fulfilled both of two criteria: (i) the sibship must have experienced low early mortality and (ii) one of the four batches of eggs from the same sibship must have experienced low late mortality, while another one must have experienced a rather strong late mortality caused by the infection. After hatching, all the larvae of these 14 batches were killed by an overdose of anaesthetics and stored in 70% ethanol until DNA extraction. We use the two selected batches of eggs each from a same sibship in a

pairwise comparison to test whether the infection changed the MHC class II allele frequency among the survivors. The batches that each experienced low early and low late mortality were used to test whether the MHC class II allele frequencies are different from Mendelian expectations, indicating nonrandom gamete fusion.

### Genetic analyses

The tail of each larvae was cut off and incubated for approximately 1 h at 45 °C in 100  $\mu\text{L}$  extraction buffer (10 mM Tris-HCl, 2 mM EDTA, 0.05 Triton X-100, and 200  $\mu\text{g mL}^{-1}$  of Proteinase K) until complete lysis. The solution was then centrifuged for 30 s at 4300 *g*, the supernatant transferred to a fresh tube, then incubated at 95 °C for 10 min, and finally stored at -20 °C until further use.

We amplified a 131 bp fragment of the MHC class II B1 locus by polymerase chain reaction (PCR) using fluorescently labelled primers (forward primer: 5'TGAAGAATG CAGAAGCATGG, label: 6FAM; reverse primer: 5'GGA GCCCTGCTCACCTGTCTTATC, label: HEX). The PCR reaction mixture (10  $\mu\text{L}$ ) contained Amplitaq Gold buffer 10x, 2.5 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{M}$  of each primer, all four dNTPs (each at 0.2 mM), 0.25 units of Amplitaq Gold polymerase (Applied Biosystems, Rotkreuz, Switzerland) and 0.5  $\mu\text{L}$  of the lysate that contained the DNA. Amplification started with 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 75 s at 72 °C. The reaction was terminated after a final primer extension for 7 min at 72 °C.

We determined the MHC class II B1-genotype of the larvae by single-stranded conformation polymorphism (SSCP) analysis on the PCR product. This technique is based on the sequence-specific electrophoretic mobility of single-stranded DNA fragments (Kim *et al.*, 1999). Genotyping was performed by nondenaturing capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA) of fluorescently labelled PCR-products as described (Binz *et al.*, 2001b). The DNA sequence of the DNA fragments obtained by PCR was determined by subcloning the fragments into pUC19 using the Sureclone Kit of Amersham Bioscience Europe GmbH (Freiburg, Germany). The sequence of the single clones was obtained by automated sequencing on an ABI Prism 3100 Genetic Analyzer. All sequences have been submitted to Genbank (Table 3b). Genomic DNA of the parents was isolated as described in Binz *et al.* (2001a)).

## Results

The average early mortality among the 14 batches of eggs was 2.3% (SE = 0.5) and not significantly different between the two batches per sibship (paired *t*-test, *t* = 0.32, d.f. = 6, n.s.). The average late mortality was

**Table 1** The seven batches of eggs (of the sibships 1–7) that experienced no or only weak selection. Number of MHC class II B1 genotypes within the sibships, early and late mortalities, and *P*-values of chi-squared Goodness-of-fit tests that each compares the observed genotype frequencies within a batch against the Mendelian null-expectancy, i.e. against equal frequencies of the genotypes.

Sibship	Number of MHC class II haplotypes	Early mortality (%)	Late mortality (%)	<i>N</i> (genotyped)	<i>P</i>
1	4	1.6	2.8	129	0.56
2	4	0.0	0.0	79	0.37
3	4	0.5	6.3	66	0.05
4	4	2.5	0.6	30	0.69
5	4	4.5	4.7	124	0.92
6	2	3.0	2.1	51	0.67
7	2	2.7	2.1	63	0.17

2.7% (SE = 0.8) for those seven batches that were not or only very weakly confronted to the infection, and 57.7% (SE = 5.5) for those that experienced strong selection by the infection ( $t = 10.9$ , d.f. = 6,  $P < 0.001$ ).

We typed a total of 1016 fish larvae for their MHC class II B1-genotypes. Ten larvae (1% of all) had nonexpected B1-genotypes (possibly recombinants or mutants) and were therefore excluded from all later analyses. In two sibships, one of the parents was homozygous which resulted in only two B1-genotypes in the offspring. All other sibships had four B1-genotypes (i.e. heterozygous parents; Table 1).

We found no evidence for a MHC-dependent gamete fusion. In all the seven batches that experienced no or only weak selection, the genotype frequencies were not significantly different from random [Table 1; Fisher combination test (Sokal & Rohlf, 1981, p. 780),  $\chi^2 = 14.4$ , d.f. = 14, n.s.]. If this non significant finding were because of a lack in statistical power, we would expect a negative correlation between the *P*-value and the number of analysed larvae per batch. However, this correlation was not significant and even positive in sign ( $r = 0.277$ , n.s.).

When we tested for possible deviations in genotype frequencies after selection by the infection, we found a strong effect in sibship 2. The effect in sibship 2 stayed statistically significant after Bonferroni correction for multiple testing. Hence, susceptibility to *P. fluorescens* depends on the MHC or closely linked loci in whitefish.

There was no detectable interaction between the epidemic and the MHC class II B1 genotype of the other six sibships (Table 2). This indicates that the interaction between the MHC class II and the infection can be quite specific. Table 3a gives the genotypes in both batches of sibship 2, and Table 3b lists the Genbank accession numbers of the amplified nucleotide sequences of the parents of sibship 2.

**Table 2** The seven batches of eggs (of the sibships 1–7) that experienced strong selection. Early mortality, late mortality (=mortality during the infection), and *P*-values of two types of frequency analyses that each test the observed genotype frequencies among the survivors within a batch against either the Mendelian null-expectancy (as in Table 1) or against the observed genotype frequencies in the noninfected batch of the same sibship each.

Sibship	Early mortality (%)	Late mortality (%)	<i>N</i> (genotyped)	<i>P</i> *	<i>P</i> **
1	1.8	42.4	78	0.62	0.42
2	2.7	46.2	74	0.0002***	0.0004***
3	0.0	73.8	54	0.30	0.16
4	0.6	63.2	32	0.86	0.69
5	2.8	69.1	71	0.38	0.42
6	1.4	39.6	81	0.58	0.59
7	7.7	69.6	74	0.82	0.24

\* $\chi^2$ -Goodness-of-fit, testing against Mendelian expectancy, i.e. equal frequencies of the genotypes.

\*\* $\chi^2$ -test for independence, testing against the frequencies in the noninfected sibgroup each (see Table 1).

\*\*\*Significant after Bonferroni-correction for multiple testing. The table gives the uncorrected *P*-values.

**Table 3** (a) The MHC class II B1 genotype frequencies of surviving embryos in the two batches of sibship 2 and (b) the names and the Genbank accession numbers of the amplified nucleotide sequences of the parents of sibship 2. Haplotype d is specified by two different PCR fragments which represents sequences from two different loci (see also Binz *et al.*, 2001a).

Genotype	ac	ad	bc	bd
(a)				
No or weak selection	13	23	21	22
Strong selection by <i>P. fluorescens</i>	30	5	15	24
	Sequence name			Genbank accession number
(b)				
Maternal haplotype a:	Cosp-B1-H-17		AF213331	
Maternal haplotype b:	Cosp-B1-H-18		AF213315	
Paternal haplotype c:	Cosp-B1-H-4b		AY150026	
Paternal haplotype d, sequence 1:	Cosp-B1-H-15		AF213329	
Paternal haplotype d, sequence 2:	Cosp-B1-H-16		AF213330	

## Discussion

Studies on inter-sexual selection often concentrate on genetic factors that are believed to be connected to host-parasite co-evolution. Recent experimental examples of general 'good genes' effects in sexual selection include Welch *et al.* (1998) and Barber *et al.* (2001), but these studies could not identify any of the genes involved. Here, we asked whether the MHC influences offspring

survival and one possible form of sexual selection. The MHC is very polymorphic in our study population of whitefish (Binz *et al.*, 2001a), as it is in most vertebrates (Parham & Ohta, 1996; Vogel *et al.*, 1999). This diversity of MHC genes could potentially be driven by pathogen-mediated selection and by sexual selection (Apanius *et al.*, 1997; Edwards & Hedrick, 1998; Penn & Potts, 1999).

Most previous studies on the interaction between the MHC and pathogens are solely based on correlative data, with no experimental control for possible confounding effects of, for example, non-MHC background genes (e.g. Briles *et al.*, 1983; Hill *et al.*, 1991; Thursz *et al.*, 1997; Paterson *et al.*, 1998; Carrington *et al.*, 1999; Langefors *et al.*, 2001). Other studies use inbred mouse strains that are congenic with respect to the MHC (e.g. Wunderlich *et al.*, 1988; Medina & North, 1998), but they do not control for potential nongenetic maternal carry-over effects, e.g. the mother's age that is known to affect offspring size and number (Girard *et al.*, 2002), or different maternal reproductive strategies that may influence the offspring's general health and vigour and hence their susceptibility to pathogens (Petrie, 1994). Moreover, some MHC-congenic lines seem to differ with respect to the mutation load on their background genes, as they experience different mortalities during their first 3 days of embryo development (Wedekind *et al.*, 1996; Carroll *et al.*, 2002). Such differences in mutation load could well interact with pathogen susceptibilities.

Comparisons within full sibship, i.e. comparing full-sibs of different MHC-types, control for potentially disturbing effects of non-MHC linked background genes or different maternal reproductive strategies while retaining an outbred genetic background (Arkush *et al.*, 2002; Lohm *et al.*, 2002). Our within-family analyses reveal that there is an interaction between the MHC class II haplotype and embryo susceptibility to *P. fluorescens*, a virulent bacterium. The interaction is as expected for the MHC (Belman *et al.*, 2002): pathogens that escape from presentation by one MHC type may not be able to escape from presentation of another MHC type. Our results are also in agreement with recent experimental findings in mice (Penn *et al.*, 2002) and other salmonids (Palti *et al.*, 2001; Lohm *et al.*, 2002; but see Arkush *et al.*, 2002).

In fish, MHC class II genes are mostly linked to each other but not to MHC class I genes (Sato *et al.*, 2000). The B1 locus that we concentrated on can therefore be seen as marker for the MHC class II haplotypes within families. Hence, the specific pathogen susceptibility that we found is linked to the combination of maternal and paternal MHC class II haplotypes. Our finding suggests that epistatic effects within the MHC class II region are possible and that they may play a role in determining the susceptibility of whitefish embryos to *P. fluorescens*. The type of host-pathogen interaction that we observed is likely to lead to frequency-dependent selection (Takahata & Nei, 1990; Smith *et al.*, 1999).

We did not find any evidence for MHC-linked gamete fusion. Effect sizes for nonrandom gamete fusion are expected to be low: in mice and rats, MHC-segregation typically deviates from Mendelian expectations by on average about 5% (Palm, 1969, 1970; Hings & Billingham, 1981, 1983, 1985; Potts *et al.*, 1991; Wedekind *et al.*, 1996; Rüllicke *et al.*, 1998). We therefore genotyped a comparatively high number of offspring within seven experimentally bred families. If we were still lacking statistical power, we would expect to see a negative correlation between the observed effect size and the sample size over the seven families. However, the corresponding correlation was not significant and even positive in sign. Hence, gamete fusion in Alpine whitefish seems to be random with respect to the MHC class II. This is in contrast to previous findings in tunicates and mice (see Introduction).

We suggest that nonrandom gamete fusion has not evolved in whitefish for three main reasons: (i) it has been suggested that the allelic specificity of the MHC could be used as a marker of the degree of relationship between two individuals (e.g. Yamazaki *et al.*, 1976; Potts *et al.*, 1994). MHC-linked sexual selection would then be a mechanism to avoid inbreeding. However, the risk of inbreeding seems to be very low in whitefish because of the usually large population sizes and nonterritorial life history of the species (Müller, 1990). Whitefish typically aggregate to large schools that wander around in the search of planktonic food. The presumably low risk of inbreeding is in sharp contrast to the situation in many simultaneous hermaphrodites or in sessile animals where nearby individuals are often close relatives, i.e. where the chance of an egg being fertilized by a sperm of a close relative would be high if no selection mechanisms prevents inbreeding (Scotfield *et al.*, 1982). (ii) The total female investment per single egg is much lower in whitefish as it is, for example, in mice. Female mice will invest into the pregnancy and lactation, while female whitefish just release thousands of relatively small eggs at the spawning site and do not show any form of brood care (Fabricius & Lindroth, 1954). If nonrandom gamete fusion involves costs, these costs would have to be weighted against all other female investments into each offspring and against the benefits of the choice. Such a cost/benefit ratio may usually be much lower in K-strategists like mice than in r-strategists like the whitefish. (iii) The costs of nonrandom gamete fusion (i.e. of refusing some sperm) may be, in general, higher in species with external fertilization than it is for internal fertilizers whose unfertilized eggs can usually wait much longer for the fertilizing sperm and in a much safer environment.

## Acknowledgments

We thank M. Burgener, L. Carroll, E. Fischer, A. Holzer and E. Schäffer for help or assistance, M. Güntert,

U. Marti and W. Potts for support and/or discussion, and two anonymous referees for useful comments on an earlier version of the paper. CW acknowledges support by the Swiss National Science Foundation and the Clöëtta Foundation.

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Received 12 May 2003; revised 4 October 2003; accepted 13 October 2003