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Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks

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Proteins of the major histocompatibility complex (MHC) play a central role in the presentation of antigens to the adaptive immune system. The MHC also influences the odour-based choice of mates in humans and several animal taxa. It has recently been shown that female three-spined sticklebacks (*Gasterosteus aculeatus*) aim at a moderately high MHC diversity in their offspring when choosing a mate. Do they optimize the immune systems of their offspring? Using three-spined sticklebacks that varied in their individual numbers of MHC class II*B* molecules, we tested, experimentally, whether allelic diversity at the MHC influences parasite resistance and immune parameters. We found that sticklebacks with low MHC diversity suffered more from parasite infection after experimental exposure to *Schistocephalus solidus* tapeworms and *Glugea anomala* microsporidians. They also showed the highest proportion of granulocytes and the strongest respiratory burst reaction, which are correlates of innate immunity. This indicates a strong activity of the innate immune system after challenge by parasites when MHC diversity is suboptimal. Individuals with very high allelic diversity at the MHC seemed inferior to those with moderately high diversity. Such a pattern is consistent with theoretical expectations of an optimal balance between the number of recognizable antigens and self-tolerance.

Keywords: major histocompatibility complex; immunocompetence; host–parasite coevolution; genetic diversity; mate choice; sexual selection

1. INTRODUCTION

The vertebrate immune system relies on two lines of defence: innate immunity and adaptive (or acquired) immunity (Janeway *et al.* 1999). Innate immunity is an efficient first protection against many pathogens, but the defence reactions are regarded as rather unspecific. To achieve a highly specific antibody-based acquired immune response, antigen-presenting cells bind foreign peptides to proteins of the major histocompatibility complex (MHC) and subsequently interact with T lymphocytes. These cells activate B lymphocytes to produce antibodies, which are specific for the antigen.

The MHC is the most polymorphic gene cluster in the human genome (Janeway *et al.* 1999). Its genetic diversity can be extremely high at the population level (Klein 1986; Apanius *et al.* 1997; Edwards & Hedrick 1998). For example, there are more than 200 alleles of some MHC loci in the human population (Janeway *et al.* 1999). By contrast, the number of different MHC molecules expressed in an individual is comparatively small. Humans typically express six different MHC class I and eight different MHC class II molecules (Janeway *et al.* 1999). Also, in many other species, loci of the MHC classes I and II are duplicated (Klein 1986). Both variation in the number of expressed loci and heterozygosity at existing loci could contribute to variability in the number of different MHC molecules per individual.

A higher number of different MHC molecules might enable the recognition of a larger spectrum of pathogen-derived antigens (Doherty & Zinkernagel 1975; Apanius *et al.* 1997). However, increasing individual MHC diversity also increases the number of possible combinations of self-antigens with MHC proteins. T cells that interact strongly with such combinations are eliminated in a process of negative selection to avoid autoreactivity, thereby reducing the T-cell repertoire that is finally available for parasite recognition (Janeway *et al.* 1999; Sebzda *et al.* 1999). Consequently, there should be an optimal number of MHC molecules in an individual (Nowak *et al.* 1992; De Boer & Perelson 1993, but see Borghans *et al.* 2003).

MHC genes influence odour preferences for certain conspecifics in humans and in several animal taxa (Yamazaki *et al.* 1976; Potts *et al.* 1991; Wedekind *et al.* 1995; Jacob *et al.* 2002). In most studies, MHC-dissimilar mating partners were preferred over similar ones, maximizing MHC heterozygosity in the offspring. However, if there is an optimal level of MHC diversity, as outlined above, females might prefer to mate with males having intermediate levels of MHC dissimilarity, aiming at optimal rather than maximal MHC heterozygosity in the offspring (Penn & Potts 1999). Jacob *et al.* (2002) recently showed that women preferred the smell of male odour donors that had more MHC-allele matches with their own alleles, and thus did not prefer the most dissimilar genotypes.

In the three-spined stickleback, *Gasterosteus aculeatus*, females optimize allelic diversity at the MHC when they choose their mates. Females of this fish species preferred males with high MHC diversity, although those females that already had a high diversity themselves tended to

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prefer males with low diversity (Reusch *et al.* 2001). A new study shows that female sticklebacks take their own MHC diversity into account when choosing a mate, aiming at an estimated offspring MHC diversity of five or six alleles (Aeschlimann *et al.* 2003). Sticklebacks with an average of five or six different MHC alleles harboured the lowest parasite loads in natural populations and after experimental exposure to three naturally abundant parasites (Wegner *et al.* 2003*a*,*b*).

We tested, experimentally, whether allelic diversity at the MHC influences immune defence against parasites. Sticklebacks that varied in their individual number of MHC class II*B* molecules were exposed repeatedly to two of their natural parasites, the tapeworm *Schistocephalus solidus* and the microsporidian *Glugea anomala*. Measurements of infection, activity of the immune system and body condition were taken nine weeks after the second parasite challenge and compared with individual MHC diversity.

2. MATERIAL AND METHODS

(a) *Sticklebacks and parasites*

Sticklebacks were laboratory-bred offspring of individuals that had been seine-netted from an interconnected natural system of lakes in Schleswig-Holstein (northern Germany) and subsequently housed and genotyped as described in Reusch *et al.* (2001). To establish variability in MHC class II*B* diversity while balancing maternal genetic background, 15 females were mated sequentially to two males each, one of which led to a high combined MHC allele diversity, while the other one yielded a low MHC diversity, thereby establishing 30 sibships. Most of the males were used for only one mating.

Thirty offspring per sibship were randomly chosen for a repeated challenge with two parasite species, the tapeworm *S. solidus* and the microsporidian *G. anomala*. All individuals were exposed as follows (figure 1). They first received one *Macrocyclops albidus* copepod (an intermediate host of *S. solidus*) that had been exposed to six *S. solidus* larvae two weeks prior to fish infection (van der Veen & Kurtz 2002). Subsequently, the same fishes were exposed for 24 h to a *G. anomala* spore suspension prepared from freshly opened cysts, which had been obtained from infected sticklebacks bred in the laboratory. To facilitate acquired immune responses, all individuals were exposed a second time, two weeks after the first exposure, to both parasite species as described above.

(b) *Infection, body condition and immunity*

Nine weeks after the second challenge, sticklebacks were dissected to screen for *S. solidus* and *G. anomala* infections and to take measures of body condition and immunity. For the present study, only 10 out of the 30 challenged offspring from each sibship were analysed. To select these 10 offspring, a first set of seven offspring was always chosen randomly from each sibship. Subsequently, the observed infection success was taken into account, i.e. the last three offspring were selected non-randomly to cover all four possible combinations of infection: (i) exposed but not infected; (ii) infected with only *S. solidus*; (iii) infected with only *G. anomala*; and (iv) infected with both parasite species. The intention was to avoid a strongly unbalanced representation of these four infection classes over the sibships. It was, however, not possible to fill all classes in all sibships. Only the randomly selected fishes were considered in the analyses of infection success, while all other results are based on all individuals.

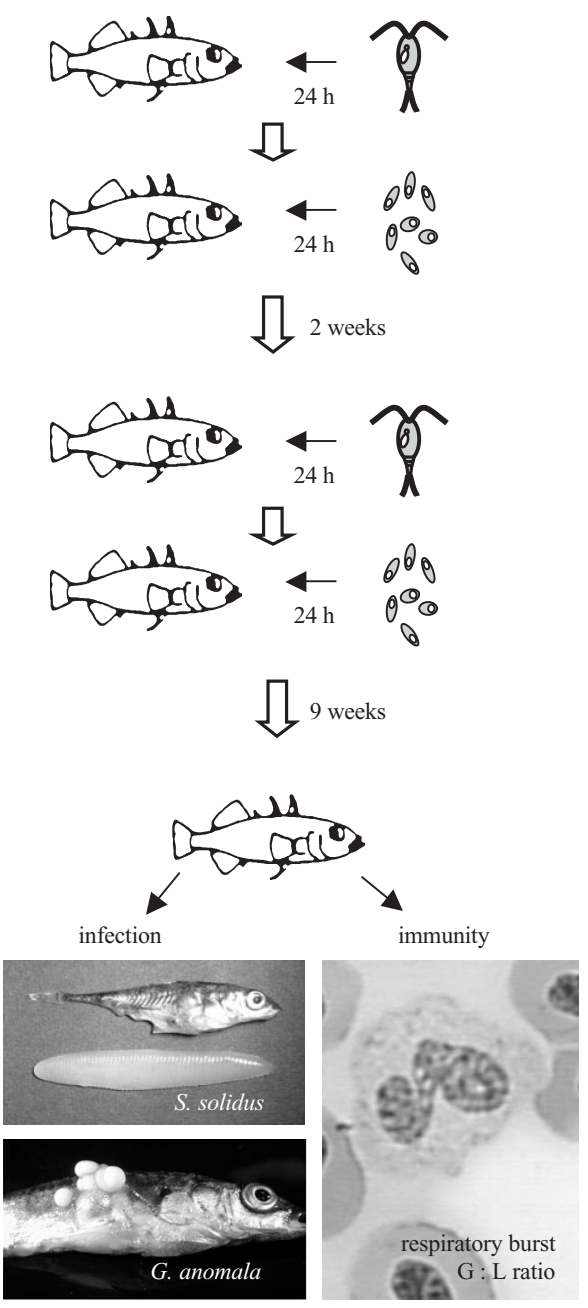

Figure 1. Experimental design. All stickleback individuals were repeatedly exposed to two parasite species *Schistocephalus solidus* tapeworms and *Glugea anomala* microsporidians. Nine weeks after the second exposure, sticklebacks were dissected to screen for parasite infection and to take measurements of immunity.

Fish body length (from the snout to the base of the tail, measured to the nearest mm) and the weight of the fishes and the tapeworms (to the nearest 0.1 mg) were determined. For all analyses fish net weight, i.e. not including the weight of the tapeworms, was considered. A parasite index (I_P) was calculated as $100 \times$ tapeworm weight/fish weight. For multiply infected fishes, the sum of the tapeworm weights was considered. The number of cysts was determined as a measure of the intensity of infection with *G. anomala*. Liver weight was recorded (to the nearest 0.1 mg) and a hepatosomatic index (I_H) was calculated as $100 \times$ liver weight/fish weight. I_H serves as a measure of

medium-term energy reserves (Chellappa *et al.* 1995). Furthermore, a fish condition factor (cf) (Frischknecht 1993) was calculated as $cf = 100 \times W/L^b$ (with fish weight W, fish length L and an exponent $b = 3.01$, as determined in a regression analysis).

To evaluate the immunological status of individual sticklebacks, we isolated leucocytes from the head kidney, the major haemopoietic and lymphoid organ of fishes (Zapata *et al.* 1996). Cell suspensions from head kidneys were prepared by forcing tissue through a sterile 40 µm nylon screen (Falcon Cell strainer 352340) into 4 ml of culture medium (RPMI-1640, Sigma R 8755 without phenol red, 10% distilled water, pH of 7.6). Cells were kept on ice. To wash cells, suspensions were centrifuged at 400*g* for 10 min at 4 °C. The cell pellet was re-suspended in 1 ml of culture medium and the cells were counted using a CASY cell counter (Schärfe Systems). As a first measure of general leucocyte activity, a leucocyte index (I_L) of the number of cells in the head kidney according to individual body weight was calculated.

As a more detailed measure, we microscopically determined the ratio of granulocytes to lymphocytes (G : L ratio) in smears of head-kidney cells from 134 out of the 166 fishes. Lymphocytes are the most important cells of the acquired immune system, while granulocytes are involved in innate immunity. The G : L ratio might therefore serve as a very rough estimate of the activity of the innate immune system in relation to the acquired immune system. Smears were not obtained from fishes with few cells, because priority was then given to measuring the 'respiratory burst' reaction (see below). For preparation of the smears, the cell suspensions were diluted 1 : 1 with foetal calf serum (Sigma). Smears were stained using a standard May–Grünwald method. Using ×1000 oil immersion, a total of 100 cells were identified per smear as granulocytes (G), lymphocytes (L) or erythrocytes, and a G : L ratio was determined. On average, the suspensions contained 26.0% lymphocytes (s.e. = 0.8%), 59.9% granulocytes (s.e. = 0.9%) and 14.1% erythrocytes (s.e. = 0.9%).

As a functional estimate of innate immune activity, we quantified the respiratory burst reaction. During the respiratory burst, reactive oxygen intermediates are generated to kill pathogens. We analysed the respiratory burst associated with phagocytosis of zymosan particles *in vitro* in a lucigenin-enhanced chemiluminescence (CL) assay (Scott & Klesius 1981). For this assay, the cell density was adjusted to 1.25×10^6 cells ml^{-1}. A volume of 160 µl of each individual cell preparation was then added to each of three wells of a CL 96-well plate. A volume of 20 µl of a 2.5 mg ml^{-1} solution of lucigenin (N,N′-dimethyl-9,9′-biacridinium dinitrate, Sigma M 8010) in phosphate-buffered saline (PBS) was added, and cell cultures were incubated for 30 min at 18 °C in a 3% CO_2 atmosphere. For induction of the respiratory burst reaction, 20 µl of a 7.5 mg ml^{-1} suspension of zymosan A from *Saccharomyces cerevisiae* (Sigma Z 4250) in PBS was added to two out of the three cell cultures of each individual, while the third well served as a control. Immediately after addition of the zymosan particles, light emission from CL, measured in relative luminescence units (RLU s^{-1}), was quantified at 20 °C on a Berthold MicroLumat Plus LB 96 V microplate luminometer. Measurements were obtained every 5 min over a time period of 3 h 30 min. For analysis, the area below the response curve (the integral, RLU) was determined with the WinGlow software delivered with the Berthold luminometer. The repeatability of this measure was 98.1%, as determined from the two replicates per fish. The mean area of the two replicates (or the single value for fishes with insufficient cells to run replicates) was used as a measure of the intensity of the respiratory burst reaction of an individual fish. Controls without zymosan generally yielded very low respiratory bursts (mean ± s.e.m. = $0.117 \pm 0.004 \times 10^6$ RLU, $n = 284$; compared with $1.465 \pm 0.052 \times 10^6$ RLU, $n = 299$, with zymosan) and were not considered in further analyses. Additional controls without lucigenin and/or without cells were included on each plate, but never led to any detectable CL.

(c) *MHC genotyping*

MHC sequence diversity was genetically determined, using spleen tissue for DNA extraction (DNeasy Tissue Kit, Qiagen). A 124 bp portion of the peptide-binding region of the MHC class II β-chain was analysed for single-strand conformation polymorphism (SSCP), using two types of reverse primers to increase the number of detectable MHC sequences (Binz *et al.* 2001; Reusch *et al.* 2001). For simplicity, we refer to these sequences as 'alleles' even though the loci of origin are unknown. PCR amplification was more problematic in these samples than in the previous studies. Finally, only individuals where amplification with both primers worked well were included, reducing the sample size to 166 individuals. In this sample, the mean number of MHC alleles per fish was 5.36 (±0.16).

(d) *Data analysis*

The total sample consisted of the 166 fishes in which MHC genotyping had been successful. However, the sample size was slightly reduced in some of the analyses, because an insufficient number of head-kidney cells were obtained from one individual, no body mass measurement had been obtained from another fish and one fish could not be sexed. To achieve normality for statistical analyses, respiratory burst was square-root transformed, I_H, I_L and the numbers of granulocytes and lymphocytes were logarithmically (ln) transformed and the G : L ratio was Box–Cox transformed, using the formula $y' = (y^{-0.4} - 1)/-0.1153$.

A multivariate analysis of variance (MANOVA) was calculated, with the respiratory burst, I_H and I_L as response variables, and including MHC diversity (number of alleles), infection status (whether or not the fishes were infected with *S. solidus* or *G. anomala*) and stickleback sex and condition (cf) as effects in the model. Interactions up to the second degree were originally included, but removed when not significant (in no case did significant interactions remain). Individual sticklebacks were used as the statistical replicates for these analyses, because MHC diversity was determined for each individual.

Nominal logistic, linear and polynomial regression models were used to analyse the effects of MHC diversity on parasite infection and immune parameters in more detail. The minima of the fitted polynomial curves were calculated by differentiation of the regression-curve equations. As a statistical control for sibship effects, identity of the father was included when necessary (maternal effects were controlled for by the double-mating design of the experiment).

For the number of *G. anomala* cysts, a generalized linear model with a negative binomial error distribution and a log-ratio link function was fitted, using Genstat 5 (Payne 2000). Confidence limits of 95% were obtained from a randomization test. For each randomization (1000 in total) we calculated the mean number of cysts for each level of MHC allele diversity. From these means, simultaneous 95% confidence intervals were calculated with a procedure implemented in the software package Genstat, which closely follows the methodology described by Hsu (1996). The observed mean values were then compared

with 95% confidence limits generated under the hypothesis of random association between MHC diversity and cyst number. For all other statistical analyses, the software JMP 4.0.4 for Macintosh (SAS Institute Inc. 2000) was used.

3. RESULTS

(a) *MHC diversity and parasite resistance*

Out of 166 sticklebacks considered in the final analysis, 32 (19.3%) were infected with only tapeworms (*S. solidus*), 40 (24.1%) with only microsporidians (*G. anomala*) and 11 (6.6%) with both parasites. The prevalence of tapeworm infection was not significantly influenced by MHC diversity (nominal logistic regression, considering only the randomly selected fishes, see § 2 for details: $\chi^2_1 = 0.542$, $p = 0.462$, $n = 124$). However, those sticklebacks that were infected with tapeworms suffered less from the infection when they had high MHC diversity: tapeworm mass (in relation to host mass, i.e. the parasite index, I_P) decreased with increasing MHC diversity. When we analysed the effect of MHC diversity on I_P in more detail, we found that a polynomial regression explained considerably more variation than did a simple linear regression (23.6% compared with 11.8%; quadratic regression: $r^2 = 0.236$, $F_{2,40} = 6.183$, $n = 43$, $p = 0.005$; significance of the quadratic term: $p = 0.017$; figure 2*a*). Sibship effects were not significant (including identity of the father into the model; effect of father: $p = 0.816$; whole model: $r^2 = 0.377$, $F_{13,29} = 1.350$, $n = 43$, $p = 0.242$). The regression remained significant when we used the mean I_P for each MHC diversity level as the statistical unit ($r^2 = 0.958$, $F_{2,3} = 34.553$, $n = 6$, $p = 0.009$). I_p was lowest in sticklebacks with allelic diversities close to the regression minimum of 6.2.

There was a slight effect of MHC diversity on the prevalence of microsporidians (nominal logistic regression: $\chi^2_1 = 2.889$, $p = 0.089$, $n = 124$, considering only the randomly selected fishes), which became significant when including the quadratic term for MHC diversity in the model ($\chi^2_2 = 6.673$, $p = 0.036$, $n = 124$). Sibship effects were not significant (effect of father: $p = 0.677$; whole model: $\chi^2_{17} = 27.062$, $p = 0.057$, $n = 124$). As a measure of the intensity of microsporidian infection the number of cysts per fish was used. Intensity seemed to show a pattern similar to the one observed for tapeworm I_P, i.e. the mean number of *G. anomala* cysts seems to be lower for sticklebacks with a moderately high number of MHC alleles (figure 2*b*). This effect of MHC diversity was significant when all fishes were considered and when the quadratic term for MHC diversity was entered into the model (generalized linear model with negative binomial distribution, without quadratic term for MHC diversity: d.f. = 1,164, deviance ratio = 2.50, $p = 0.116$, $n = 166$; including the quadratic term: d.f. = 2,163, deviance ratio = 10.23, $p < 0.001$, $n = 166$). However, it was not significant when only infected individuals were considered (d.f. = 1,49, deviance ratio = 0.09, $p = 0.761$, $n = 51$; including the quadratic term: d.f. = 2,48, deviance ratio = 1.24, $p = 0.290$, $n = 51$).

(b) *MHC diversity and immunity*

MHC diversity did not significantly influence metabolic condition in terms of I_H (table 1). The head-kidney leucocyte index I_L was only weakly affected by MHC diversity (table 1). By contrast, MHC diversity strongly influenced the G : L ratio. Sticklebacks with high MHC diversity showed a lower G : L ratio. A polynomial explained more variation than a linear term (15.3% compared with 7.5%; polynomial regression: $r^2 = 0.153$, $F_{2,131} = 11.816$, $n = 134$, $p < 0.0001$; significance of the

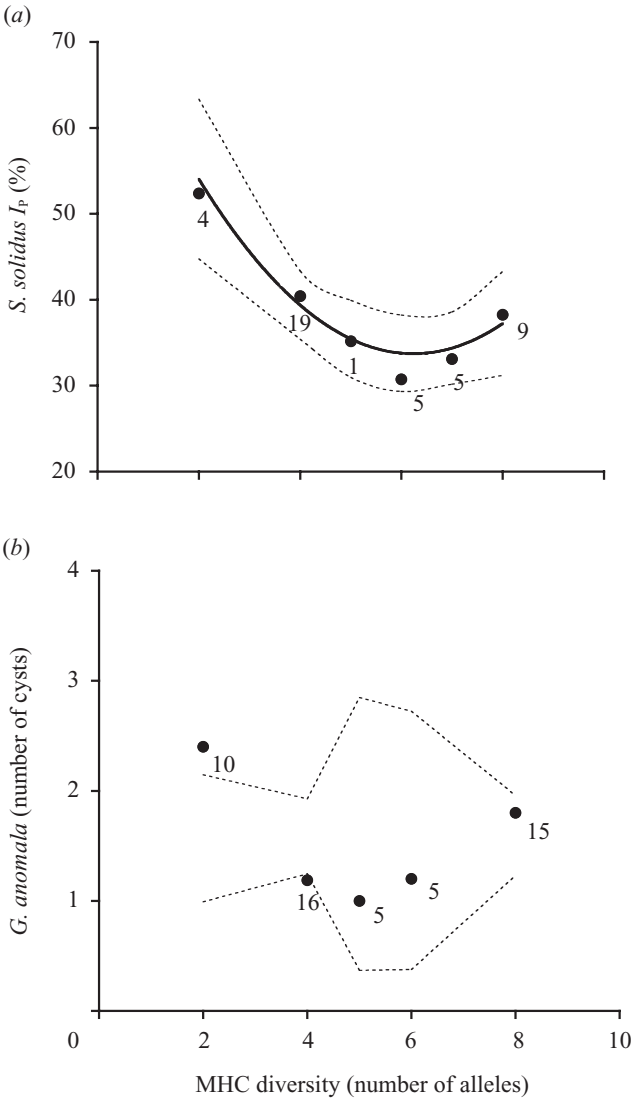

Figure 2. (*a*) *Schistocephalus solidus* parasite index (I_P) of stickleback fishes ($n = 43$) in relation to individual MHC class II*B* allele diversity. For each level of MHC diversity, means are shown for sticklebacks infected with *S. solidus* after repeated experimental exposure to both *S. solidus* tapeworms and *Glugea anomala* microsporidians. Numbers next to data points indicate sample size, the solid line indicates a polynomial regression ($I_P = 45.08 - 1.96 \times \mathrm{MHC} + 1.13 \times (\mathrm{MHC} - 5.37)^2$), and broken lines show 95% confidence limits. (*b*) Number of *G. anomala* cysts of sticklebacks ($n = 51$) in relation to individual MHC class II*B* allele diversity. For each level of MHC diversity, means are shown for sticklebacks infected with *G. anomala* after repeated experimental exposure to both parasites. Numbers next to data points indicate sample size, and the broken lines indicate 95% confidence limits for a random association between MHC and cyst number, obtained from a randomization test (i.e. individuals with two MHC alleles deviate from a random association).

Table 1. Multivariate analysis of variance (MANOVA) for the effect of MHC diversity, tapeworm infection (*Schistocephalus solidus*), microsporidian infection (*Glugea anomala*), fish gender (sex) and condition factor (cf) on the hepatosomatic index I_H (ln transformed), the relative number of head-kidney leucocytes I_L (10^6 cells g^{-1}, ln transformed) and the respiratory burst (RLU, square root transformed) of head-kidney cells of sticklebacks ($n = 163$).

	response variable							
	I_H		I_L		respiratory burst		whole model	
effect	$F_{1,157}$	p	$F_{1,157}$	p	$F_{1,157}$	p	$F_{3,155}$	p
MHC diversity	0.560	0.455	3.219	0.075	13.731	< 0.001	5.520	0.001
S. solidus	51.956	< 0.001	0.036	0.850	4.261	0.041	21.380	< 0.001
G. anomala	1.057	0.305	0.588	0.444	1.020	0.314	0.985	0.402
sex	15.209	< 0.001	6.559	0.011	4.324	0.039	6.416	< 0.001
cf	0.038	0.847	0.653	0.420	4.253	0.041	1.547	0.205
	$F_{5,157} = 17.900$ $p < 0.001$		$F_{5,157} = 2.467$ $p = 0.035$		$F_{5,157} = 5.143$ $p < 0.001$		Wilks' $\lambda = 0.521$ $p < 0.001$	

quadratic term: $p = 0.0007$). For sticklebacks infected with *S. solidus* or with *G. anomala*, only polynomial regressions remained significant (polynomial regressions: *S. solidus*, $r^2 = 0.209$, $F_{2,28} = 3.710$, $n = 31$, $p = 0.037$; *G. anomala*, $r^2 = 0.367$, $F_{2,39} = 11.316$, $n = 42$, $p = 0.0001$). Infection with *S. solidus* or with *G. anomala* increased the G : L ratio ($F_{1,132} = 4.910$, $n = 134$, $p = 0.028$, and $F_{1,132} = 4.927$, $n = 134$, $p = 0.028$, respectively).

Was the effect of MHC diversity on the G : L ratio mainly mediated by differences in the numbers of granulocytes or lymphocytes? Using the head-kidney cell counts in combination with the relative numbers of granulocytes and lymphocytes to calculate their absolute numbers (adjusted for fish weight), we found a significant effect of MHC diversity on granulocytes, but not on lymphocytes (polynomial regressions: granulocytes, $r^2 = 0.058$, $F_{2,131} = 3.998$, $n = 134$, $p = 0.021$; lymphocytes, $r^2 = 0.007$, $F_{2,131} = 0.487$, $n = 134$, $p = 0.615$).

Did the increased proportion of granulocytes in individuals with low MHC diversity result in any functional differences, i.e. an increased respiratory burst reaction? MHC diversity strongly influenced the respiratory burst reaction (table 1). Sticklebacks with few MHC alleles showed a stronger respiratory burst. For a more detailed analysis of this effect, respiratory burst was plotted against MHC diversity (figure 3). When fitting a linear regression line, MHC diversity explained 7.1% of the variation in respiratory burst. However, a quadratic curve increased the fit considerably, accounting for 13.0% of the variation (polynomial regression: $r^2 = 0.130$, $F_{2,162} = 12.075$, $n = 165$, $p < 0.0001$; significance of the quadratic term: $p = 0.001$). Sibship effects were significant, but the effect of MHC diversity was not reduced when these were included (effect of father: $p < 0.0001$; MHC diversity: $p = 0.008$ and $p < 0.0001$, for the linear and the quadratic terms, respectively; whole model: $r^2 = 0.517$, $F_{17,147} = 9.249$, $n = 165$, $p < 0.0001$). When using mean respiratory burst for each level of MHC diversity, the polynomial regression was still significant ($r^2 = 0.782$, $F_{2,4} = 7.163$, $n = 7$, $p = 0.048$). The lowest respiratory burst values were obtained at an allelic diversity of 6.4.

Tapeworm-infected sticklebacks had a significantly increased respiratory burst compared with uninfected ones (table 1). However, the effects of MHC diversity and parasite infection were not confounded in our study. When analysing infected and non-infected sticklebacks separately, quadratic regressions fitted the data better than linear regressions for all the groups. The regressions were significant for all groups, except for sticklebacks infected with *S. solidus*. Here, a linear regression was not significant, while the quadratic curve was almost significant ($r^2 = 0.132$, $F_{2,39} = 2.978$, $n = 42$, $p = 0.063$).

The higher the proportion of granulocytes, the stronger was the respiratory burst (linear regression: $r^2 = 0.106$, $F_{1,132} = 15.607$, $n = 134$, $p = 0.0001$). However, the G : L ratio alone was not sufficient to explain the effect of MHC

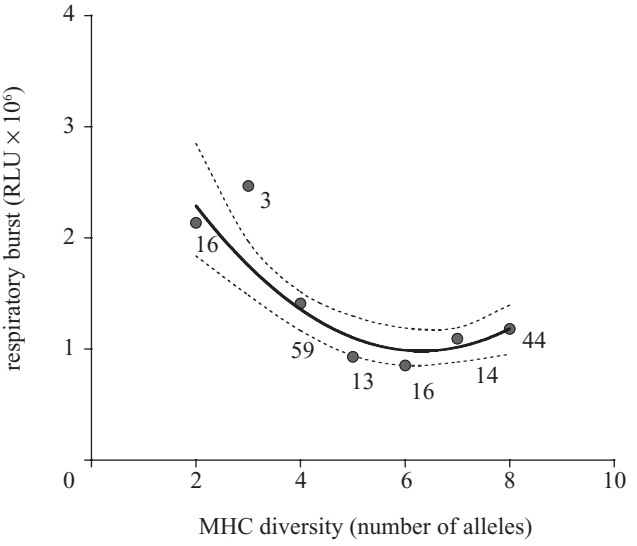

Figure 3. Respiratory burst of head-kidney leucocytes of sticklebacks ($n = 165$), in relation to MHC class II*B* allele diversity. Fishes had been exposed to both *Schistocephalus solidus* tapeworms and *Glugea anomala* microsporidians. Respiratory burst induced by phagocytosis of zymosan particles was measured in relative luminescence units (RLU) by a lucigenin-enhanced chemiluminescence assay. Means (transformed back from square-root-transformed values) are shown for each level of MHC diversity. Numbers next to data points indicate sample size, the solid line indicates a polynomial regression ($\sqrt{\text{respiratory burst}} = 1321 - 54.18 \times \text{MHC} + 27.37 \times (\text{MHC} - 5.36)^2$), and broken lines show 95% confidence limits.

diversity on the respiratory burst. When using the residuals from the above regression as the dependent variable, the effect of MHC diversity remained significant (polynomial regression: $r^2 = 0.062$, $F_{2,131} = 4.324$, $n = 134$, $p = 0.015$). This indicates that the effect of MHC diversity on the respiratory burst was mediated to some extent by the G : L ratio, but also by other factors, such as the activation status of the granulocytes.

4. DISCUSSION

Based on patterns of parasite burden in the field (Wegner *et al.* 2003*b*) and mate preference of females (Reusch *et al.* 2001), we expected that sticklebacks with a moderately high allelic diversity at the MHC might suffer least from parasite infection after experimental exposure to the tapeworm *S. solidus* and the microsporidian *G. anomala*. We found that MHC diversity correlated negatively with tapeworm burden, while there was no significant effect of MHC diversity on the probability of tapeworm infection. An MHC-based acquired immune response might not eliminate the worms completely, but could reduce the growth of the worms, thereby limiting the fitness reduction associated with the infection (Arme & Owen 1967; Milinski 1985). We might not see any effect of MHC on the probability of a first infection, because acquired immunity takes time to develop, probably longer in fishes than in mammals (Nie & Hoole 1999). Thus, the time between the first and second challenges could also have been too short. Moreover, different immune mechanisms could be involved in preventing an infection and in limiting the degree of the infection. Innate immune functions might be most important for destroying tapeworms at an early stage, while antibodies might be directed against older worms to limit their growth.

In contrast to tapeworm prevalence, the probability of infection with *G. anomala* microsporidians showed a significant association with MHC diversity. Also, the intensity of infection seemed to be slightly influenced by MHC diversity. Taken together, sticklebacks with low MHC diversity clearly suffered more from the infections, indicating a positive effect of allelic diversity at the MHC. Given that three-spined sticklebacks seem to possess up to six MHC class II*B* loci (Sato *et al.* 1998; Binz *et al.* 2001), diversity in the number of MHC alleles per individual could, in theory, be created both by differing numbers of loci and by heterozygosity at existing loci. Variation in number of loci has been demonstrated for other fish species (Malaga-Trillo *et al.* 1998; Figueroa *et al.* 2001). At the present time, we cannot discriminate between these two potential causes, and therefore concentrated on the number of different alleles per individual as a compound estimate.

Based on parasite burden in the field and female mating decisions, we expected increasing parasitization for individuals with more than six alleles. Such an optimum can best be described with a polynomial regression, and inclusion of a quadratic term improved the model fit for tapeworm burden and for *Glugea* infection. The optimal allelic diversity found in the present study (six alleles) closely resembles the mean number of MHC alleles per individual found in a natural population (5.8; Reusch *et al.* 2001) and the optimum allelic diversity with regard to parasite burden in the field (5.2; Wegner *et al.* 2003*b*). This suggests that there is indeed an optimum parasite resistance at a moderately high MHC diversity. However, further experimental data including data from more sticklebacks with high numbers of MHC alleles would be necessary to corroborate this finding for these two parasite species. Recent experimental evidence with three other parasites, *Diplostomum spathaceum*, *Camallanus lacustris* and *Anguillicola crassus*, supports our hypothesis that optimal rather than maximal MHC diversity confers the highest level of parasite resistance and therefore the highest fitness (Wegner *et al.* 2003*a*).

We do not know whether all MHC alleles that were detected with the SSCP method are expressed. However, out of 15 sequences amplified from genomic DNA 14 corresponding sequences were also found in reverse transcribed messenger RNA (K. M. Wegner and T. B. H. Reusch, unpublished data). On the other hand, the SSCP method tends to underestimate the true diversity, because small differences between alleles might not be resolved (Binz *et al.* 2001; Reusch *et al.* 2001). The allele numbers that are given here should therefore be viewed as correlates of the true MHC diversity. Nevertheless, the previous arguments may have increased the variance in our results, but cannot bias the effect, and are thus conservative for our findings. Moreover, the study by Wegner *et al.* (2003*a*), which came to similar conclusions, was based on expressed MHC sequences.

How does MHC diversity and infection status relate to immune responses? Individuals with low allelic diversity showed a higher proportion of granulocytes. MHC diversity had an effect on the number of granulocytes, but not lymphocytes, in the head kidney. This indicates that appropriate T- and B-lymphocyte clones might proliferate upon infection in MHC-optimal fishes, but the absolute number of lymphocytes seems to remain fairly unaffected. Granulocytes, by contrast, seem to increase in number in MHC-suboptimal fishes, indicating a stronger activity of the innate immune system. Unfortunately, apart from these cell counts, we do not have any direct measures of adaptive immune responses. However, the innate and the adaptive immune systems are mutually regulated (Janeway *et al.* 1999; Luster 2002). We might, therefore, expect that individuals with an optimal adaptive immune system (e.g. optimal MHC diversity) could reduce the expression of innate immune traits. Consistent with this expectation, we found that sticklebacks with low MHC diversity had a more strongly activated respiratory burst, a measure of the activity of the innate immune system. This measure was lowest for sticklebacks with six alleles. However, more data on individuals with high allele numbers would be needed to ensure that there is a minimum activity of the respiratory burst coinciding with the peak parasite resistance at six alleles.

We assume that the effect of MHC on innate immunity is based on a rather indirect downregulation of innate immunity when the adaptive immune response works well. However, the opposite could also be expected. Given that energy limitation determines investment in immunity, individuals in better condition should be able to increase both lines of defence. Yet our data indicate that individuals with suboptimal MHC diversity are unable to turn an initial innate immune response into an acquired response,

which might lead to a long-lasting strong activation of innate immune traits (Janeway *et al.* 1999; Luster 2002). A strong expression of innate immunity can be costly. Immune reactions themselves have been shown to be energetically costly (Lochmiller & Deerenberg 2000). Our data might point to such costs, as tapeworm-infected sticklebacks had a strongly reduced metabolic condition, I_H (3.44 versus 5.03) compared with uninfected ones (table 1), which might be a consequence of the infection itself, the activated immune system, or both.

Probably even more important than metabolic costs, immune defence can have its own costs (von Schantz *et al*. 1999). Such costs can, for example, arise from specific immunity directed against self-antigens in autoimmune disorders (Janeway *et al.* 1999), or from collateral damage caused by unspecific defences. In particular, reactive oxygen intermediates that are produced during the respiratory burst can contribute to ageing, cancer and immune or brain disorders (Ames *et al.* 1993). Among the mechanisms that restrict the harmful side-effects of reactive oxygen, radical scavengers such as carotenoids have attracted much attention (Lozano 1994; Olson & Owens 1998). Carotenoid-based male breeding signals play an important role during female choice in sticklebacks and in many other animal taxa (Milinski & Bakker 1990; Olson & Owens 1998). Female sticklebacks prefer males with bright red throats (Milinski & Bakker 1990). These carry fewer parasites after experimental infection with *Ichthyophthirius multifiliis* (Milinski & Bakker 1991), and have offspring that are more resistant to *S. solidus* (Barber *et al.* 2001). Our results give a possible explanation for these findings. Males with optimal immunogenetics might decrease their respiratory burst, thereby reducing the demands on oxygen scavengers such as carotenoids to buffer oxidative stress. Surplus carotenoids could then be allocated to breeding ornamentation. This scenario proposes a mate-choice mechanism that uses both visual and olfactory cues.

The present study gives experimental evidence for a beneficial effect of high individual MHC diversity with regard to parasite resistance. It further indicates that a moderately high diversity leads to optimal immune performance. A few other studies showed selective advantages of MHC heterozygosity, potentially leading to benefits of MHC-based mate choice (Penn 2002; Penn *et al.* 2002). However, the choice of MHC-dissimilar mating partners can also promote inbreeding avoidance (Penn 2002). Studies in fish species demonstrate the relevance of MHC for resistance, while inbreeding avoidance seems not to be particularly important (Landry *et al.* 2001; Langefors *et al.* 2001). Choosing optimal diversity at the MHC may nevertheless be a strategy of optimal outbreeding (Bateson 1983), if the MHC diversity is linked to general genomic diversity. However, the number of MHC alleles per individual was not correlated with genome diversity measured at seven microsatellite loci in a previous study in the same stickleback population (Reusch *et al.* 2001). Furthermore, in contrast to other species (such as mice or humans) where MHC-dependent mate-choice patterns have been shown, the risk of mating with close kin is low in natural populations of sticklebacks (Steck *et al.* 1999; Reusch *et al.* 2001). Thus, mate choice seems to aim at optimizing offspring immunity in sticklebacks. Our study suggests consequences of mate choice for immunity that have the potential to be important in other vertebrate species.

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