Genetica (2012) 140:407–420
DOI 10.1007/s10709-012-9689-y

# The functional importance of sequence versus expression variability of MHC alleles in parasite resistance

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Received: 28 March 2012 / Accepted: 12 November 2012 / Published online: 24 November 2012
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**Abstract** Understanding selection processes driving the pronounced allelic polymorphism of the major histocompatibility complex (MHC) genes and its functional associations to parasite load have been the focus of many recent wildlife studies. Two main selection scenarios are currently debated which explain the susceptibility or resistance to parasite infections either by the effects of (1) specific MHC alleles which are selected frequency-dependent in space and time or (2) a heterozygote or divergent allele advantage. So far, most studies have focused only on structural variance in co-evolutionary processes although this might not be the only trait subject to natural selection. In the present study, we analysed structural variance stretching from exon1 through exon3 of MHC class II *DRB* genes as well as genotypic expression variance in relation to the gastrointestinal helminth prevalence and infection intensity in wild yellow-necked mice (*Apodemus flavicollis*). We found support for the functional importance of specific alleles both on the sequence and expression level. By resampling a previously investigated study population we identified specific MHC alleles affected by temporal shifts in parasite pressure and recorded associated changes in allele frequencies. The allele *Apfl-DRB**23 was associated with resistance to infections by the oxyurid nematode *Syphacia stroma* and at the same time with susceptibility to cestode infection intensity. In line with our expectation, MHC mRNA transcript levels tended to be higher in cestode-infected animals carrying the allele *Apfl-DRB**23. However, no support for a heterozygote or divergent allele advantage on the sequence or expression level was detected. The individual amino acid distance of genotypes did not explain individual differences in parasite loads and the genetic distance had no effect on MHC genotype expression. For ongoing studies on the functional importance of expression variance in parasite resistance, allele-specific expression data would be preferable.

**Keywords** Immune genes · Sequence variability · Gene expression · Gastrointestinal helminths · *Apodemus flavicollis* · Rodent

## Introduction

The genes of the major histocompatibility complex (MHC) have drawn much attention in evolutionary biology as they are known to play a fundamental role in parasite resistance and fitness-relevant life-history decisions (Sommer 2005; Schad et al. 2012). The MHC is a multigene family and its 'classical' genes, class I and II, code for membrane-anchored glycoproteins that present antigens to T cells (Hughes and Yeager 1998). They show, in most species, a high allelic variety and have often been subject to recent gene duplication events (Hughes and Yeager 1998; Axtner and Sommer 2007; Meyer-Lucht et al. 2008; Lenz et al. 2009). Mutation, recombination, gene conversion and

**Electronic supplementary material** The online version of this article (doi:10.1007/s10709-012-9689-y) contains supplementary material, which is available to authorized users.

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birth-and-death evolution of genes are believed to be the genetic mechanisms responsible for the generation of new alleles (Parham and Otha 1996; Nei et al. 1997; Edwards and Hedrick 1998; Martinsohn et al. 1999; Nei and Rooney 2005). The exceptionally high allelic variation of MHC molecules is mainly restricted to the functional important antigen binding sites (ABS), whereas other parts of the mature protein are relatively conserved (Hughes and Nei 1988, 1989). The ABS are under positive selective pressure and represent the functionally most important part of the MHC, whereas other regions are exposed to purifying selection that maintains major structural integrity (Hughes et al. 1994; Nei 2005). This was revealed by crystal analysis of the human *DR1* molecule (Brown et al. 1993) and its mouse homologue *H2-E* (Fremont et al. 1996). They illustrated that antigens are bound by genetically conserved MHC encoded hydrogen bonds to the peptide backbone of the antigen and additionally by four to five pockets that bind side chains of the antigen. These pockets are composed of the highly polymorphic ABS of the β chain, and determine its three-dimensional structure and its binding affinity. The high ABS variation is required to recognize a large repertoire of antigens, enhancing the potential immune responses within an individual or across individuals in a population (Brown et al. 1993; Fremont et al. 1996; McFarland et al. 2005).

It is widely agreed that parasite mediated selection is the major driving force in maintaining MHC polymorphism (Doherty and Zinkernagel 1975; Bernatchez and Landry 2003; Piertney and Oliver 2006). In addition, reproductive mechanisms such as disassortative mating and maternal-foetal interactions have been suggested as alternative or complementary mechanisms maintaining MHC diversity (Penn and Potts 1999). Two main selection scenarios explaining susceptibility or resistance to parasite infections which in turn might also affect life history decisions are currently debated. In one scenario susceptibility related MHC alleles are selected in a frequency-dependent manner in space and time {*negative frequency dependent selection*, *rare-allele advantage* (Takahata and Nei 1990; Slade and McCallum 1992), *fluctuating selection* (Hedrick et al. 1987; Hill et al. 1991)}. Alternatively, a *heterozygote advantage* (Doherty and Zinkernagel 1975), or its specific form a '*divergent-allele advantage*' caused by highly dissimilar alleles (Doherty and Zinkernagel 1975; Hughes and Nei 1988) has been suggested. The relative importance of these non-exclusive hypotheses in maintaining adaptive genetic variation at the MHC is still a matter of debate (reviewed by Bernatchez and Landry 2003; Sommer 2005; Piertney and Oliver 2006; Spurgin and Richardson 2010). Both hypotheses predict positive or negative associations between certain parasites or pathogens and specific MHC alleles or genotypes, which change over time, making it often difficult to distinguish between these mechanisms in real-life models (Spurgin and Richardson 2010). Indeed, several studies found such associations in wild populations (Deter et al. 2008; Kloch et al. 2010; Schwensow et al. 2010; Schad et al. 2012). Other studies have also shown an advantage of MHC heterozygote individuals (McClelland et al. 2003; Froeschke and Sommer 2005; Oliver et al. 2009) or for individuals with a more divergent MHC repertoire (Lenz et al. 2009).

So far, most studies investigating parasite-mediated selection have focussed on the sequence variability of the nucleic acid sequences encoding MHC genes. However, there is growing evidence that differences on the transcriptomic level of genetic components are also of evolutionary importance (Oleksiak et al. 2002; Morley et al. 2004; Ouborg and Vriezen 2007). Expression of MHC genes is generally induced upon infection (Gardiola and Maffei 1993) and the amount of activated T cells depends on the amount of MHC class II expression. However, studies have also indicated that some pathogens actively inhibit the expression of MHC class II genes as a strategy to evade immune reaction (Wojciechowski et al. 1999; Zhong et al. 1999; Noss et al. 2000; Lüder et al. 2003; Pai et al. 2003; Axtner and Sommer 2011). Furthermore, differential expression of MHC class II haplotypes can skew the cytokine response (Th1–Th2 balance) of T cells (Baumgart et al. 1998). Consequently, a diminished MHC expression can influence the susceptibility to pathogens independent of the structural sequence variation in functionally important parts of the MHC (Ting and Trowsdale 2002). Therefore it becomes evident that structural variation alone might not account for disease associations in the MHC class II region (Handunnetthi et al. 2010). Although often recommended (Sommer 2005; Piertney and Oliver 2006; Spurgin and Richardson 2010), MHC gene expression analysis in in situ ecological and evolutionary studies are still rare. To date, only a few attempts have been made to measure MHC gene expression in non-classical model species, such as sea bass (Buonocore et al. 2007), three-spined sticklebacks (Wegner et al. 2006), minks (Bowen et al. 2007), Steller sea lions (Bowen et al. 2006), pallid Atlantic forest rats (Schwensow et al. 2011) or yellow-necked mice (Axtner and Sommer 2011). Methodological difficulties might be one reason why gene expression studies are still rare in wildlife studies. Non-model species generally lack genomic background information that is needed to apply already developed techniques in model organisms (Ouborg and Vriezen 2007).

In the present study, we used a previously established real-time PCR method (Axtner and Sommer 2009) to investigate the role of MHC expression in pathogen resistance in liver samples of the yellow-necked mouse, *Apodemus flavicollis*. The short generation time of mice

make adaptation processes visible within a short time scale. The gastrointestinal parasite fauna of *A. flavicollis* is well characterized. It is dominated by the trichostrongylid nematode *Heligmosomoides polygyrus* inhabiting the small intestine (Ferrari et al. 2004, 2009; Klimpel et al. 2007). This nematode has a direct lifecycle and involves both free-living and parasitic stages (Anderson 2000). Its infective L3 larvae penetrate the duodenal wall, encyst and moult twice until they return as adult nematodes to live in the intestinal lumen where they feed on intestinal tissue (Bansemir and Sukhdeo 1994). Other commonly found helminths include *Trichuris muris*, a whipworm with a similar life cycle, except that it is embeds in the mucosa of the caecum, and *Syphacia stroma,* a pinworm, which lives and feeds in the gut lumen at all stages in the life-cycle.

Our previous studies have provided several lines of support for strong selection acting on the structural variation of the MHC DRB exon 2 of *A. flavicollis*. The number of MHC alleles in a population is related to the gastrointestinal helminth load (Meyer-Lucht and Sommer 2009). Evidence for trans-species mode of evolution (i.e., the preservation of alleles or their lineages over species barriers, Klein 1987; Klein et al. 1998), positive selection acting on the ABS (Musolf et al. 2004), and associations of specific MHC alleles with parasite load (Meyer-Lucht and Sommer 2005) have been detected.

We chose to measure MHC expression in the liver because, besides its physiological function, the liver acts as a filter for antigens that enter the organism from the gastrointestinal tract and mediates tolerance to harmless and recognition of harmful antigens (Racanelli and Rehermann 2006; Selmi et al. 2007; Nemeth et al. 2009; Tiegs and Lohse 2009). Hepatic cells are capable of MHC class II presentation in the case of inflammation (Herkel et al. 2003), but also most of the non-hepatocytes, which can make up to 40 % of liver cells, are antigen-presenting cells (Racanelli and Rehermann 2006).

The specific aim of the present study was to investigate whether, in addition to MHC sequence variability, differences in MHC expression also contribute to pathogen resistance in *A. flavicollis*. We resampled a previously investigated population (Meyer-Lucht and Sommer 2009), quantified the individual burden with gastro-intestinal helminths and genotyped 363 bp long messenger RNA transcripts, comprising the complete second exon of the MHC class II DRB gene. We employed quantitative real-time PCR (qPCR) to test for different expression levels of MHC class II genotypes. As a sign of parasite-driven selection on specific alleles, we expected that temporal changes in the prevalence of parasites would be associated with frequency shifts in MHC alleles associated with parasite resistance. Since it is assumed that higher levels of MHC mRNA transcripts lead to an increased immune activation (Handunnetthi et al. 2010), we expected to observe a higher MHC class II expression in infected compared to non-infected individuals. This should hold true at least for infections by parasite species, which do not actively inhibit the expression of MHC class II genes. In case of a heterozygote advantage, heterozygous individuals should have lower parasite loads than homozygous ones or should show differences in their MHC expression. The divergent allele advantage hypothesis would be supported if the individual amino acid distance correlates with individual differences in the number of different helminth infections or the overall parasite burden by multiple infections which in turn are associated with the MHC genotype expression.

## Methods

### Genetic and parasitological sample collection

Sherman live traps were used to capture yellow-necked mice (*A. flavicollis*) in a single population in 2004 (Meyer-Lucht and Sommer 2009) and 2008 (present study) in a deciduous forest situated 35 km North–East of the city of Hamburg, Northern Germany. Only adult animals with a body mass over 16 g (Jüdes 1979), neither pregnant nor lactating, were sampled. In 2008, animals were anesthetized by inhalation of isoflurane (Forene®, Abbott AG, Switzerland) and immediately sacrificed by cervical dislocation. Liver was stored in RNA-Later (Sigma-Aldrich Chemie GmbH., Germany) for 24 h at 4 °C and subsequently frozen at −20 °C until RNA extraction. The whole intestinal tract was stored in 75 % ethanol for helminthological screenings under a microscope. Nematodes were assigned at least to the genus level, whereas all cestodes could not be further distinguished. Infection intensity (number of worms per individual) was assessed by worm counts in different compartments of the digestive tract (gut, small intestine, caecum and colon). All research reported in this manuscript was in accord to the legal requirements of Germany and complied with the protocols approved by the responsible state office for Agriculture, Environment and Rural Areas of Schleswig–Holstein (Reference No: LANU 315/5327.74.1.6).

### Nucleic acid extraction and cDNA synthesis

Genomic DNA was extracted from ear tissue samples with the DNeasy tissue kit (Qiagen). Total RNA was extracted from liver samples. The whole liver was homogenized (2 × 10 s at 5,000 rpm, Precellys, Bertin Technologies) in QIAzol lyses reagent (Qiagen) with 1.4 mm ceramic beads. From each homogenate, we placed 0.5 ml in two 1.5 ml

tubes and treated each of these aliquots separately as independent replicates A and B for the subsequent procedures. Total RNA was extracted following the QIAzol lyses reagent protocol and dissolved in 87.5 µl of water. To eliminate genomic DNA contaminants, subsequent digestions were performed with DNase I (RNase-free DNase Kit, Qiagen GmbH, Germany), followed by a clean-up using RNeasy spin columns (Qiagen GmbH, Germany) according to the manufacturer's protocol. Total RNA was eluted in 100 µl of water and its amount and purity was assessed three times by a Nanodrop 1000 (Thermo Fisher Scientific Inc., USA) and averaged. RNA quality was checked on agarose gels by electrophoresis. 2 µg of total RNA was reverse transcribed with Oligo-$dT_{18}$ primers (5 µM) in a 20 µl reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche Holding GmbH, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was then diluted 1:3 with distilled water prior to quantitative real-time RT PCR (qPCR).

## MHC genotyping

The variability of the functionally most important part of MHC class II genes, DRB exon 2, was assessed using genomic DNA (gDNA) and their expression verified by mRNA transcripts (cDNA). The gDNA of each individual was genotyped with the primer pair GH46 and JS2 (218 bp, Supplemental Figure 1) according to Meyer-Lucht and Sommer (2005). The PCRs were performed in a 20 µl reaction volume and PCR products were checked upon 1.5 % agarose gels. Single-stranded conformation polymorphism (SSCP) analysis and cycle sequencing were applied to detect MHC class II DRB alleles. Details of SSCP analysis were described previously (Meyer-Lucht and Sommer 2005). 2 µl of the re-amplified single SSCP bands were treated with 0.25U FastAP™ thermo sensitive alkaline phosphatase and 4U of Exonuclease I (both Fermentas) to remove unincorporated primers and to degrade unincorporated nucleotides prior to sequencing of PCR products. The samples were incubated for 15 s at 37 °C and the reaction was stopped by heating up to 85 °C for 15 min. 2 µl of sequencing buffer, as well as 1 µl of BigDye v3.1 (Applied Biosystems) and 1 mM primer was added to the sequencing reaction. Cycle sequencing was done for 20 cycles consisting of 96 °C for 10 s, 52 °C for 10 s and 60 °C for 90 s. The sequencing products were cleaned-up using BigDye XTerminator Purification Kit (Applied Biosystems) and subsequently analysed on a 3130×l Genetic Analyser (Applied Biosystems). Obtained sequences were visually edited and aligned using the alignment editor embedded in MEGA 4.0 (Tamura et al. 2007).

To determine whether all alleles identified by gDNA are transcribed, we used cDNA as a template to amplify a 365 bp long fragment using the primers JA-Apfl-10 and JSex3-DRB (Supplemental Figure 1). The amplicons comprised parts of the first and third exon as well as the complete second exon of the MHC class II DRB gene. For a few individuals, different primer combinations (JA-Apfl-10/JS2, JA-Apfl-11/JS2 and JA-Apfl-06/JSex3-DRB; Supplemental Figure 1) were required which resulted in shorter amplification products. To obtain the sequences of the longer amplification products a molecular cloning approach was required. We purified PCR products (Cycle pure, Peqlab), and cloned them into a pCR®4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen). We picked at least eight clones per PCR (max. 32 clones). We used the vector primers T7 and M13rev to reamplify recombinant clones and sequenced them as described above. We accepted a clone sequence when: (1) it occurred in two different individuals or (2) it appeared in three independent PCRs.

## MHC gene expression

Quantitative PCR was performed for the MHC DRB gene, as well as for four reference genes (ribosomal protein S18, calnexin, cytoplasmic actin gamma 1, phosphoglycerate kinase 1) on a Rotor Gene 3000 (Corbett Research). Details on reference gene validation and reference gene primers have been described previously (Axtner and Sommer 2009). The relative quantification of the gene of interest to reference genes allows the comparison among individuals under the assumption that the reference genes are more or less equally expressed among individuals (Thellin et al. 1999). It is still the most accurate way to detect expression differences between individuals because it controls for artificial variation, e.g. due to differences in the amount of sample, RNA extraction or reverse transcription efficiency (Huggett et al. 2005). Intron-spanning primers were used to avoid the amplification of genomic DNA contaminants. A 172 bp long fragment of the MHC DRB gene was amplified with the primers JA-Apfl-6 and JSex3-DRB (Supplemental Figure 1). To account for intra-run differences all qPCR reactions were run in triplicates for each replicate A and B with a no-template control to check for contaminations. To avoid inter-run variation the replicates A and B of each animal were analysed in the same run. Each tube contained 2.5 µl of cDNA template, 12.5 µl SensiMix™ SYBR® No-ROX (Bioline), 0.5 µl primer (20 µM) and 9.5 µl $dH_2O$. The qPCR conditions were 10 min at 95 °C and 45 cycles of each 95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 s. Melting curve analysis was performed from 65° to 95 °C in 0.5 °C steps each lasting 5 s to confirm presence of a single product and absence of primer-dimers.

The run-specific PCR efficiency for each gene was calculated using the program LinRegPCR 11.6 (Ramakers et al. 2003; Ruijter et al. 2009). A threshold was set manually at 0.5 of the logarithm of the normalized fluorescence. We calculated for each tube in each run the expression value (Q) for each gene out of the individual quantitation cycle (Cq) and the mean amplification rate (E) for each gene in each run by $Q = E^{-Cq}$. The data were inspected visually for Cq or expression outliers and the arithmetic mean of the six expression values Q per individual sample (triplicates of each replicate A and B) were calculated. The MHC expression value was normalized to the geometric mean of the four reference genes (Vandesompele et al. 2002). We log-transformed the normalized relative MHC gene expression levels to attribute equal weight to conditions with over-expression or under-expression and to diminish the influence of outlier values (Willems et al. 2008). To demonstrate the reproducibility of our results, the ratio of the normalized log transformed expression of both replicates (A and B) of each animal was calculated and we accepted only values between 0.85 and 1.15.

## Data analyses

We compared the helminth prevalence in samples collected in 2004 (Meyer-Lucht and Sommer 2005) and 2008 (this study) by an exact unconditional test (Reiczigel et al. 2008; Rózsa et al. 2010) implemented in Quantitative Parasitology 3.0 (Reiczigel and Rózsa 2005).

Arlequin 3.5.1.2 (Excoffier and Lischer 2010) was used to test for deviations from Hardy–Weinberg expectations. In order to detect evidence for positive selection, two different approaches were used. First, we calculated the non-synonymous ($d_N$) and synonymous ($d_S$) substitution rate using MEGA 4.0 (Tamura et al. 2007) and their ratio ω following the method of Nei and Gojobory (1986) with Jukes–Cantor (1969) correction for multiple substitution in pair wise comparisons for the total sequence length spanning exon 1 to exon 3 (365 bp). Substitutions rates were also analysed within and outside the ABS of exon 2 inferred from crystal analysis of the human DR1 molecule (Brown et al. 1993) and standard errors were calculated by bootstrapping with 500 replicates. ω ($d_N/d_S$) was tested to be significantly larger than one with a *Z* test implemented in MEGA 4.0 (Tamura et al. 2007). The second approach consisted of a maximum likelihood analysis using the program CODEML integrated in the software package PAML 4.4 (Yang 2007). It allows the detection of species-specific positively selected sites (PSS) independent of assumptions derived from the human HLA molecule. CODEML estimates ω among sites by applying different models of codon evolution using maximum likelihood procedures. Models M7 and M8 were applied, where the first represents the null model assuming neutral evolution and the latter represents the alternative model allowing for positive selection. Both models assume a beta distribution of ω. The fit of both models on the observed data was tested with a likelihood ratio test with D = −2*log (likelihood (M7)/likelihood (M8)). The probability distribution of the test statistic can be approximated by a Chi square distribution with $|(df_{M7} - df_{M8})|$ degrees of freedom (*df*), which, in our case, was two. PSS and the probabilities for the site classes in the model M8 were calculated by Bayes empirical Bayes (BEB). The genetic distance between individual alleles were calculated by the number of amino acid substitutions using the Poisson model implemented in MEGA 4.0 (Tamura et al. 2007).

All further statistical analysis and graphics were performed with R (ver. 2.14.1, R Development Core Team 2011). We compared the MHC allele frequencies observed in individuals sampled in 2004 (Meyer-Lucht and Sommer 2005) with those collected in 2008 (this study) by a Kolmogorov-Smirnoff goodness-of-fit test. It is based on the maximum absolute difference between the two observed cumulative distributions of both samples. If this difference is significantly large, the two distributions are considered different.

To analyse the effects of different MHC alleles on the prevalence (presence/absence) of parasitic infections, we calculated odds ratio tests based on a conditional maximum likelihood estimate. Thereby, the odds ratios of the most common MHC alleles (>0.05 relative frequency) in relation to each of the four most common parasites (>ten individuals) were included. No relation would result in an odd = 1, odd < 1 indicates a negative association, odd >1 a positive association. A 95 % confidence interval for each odds ratio was calculated and a two-sided Fisher exact test was used to test whether the odds ratio was significantly different from one.

To study whether different MHC alleles also have a significant influence upon helminth infection intensity (number of worms) generalized linear models were employed using the specific MHC alleles as predictors. We included only the most common alleles (>0.05 relative frequency) and merged all other alleles to a single group. As the data were over dispersed we tested for deviance from a negative binomial distribution (Crawley 2007). In the case of deviance, generalized linear models with a quasi-poisson error distribution were used.

We tested whether genotypes consisting of alleles which were significantly associated with helminth burden differed in their MHC expression. Therefore the mRNA transcriptional levels between infected and uninfected individuals carrying these alleles were compared using an exact Wilcoxon rank sum test. Furthermore we tested if homozygote and heterozygote individuals differ in the number of

different helminth infections, in their total infection intensity or MHC expression by a Wilcoxon rank sum test. We also tested whether the amino acid distance or expression levels correlated with the infection intensity per parasite species and the total infection intensity using Spearman rank correlations. The total parasite burden was calculated by summing the normalised infection intensities of the four most common helminth species. As the number of parasites per host and thus the infection intensities varied a lot between the different helminth species we normalised the number of worms of each parasite species by dividing the individual worm counts by the observed maximal infection intensity with this species and multiplying by a 100.

## Results

### Sample collection 2004 and 2008

In 2004, Meyer-Lucht and Sommer life-trapped 22 individuals and took ear tissue and faeces samples (Meyer-Lucht and Sommer 2005). The parasite burden of all those 22 samples was assessed via faecal egg counts and all individuals were genotyped based on gDNA. In 2008, we collected 71 individuals. No family units were collected (microsatellite data not shown).

### Changes in parasite load between 2004 and 2008

All five parasite species observed in 2004 (Meyer-Lucht and Sommer 2005) were also detected in 2008. In 2008, a sixth helminth species was found but it was present in only a single individual (Table 1). We noted the nematodes *T. muris* (Trichuridae) and *Aonchotheca* sp. (Capillaridae) both belonging to the order Rhabditida, *Mastophorus* sp. (Spirocercidae, Spiruridea), *S. stroma* (Oxyuridae, Oxyurida) and *Heligmosomoides polygyrus* (Heligmosomoidae, Strongylida). Cestodes could not be distinguished to the family level and were treated as one group. In only six out of 69 animals sampled in 2008 no infections were detected. Four helminths occurred in more than 20 % of the individuals (*H. polygyrus*, *S. stroma*, *T. muris* and cestodes). Helminth richness ranged from 0 to 4 with a mean of $1.71 \pm 0.98$. Significant changes in the prevalence between 2004 and 2008 were observed in three nematode species. The prevalence of the nematodes *T. muris* and *S. stroma* increased from 2004 to 2008 by 24 and 30 %, respectively, whereas the prevalence of the trichurid nematode Aonchotheca sp. and cestodes decreased by 21 and 19 %, respectively (Table 1).

**Table 1** Differences in the prevalence and the corresponding *p* values of different helminth species detected in *A. flavicollis* in the years 2004 and 2008

| Species | Prevalence | | *p* |
|---|---|---|---|
| | 2004 | 2008 | |
| *Aonchotheca* sp. | 0.273 | 0.058 | 0.008 |
| Cestodes | 0.455 | 0.261 | 0.093 |
| *H. polygyrus* | 0.318 | 0.551 | 0.062 |
| *Mastophorus* sp. | – | 0.006 | – |
| *S. stroma* | 0.136 | 0.464 | 0.005 |
| *T. muris* | 0.091 | 0.333 | 0.026 |

### MHC sequence variability and selection pattern

The genotyping of the second exon (217 bp) of the MHC class II *DRB* gene using genomic DNA revealed 33 different MHC alleles in 71 *A. flavicollis* sampled in 2008. Eleven of these MHC alleles have already been observed in genomic DNA samples of animals trapped in 2004 (Musolf et al. 2004; Meyer-Lucht and Sommer 2005). *Apfl-DRB**78 could only be amplified from cDNA but not from gDNA, probably due to mutation at the primer binding site of GH46 that lies partially in the first intron (Supplemental Figure 2). The expression of all alleles was confirmed by cDNA analyses (365 bp), except for *Apfl-DRB**37. Animals carrying *Apfl-DRB**37 were discarded from further analyses, which reduces the samples size to 69 individuals. Melting curve analyses of our real-time PCR product showed only products of similar length and revealed no splicing variants. All sequences were submitted to GenBank (accession numbers JQ858340-JQ858371).

The 32 different nucleotide sequences coded for 30 different amino acid sequences (Supplemental Figure 2). The nucleotide sequences *Apfl-DRB**03a and *Apfl-DRB**03b differed by a single synonymous substitution at nucleotide position 290 as well as the nucleotide sequences of *Apfl-DRB**09a and *Apfl-DRB**09b at position 113. No more than two alleles per individual were identified suggesting that a single *DRB* locus was amplified. Thirty-four out of 121 amino acid positions were variable. Alleles varied between one and 25 amino acids (mean $= 16 \pm 5$). Forty-seven different genotypes were observed (average frequency: $1.47 \pm 1.1$, max: 6). Genotype frequencies did not differ from Hardy–Weinberg expectations ($H_{obs} = 0.90$, $H_{exp} = 0.88$, $p = 0.546$).

Signatures of long-term (historical) positive selection have been detected by different test approaches. Elevated rates of non-synonymous ($d_N$) over synonymous ($d_S$) substitutions were restricted to exon 2 (Supplemental Figure 3) and were significantly higher in ABS inferred from the human DR1 molecule (Brown et al. 1993), but not in

non-ABS (Supplemental Table 1). Species-specific analyses using CODEML also indicated strong positive selection. The likelihood ratio test revealed that the alternative model M8, which allowed for positive selection fitted much better to the data than the null model M7 assuming neutral evolution (LRT $\chi^2 = 154.43$, $df = 2$; $p < 0.001$). CODEML identified 16 PSS, most of them in close vicinity to one of the residues that form the antigen binding grove in the human DR1 protein (Brown et al. 1993) (Supplemental Figure 3). Also all PSS were restricted to the second exon. The partial sequences of the first and third exon were highly conserved.

## Changes in MHC allele frequencies between 2004 and 2008

In 2004, 17 different alleles were found in 22 individuals (Meyer-Lucht and Sommer 2005) from which 14 were also among the 33 alleles detected in 2008. The rare alleles *Apfl-DRB**24 and *Apfl-DRB**27 present in only a single individual each in 2004 were not observed in the 69 analysed samples from 2008 (Fig. 1). Most alleles were detected in only a few individuals and only four MHC alleles (*Apfl-DRB**01, *03, *21 and *23) had a relative frequency >0.05. A Kolmogorov-Smirnoff goodness of fit test revealed significant differences in the frequencies of all observed alleles between the 2 years ($p = 0.005$). Changes of more than 5 % between 2004 and 2008 were detected in *Apfl-DRB**03 (+12 %), as well as for *Apfl-DRB**23 (−10 %) and *Apfl-DRB**26 (−6 %).

## Association of specific MHC alleles and helminth infections

Investigations of the effects of the most common MHC alleles (*Apfl-DRB**01, *03, *21 and *23, >0.05 relative frequency) on the prevalence of the most common helminths (cestodes, *H. polygyrus*, *S. stroma*, *T. muris*, present in >10 individuals) revealed that animals carrying the allele *Apfl-DRB**23 had a reduced probability of being infected with the oxyurid nematode *S. stroma* (Table 2, Bonferroni non-significant). In addition, *Apfl-DRB**23 showed a strong positive association with the infection intensity of cestodes ($p = 0.0066$). *Apfl-DRB**21 showed a strong tendency ($p = 0.0505$) to be positively associated with the infection intensity of *H. polygyrus* (Table 3). The infection intensity of the nematodes *S. stroma* or *T. muris* could not be explained with the occurrence of the four most common MHC alleles.

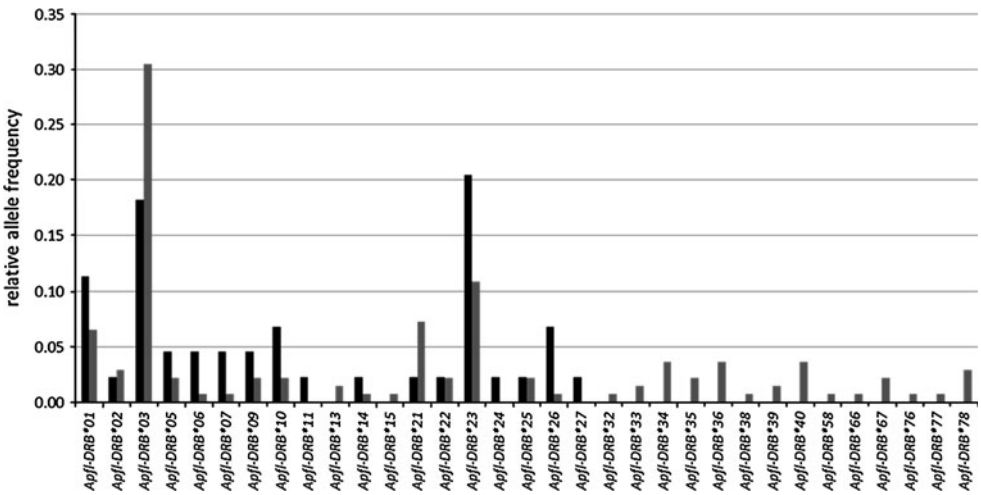


**Fig. 1** Temporal change of relative MHC allele frequencies between 2004 (22 individuals, *black bars*) and 2008 (69 individuals, *grey bars*)

**Table 2** Effects of specific MHC alleles on the prevalence (presence/absence) of parasitic infections

| Allele | *H. polygyrus* | | | *S. stroma* | | | *T. muris* | | | Cestodes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OR | CI | *p* | OR | CI | *p* | OR | CI | *p* | OR | CI | *p* |
| *Apfl-DRB**01 | 0.62 | 0.11–3.18 | 0.721 | 1.52 | 0.29–8.45 | 0.723 | 0.15 | 0.55–16.25 | 0.148 | 0.94 | 0.09–5.75 | 1 |
| *Apfl-DRB**03 | 0.55 | 0.18–1.61 | 0.329 | 1.99 | 0.67–6.00 | 0.224 | 0.35 | 0.11–1.08 | 0. 070 | 1.76 | 0.51–6.66 | 0.410 |
| *Apfl-DRB**21 | 0.50 | 0.09–2.34 | 0.327 | 1.87 | 0.40–10.08 | 0.496 | 0.46 | 0.04–2.59 | 0.477 | 1.25 | 0.19–6.42 | 0.714 |
| *Apfl-DRB**23 | 1.17 | 0.28–5.27 | 1 | 0.18 | 0.02–0.98 | 0.028 | 1.54 | 0.34–6.55 | 0.517 | 2.38 | 0.51–10.53 | 0.276 |

The odds ratios (OR), their 95 % confidence interval (CI) and the corresponding *p* values are shown. All results did not reach Bonferroni significance of $\alpha = 0.003$

**Table 3** Effects of the most common MHC alleles (>0.05 relative frequency) on the infection intensity caused by gastrointestinal helminths in individuals sampled in 2008 indicated by maximal models

| *H. polygyrus* | Estimate | SE | *z* value | *p* |
|---|---|---|---|---|
| Intercept | 0.3557 | 0.9015 | 0.395 | 0.693 |
| *Apfl-DRB**01 | −0.2742 | 0.7169 | −0.382 | 0.702 |
| *Apfl-DRB**03 | −0.1897 | 0.5062 | −0.375 | 0.708 |
| *Apfl-DRB**21 | 1.3790 | 0.7052 | 1.955 | 0.051 |
| *Apfl-DRB**23 | −0.2576 | 0.7706 | −0.334 | 0.738 |
| Other alleles | 0.5660 | 0.6974 | 0.812 | 0.417 |
| Theta | | 0.4096 | | |
| Null deviance | | 70.801 | 68 *df* | |
| Residual deviance | | 65.456 | 63 *df* | |
| *S. stroma* | Estimate | SE | *t* value | *p* |
| Intercept | −0.435 | 2.762 | −0.158 | 0.875 |
| *Apfl-DRB**01 | 2.737 | 1.434 | 1.909 | 0.061 |
| *Apfl-DRB**03 | 1.533 | 1.418 | 1.081 | 0.284 |
| *Apfl-DRB**21 | 2.324 | 1.497 | 1.553 | 0.126 |
| *Apfl-DRB**23 | −1.488 | 4.055 | −0.367 | 0.715 |
| Other allele | 1.895 | 1.594 | 1.188 | 0.239 |
| Null deviance | | 5987.5 | 68 *df* | |
| Residual deviance | | 4636.8 | 63 *df* | |
| *T. muris* | Estimate | SE | *z* value | *p* |
| Intercept | −1.1515 | 0.9871 | −1.166 | 0.243 |
| *Apfl-DRB**01 | 1.2019 | 0.6868 | 1.750 | 0.080 |
| *Apfl-DRB**03 | −0.1496 | 0.5413 | −0.276 | 0.782 |
| *Apfl-DRB**21 | −0.7931 | 0.9628 | −0.824 | 0.410 |
| *Apfl-DRB**23 | 0.7431 | 0.7844 | 0.947 | 0.344 |
| Other allele | 0.7753 | 0.7676 | 1.010 | 0.312 |
| Theta | | 0.565 | | |
| Null deviance | | 62.549 | 68 *df* | |
| Residual deviance | | 53.906 | 63 *df* | |
| Cestodes | Estimate | SE | *t* value | *p* |
| Intercept | −5.857 | 3.074 | −1.905 | 0.061 |
| *Apfl-DRB**01 | 1.118 | 2.146 | 0.521 | 0.604 |
| *Apfl-DRB**03 | 2.264 | 1.568 | 1.444 | 0.154 |
| *Apfl-DRB**21 | 3.204 | 1.667 | 1.921 | 0.059 |
| *Apfl-DRB**23 | 4.459 | 1.588 | 2.809 | **0.007** |
| Other allele | 3.927 | 1.658 | 2.369 | **0.021** |
| Null deviance | | 415.19 | 68 *df* | |
| Residual deviance | | 252.97 | 63 *df* | |

The estimates denote the effect of the particular factor on the dependent variable

Bold numbers denote significance levels < 0.05

For six of our 69 samples we were unable to find congruent results in gene expression according to our strict quality control protocol, even after several experimental repeats. Thus we excluded them and restricted our MHC expression analyses to 63 animals. MHC *DRB* gene expression showed considerable variation (Fig. 2) and the genotype comprising the *Apfl*-DRB*23 had the lowest expression. Nevertheless the expression of the most common alleles did not differ significantly from each other (Kruskal–Wallis test, $\chi^2 = 2$, $df = 2$, $p = 0.3679$).

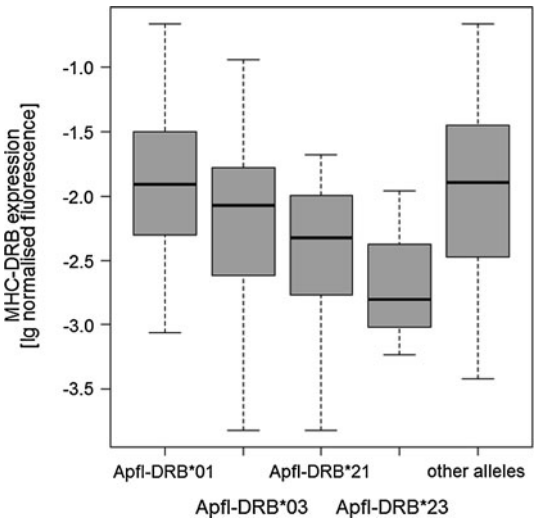


**Fig. 2** MHC gene expressions of the most common alleles (>0.05 relative frequency). Indicated are normalized log transformed values

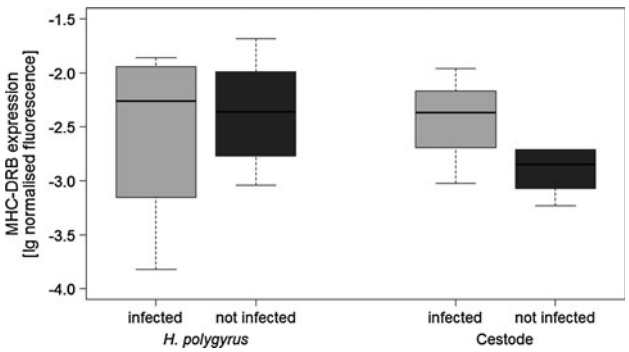


**Fig. 3** MHC DRB gene expression in individuals carrying the allele *Apfl-DRB**21 or *Apfl-DRB**23 in relation to the prevalence by *H. polygyrus* and cestodes, respectively

We tested whether genotypes consisting of these alleles significantly associated with helminth burden differed in their MHC expression and compared the mRNA transcriptional levels between infected and uninfected individuals. We could not test Apfl-*DRB**23 expression in relation to *S. stroma* infection due to missing expression data for the infected animals. In line with our expectation, MHC mRNA transcript levels tended to be higher in cestode-infected animals carrying the allele *Apfl-DRB**23 compared to non-infected animals, though the difference

was not significant, probably due to sample size limitations (MWU test: N = 9, $p_{\text{Apfl-DRB*23}} = 0.302$; Fig. 3). No significant difference in the expression level of genotypes comprising the allele *Apfl-DRB**21 in relation to *H. polygyrus* infection was detected (N = 10, $p_{Apfl\text{-}DRB*21} = 0.819$; Fig. 3). The genotype expression comprising the allele *Apfl-DRB**21 or *23 did not correlate with the infection intensity per parasite species (Spearman rank correlations: all $p > 0.2$).

### Association of MHC heterozygosity or allele divergence and helminth infections

Homozygote and heterozygote individuals did not differ in the number of different helminth infections (exact Wilcoxon rank sum test, W = 221, $p = 0.934$; Fig. 4a) or in their total helminth burden (W = 210, $p = 0.904$; Fig. 4b). They also showed no differences in their expression levels (W = 124, $p = 0.1178$, Fig. 4c).

The mean amino acid distance in the observed genotypes was 0.119 ± 0.053 (max: 0.198). There was no difference in the amino acid distance between genotypes that occurred only once and the more frequent ones ($\text{mean}_{n1} = 0.113$, $\text{mean}_{nx} = 0.129$; $t$ test, $t = -0.79$, $df = 14.3$, $p = 0.44$). The amino acid distance had no influence upon the number of different helminth infections per individual (Spearman rank correlation, roh = 0.068, $p = 0.579$), the normalised infection intensity of the four most common helminth species (Spearman rank correlation, $\alpha_{\text{Bonferroni}} = 0.01$, $\text{roh}_{\text{H. polygyrus}} = 0.026$, $p = 0.035$; $\text{roh}_{\text{S. stroma}} = -0.0114$, $p = 0.926$; $\text{roh}_{\text{T. muris}} = -0.1373$, $p = 0.261$; $\text{roh}_{\text{cestodes}} = 0.0120$; $p = 0.871$, $\text{roh}_{\text{total}} = -0.021$, $p = 0.862$, Fig. 5a) as well as on the total infection intensity (roh = −0.0213, $p = 0.8619$, Fig. 5a). Also the MHC gene expression did not correlate with the parasite burden (Spearman rank correlation, roh = −0.1069, $p = 0.4045$, Fig. 5b) and the amino acid distance (Pearson correlation, $t = 1.665$, $df = 61$, $p = 0.101$, Fig. 5c). We repeated all analyses by calculating the genetic distance of PSS sites only. No significant differences were observed (data not shown).

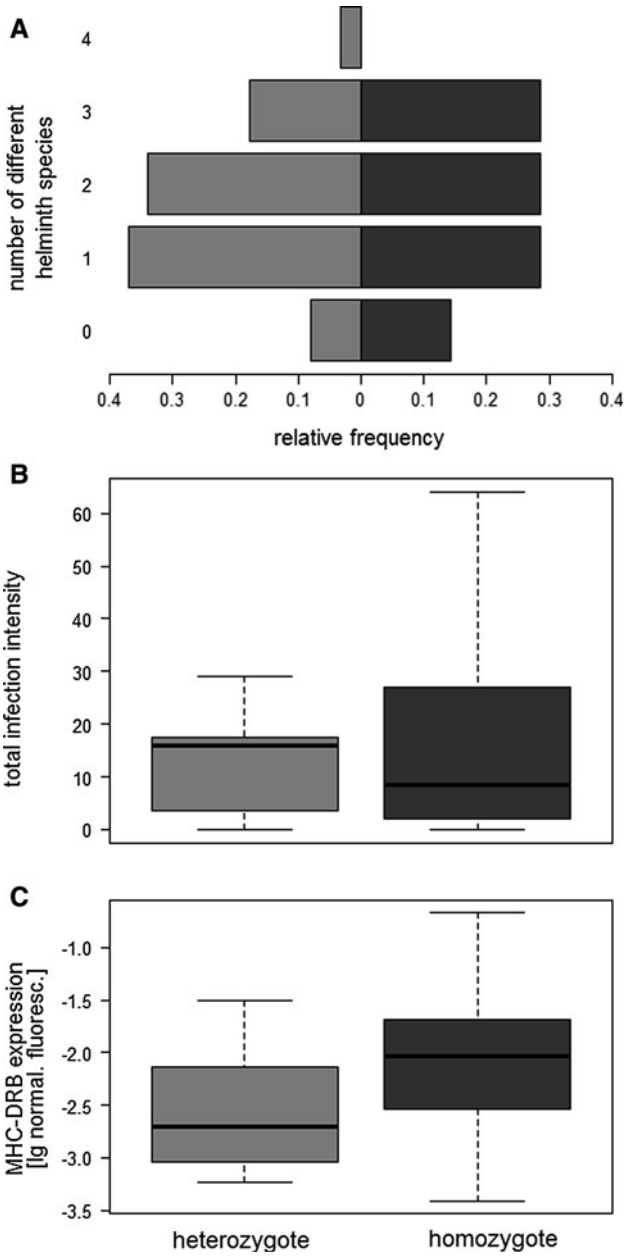


**Fig. 4** **a** Number of different helminth infections, **b** total infection intensity and **c** MHC genotype expression in homo- and heterozygote individuals. No difference between homo- and heterozygote individuals were observed

## Discussion

### MHC sequence variability and selection pattern

In this study we analysed the complete second exon of the MHC class II *DRB* gene plus adjacent parts of the first and the third exon (363 bp) based on mRNA transcripts in a wild population of *A. flavicollis*. A fragment of the second exon has been described on the genomic level before (Musolf et al. 2004; Meyer-Lucht and Sommer 2005, 2009). We could confirm the transcription and thus most likely the functionality of all new alleles, as well as for many of the already described ones. As in previous studies, our present study revealed a high $d_N/d_S$ ratio in ABS but not in non-ABS providing evidence for historical positive selection (Musolf et al. 2004; Meyer-Lucht and Sommer 2005, 2009). Accordingly, all of our identified 16 species-specific PSS were located within the second exon and were congruent with or in close vicinity to ABS of the human

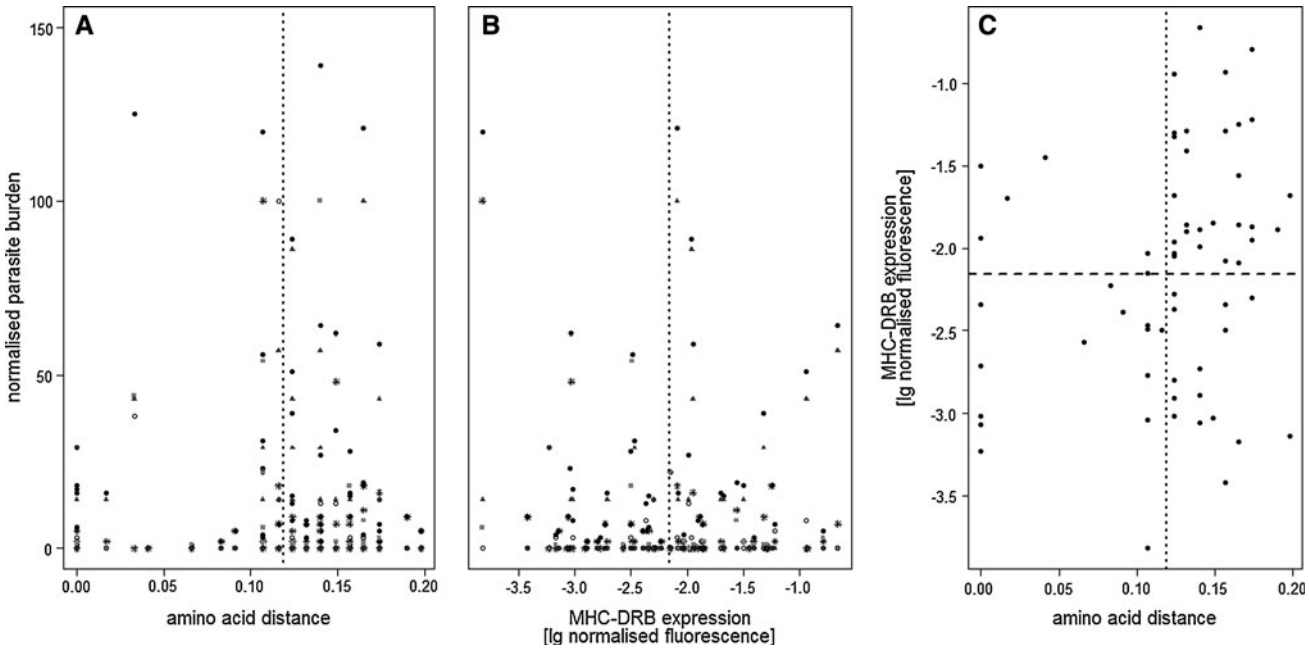


**Fig. 5** Normalised infection intensity of the four most common helminth species as well as total infection intensity in relation to **a** amino acid distance and **b** MHC class II *DRB* gene expression. **c** MHC class II *DRB* gene expression in relation to amino acid distance. No significant correlations were observed. *Dashed lines* denote the arithmetic mean. *Black dots* denote total infection intensity, *asterisk* the normalised infection intensity with *H. polygyrus*, *grey squares* the with *S. stroma*, *triangles* with *T. muris* and *white circles* with cestodes

*DR* protein antigen (Brown et al. 1993). The ABS are under positive selective pressure and represent the functionally most important part of the MHC, whereas other regions are exposed to purifying selection that maintains major structural integrity (Hughes et al. 1994; Nei 2005). In line with expectations we could demonstrate that the parts of the first and third exon were highly conserved. Thus, the single amplified *DRB* locus of *A. flavicollis* provides an excellent wildlife model to investigate the functional importance of structural, as well as expression variance, of classical MHC class II genes under natural selection conditions. Most studies in mammalian wildlife species do not confirm the expected variability pattern across DRB exons and do not use cDNA. This bears the risk of taking alleles from non-functional MHC loci (i.e. pseudogenes) into account, which might lead to wrong conclusions (Axtner and Sommer 2007). Moreover, alternative splicing might have an effect on functionality. Recent studies in salamanders of the genus *Ambystoma* observed mRNA transcripts produced by alternative splicing that lack the protein binding region (Laurens et al. 2001; Bulut et al. 2008). The authors still assume functionality for these variants as they are persistent for more than three million years (Bulut et al. 2008) probably due to positive selection (Lareau et al. 2004). A functionality of splicing variants would change selection pressures upon the nucleotide sequences. Our melting curve analyses consistently revealed PCR products of similar length in *A. flavicollis* and to the best of our knowledge no MHC class II splice variants are known for rodents.

## Association of specific MHC alleles and helminth infections

By comparing samples from 2004 and 2008, we were able to show that MHC allele frequencies do change within 5 years, which demonstrates that this can happen in a rather short time or even in consecutive generations (Westerdahl 2004; Charbonnel and Pemberton 2005; Eizaguirre et al. 2012). It must be noted, however, that the sample size of 2004 was relatively small and we restricted our analyses to the most common alleles only. The genotypes in 2008 were clearly dominated by the allele *Apfl-DRB**03, which was already the second most common allele in 2004. Its frequency increased by more than 12 % and it occurred in more than half of the individuals. Neither in 2004 nor in 2008, was an association of *Apfl-DRB**03 with any kind of gastrointestinal helminth infection detected, which might suggest a strong selective advantage of *Apfl-DRB**03 against other parasites or pathogens we did not control for.

The second most frequent allele in 2004, *Apfl-DRB**23, was negatively (but not Bonferroni-significant) associated

with *S. stroma* prevalence and at the same time positively associated with cestode infection intensity. Such pleiotropic effects have also been demonstrated in another recent rodent study (Froeschke and Sommer 2012). As *Apfl-DRB**23 lost about 10 % in frequency, one could speculate that cestode infection might exert a stronger selection pressure than *S. stroma* infections. Indeed, the prevalence of cestodes decreased by 19 % between 2004 and 2008. In 2004, *Apfl-DRB**23 was negatively associated with faecal egg counts of heligmosomoid nematodes but not with *S. stroma* nor cestode infections (Meyer-Lucht and Sommer 2005). Changes in the functionality of specific MHC alleles can be the result of fluctuating selection by temporal and spatial inconsistencies of a parasite community (Hedrick et al. 1987). Moreover, in 2004 non-invasive faecal egg counts were used to investigate gastrointestinal infections. Although both methods provide highly correlative results for many gastrointestinal nematodes (Seivwreight et al. 2004; Froeschke et al. 2010), faecal egg counts might underestimate *Syphacia* sp. infections. This parasite does not deposit its eggs in the intestine, instead it moves through the anal opening and cements its eggs to the perinanal skin (Brown and Rosenthal 1997).

The third most common allele in 2004, *Apfl-DRB**01, decreased in frequency but showed no association to helminthic parasite burden. *Apfl-DRB**21 increased in frequency and showed a marginal significant ($p = 0.0505$) positive association with the infection intensity of *H. polygyrus*. Our recent study suggests that *H. polygyrus* acts as an immune-suppressive and causes a systemic reduction of the MHC *DRB* gene expression as an immune evasion strategy (Axtner and Sommer 2011), as has been suggested for other microparasites (LeibundGut-Landmann et al. 2004). Several studies have shown that pathogens can actively inhibit MHC expression (Wojciechowski et al. 1999; Zhong et al. 1999; Noss et al. 2000). If *H. polygyrus* is able to interfere with the general MHC transcription to avoid immune recognition, it might not exert a strong selective pressure upon the structural sequence variability of MHC antigen binding motifs.

Many genes show heritable variation in their expression levels and as natural selection can act on these traits, this might be of evolutionary importance (Oleksiak et al. 2002; Morley et al. 2004; Ouborg and Vriezen 2007). Wegner et al. (2006) suggested that certain MHC haplotypes differ in their expression level and heritable nucleotide polymorphism has been found in the MHC promoter region in mouse (Mitchison and Roes 2002), horse (Díaz et al. 2005) and primates, showing indices of balancing selection (Loisel et al. 2006). The importance of differences in MHC expression becomes evident in autoimmune diseases (Heldt et al. 2003) and might also play a role in parasite resistance. As we expected, higher MHC mRNA transcription was observed in animals carrying the allele *Apfl-DRB**23 and being infected with cestodes. The expression of genotypes comprising the allele Apfl-DRB*21 were lower than others and was not influenced by the prevalence of *H. polygyrus*. The potential ability of *H. polygyrus* to suppress MHC class II expression (Axtner and Sommer 2011) might open a gateway for co-infections independent from the MHC binding motifs of the host. However, these results were not significant probably due to sample size limitations. It is likely that much larger sample sizes are required to account for many confounding factors affecting gene expression under natural selection conditions. Moreover, we could only investigate expression differences of genotypes. Due to the high allelic diversity at the functional important antigen-binding sites (ABS) on one hand, but sequence similarity outside the ABS on the other hand, it was not possible to design allele-specific primers to measure individual allelic expression in this species.

### Association of MHC heterozygosity or allele divergence and helminth infections

A high allelic diversity is normally accompanied by a high level of heterozygote individuals. In our study, neither level of heterozygosity was beyond expectation, nor did homozygote individuals showed any unusual features in parasitic burden or MHC expression.

Lenz (2011) showed that divergent alleles can provide a selective advantage in terms of helminth resistance by the ability to bind a higher variety of antigens, which was also reflected in a better body condition (Lenz et al. 2009). In our data, the amino acid distance had no effect upon the number of different helminth infections and overall worm burden by multiple infections of an individual. Furthermore, the genetic distance between individual alleles had no effect on the MHC expression, which in turn did also not correlate with the parasite load. Thus we did not find any evidence for either heterozygote nor divergent allele advantage in our data.

## Conclusion

In our study in *A. flavicollis* we demonstrated clear signs of positive selection shaping the classical structural variance of MHC class II polymorphism. By resampling a previously investigated study population we identified specific MHC alleles affected by temporal shifts in parasite pressure and recorded associated changes in allele frequencies. We found support for the functional importance of specific alleles both on the sequence and probably also on the expression level. The latter was not significant due to sample size limitations in relation to allelic diversity. No evidence for a heterozygote

or divergent allele advantage on the sequence or expression level was detected. Our study suggests that not only structural variance of MHC genes might be the target of co-evolutionary processes. Also, expression variance might be of functional importance in parasite resistance. However, large sample sizes are required to disentangle these effects in wildlife species confronted by many confounding parameters under natural selection conditions. For ongoing studies, wildlife species with low MHC allelic diversity would be desirable, so that measurements of allele-specific expression might be feasible.

**Acknowledgments** This study was funded by the Federal Ministry of Education and Research (BMBF). We would like to thank A. Drews, state office for Agriculture, Environment and Rural Areas of Schleswig–Holstein and J. Stäcker, district forester for their support and permission to carry out this study, A. Schmid for essential laboratory work, Y Meyer-Lucht for comments on the first draft of this manuscript, and Megan L Steele for language corrections. We are grateful for the comments and suggestions of the associate editor and anonymous referees which considerably improved the manuscript.

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