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MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach

Joachim Kurtz*, K. Mathias Wegner†, Martin Kalbe, Thorsten B. H. Reusch‡, Helmut Schaschl¶, Dennis Hasselquist‖ and Manfred Milinski

Department of Evolutionary Ecology, Max Planck Institute of Limnology, August-Thienemann-Strasse 2, 24306 Plön, Germany

Individual variation in the susceptibility to infection may result from the varying ability of hosts to specifically recognize different parasite strains. Alternatively, there could be individual host differences in fitness costs of immune defence. Although, these two explanations are not mutually exclusive, they have so far been treated in separate experimental approaches. To analyse potential relationships, we studied body condition and oxidative stress, which may reflect costs of immunity, in three-spined sticklebacks that had been experimentally exposed to three species of naturally occurring parasite. These sticklebacks differed in a trait, which is crucial to specific parasite defence, i.e. individual genetic diversity at major histocompatibility complex (MHC) class IIB loci. Oxidative stress was quantified as tissue acrolein, a technique that has been applied to questions of immuno-ecology for the first time. We measured gene expression at the MHC and other estimates of immune activation. We found that fish with high levels of MHC expression had poor condition and elevated oxidative stress. These results indicate that MHC-based specific immunity is connected with oxidative stress. They could, thus, also be relevant in the broader context of the evolution of sexually selected signals that are based on carotenoids and are, thus supposed to reflect oxidative stress resistance.

Keywords: major histocompatability complex; immunocompetence; oxidative stress; parasite resistance; coevolution; three-spined stickleback

1. INTRODUCTION

Individuals often vary strongly in their resistance to infectious diseases (Frank 2002; Schmid-Hempel 2003). From an evolutionary perspective, such variation has usually been explained by two factors: (i) specific immunological compatibility may render hosts that are resistant to one strain or species of pathogen susceptible to another (for review see Schmid-Hempel & Ebert 2003). (ii) Since immune reactions are costly to produce, hosts that cannot afford to raise efficient defence will be more susceptible (for review see Sheldon & Verhulst 1996; Schmid-Hempel 2003). Although these two explanations are not mutually exclusive, they have so far been treated in separate experimental approaches. For example, variance in resistance that is based on genes of the major histocompatibility complex (MHC) is usually regarded as evidence for the first cause of variability (for review see Apanius *et al.* 1997; Penn & Potts 1999; Wegner *et al.* 2004). The MHC is central to specific responses of the acquired immune system of vertebrates. Investment in costly immune defence provides some good examples for the second cause of variability in both vertebrates and invertebrates (Moret & Schmid-Hempel 2000; Rolff & Siva-Jothy 2003).

Both innate and adaptive immunity can be costly, and both parts of the immune system are connected (Dixon & Stet 2001; Goldstein 2004), so that specificity in conjunction with the costs of defence should determine host resistance. Here, we want to test whether MHC-based specific immunity is connected to the potential costs of immunity. Such costs may come as energetic expenditure on immune defence (e.g. Ots *et al.* 2001) and as immunopathology (Graham *et al.* 2005), e.g. through 'oxidative stress' (von Schantz *et al.* 1999; Finkel & Holbrook 2000).

Normal intracellular metabolism in mitochondria is one important source of oxidative stress (Finkel & Holbrook 2000). However, oxidative damage can also more directly result from activated immune reactions, such as the 'respiratory burst', during which reactive oxygen species (ROS, 'free radicals') are produced (Chanock *et al.* 1994; Bogdan *et al.* 2000; Fang 2004). Although directed against pathogens, the effect might be difficult to restrict to its target and then cause oxidative stress, i.e. detrimental for the host (von Schantz *et al.* 1999; Nathan & Shiloh 2000). ROS produced during an immune response have been shown to lead to host tissue damage. For example, the inhibition of ROS-mediated processes can reduce mortality in mice with virus-induced pneumonia (Akaike *et al.* 1996), and eradication of

* Author and address for correspondence: ETH Zürich, Ecology & Evolution, ETH-Zentrum CHN J12.1, Universitätsstrasse 16, 8092 Zürich, Switzerland (joachim.kurtz@env.ethz.ch).
† Present address: ETH Zürich, Ecology & Evolution, ETH-Zentrum CHN H72, Universitätsstrasse 16, 8092 Zürich, Switzerland.
‡ Present address: Institut für Evolution und Biodiversität, Universität Münster, Hüfferstrasse 1, 48149 Münster, Germany.
¶ Present address: Konrad-Lorenz Institute for Ethology, Savoyenstrasse 1a, 1160 Vienna, Austria.
‖ Present address: Department of Animal Ecology, Lund University, Ecology Building, 223 62 Lund, Sweden.

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Helicobacter pylori attenuates oxidative stress in the human gastric mucosa (Pignatelli *et al.* 2001).

Three-spined sticklebacks (*Gasterosteus aculeatus*) have recently become a prime model system for studying evolutionary and ecological aspects of parasite resistance (Milinski & Bakker 1990; Barber *et al.* 2001; Reusch *et al.* 2001; Wegner *et al.* 2003; Kurtz *et al.* 2004). Stickleback individuals with either low or high number of MHC class IIB sequence variants suffer more from parasite infection than fish with an intermediate number (Wegner *et al.* 2003; Kurtz *et al.* 2004), which might result from the balance between antigen recognition and self-tolerance (Nowak *et al.* 1992).

In order to relate MHC-mediated specific defence to oxidative stress, we have now obtained immunological and physiological measures from a sub-sample of sticklebacks that have previously been used in an experiment that demonstrated enhanced parasite resistance in fish with an optimal (i.e. intermediate) number of MHC class II variants (Wegner *et al.* 2003). Using these fish ensured that the MHC-based immune system had influenced the infection. Moreover, we recently found that sticklebacks with low diversity in the MHC have higher levels of MHC gene expression (Wegner *et al.* in press). This suggests that they might compensate for the lack in 'quality' or diversity of alleles by raising the 'quantity' of MHC proteins that are available for the presentation of antigens. MHC expression may, thus represent an important, as yet disregarded, cost of the activated immune response, in particular in fish with non-optimal MHC diversity. Further, we found previously that non-optimality of individual MHC variation results in an increased respiratory burst reaction against parasites (Kurtz *et al.* 2004). If respiratory burst leads to oxidative stress, this should be costly for the host.

Taken together, these previous results suggest that specific MHC-mediated defence and costs of immunity are related in sticklebacks. We, therefore, now test the following predictions: costs of mounting an immune response towards parasitic exposure should be higher in individuals, which (i) do not possess the optimal (i.e. intermediate) number of MHC variants and/or (ii) most strongly increased their MHC expression. Such costs may mainly occur as energy depletion or as oxidative stress, which we estimated from body condition indices and from tissue acrolein levels, respectively (Chellappa *et al.* 1995; Uchida *et al.* 1998; Uchida 1999). To identify potential mediators of oxidative stress, we obtained additional measures of immune activity such as the respiratory burst.

2. MATERIAL AND METHODS

(a) *Parasites: exposure of sticklebacks and screening of the infection*

For experimental exposure of sticklebacks, we used the nematodes *Camallanus lacustris* and *Anguillicola crassus* and the trematode *Diplostomum pseudospathaceum*, all obtained from populations connected to the host population. The two nematode species are trophically transmitted via copepods. To raise the infective nematode larvae in the lab, *Macrocyclops albidus* copepods were individually infected with *A. crassus*, obtained from swim bladders of adult European eels (*Anguilla anguilla*), or with *C. lacustris*, obtained from guts of adult perch (*Perca fluviatilis*), respectively. Infective cercariae of *D. pseudospathaceum* were obtained from the first intermediate host, the freshwater snail *Lymnaea stagnalis*. In a previous publication (Wegner *et al.* 2003), this species of *Diplostomum* was denoted as *D. spathaceum*, but more careful recent morphological determination revealed that it is the species *D. pseudospathaceum*.

The sticklebacks used in this study were a subset of fish used for previous experiments (Wegner *et al.* 2003). They were lab-bred offspring of fish caught from a large population in the 'Große Plöner See', northern Germany. Fish were reared in water at a temperature of 18 °C and 14 h light per day, which corresponds to natural breeding conditions. Four fish families (of six used in the previous study) contributed 30 individuals each to the present experiment. Five individuals from each family were randomly assigned as controls while each of the remaining 25 fish was exposed either once (10 fish) or twice (15 fish) to all three species of parasites. The mean exposure doses were *ca* 10.21 ($\pm$0.07)/9.93 ($\pm$0.05) *A. crassus*, 2.83 ($\pm$0.14)/6.08 ($\pm$0.02) *C. lacustris* and exactly 20/40 *D. pseudospathaceum* for the first/second exposure, respectively. These treatments originally aimed at creating different exposure regimes. However, since there was no significant effect on the parameters considered here, we did not consider these treatments further.

Fish were dissected in the third week after the second exposure, one sibship (i.e. 30 fish) per day. Fish standard length (to the nearest mm) and weight (to the nearest 0.1 mg) were determined, spleens were weighed and immediately transferred into RNAlater solution (Qiagen) for RNA extraction (see below), head kidneys were kept on ice and used immediately for the preparation of leucocytes for the immune assays (see below), and livers were weighed and frozen at -70 °C for the quantification of oxidative stress (see §2*d*). Gut (*C. lacustris*), swim bladder wall (*A. crassus*) and eye lenses (*D. pseudospathaceum*) of the sticklebacks were then carefully screened for infections with the three species of parasite.

(b) *Body condition of the sticklebacks*

As a measure of energy reserves, we quantified the hepatosomatic index I_H, i.e. the weight of the liver (to 0.1 mg) relative to body weight, as $100\times$liver weight/fish weight. I_H is frequently used in fish as an estimate of medium term energy reserves, i.e. the metabolic condition (Chellappa *et al.* 1995). This measure is thus, more relevant here than the also widely used weight–size relationships. However, such a standard body condition factor (CF) was also calculated here according to Frischknecht (1993) as $CF = 100 \times W/L^b$ (the coefficient b is the slope obtained from a linear regression of log-transformed values of W and L).

(c) *Immune defence: estimates of innate immunity of sticklebacks*

To analyse the activity of the immune system of the sticklebacks, we obtained diverse measures from each fish and determined a spleen index I_S, i.e. the weight of the spleen according to body weight. In bony fish, the spleen is an important peripheral lymphoid organ, i.e. it is mainly involved in adaptive immunity. However, the head kidney is the major immune organ of bony fish (Zapata 1996). Thus, we estimated the overall immune activity from a leukocyte index I_L, i.e. the number of leukocytes obtained from the head kidney according to individual body weight. Moreover, as a measure of the relative activity of the innate immune

system, we determined the $G:L$ ratio, i.e. the proportion of granulocytes (cells involved in innate defence) in relation to lymphocytes (cells involved in adaptive immunity) in the head kidney. Finally, as a functional estimate of the level of activity of the innate immune system, we quantified the respiratory burst reaction of head kidney cells in an *in vitro* assay.

To obtain these measures, we isolated leucocytes from the head kidneys as previously described (Kurtz *et al.* 2004). Differential cell counts were obtained on a Becton Dickinson FACSCalibur™ flow cytometer using the CellQuest Pro 4.02 software. All the samples were supplemented with propidium iodide (2 mg l^{-1}, Sigma Aldrich) to detect dead cells. Forward- and side-scatter values (FSC/SSC characteristics) of at least 10 000 cells were acquired in linear mode; fluorescence intensities at wavelengths of 530 and 585 nm were acquired at log-scale. Cellular debris with low-FSC characteristics and dead cells (propidium iodide positive) were excluded from further evaluation. Different cellular subsets were denoted as lymphocytes (low FSC/low SSC) and granulocytes (high FSC/high SSC; Scharsack *et al.* 2004). Absolute cell counts were determined with the standard cell dilution assay (Pechhold *et al.* 1994) in a modified form, by addition of 2×10^5 green fluorescent particles (4 μm, Polyscience, USA) to each tube, as a standard for counting (Scharsack *et al.* 2004). Cell density was adjusted to 1.25×10^6 live cells ml^{-1}, corresponding to 2×10^5 cells per assay, and respiratory burst associated with phagocytosis of zymosan particles was measured *in vitro* in a lucigenin-enhanced chemiluminescence (CL) assay modified from Scott & Klesius (1981), as described in Kurtz *et al.* (2004).

(d) *Oxidative stress: measuring protein-bound acrolein in liver tissue*

For measuring oxidative stress in stickleback tissue, we adapted a technique that is based on the detection of protein-bound acrolein, a marker of oxidative protein damage (Uchida 1999; Uchida *et al.* 1998). Acrolein (CH_2=CH–CHO) is ubiquitously generated in biological systems through lipid peroxidation. It is accumulated as a stable product, covalently bound to protein, mainly as an acrolein–lysine adduct (FDP-lysine).

To produce tissue homogenates, previously weighed stickleback livers were added to in 0.01 M phosphate buffered saline, pH 7.4 (PBS, SIGMA, P 3813), adjusted to 10 mg fresh weight per millilitre. Tissue was disrupted by sonication (for 2 min; output level 3, duty cycle 50%) in a Branson Ultrasonic Disruptor Sonifier W-250 with a high-intensity cup horn (001-059-046, Heinemann, Schwäbisch Gmünd, Germany), under constant cooling to avoid over-heating of the samples. Insoluble material was removed by centrifugation at 4500 g for 10 min at 4 °C. Protein content in the supernatant was determined (Roti Nanoquant, K 880.1, Carl Roth GmbH & Co., Karlsruhe, Germany) and then adjusted to 30 μg protein ml^{-1} PBS for the subsequent ELISA.

A standard indirect ELISA protocol, using the monoclonal antibody mAb5F6 as the primary antibody was used, following Uchida *et al.* (1998): for 'coating', each well of a 96 well plate (Nunc Maxisorp) was filled with 100 μl of the liver tissue homogenate (3 μg protein per well) prepared as described above. All samples (including controls) were added to the plate in duplicate. On each plate, we included as a positive control, acrolein-modified BSA, prepared by the reaction of 1 mg BSA with 10 mM acrolein in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37 °C. As a negative control, we added unmodified BSA. Plates were incubated overnight at 4 °C and plates subsequently 'washed' three times with 0.1 M TBS–Tween (Tris buffered saline, containing 0.05% Tween 20, 0.138 M NaCl, 0.0027 M KCl, pH 7.8). For 'blocking', the plate was incubated for 30 min at 37 °C with 200 μl 1% BSA in TBS–Tween per well. After 'washing' (three times as described above), 100 μl of the monoclonal antibody mAb5F6, diluted 1 : 1000 in TBS–Tween, were added and incubated for 60 min at 37 °C. After 'washing' (three times), 200 μl of a horse radish peroxidase (HRP) conjugated anti-mouse IgG produced in rabbit (SIGMA, A 9044), diluted 1 : 10 000 in TBS–Tween was added as the secondary antibody, and incubated for 60 min at 37 °C. After 'washing' (three times), 200 μl of the substrate solution (SIGMA Fast OPD, P 9187) was added and the reaction kinetic was recorded for 60 min at 450 nm on a BIO-TEK PowerWave×Select microplate scanning spectrophotometer (BIO-TEK Instruments, Inc., Winooski, Vermont, USA), using the BIO-TEK KC4 (2000) software. Means of the duplicate values reached after 30 min, with the BSA controls subtracted to account for differences among plates, were used for further analyses. We further affirmed the reliability and specificity of the ELISA method in additional tests with serial dilutions of the primary antibody, and with competition assays, where the primary antibody was pre-incubated with acrolein-modified KLH at 4 °C over night.

(e) *MHC: individual genetic diversity and gene expression*

MHC class IIB sequence diversity was genetically determined, using mRNA extracted from spleen tissue (RNeasy, Qiagen, Hilden) and reverse transcribed into cDNA (Omniscript RT, Qiagen; for details, see Wegner *et al.* in press). A 124 base pair (bp) long 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). Due to recent gene duplication, potentially in conjunction with inter-locus gene conversion, the exact locus designation of these MHC class IIB sequences cannot be easily identified through PCR-based strategies (Reusch *et al.* 2004). We, thus, refer to these sequences as 'variants', which were denoted as 'alleles' in previous publications (Reusch *et al.* 2001; Kurtz *et al.* 2004).

Expression levels were measured by a real time PCR assay comparing the template quantity of MHC class II transcripts against the constitutive house-keeping gene β-actin, i.e. the difference between the cycles at which the respective PCR reactions enter the exponential phase (ΔCT). Real time PCR was performed on an ABI SDS 7000 (Applied Biosystems) with ABsolute QPCR SYBR© green master mix (Abgene) amplifying a 105 bp stretch from the exon 2 of MHC class IIB covering all known MHC class IIB sequences of sticklebacks and a 260 bp stretch of the β-actin gene (for details see Wegner *et al.* in press).

(f) *Data analysis*

The original sample consisted of four fish families with 30 fish each, i.e. 120 fish. Two fish died before the end of the experiment, two fish originally assigned to the control group were exposed by mistake and three fish were mixed up, reducing the total sample to 113 individuals. The sample size was further reduced in some of the analyses, since we did not

Table 1. Relation of immunological and morphological parameters to metabolic condition (left) and oxidative stress (right) of parasitized sticklebacks. (Data refer to mixed models (whole model: I_H, $r^2=0.26$; oxidative stress, $r^2=0.38$, $n=104$) that further included fish family as a random factor, which explained 16.5 and 22.3% of the variance (REML estimates) for I_H and oxidative stress, respectively. Bold type indicates a significance of $p<0.05$.)

	metabolic condition (I_H)				oxidative stress (liver acrolein, OD 450)			
factors	d.f.	SS	F	p	d.f.	SS	F	p
fish sex	1	0.169	2.659	0.106	1	0.0000	0.000	0.994
MHC diversity	1	0.032	0.496	0.483	1	0.0048	1.691	0.197
MHC expression (ΔCT)	1	0.614	9.663	**0.003**	1	0.0381	13.362	**<0.001**
spleen index (I_S)	1	0.150	2.352	0.129	1	0.0000	0.024	0.877
head kidney index (I_L)	1	0.255	4.015	**0.048**	1	0.0027	0.946	0.333
$G:L$ ratio	1	0.002	0.026	0.872	1	0.0058	2.048	0.156
respiratory burst	1	0.165	2.603	0.110	1	0.0019	0.679	0.412

obtain sufficient amount of head kidney leucocytes for the analysis of respiratory burst from three individuals, determination of the number of MHC variants by SSCP analysis was unsuccessful in six individuals, and quantification of MHC expression was unsuccessful in seven individuals. These groups overlap partly, so that the sample sizes may vary for the statistical analyses used, depending on the variables included in the corresponding statistical model.

To achieve normality for statistical analyses, respiratory burst was square-root transformed, I_H, I_L and I_S were logarithmically (ln) transformed, the $G:L$ ratio and the CF were Box–Cox transformed. Oxidative stress was normally distributed without transformation, and MHC expression ΔCT showed only a slight deviation from normality and was left untransformed.

Fish families differed in most of the parameters studies here. However, we were not primarily interested in such potential genetic effects here, and our experimental design was thus not optimized accordingly. Rather, here we want to control statistically for such effects. As a rule, we thus always calculated mixed effect models that included fish family as a random effect, however, without reporting the statistical details for this factor. We used standard least square fitting with the restricted maximum likelihood (REML) approach. All analyses were carried out in JMP 5.1 for Macintosh (1989–2003 SAS Institute Inc).

3. RESULTS

(a) *Influence of infection and fish gender on immune function and body condition*

Of all fish exposed to the three parasites, 75.0% were infected with *C. lacustris*, 87.5% with *D. pseudospathaceum* and 47.9% with *A. crassus*. Parasite infection did not strongly influence the immune traits that were measured here, except for an increased $G:L$ ratio in fish infected with *D. pseudospathaceum* ($F_{1,108}=5.283$, $p=0.024$). Fish infected with *C. lacustris* had reduced metabolic body condition, I_H ($F_{1,108}=7.282$, $p=0.008$).

There was a trend for female sticklebacks to have slightly higher values in several measures of immunity, which was significant for I_L ($F_{1,108}=7.635$, $p=0.007$) and I_S ($F_{1,108}=4.350$, $p=0.040$). This is in line with the general pattern observed among vertebrates (Zuk & McKean 1996). Females also had higher measures of body condition, I_H ($F_{1,108}=7.033$, $p=0.009$) and CF ($F_{1,108}=10.314$, $p=0.002$), so that we included fish sex into the statistical models below. However, there were no gender differences in MHC expression ΔCT ($F_{1,105}=0.024$, $p=0.877$).

(b) *Protein-bound acrolein as a marker for oxidative stress*

We quantified oxidative stress with an ELISA for protein-bound acrolein in livers of the experimental fish. The values obtained were substantially lower than the positive control (BSA, chemically modified with acrolein), which reached OD 450 values above 3, but markedly higher than the negative control (non-modified BSA), except for only two samples, which lead to two negative OD 450 values after subtraction of the control.

Oxidative stress correlated negatively with both measures of body condition, i.e. the CF ($F_{1,108}=4.913$, $p=0.029$) and metabolic condition I_H ($F_{1,108}=19.955$, $p<0.0001$). By contrast, there were no significant differences in oxidative stress resulting from infection with the three parasite species ($p>0.15$). Oxidative stress was not significantly related to the strength of the respiratory burst reaction ($F_{1,105}=3.605$, $p=0.060$). Moreover, there were no gender differences with regard to oxidative stress ($F_{1,108}=0.797$, $p=0.374$).

(c) *Relation between immune activity, condition and oxidative stress*

We expected that increased activity of the immune system could have detrimental effects on host body condition and might intensify oxidative stress. To identify relevant immunological parameters, we first calculated mixed models with I_H or oxidative stress as the dependent variable, all immune measures as factors, and the factors fish gender and fish family (the latter as a random effect), to control for genetic background. In these full models, the head kidney index I_L was weakly positively correlated with the metabolic condition I_H, and one of the immune parameters, MHC expression ΔCT, had a highly significant effect on both I_H and oxidative stress (table 1). We then examined the association between MHC expression and both fish condition and oxidative stress more closely with mixed models containing only the respective factor and fish family (as a random effect): fish with higher levels of MHC expression ΔCT had both poorer metabolic condition I_H ($F_{1,105}=8.529$, $p=0.004$, $r^2=0.13$; figure 1) and more oxidative stress ($F_{1,105}=12.406$, $p<0.001$, $r^2=0.30$; figure 2).

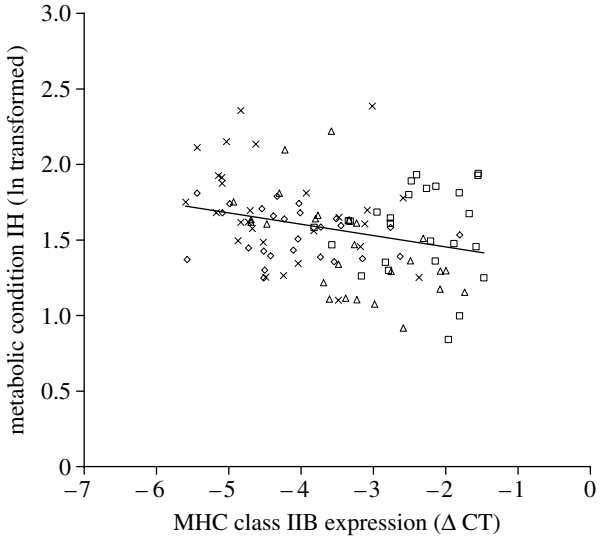

Figure 1. Metabolic condition, measured as the hepatosomatic index I_H, of sticklebacks in relation to gene expression at the MHC class IIB, after experimental exposure to three species of helminth parasites. Individual fish ($n=110$) stem from four fish families, as indicated by different markers.

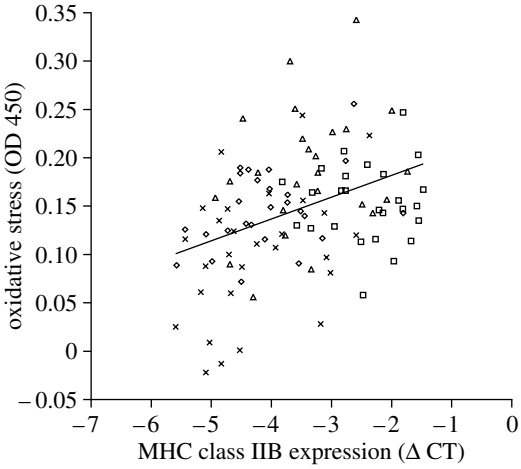

Figure 2. Oxidative stress in stickleback liver tissue (acrolein measured from the OD 450 in an ELISA) in relation to gene expression at the MHC class IIB. Different markers indicate fish families.

The full model did not show any significant effect of individual diversity at the MHC on oxidative stress. Yet this factor deserves further attention, since our expectation was that fish with optimal individual diversity might suffer less from oxidative stress. Based on the expectation of an optimum curve, we calculated a reduced model containing as factors only MHC diversity, its squared term and fish family (as a random effect). In this model, only the linear term was significant (MHC diversity, $F_{1,103}=4.159$, $p=0.044$; squared MHC diversity, $F_{1,103}=1.517$, $p=0.221$, $r^2=0.25$; figure 3). Yet, in the only family where individuals with the high number of nine MHC variants were present, squared MHC diversity was significant (MHC diversity, $F_{1,23}=2.768$, $p=0.110$; squared MHC diversity, $F_{1,23}=6.200$, $p=0.020$).

4. DISCUSSION

We found that the level of expression of MHC class IIB genes correlated negatively with metabolic body condition and positively with oxidative stress in three-spined sticklebacks that were experimentally exposed to three species of naturally occurring parasite. While the MHC is a prime example of genes that influence specific parasite resistance, metabolic condition and oxidative stress are indicative of costs of parasite defence. Therefore, our study implies that specific host–parasite interactions are intimately connected with the costs of immunity, and a malfunctioning specific defence system might exaggerate the costs associated with its use.

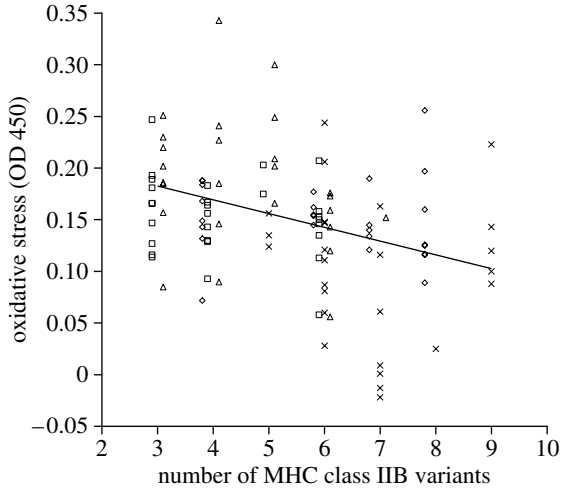

Figure 3. Oxidative stress in stickleback liver tissue, in relation to individual genetic diversity at the MHC IIB. Different markers indicate fish families, which are shown slightly offset to facilitate visibility.

To quantify oxidative damage, we here adapted an assay for the measurement of protein-bound acrolein to stickleback liver tissue homogenates (Uchida *et al.* 1998). This measure of oxidative stress has previously been used in medical contexts only (Uchida 1999), and was here applied to questions of immuno-ecology for the first time. As a stable end product resulting from oxidative stress, acrolein directly relates to accumulating long-term tissue damage. By contrast, previous methods used in an ecological context were based on the level of host counter measures as rather indirect estimates of oxidative stress (Blount *et al.* 2003; Alonso-Alvarez *et al.* 2004; Horak *et al.* 2004). A recent eco-toxicological study in sticklebacks shows that such classical indicators may only be affected transiently during continued exposure to oxidative stress (Sanchez *et al.* 2005).

In the current study, long-term oxidative damage correlated negatively with measures of body condition. This suggests that fish in poor condition also suffer from elevated oxidative stress. As predicted, oxidative stress correlated positively with a marker of immune system activation, the level of expression of MHC class II genes upon experimental exposure to parasites. However, since we cannot directly manipulate MHC expression levels in sticklebacks, we are dealing with a correlative response here, rather than an experimental treatment and the results must, therefore, be interpreted with caution. MHC expression was a highly significant predictor of both metabolic condition and oxidative stress of the sticklebacks (table 1), yet it explained only a small proportion of the total variance. Not surprisingly, parameters that are not considered here seem to influence these complex

measures of body condition, such as genetic factors (contributing to the family effect) other than MHC, further immune parameters, or factors unrelated to immunity.

The precise nature of a potential mechanistic link between the defence system and oxidative stress is as yet unclear. We could imagine three non-exclusive explanations. First, there might be a direct link between MHC expression and oxidative stress. Components of the adaptive immune system have been hypothesized to use oxidative effectors, and a high level of their activity could, thus, potentially lead to oxidative stress (Bogdan *et al.* 2000; Wentworth *et al.* 2002). Yet, the most important sources of ROS during vertebrate immune defence are cells of the innate immune system, such as polymorphonuclear leukocytes and macrophages (Nathan & Shiloh 2000).

Our second possible explanation is, therefore, that malfunctioning of MHC-mediated specific immunity could lead to sustained activity of innate immune functions that could then cause oxidative stress. However, contrary to expectation from a previous study (Kurtz *et al.* 2004), respiratory burst (which is an important ROS-producing innate immune reaction) was not related to oxidative damage. The previous results were obtained with different parasite species, the tapeworm *Schistocephalus solidus* and the microsporidian *Glugea anomala*. For *S. solidus*, there is an experimental evidence that the respiratory burst is a relevant immune reaction (Kurtz *et al.* 2004; Scharsack *et al.* 2004; Hammerschmidt & Kurtz 2005*a*,*b*). By contrast, although we do know that the immune system is efficient in defence against the three parasite species in the current experiment (Wegner *et al.* 2003), it is as yet unknown precisely which immune reactions are the most suitable defences. Contrary to *S. solidus*, which lives in the body cavity of its fish host, all the three parasites that were studied here reside in body compartments. Thus, defence might rely on more localized or more complex immune traits, which may not result in any of the systemic immunological effects that we studied here.

Finally, any increase in metabolism alone can lead to the production of excess oxidative stress (Finkel & Holbrook 2000). The increased energy demand that is often associated with an activated immune system (Ots *et al.* 2001; Råberg *et al.* 2002; Freitak *et al.* 2003; Martin *et al.* 2003) may thus be an alternative explanation for the here observed association of MHC expression with both reduced metabolic condition and intensified oxidative stress.

Counter to expectation, we did not observe a clear relationship between individual MHC diversity and metabolic condition or oxidative damage, although there was a trend for more severe oxidative stress in fish with low numbers of MHC variants (figure 3). In contrast to previous studies, that showed reduced resistance in fish with either too low or too high numbers of MHC variants (Wegner *et al.* 2003; Kurtz *et al.* 2004), the current dataset might not have been adequate for testing the idea of increased immune costs at higher than optimal numbers of MHC sequence variants, since only two fish broods contained enough individuals with more than six MHC variants, which is the optimal number predicted from previous studies. After all, in one of those families, increased oxidative stress with above-optimal MHC diversity was observed. This might indicate the lowest oxidative stress at intermediate levels of MHC diversity, but deserves further studies to obtain more conclusive results.

Our result of increased oxidative stress associated with immune activation could also be relevant for understanding the evolution of sexually selected traits that are based on antioxidants such as carotenoids. Based on their dual role in both oxidative stress protection and the production of sexual signals, carotenoids have been considered good examples of honest signalling (Folstad & Karter 1992; Lozano 1994; von Schantz *et al.* 1999). According to these concepts, carotenoid-based ornaments may reflect an individual's well-functioning immune system that does not give rise to excess oxidative stress. In line with this hypothesis, recent studies in birds showed that experimental immune activation impedes the expression of carotenoid-based sexual signals (Faivre *et al.* 2003; Alonso-Alvarez *et al.* 2004). However, antioxidant defences were not directly influenced (Alonso-Alvarez *et al.* 2004). In the current study, oxidative stress was related to the activity of MHC-based immunity. Thus, sticklebacks with low-MHC expression should have reduced demands for antioxidants to buffer oxidative stress (von Schantz *et al.* 1999). Whether surplus carotenoids could then be allocated to breeding ornamentation, i.e. the red throat of male sticklebacks, which contains carotenoids (Wedekind *et al.* 1998) and reflects heritable parasite resistance (Milinski & Bakker 1990; Barber *et al.* 2001), remains to be shown in future studies. If so, sticklebacks might use multiple signals to assess their partners' immune system (Wedekind 1992; van Doorn & Weissing 2004): odour cues, which directly signal MHC genetics (Reusch *et al.* 2001; Milinski *et al.* 2005), and breeding coloration, which might then represent a composite signal of oxidative stress.

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