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Relative importance of MHC and genetic background for parasite load in a field experiment

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ABSTRACT

Question: How do major histocompatibility complex (MHC) genes influence parasite load in a natural experiment?

Methods: We crossed three-spined sticklebacks from a river habitat with genetically distinct lake sticklebacks and inter-crossed the resulting F1 hybrids. F2 offspring segregate into pure river or pure lake MHC genotypes and into two hybrid genotypes, while randomizing the background genome across all MHC genotypes.

Experiment: In outdoor cages, we exposed fish reciprocally to the natural parasite communities of the river and lake habitats.

Results: MHC genotype did not significantly influence parasite load. In contrast, genomic background explained a significant percentage of the variation in parasite load.

Keywords: adaptive and innate immune system, *Gasterosteus aculeatus*, major histocompatibility complex, parasite load.

INTRODUCTION

Genes of the major histocompatibility complex (MHC) play a key role in the activation of the adaptive immune system in vertebrates (Janeway *et al.*, 1999). The great interest devoted to the impact of MHC genes on parasite resistance is therefore not surprising. Specific MHC alleles are associated with disease resistance or susceptibility (for a review, see Apanius *et al.*, 1997). In three-spined sticklebacks, individuals with intermediate numbers of MHC alleles had the lowest parasite load (Wegner *et al.*, 2003a, 2003b) and parasite growth (Kurtz *et al.*, 2004). MHC congenic mice (i.e. inbred strains that differ only in their MHC haplotype) have been widely used to experimentally test the influence of MHC genes on resistance to various diseases (for a review, see Apanius *et al.*, 1997). However, differential accumulation of mutations in different strains can constrain conclusions arrived at in experiments with congenic animals (Carroll and Potts, 2001). Experiments using second-generation mice (F2) obtained from crosses between different

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parental strains and inter-crossing the F1 generation to extract the original MHC haplotypes avoid this problem by randomizing any background mutations. Such studies demonstrate the effect of MHC genes on pathogen susceptibility (Lohm *et al.*, 2002; Penn *et al.*, 2002).

Despite the interest in MHC genes, no direct experimental evidence exists showing the influence of MHC genes on parasite load in a natural habitat. An experiment in a natural habitat has the obvious advantage of covering the full range of natural parasite species. Using only single parasite strains or species can be problematic, as the fitness of an individual parasite might depend on potentially co-infecting parasites (Brown *et al.*, 2002). Furthermore, whether or not a difference in susceptibility between different MHC haplotypes exists depends on the parasite species (for a review, see Apanius *et al.*, 1997). In a laboratory experiment, the number of MHC alleles only influenced parasite load when fish were exposed to several parasite species (Wegner *et al.*, 2003a). Furthermore, an experiment in natural habitat avoids possible artificial conditions of laboratory experiments. It also preserves the strength of an experimental manipulation lacking in correlative studies.

Three-spined sticklebacks (*Gasterosteus aculeatus*) present an ideal system to determine whether MHC genes influence the parasite load in the field. We take advantage of populations derived from river and lake habitats, since river and lake sticklebacks have almost completely divergent MHC genotypes, as revealed by sequence comparison (T.B.H. Reusch, K.M. Wegner, M. Kalbe and W.T. Stam, unpublished data). Gene flow between the two habitat types is practically non-existent (Reusch *et al.*, 2001b). This genetic divergence allowed us to obtain replicated F2 siblings with completely divergent MHC genotypes by crossing river fish with lake fish and inter-crossing the F1 hybrids. In each family, F2 offspring will segregate into pure river and pure lake MHC genotypes as well as into the two possible hybrid MHC genotypes that carry a common background genome randomized over MHC genotypes. In exposing the F2 fish to the natural parasite communities in the river and lake, we tested for an effect of MHC genotype on parasite load in the natural habitat. In using several hybrid families, we tested the effect of different background genomes (= family effect). The parasite communities in the two habitat types differ, with lake fish harbouring more parasite species than river fish (Kalbe *et al.*, 2002).

Genes closely linked to the MHC region do not segregate independently from the MHC genes during meiosis. For three-spined sticklebacks, Peichel *et al.* (2001) showed that 26 linkage groups exist. For example, MHC class I and II genes are on different linkage groups (Sato *et al.*, 2000), allowing the effects of the two classes to be separated. In this study, we concentrated on the MHC class II genes, as they are critical for activation of the immune system against most extracellular parasites (Penn and Potts, 1999). Furthermore, crossing-over events (recombination) within the MHC class II genes do happen (Reusch *et al.*, 2004). Thus, the combined effect of segregation and crossing over should result in an acceptable randomization level of the background genome over the MHC genotype.

Besides examining whether the difference in MHC sequence influences parasite load, we also tested whether MHC genotypes are locally adapted, as organisms are often found to be locally adapted (Lively, 1989; Ebert, 1994). Additionally, we examined whether there is a 'general' local adaptation of the fish to their natural habitat not restricted to the MHC genotype. To answer this question, we also exposed pure lake and river lines, bred over two generations, reciprocally in the two habitats.

Despite the important role of MHC genes for parasite resistance, the existing evidence for MHC-dependent parasite resistance in natural populations is only correlative. In decoupling the MHC genotype from the background genome, our experimental design

allowed us to study explicitly the influence of MHC genotype on parasite load and of MHC origin for local adaptation in a natural habitat.

MATERIALS AND METHODS

Fish-rearing conditions

We caught three-spined sticklebacks from one river (the River Schwale) and one lake (Lake Vierersee) from two different drainage systems in northern Germany in autumn 2002. We kept fish in 16-litre tanks at a density of around 20 fish per tank with continuous water exchange in 'winter' conditions (6°C, 8 h light). We fed the fish with frozen red mosquito larvae. In spring 2003, we transferred fish for 2 weeks to 'spring' conditions (12°C, 12 h light) and subsequently to 'summer' conditions (18°C, 16 h light). We isolated males in 16-litre tanks and offered artificial nesting material. We kept females at a density of five fish per tank. We introduced a female ready to spawn with the male and, after successful mating, we transferred eggs to individual tanks. We fed the hatching offspring of the F1 generation with live *Artemia salina* for the first 2 months, and we kept all offspring of one family in one tank. After this period, we fed fish with frozen red mosquito larvae, and kept fish at a density of around 20 fish per tank. In autumn 2003, we passed F1 fish through an artificial winter (2 weeks in 'spring' conditions, followed by 4 weeks in 'winter' conditions, then again 2 weeks in 'spring' conditions) and subsequently transferred the fish to 'summer' conditions for breeding of F2 offspring. After 7 months of growth in 'summer' conditions at a density of around 20 fish per tank, we used these F2 fish for the experiment.

Breeding design

Figure 1 shows the breeding design we used to obtain fish with different MHC genotypes and a randomized background genome over MHC genotypes. We produced eight F1 hybrid families. Each hybrid family originated from a cross between a wild-caught river fish and a lake fish. Each parental fish was only used once. In four crosses the males came from the river and the females from the lake, and in the other four crosses the males came from the lake and the females from the river. From each of the resulting eight hybrid F1 families, we chose one fish, either a male or a female. We used each of the eight fish only once to form four male–female pairs to produce four F2 families (crosses took place only between fish where both had either a river father or both had a lake father). F2 fish from these four families are hereafter denoted as hybrid families. Fish with both MHC haplotypes originating from river fish are referred to as RR fish, fish with two lake haplotypes as LL fish and fish with mixed haplotypes as LR fish. How we identified the different MHC genotypes is described below. In contrast to these three classes of MHC origin (LL, RR and LR), 16 possible genotypes exist in the four F2 hybrid families, four in each family. The genotypes are characterized as LL*n*, RR*n*, LR*n* and RL*n*, with *n* as family number 1–4. The first letter stands for the haplotype inherited from the F1 father, the second letter for the haplotype inherited from the F1 mother. L and R represent the lake or river origin of the haplotype. As the MHC genotypes differ almost completely in the two habitats, this design resulted in F2 offspring segregating within each family into four distinct MHC genotypes with a shared randomized background genome.

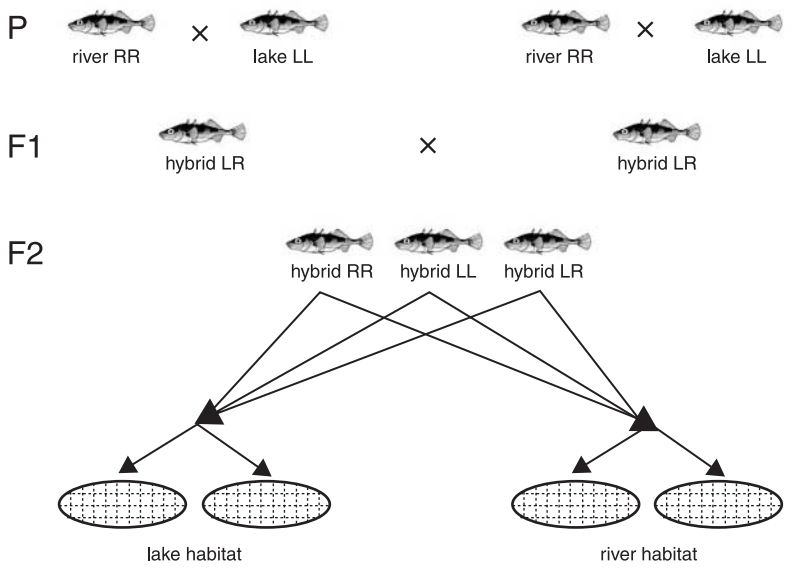

Fig. 1. Breeding design of one hybrid family. River, lake or hybrid fish describe the origin of the background genome. The capital letters stand for the origin of the MHC: RR for river MHC, LL for lake MHC and LR for hybrid MHC from lake and river. Arrows indicate assignment to the four cages (ovals).

To further obtain pure river fish, we produced six crosses between wild-caught river fish. From each of the six F1 families, we chose one fish. We used each of the six fish only once to form three pairs to produce three F2 pure river families (referred to as PR fish). The same procedure with lake fish yielded three F2 pure lake families (referred to as PL fish).

The field exposure

Within a single hybrid family, we selected five to eight fish (numbers depended on the family) with an LL MHC origin, five to eight fish with an RR MHC origin and five to eight fish with an LR MHC origin (MHC genotyping explained below). We inter-mixed the selected fish of all three MHC origins in a single aquarium. Then we randomly distributed the fish into one of two cages and placed the cages in the lake (Fig. 1). From the same family, we inter-mixed five to eight fish of each MHC origin in a second aquarium, and randomly distributed them to two cages and placed the cages in the river (Fig. 1). We repeated this procedure for the remaining three hybrid families by distributing each family to four new cages (16 cages in total). This resulted in having only fish of the same hybrid family in the same cage, so that the three different MHC origins and the four genotypes of the same family experienced the same environment. Any cage effect was thus randomized over the four cages used for each family. The numbers of fish with the same MHC origin included per family and habitat varied from five to eight, as different MHC origins were not equally available. Fish with an LR MHC origin included two different genotypes within each family: LR*n* and RL*n* MHC genotypes. The two genotypes were not represented in equal numbers within each family. Per family we included six to thirteen LR*n* fish and two

to six RL*n* fish. On average, a single genotype was represented by 9.1 fish distributed in the two habitats.

Additionally, in each cage we released two fish of each pure river family and of each pure lake family. This resulted in 14–16 fish in each cage. Before release, we measured size and weight for every fish.

We released the F2 fish in cages placed in the river and lake, near the two catching areas of the parental generation, in August 2004. In the river, we placed eight cages at intervals of 10 m in the middle of the river (the river bed is 2 m wide and 0.5 m deep). In the lake, we placed eight cages 10 m offshore at a depth of 1 m at intervals of 10 m. The cages were pipe-shaped (1 m long and 40 cm in diameter) and covered with wire netting of 4 mm mesh size.

MHC genotyping and individual characterization

We extracted DNA of each F2 individual from a small piece of dorsal spine as described in Reusch *et al.* (2001b). To identify the different MHC class II genotypes, we combined the discrimination effect of the binding specificity of eight newly developed primers by using the single-stranded conformation polymorphism (SSCP) method. Each of the eight primers used amplifies a unique subset of MHC class IIB sequences. We further separated each subset using single-stranded conformation polymorphism on a capillary sequencer (Binz *et al.*, 2001). [For details of the primer development and polymerase chain reaction conditions, see Wegner (2004).] We preferred the eight new primers over the two old primers used in Reusch *et al.* (2001a), Wegner *et al.* (2003a, 2003b) and Kurtz *et al.* (2004) because, with the two old primers, we could not distinguish all four genotypes in all four hybrid families. For simplicity, we refer to different sequences as being different alleles, although they may stem from different loci. With the aid of this newly developed technique, within each hybrid family we can distinguish all four possible MHC genotypes.

Using five polymorphic microsatellites, all experimental fish got an individual identity to discriminate them after the experiment for measuring growth and change in weight during the experiment. We used the microsatellites Gac1097, Gac1125, Gac4170, Gac5196 and Gac7033 developed by Largiader *et al.* (1999) and modified by Reusch *et al.* (2001b).

Parasite load

We collected fish 8 weeks after release. At that time, fish were still sexually immature. We measured size and weight again, and we checked fish for parasite infection. We screened the surface and all inner organs, including eyes and gills [for details on parasite screening, see Kalbe *et al.* (2002)]. We found 15 macroparasites and recorded their numbers quantitatively except for *Trichodina* sp. (Table 1). For *Trichodina* sp. (Ciliata), we estimated the number of parasites to be 0, 10, 50 or 100 on the visible parts of the fins. An estimation of *Trichodina* sp. numbers was possible, as fish were not heavily infected and numbers did not exceed 100 individuals per fish. Both *Trichodina* sp. and *Gyrodactylus* sp. (Monogenea) can reproduce on the fish. Therefore, numbers found on the fish are probably not equivalent to numbers actually infecting the fish.

For every individual fish we calculated an overall parasite load averaged over all parasite species. Some parasite species occur at much higher numbers per fish than other species. These species would have a strong influence on the overall parasite load. To correct for this

effect, we used relative numbers for a given parasite species and habitat, calculated as follows. For each parasite species, we recorded the maximal number found on a single fish in the experimental population separately for both habitats. Then we divided number of individuals of one parasite species found on one fish with the maximal number of the corresponding parasite species. We did this for every parasite species and habitat type separately. Fish not infected by one parasite scored a zero for that parasite species. To calculate the parasite load of one fish, we took the average over all relative numbers of parasite species actually present in the respective habitat. We also calculated the parasite load using the Shannon diversity index calculated separately for each fish (Magurran, 1988) and a normalized value (for every fish, the deviation from the mean abundance of a given parasite species in a given population was expressed in units of standard deviation and averaged over all parasite species present in a given habitat). The results obtained with the two alternative parasite loads were consistent with the original parasite load calculated with relative numbers and are not reported.

To determine whether the MHC genotype had an influence on particular parasites in the hybrid lines, we focused on two parasite species with a high prevalence (> 60%), the specialist *Gyrodactylus* sp. (river habitat) that is mainly found on three-spined sticklebacks and the generalist *Diplostomum* sp. (lake habitat) that also infects several other fish species (Table 1). We then analysed two parasite species with a low prevalence (< 30%), the specialist *Proteocephalus filicollis* (analysed solely for the river habitat, as only five fish were parasitized by this species in the lake habitat) and the generalist *Valipora campylancristrota* (lake habitat).

Table 1. Parasite species name, class name, prevalence (expressed as a percentage, P%), mean abundance per fish (expressed as the average number of individual parasites per fish, including zero counts for fish not infected with the respective parasite species) and maximal numbers found on a single fish (max) for the two habitats separately

		River habitat			Lake habitat		
Parasite species	Class	P%	Mean	Max.	P%	Mean	Max.
Proteocephalus filicollis	Cestoda	27.27	0.52	8	5.88	0.06	1
Trichodina sp.	Ciliata	2.48	0.25	10	68.91	21.85	100
Anguillicola crassus	Nematoda	0.83	0.01	1	15.97	0.21	3
Raphidascaris acus	Nematoda	13.22	0.17	3	0.84	0.02	2
Gyrodactylus sp.	Monogenea	69.42	4.6	38			
Acanthocephalus lucii	Acanthocephala				0.84	0.01	1
Paradilepis scolecina	Cestoda				2.52	0.03	1
Valipora campylancristrota	Cestoda				20.17	0.39	5
Argulus foliaceus	Crustacea				12.61	0.18	4
Apatemon cobititdis	Digenea				1.68	0.03	2
Cyathocotyle prussica	Digenea				5.04	0.07	3
Diplostomum sp.	Digenea				100	18.8	64
Echinochasmus	Digenea				18.49	0.19	2
Tylodelphis calvata	Digenea				6.72	0.24	11
Camallanus lacustris	Nematoda				23.53	0.32	4

Data analysis

We assessed the influence of the 16 different MHC genotypes in the hybrid lines on parasite load by nesting the MHC genotypes within the family effect. We performed this analysis of variance (ANOVA) using the residuals taken from a one-way ANOVA with habitat as a fixed factor to control for habitat effect. We treated MHC genotype and family as random factors. To test for MHC local adaptation in the hybrid lines, we determined the interaction between origin of MHC (LL or RR) and the habitat of exposure from an ANOVA model. MHC origin was fully crossed with the factors habitat and family. To test for local adaptation in general, we measured the interaction between origin (lake, river or hybrid lines) and the habitat of exposure. Family effect was nested within origin, but not within habitat of exposure, resulting in a split-plot design. Family effect was controlled for in this design, but family effect statistics are not shown, as drawing conclusions from the nested factor statistics is not recommended in such a split-plot design (Zar, 1999). We further tested for a correlation between number of MHC alleles and parasite load. We tested the correlations using the residuals obtained from a two-way ANOVA, including the factors habitat of exposure and family as described in Wegner *et al.* (2003a). We first did this correlation with pure lake lines only and then for all fish, as Wegner *et al.* (2003a) and Kurtz *et al.* (2004) also used lake lines in their experiments. For all analyses we tested residuals graphically for normal distribution (Zar, 1999) and where necessary we used log or square root transformations.

RESULTS

MHC genotypes

In total, we found 38 different SSCP signals, each probably corresponding to different MHC alleles in the experimental fish. We did not find any crossing-over events within the MHC region. In the hybrid lines, we could clearly distinguish the four possible MHC genotypes within each family in all cases. Also, considering each of the eight primers singly, MHC genotypes differed markedly within each family. In three families, RR and LL MHC genotypes did not share any alleles and in one family only one allele was shared. Comparing over all four families, MHC genotypes differed from each other at least for two alleles, except for one case of two identical LL MHC genotypes.

In the pure lines, MHC genotypes did not differ within each family as markedly as in the hybrid lines. In three families, we could only distinguish two or three different MHC genotypes of the four possible genotypes in F2 fish. In case of the pure lines, we could not exclude the possibility that our MHC discrimination technique failed to distinguish between different MHC sequences. We therefore used the pure lines solely to test for 'general' local adaptation and not to test for an influence of MHC genotype on parasite load.

Fish condition

In the hybrid lines, MHC origin (RR, LL or LR) had no significant influence on length or mass of fish before the start of the experiment (length: $F_{2,115} = 0.02$, $P = 0.9796$; mass: $F_{3,114} = 0.40$, $P = 0.6737$). Of the 242 released fish, 11 died in the river cages and 12 in the lake cages during the experiment. Neither changes in length ($F_{1,214} = 0.20$, $P = 0.6561$) nor mass ($F_{1,214} = 0.06$, $P = 0.7993$) were correlated with parasite load. Details of the parasite infections are summarized in Table 1.

Influence of MHC genotype on total parasite load

The different MHC genotypes within each family did not significantly influence parasite load (Fig. 2). The null hypothesis of no influence of the MHC genotype on parasite load was not rejected with a power of 56% (at $\alpha = 0.05$). Only one MHC genotype, RL4 in the fourth family represented by only two fish, seemed to have a higher parasite load than the other genotypes. In contrast, family-specific genetic background did influence parasite load (Fig. 2). Family explained 8.36% of the total variance and MHC genotype 0.16%. The family raw-effect size (= 0.027) is 1.8 times higher than the raw-effect size of MHC genotype (= 0.015). Details of this ANOVA with MHC genotype nested within family and controlling for habitat effects are shown in Table 2a.

Influence of MHC genotype on parasite load of single species

MHC genotype had no significant effect on the number of all four parasite species analysed separately (Table 2b–e). Consistent with the results obtained with the total parasite load, in three of the four cases family had a significant effect (Table 2b–e).

Local adaptation

We did not find any evidence for MHC local adaptation in the hybrid lines – that is, no statistically significant interaction existed between habitat of exposure (river or lake) and

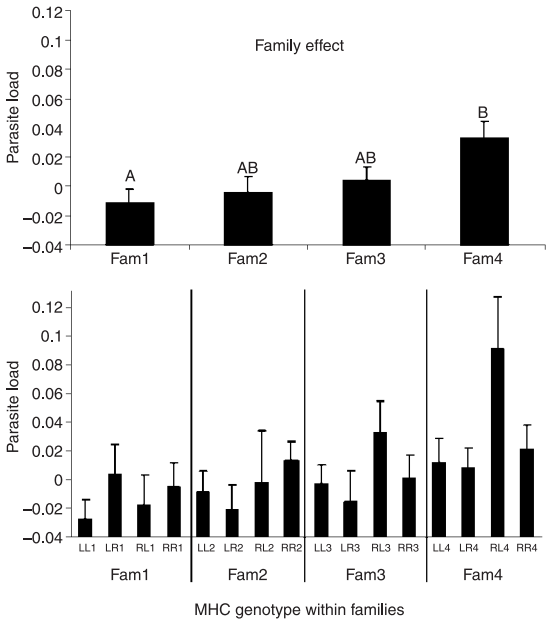

Fig. 2. Effect of family and MHC genotype on parasite load. The upper panel shows mean parasite load of sticklebacks in the four hybrid families, exposed in the two habitats. Bars marked with different letters are significantly different after a Tukey HSD multiple comparison test. The lower panel shows the parasite load for the 16 MHC genotypes in the four hybrid families. Bars show least square means (± standard errors), computed from residuals of an ANOVA including habitat effect.

Table 2. Statistical details for MHC genotype and family effect and for the local adaptation analysis

	Factor	d.f.	*F*	*P*
Effect of MHC genotype and family on parasite load				
(a) Combined parasite load	MHC genotype	12,125	1.01	0.4397
	Family	3,20	3.27	0.0423
(b) *Gyrodactylus* sp.	MHC genotype	12,55	0.53	0.8855
	Family	3,29	1.23	0.3158
(c) *Diplostomum* sp.	MHC genotype	12,54	0.98	0.4804
	Family	3,21	10.42	0.0002
(d) *Proteocephalus filicollis*	MHC genotype	12,55	0.33	0.9802
	Family	3,40	5.62	0.0026
(e) *Valipora campylancristrota*	MHC genotype	12,54	1.22	0.2924
	Family	3,19	3.60	0.0322
Local adaptation				
(f) MHC	Habitat	1,76	6.83	0.0108
	MHC origin	1,76	1.91	0.1705
	Family	3,76	1.62	0.1913
	Habitat × MHC origin	1,76	0.60	0.4422
	Habitat × family	3,76	2.50	0.0655
	MHC origin × family	3,76	0.07	0.9748
	Habitat × MHC origin × family	3,76	1.75	0.1641
(g) Background	Habitat	1,90	2.37	0.1269
	General origin	1,4	7.82	0.0484
	Habitat × general origin	1,90	2.63	0.1083

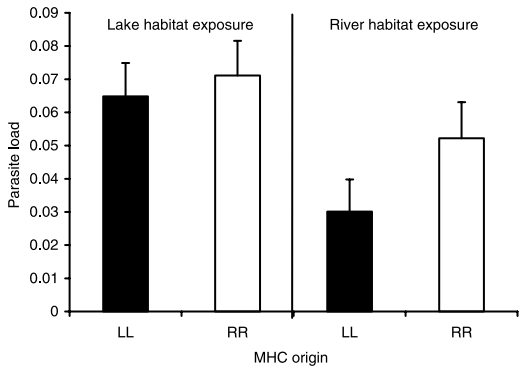

Fig. 3. Local adaptation of MHC origin. Parasite load of F2 hybrid sticklebacks, carrying MHC genotypes of different origin (LL = lake, RR = river) and exposed to parasites in the two habitat types. Bars show least square means (± standard errors), controlling for family effects.

origin of MHC (RR or LL) (Fig. 3). Parasite load was higher in the lake habitat than in the river habitat. This was not a surprise, as the mean abundance of the different parasite species was much higher in the lake habitat than in the river habitat. MHC origin did not

influence parasite load and there was no family effect. Details of this fully crossed three-way ANOVA are shown in Table 2f. The parasite load of fish with a mixed MHC from the river and the lake (LR) did not differ significantly from that of fish with an LL or RR MHC ($F_{2,117} = 1.04$, $P = 0.3571$; habitat and family effect included in the model, statistical details not shown).

In a test for 'general' local adaptation using the pure lines, we also did not find a significant interaction between habitat of origin (pure lake or pure river) and habitat of exposure. Habitat of exposure had no significant effect. Fish from the pure river lines had a significantly higher parasite load than fish from pure lake lines. Statistical details of the split-plot design are shown in Table 2g. There was also no significant interaction between habitat of origin and habitat of exposure using the pure lines and the hybrid lines (habitat × general origin: $F_{2,226} = 2.08$, $P = 0.1270$; habitat, general origin and family effect included in the model, statistical details not shown).

Number of MHC alleles

Fish of the hybrid lines with an RR MHC had on average 6.5 ± 0.20 (± standard error) different alleles; LL MHC fish had 6.0 ± 0.23 alleles, LR MHC fish had 8.4 ± 0.18 alleles and RL MHC fish had 7.4 ± 0.16 alleles. Pure river fish had on average 6.9 ± 0.24 different alleles, lake fish 7.3 ± 0.27 different alleles. The number of different MHC alleles in the pure lake lines only did not correlate with parasite load in either a linear ($F_{1,38} = 0.23$, $P = 0.6374$) or in a quadratic polynomial way ($F_{2,37} = 0.43$, $P = 0.6554$), when controlling for both habitat and family effects. Using all fish, the number of different MHC alleles did not correlate linearly ($F_{1,224} = 0.04$, $P = 0.8512$) or in a quadratic way ($F_{2,223} = 0.78$, $P = 0.4619$).

Parasites in wild-caught fish

To compare parasite infection of the experimental fish with naturally occurring fish, we caught 30 wild fish near the cages, 15 in the river and 15 in the lake, during the experimental period and screened for parasites. Thirteen of the 15 parasite species found in wild fish we also recorded for the experimental fish. The maximal numbers found in single wild fish exceeded those found in single experimental fish for only two parasite species.

DISCUSSION

Despite the increasing interest in the relevance of MHC genes for resistance against parasites, experimental evidence for an effect of MHC genotype on parasite load in natural habitats does not exist. Here we performed an experiment in which we exposed sticklebacks to the natural parasite fauna of a river and a lake habitat. Contrary to expectations, we did not find any influence of the MHC genotype on parasite load. Instead, the genomic background was more important for explaining parasite load than the MHC genotype.

To test whether MHC genotype has an influence on parasite load of a diverse and naturally occurring parasite community, two basic conditions have to be met. First, experimental individuals have to differ in their MHC genotype independent of their genetic background. We achieved this in a two-generation breeding design, where we obtained replicated F2 fish that belonged to the same family but had completely different MHC

genotypes. Second, experimental individuals must be challenged by a diverse natural parasite community. This was achieved by releasing the experimental fish into field enclosures placed in the natural habitats. The parasite loads of experimental fish were very similar to those of naturally occurring fish, as we recorded 13 of the 15 parasite species found in wild fish in the experimental fish also. The parasite community did not only comprise several parasite species, but the within-species diversity was probably also high. For example, we showed that naturally occurring three-spined sticklebacks from a lake harboured on average 22 different clones of *Diplostomum pseudospathaceum* per fish (Rauch *et al.*, 2005).

The vertebrate immune system can be subdivided into the innate and adaptive immune system (Janeway *et al.*, 1999). The innate immune system is a fast and effective first line of defence against many pathogens, but is regarded as rather unspecific (Dixon and Stet, 2001). However, recent studies indicate that invertebrates, thought to rely only on innate immunity, possess a specific defence and memory (for a review, see Kurtz, 2005). The adaptive immune defence achieves its extraordinary specificity by somatic rearrangement of genes that encode antigen receptors [i.e. T-cell receptors and antibodies (Kurtz, 2005)]. For the activation of an adaptive immune response, MHC genes play a key role because they present peptides derived from pathogens to T-cells. The MHC can be subdivided in two main classes. MHC class I encoded proteins mainly present peptides of intracellular parasites, such as many viral or bacterial pathogens (Penn and Potts, 1999). This normally results in destruction of the infected cell by cytotoxic T-cells. In contrast, MHC class II encoded proteins mainly present peptides derived from extracellular parasites, such as extracellular bacteria and macroparasites (Penn and Potts, 1999). If the foreign peptide bound to the MHC protein is recognized by T-cells, a complex immune reaction activation is initiated. Obviously, MHC class II genes are an important part of the immune system, yet they are only one part in the network. A variety of different mechanisms and pathways exists for defence against pathogens. In this study, the background genome had a greater influence in explaining parasite load than the MHC class II genes. This influence of the background genome may involve parts of the MHC class II immune reaction cascade that are not necessarily genetically linked to the MHC region. It might further comprise other parts of the adaptive immune system, including the innate immune system, as well as interactions between different mechanisms and pathways.

Interactions between MHC genes and genetic background can lead to dramatic changes in parasite resistance (Apanius *et al.*, 1997). Such interactions between MHC genes and the genetic background alone are not sufficient to explain the family effect found in this study. For every genetic background, our design had four different MHC genotypes. This leads to a randomization of the MHC genotype effect over the family effect. Furthermore, it has been shown that products produced by the innate immune system influence the adaptive immune system (Belardelli and Ferrantini, 2002). Through the randomization of the background genome over the MHC genotypes, parts of the river genetic background get connected with lake MHC genotypes and vice versa. If the combination of river and lake parts of the immune system prevents the activation of the MHC genes, no influence of the MHC genotypes on parasite load could be expected. However, in that case we would expect that the pure river and lake lines would have a lower parasite load than the hybrid lines, which was not the case. Researchers comparing the relative importance of MHC genes on resistance against single parasite species reported that MHC haplotypes explained between 1.6% and 14% of the total phenotypic variance found (Apanius *et al.*, 1997). In our study, MHC genotype did not explain a significant percentage of the variance in parasite load; in contrast, genetic background accounted for 8.36% of the variance.

Experiments with congenic mice revealed that a specific MHC haplotype either increased or decreased susceptibility depending on the parasite population used (Apanius *et al.*, 1997). If in this study a certain MHC genotype conferred an increased resistance to some parasite species while simultaneously decreasing resistance to another, the combined parasite load measure would yield an intermediate value. However, for the four parasite species analysed separately, we did not find an influence of MHC genotype on parasite load.

Many researchers have reported an influence of MHC genes on parasite load (for a review, see Apanius *et al.*, 1997). In this paper, however, we did not find such an influence. To rule out a false-negative finding, we also have to consider possible limitations of the experimental design. First, the experimental period of 8 weeks might have been too short to result in a marked influence of the MHC genotype on parasite load. MHC genes are part of the adaptive immune system, which needs time to build up a defence. However, in a laboratory experiment, which showed an influence of the number of MHC alleles on parasite load in sticklebacks, the time between the first and last infection was also 8 weeks (Wegner *et al.*, 2003a). Furthermore, in this experiment, the immune system was only challenged for two times with parasites. In our study, parasite challenge was probably continuous, so that the adaptive immune system received many signals for activation. Second, if parasite infections might have occurred almost exclusively in the last weeks of the experiment, the adaptive immune system would not have had enough time to become activated. However, parasite infection is probably more likely to decrease when approaching the winter season as water temperature decreases. For example, infection rates of the trematode *Diplostomum spathaceum* in rainbow trout decreased with decreasing water temperature in a laboratory experiment (Stables and Chappell, 1986). Third, if parasite pressure were to decline dramatically in the last weeks of the experiment, there would be very few new infections during the time when the adaptive immune system is activated. This would imply that the parasites we found infected the fish very early in the experiment and that they persisted within the fish until the end of the experiment. This would, however, give the adaptive immune system the opportunity to react against those parasites in the last weeks of the experiment, when it had enough time to build up a defence. Fourth, fish residing inside the cages might have experienced a lower exposure to parasites than free-living fish, potentially resulting in insufficient parasite pressure. This seemed not to be the case, as the parasite load of the experimental fish was very similar to that of free-living fish caught during the experimental period.

We did not find local adaptation of parasites to local MHC genotypes, as fish with a MHC originating from the river (RR) had in both habitats a slightly higher parasite load than fish with a MHC originating from the lake (LL). Similarly, we did not find a 'general' local adaptation using the pure lines, as pure river fish had a higher parasite load than lake fish in both habitats. One possible explanation is that lake fish are, in general, exposed to a higher parasite pressure, as the number of parasite species is higher in the lake habitat (Kalbe *et al.*, 2002). As a consequence, lake fish may have in general a better defence against parasites without being especially adapted to their home parasites. Kalbe and Kurtz (2006) showed that sticklebacks from a lake population had a higher immunocompetence (respiratory burst activity and spleen size) than sticklebacks from a river population.

Two recent laboratory experiments with sticklebacks have reported that fish with an intermediate number of MHC alleles had the lowest parasite load or parasite growth (Wegner *et al.*, 2003a; Kurtz *et al.*, 2004). In a field experiment (K.M. Wegner, M. Kalbe and T.B.H. Reusch, unpublished data), it was shown that fish with an intermediate number of alleles had the highest survival rate. Furthermore, birds with an intermediate number of MHC alleles had the largest clutch size

(Bonneaud *et al.*, 2004). In this study, we did not find a correlation between parasite load and number of MHC alleles. However, we did not design our breeding programme to maximize allele number diversity. Analysing only the pure lake lines to compare the results with the two laboratory studies, which also used lake offspring, the number of alleles varied only from 6 to 9. In contrast, experimental designs by Wegner *et al.* (2003a) and Kurtz *et al.* (2004) aimed to maximize segregation above and below optimal number of MHC alleles. This resulted in the number of alleles varying from 3 to 9 and 2 to 8, respectively. In the present study, the ability to detect an effect of numbers of different alleles is clearly restricted with such a small allelic variance. Note that the average number of alleles is slightly higher in this study than reported previously (Reusch *et al.*, 2001a; Wegner *et al.*, 2003b; Kurtz *et al.*, 2004). This is consistent with the predicted ability of the novel MHC typing system applied in this study, which uses eight primer pairs and can amplify around 20% more different MHC sequence variants than the previously published method using only two primer pairs.

In summary, we have shown in a field experiment that the genetic background is more important than the MHC genotype in influencing parasite load. Despite the demonstrated influence of MHC genes on immunity, the present results highlight the importance of extending studies on the genetic basis of parasite resistance to different regions of the genetic background located beyond the MHC.

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