Immune gene expression and diversity in relation to gastrointestinal parasite burden in small mammals

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Doctoral Thesis Jan Axtner

To those who kept believing.

Content

Preface

Most studies on the functional importance of immune gene variation (Major histocompatibility complex, MHC) in parasite resistance focus on the role of sequence variability in regions responsible for antigen binding and presentation. The aim of my doctoral thesis was to investigate the relevance of transcriptional differences of adaptive MHC variation in natural populations of small mammals in relation to parasitic infections. This thesis comprises of five published articles, myself being first or second author which can be read independently (Axtner and Sommer 2009, 2011, 2012; Weyrich et al. 2010; Schwensow et al. 2011). All articles have co-authors and are therefore written in the first person plural. The individual contribution of each co-author to the publications is stated at the end of the individual article summaries.

Summary

One of the most fascinating aspects of biology is the mutual evolutionary relationship between hosts and their parasites. Their perpetual, complex coexistence is characterized by a reciprocal interplay that has produced some of the most astounding adaptations in nature. One adaptation is the extraordinary degree of genetic diversity observed in some immune genes of vertebrates, the so called major histocompatibility complex (MHC). MHC genes encode proteins that are responsible for the recognition of foreign antigens and the triggering of a subsequent, adequate immune response of the organism. Thus they hold a key position in the immune system of vertebrates – and hence of us human beings, where the MHC is known as human leucocyte antigen (HLA). It is believed that the extraordinary genetic diversity of MHC genes is shaped by adaptive selectional processes in response to the reoccurring adaptations of parasites and pathogens. Therefore it is assumed that natural populations that suffer a loss of this genetic diversity face a higher potential extinction risk as they are thought to be more vulnerable to parasites, pathogens and transmitted diseases. Indeed, in recent years a large number of MHC studies were performed in a wide range of wildlife species aiming to understand the role of immunegene diversity in parasite resistance under natural selection conditions. Methodically, most of this work with very few exceptions has focussed only upon the structural, i.e. sequence diversity of regions responsible for antigen binding and presentation. Most of these studies found evidence that MHC gene variation did indeed underlie adaptive processes and that an individual's allelic diversity explains parasite and pathogen resistance to a large extent. Nevertheless, our understanding of the effective mechanisms is incomplete.

A neglected, but potentially highly relevant component concerns the transcriptional differences of MHC alleles. Indeed, differences in the expression levels MHC alleles and their potential functional importance have remained unstudied. This is due to the fact that reliable measurements of different levels of messenger RNA via quantitative real-time PCR only became feasible around ten years ago, but the highly sensitive but also costly method was not well established for wildlife species. The idea that also transcriptional differences might play an important role relies on the fact that lower MHC gene expression is tantamount with reduced induction of CD4+ T helper cells and thus with a reduced immune response. Hence, MHC gene expression might be an important aspect in the antagonistic interplay between host and parasites.

My dissertation studies are dealing with the antagonistic interplay between host and parasites. I included the expression of MHC genes and of immune regulative cytokines as additional factors to reveal the functional importance of MHC diversity. I studied MHC diversity in two unrelated free-ranging rodent species, the South-American rodent *Delomys sublineatus* and in the European yellow-necked mouse, *Apodemus flavicollis*, in association with their gastrointestinal helminths under natural selection conditions. I sampled a single population of each study species to reduce interpopulationary differences in MHC adaptions.

I caught *D. sublineatus* in the Brazilian coastal forest Mata Atlântica in the landscape around Tapiraí, State São Paulo. I trapped *A. flavicollis* in Northern Germany, 35km north-east of the city Hamburg. In total, I captured and examined 24 *D. sublineatus* and 71 *A. flavicollis* individuals and detected nine different helminth species in *D. sublineatus* and six in *A. flavicollis*. I established the method of relative quantification of mRNA on liver and spleen samples of both species in our laboratory. Accordingly, the amount of mRNA of a gene of interest is measured relative to the amount of mRNA of several reference genes, whose expression profiles have to be relative constant in time. As there was no available information on nucleic sequences of potential reference genes in both species, PCR primer systems that were established in laboratory mice have to be tested and adapted for both non-model organisms. In the due course, sets of stable reference genes for both species were found and thus the preconditions for reliable measurements of mRNA levels established.

For *D. sublineatus* it could be demonstrated that helminth infection elicits aspects of a typical Th2 immune response. Whereas mRNA levels of the cytokine interleukin *Il4* increased with infection intensity by strongyle nematodes neither MHC nor cytokine expression played a significant role in *D. sublineatus*.

For *A. flavicollis* I found a negative association between the parasitic nematode *Heligmosomoides polygyrus* and hepatic MHC mRNA levels. As a lower MHC expression entails a lower immune response, this could be evidence for an immune evasive strategy of the nematode, as it has been suggested for many micro-parasites. This implies that *H. polygyrus* is capable to interfere actively with the MHC transcription. Indeed, this parasite species has long been suspected to be immunosuppressive, e.g. by induction of regulatory T-helper cells that respond with a higher interleukin *Il10* and tumor necrosis factor *Tgfb* production. Both cytokines in turn cause an abated MHC expression. By disabling recognition by the MHC molecule *H. polygyrus* might be able to prevent an activation of the immune system. Indeed, I found a strong tendency in animals carrying the allele Apfl-*DRB**23 to have an increased infection intensity with *H. polygyrus*. Furthermore, I found positive and negative associations between specific MHC alleles and other helminth species, as well as typical signs of positive selection acting on the nucleic sequences of the MHC. The latter was evident by an elevated rate of non-synonymous to synonymous substitutions in the MHC sequences of exon 2 encoding the functionally important antigen binding sites whereas the first and third exons of the MHC *DRB* gene were highly conserved.

In conclusion, the studies in this thesis demonstrate that valid procedures to quantify expression of immune relevant genes are also feasible in non-model wildlife organisms. In addition to structural MHC diversity, also MHC gene expression should be considered to obtain a more complete picture on host-pathogen coevolutionary selection processes. This is especially true if parasites are able to interfere with systemic MHC expression. In this case advantageous or disadvantageous effects of allelic binding motifs are abated and host-parasite co-evolutionary processes might not only be

reflected by the structural variance of MHC alleles. As in previous studies, I also found ample evidence for the importance of structural variance in MHC genes reflected by high allelic diversity, strong indices of selection in the past and contemporary associations to parasite infection and recent shifts in allelic frequencies.

My studies indicated that in addition to structural sequence variance also MHC gene expression might play an important role in antagonistic coevolution though the interplay is not clear yet. The study suggested that it depends on the specific parasite species involved as some parasite species are able to interfere with the expression pattern of immune relevant genes facilitating the gateway for further parasite infections. Furthermore heritable expression differences of MHC alleles are known, but their effect on susceptibility to parasites is still unclear and leaves an open field for future studies.

Zusammenfassung

Einer der faszinierendsten Aspekte der Biologie ist sicherlich die wechselseitige evolutive Beziehung zwischen Wirten und ihren Parasiten. Deren fortwährendes und komplexes Miteinander ist durch ständige gegenseitige Beeinflussung geprägt und hat einige der erstaunlichsten Anpassungen in der Natur hervorgebracht. Die außergewöhnlich hohe genetische Vielfalt einiger Immungene bei Wirbeltieren, des Haupthistokompatibilitätskomplex oder im Englischen “major histocompatibility complex (MHC)“, ist eine derartige Anpassung. Eine der Hauptaufgaben von MHC-kodierten Proteinen ist die Erkennung körperfremder Moleküle und das Einleiten einer sich anschließenden adäquaten Gegenantwort des Organismus. MHC-Gene nehmen damit eine Schlüsselrolle im Immunsystem der Wirbeltiere und damit auch von uns Menschen ein, bei denen dieser Genkomplex auch als auch als „human leucocyte antigen (HLA)“ bezeichnet wird. Man nimmt an, dass diese außergewöhnliche Vielfalt der MHC-Gene durch adaptive Selektion geformt und erhalten wird und sie eine Antwort auf die sich ständig anpassenden Parasiten und Krankheitserreger ist. Daher wird auch vermutet, dass natürliche Populationen, die diese genetische Vielfalt aufgrund äußerer Einflüsse verloren haben, einem erhöhten Aussterberisiko unterliegen könnten, da sie möglicherweise anfälliger für Parasiten und Pathogene sein könnten. Als Konsequenz daraus wurden zahlreiche MHC-Studien an Wildtieren durchgeführt, die die Rolle der genetischen Vielfalt hinsichtlich der Parasitenresistenz unter natürlichen Selektionsbedingungen untersuchten. Von wenigen Ausnahmen abgesehen, befassten sich die meisten Studien dieser Art mit der Variabilität der Nukleinsäuren in Bereichen, die in der Antigenbindung eine entscheidende Rolle spielen, d.h. mit der strukturellen Vielfalt der MHC Gene. Viele dieser Arbeiten haben Hinweise dafür gefunden, dass die MHC-Gene einer adaptiven Selektion unterliegen und die individuelle Allelausstattung eines Tieres einen Großteil von dessen Parasiten- und Pathogenbelastung erklären kann. Trotzdem ist unser Verständnis über die wirkenden Mechanismen teilweise noch lückenhaft.

Ein bis dato stark vernachlässigter aber möglicherweise sehr entscheidender Aspekt hierbei sind eventuelle transkriptionale Unterschiede der MHC Allele, d.h. Unterschiede in ihrer Genexpression. Die Idee, dass transkriptionale Unterschiede ein wichtiger Aspekt im antagonistischen Zusammenspiel zwischen Wirt und Parasit sein könnten beruht auf der Tatsache, dass eine geringere MHC Genexpression gleichbedeutend mit einer geringeren Aktivierung von CD4+ T-Helferzellen und somit einer verminderten Immunantwort ist. Tatsächlich wurde dieser Aspekt und seine womöglich funktionelle Bedeutung bis dato kaum untersucht. Dies lag vor allem daran, dass die Messung unterschiedlicher Mengen von Boten-RNA mittels quantitativer ‚real-time PCR‘ zwar seit ungefähr zehn Jahren verlässlich funktioniert, diese äußerst empfindliche und zugleich teure Methode für Modelorganismen, nicht jedoch für Wildtiere nicht hinreichend etabliert war.

Meine Dissertation behandelt die antagonistische Wechselbeziehung zwischen Wirt und Parasit und zieht dabei neben der genotypischen Diversität von MHC-Genen auch deren Expression sowie die Genexpression immunregulativer Zytokine mit in Betracht. Ich habe dies an zwei nicht näher verwandten, frei lebenden Kleinsäugerarten, unter natürlichen Selektionsbedingungen untersucht und in Bezug zu deren individueller Belastung mit parasitären, gastrointestinalen Helminthen betrachtet. Ich habe jeweils eine einzige Population des südamerikanischen Nagers *Delomys sublineatus*, sowie der Europäischen Gelbhalsmaus *Apodemus flavicollis* beprobt, um eventuelle Unterschiede zwischen Populationen der jeweiligen Art zu vermeiden. *D. sublineatus* habe ich im brasilianischen Küstenregenwald, der Mata Atlântica, in der Gegend um die Stadt Tapiraí im Bundesstaat São Paulo gefangen. *A. flavicollis* wurde in Norddeutschland, ca. 35km nordöstlich der Stadt Hamburg gefangen. Insgesamt habe ich 24 *D. sublineatus* in Brasilien, sowie 71 *A. flavicollis* in Deutschland gefangen und untersucht. Neun unterschiedliche Helminthen wurden in *D. sublineatus* und sechs in *A. flavicollis* unterschieden. Anhand von Leber und Milzproben beider Arten habe ich zunächst die Methode der ‚real-time PCR' und der relativen Quantifizierung von mRNA in unserem Labor etabliert. Bei dieser Methode wird die Menge an mRNA eines Genes relativ zur mRNA-Menge mehrerer Referenzgene gemessen, deren Expression in einer Zelle über die Zeit gesehen relativ konstant sein sollte. Da es zum Zeitpunkt der Studie keine Sequenzinformation über potentielle Referenzgene für beide Arten gab, wurden bereits für Labormaus etablierte PCR-Primersysteme an beiden Arten getestet. Für beide Arten konnten so entsprechend stabile Referenzgene gefunden und somit die Grundvoraussetzung für zuverlässige Genexpressionsmessungen geschaffen werden. Mit den Arbeiten konnte für *D. sublineatus* gezeigt werden, dass Helminthenbefall eine typische Th2 Immunantwort induziert, und dass der Zytokin *Il4* Gehalt mit Befallsintensität strongyler Nematoden zunimmt. Es wurde für *D. sublineatus* kein signifikanter Zusammenhang zwischen MHC Expression oder anderen Zytokinen mit Helminthenbefall gefunden.

In *A. flavicollis* wurde ein negativer Zusammenhang zwischen haptischer MHC-Expression und dem parasitären Nematoden *Heligmosomoides polygyrus* festgestellt. Da eine verringerte MHC Expression eine verminderte Immunantwort bedeutet, könnte dies auf eine Immunvermeidungsstrategie des Nematoden hindeuten, wie sie bereits für zahlreiche Mikroparasiten vermutet wurde. Dies setzt voraus, dass *H. polygyrus* aktiv in die MHC-Transkription einzugreifen vermag. Seit langem wird vermutet, dass diese Art immunsuppressiv auf ihren Wirt einwirkt, z.B. durch Induktion von regulatorischen T-Helferzellen, die dann mit einer erhöhten *Il10*- und *Tgfb*-Ausschüttung reagieren. Diese beiden Zytokine wiederum bewirken eine verminderte MHC-Expression. Indem *H. polygyrus* eine Erkennung durch den MHC verhindert könnte er eine Aktivierung des Immunsystems des Wirtes vermeiden. Dennoch erwiesen Tiere, die das MHC-Allel Apfl-*DRB**23 trugen, in meiner Studie eine starke Tendenz zu einer erhöhten Infektionsintensität mit *H. polygyrus* auf. Des Weiteren fand ich typische positive und negative Assoziationen zwischen

MHC-Allelen und anderen Helminthenarten, sowie Zeichen eines positiven Selektionsdruckes auf den MHC-Sequenzen. Letzteres wurde durch eine erhöhte Rate nicht-synonymer zu synonymer Mutationen deutlich. Die nicht-synonymen Veränderungen waren auf bestimmte Positionen innerhalb des zweiten Exons des *DRB*-Genes beschränkt, wohingegen die untersuchten Bereiche des ersten und dritten Exons stark konserviert vorlagen. Diese variablen Positionen kodieren meist Schlüsselstellen im Bereich der Antigenbindungsstelle im MHC Molekül.

Zusammenfassend zeigt diese Arbeit, dass Genexpressionsstudien auch an Wildtieren durchgeführt und verlässliche Daten erzeugt werden können. Zusätzlich zur strukturellen Vielfalt sollten zukünftig auch mögliche Genexpressionsunterschiede bei MHC-Studien berücksichtigt werden, um ein kompletteres Bild der koevolutiven Wirt-Parasiten-Beziehungen zeichnen zu können. Dies ist vor allem dann von evolutiver Bedeutung, wenn die Parasiten in der Lage sind die MHC Expression aktiv zu beeinflussen. Dies hieße jedoch auch, dass immunsuppremierende Parasiten die vor- und nachteilige Effekte unterschiedlicher Erkennungsmotive einzelner MHC-Allele vermindern würden und in diesen Fällen die strukturelle MHC-Vielfalt nicht die antagonistischen Wirt-Parasiten-Beziehungen wiederspiegelt. Nichtsdestotrotz habe ich vielerlei Hinweise auf die enorme Bedeutung der strukturellen Vielfalt der MHC-Gene gefunden, die sich in einer außerordentlichen Alleldiversität, starke Anzeichen von Selektion in der Vergangenheit sowie gegenwärtige Assoziationen mit Parasiteninfektionen und Wechseln in Allelfrequenzen zeigen.

Die Studien konnten nicht die exakte Bedeutung von MHC-Genexpression in der antagonistischen Koevolution definieren, aber sie konnten zeigen dass diese Bedeutung stark von den jeweils beteiligten Partnern abzuhängen vermag. Zudem sind vererbbare Expressionsunterschiede von MHC-Allelen bekannt, ihre Bedeutung für die Anfälligkeit gegenüber Parasiten ist jedoch noch nicht geklärt und lässt noch ein weites Feld für zukünftige Studien.

Introduction

Parasites are organisms that live in or on another organism and take their nourishment from their host which might be associated with fitness deficits. They account for at least half of the world's species diversity and are omnipresent, so that natural populations are permanently exposed to them (Windsor 1998; Poulin and Morand 2000). As parasites can have a strong influence upon the survival, condition and the fecundity of their individual host (Anderson and May 1979; May and Anderson 1979; Hurd 2001) they act as potent selective agents and are of evolutionary importance in natural populations (Altizer et al. 2003). Both, host and parasite are bound in an antagonistic coevolution that leads to an on-going counter adaptation in both, known as the 'Red Queen' hypothesis (van Valen 1973; Salathé et al. 2008). Their perpetual arms race can lead to local adaptations and even speciation events (Huyse et al. 2005; Eizaguirre and Lenz 2010). While the parasite population tries to evade recognition and activation of the host's immune system, the host struggles to detect parasites and to mount an adequate immune defence (Sorci et al. 1997). The interactions of both antagonists are quite numerous and complex and many different genes have been found to be involved in parasite defence (Acevedo-Whitehouse and Cunningham 2006).

MHC

The most important resistance related genes are the ones belonging to the major histocompatibility complex (MHC) where ample associations between genetic make-up and susceptibility or resistance to parasites has been demonstrated (e.g. Meyer-Lucht and Sommer 2005; Bonneaud et al. 2006; Axtner and Sommer 2007; Tollenaere et al. 2008; Schwensow et al. 2010). Classical MHC genes code for membrane-standing glycoproteins that are key receptor molecules responsible for the recognition and binding of foreign antigens (Hughes and Yeager 1998). Loaded MHC molecules play a crucial role in the immune system as they are recognized by T lymphocytes that initiate the appropriate immune response. Two groups of classical MHC genes are distinguished, class I and class II genes. They are defined by their expression patterns and the origin of their loaded antigens. Class I genes are constantly expressed in every somatic cell and their molecules present endogenously derived antigens to cytotoxic T cells. Class II genes have a more restricted expression pattern as they are mainly presented by antigen presenting cells of the immune system such as dendritic cells, B cells or macrophages. Some other cell types like hepatocytes can express class II genes upon induction with interferon γ (Ting and Trowsdale 2002; Herkel et al. 2003). MHC class II molecules present their exogenous derived antigens to $CD4^+$ T helper cells which in turn release cytokines triggering an adequate immune response.

The MHC has attracted a lot of attention in infection biology and evolutionary studies dealing with host-parasite interactions in the past two decades. Its classical genes show an extraordinary high level of genetic diversity that is interpreted to be a result of the antagonistic coevolution between

host and parasite and is suggested to be maintained by parasite mediated balancing selection (Doherty and Zinkernagel 1975; Bernatchez and Landry 2003; Piertney and Oliver 2006; Spurgin and Richardson 2010). Many different selection mechanisms have been proposed to be involved in the maintenance of the genetic diversity of classical MHC genes and their relative individual importance is still subject to debate (Piertney and Oliver 2006; Spurgin and Richardson 2010). The most established hypotheses are heterozygote advantage (Doherty and Zinkernagel 1975), divergent allele advantage hypothesis (Doherty and Zinkernagel 1975; Hughes and Nei 1988), negative frequency dependent selection, (Takahata and Nei 1990; Slade and McCallum 1992) and/or fluctuating selection (Hedrick et al. 1987). However, they are non-exclusively and many studies have focussed on the levels of MHC polymorphism in wildlife to unravel the importance of the respective hypotheses in the preservation of genetic diversity of MHC (for reviews see Bernatchez and Landry 2003; Sommer 2005; Piertney and Oliver 2006; Spurgin and Richardson 2010). But still there is the need for more research to understand the complex effective mechanisms acting in this evolutionary process and of which our understanding is yet incomplete.

MHC and infection

By recognising and binding foreign antigens MHC molecules are in the first defence line of the adaptive immune system. Therefore they would provide an adequate target for parasites and pathogens to attack, if they run an immune evasive strategy. For micro-parasites like *Chlamydia* sp., *Mycobacterium* sp. or *Toxoplasma* sp. it was demonstrated that they extenuate the host's MHC class II expression (Wojciechowski et al. 1999; Zhong et al. 1999; Noss et al. 2000; Lüder et al. 2003; Pai et al. 2003) most likely to avoid immune cell activation and CD4+ T cell initiation (LeibundGut-Landmann et al. 2004). So far, such a mechanism has not been described for macro-parasites like helminths, although helminths are described as potent regulators of the host's immune system (Maizels and Yazdanbakhsh 2003; Maizels et al. 2004; Jackson et al. 2008). Helminths can have a significant impact on individual fitness and mortality (Deter et al. 2007; Pedersen and Greives 2008) and thus produce a strong selective pressure on the host immune system including the hosts' MHC genes. Therefore they are well suited candidates to study parasite-mediated selection on the MHC sequence variance and MHC gene expression.

Helminths are a paraphyletic group of parasitic worms comprising nematodes, cestodes, and trematodes, which can cause chronic infestations. Even though helminth species have evolved independently, they generally induce a similar stereotypic T-helper (TH) 2-type response in the host species (Maizels et al. 2004; Maizels 2005; Anthony et al. 2007; Jackson et al. 2008). This T_H2-type response is hallmarked by elevations of typical T_H2 cytokines (Kreider et al. 2007; Harris and Gause 2011). Furthermore, helminth infection induces alternatively activated macrophages that differ in their surface and secreted molecules from classical activated macrophages in T_H1 responses

(Anthony et al. 2006; Kreider et al. 2007) and it induces regulatory T (T_{reg}) cells (Maizels and Yazdanbakhsh 2003; Maizels et al. 2004; Jackson et al. 2008). The alternatively activated macrophages have an anti-inflammatory effect on T_H1-type responses, assist wound healing and promote T_H2 components responsible for worm expulsion (Kreider et al. 2007; Moreau and Chauvin 2010). T_{reg} cells produce elevated levels of immunosuppressive cytokines such as transforming growth factor β (*Tgfb*) and interleukin 10 (*Il10*) (Metwali et al. 2006; Finney et al. 2007). Both cytokines were demonstrated to reduce presentation of MHC class II genes in antigen presenting cells (Knolle et al. 1998; Romieu-Mourez et al. 2007). Their potential role is to counter an interferon γ induced inflammatory T_H1 reaction that might cause damage to the own organism itself if it is not controlled (T_H1/T_H2 balance).

Most of the previous studies on the role of MHC diversity in parasite resistance focussed only on the structural variability of the nucleic acid sequences encoding different MHC alleles and their potential evolutionary importance. There is growing evidence that differences at the transcriptional level of genetic components are also of evolutionary significance (Oleksiak et al. 2002; Morley et al. 2004; Ouborg and Vriezen 2007). Structural variation of MHC alleles alone cannot fully account for disease associations (Handunnetthi et al. 2010). As a reduced MHC expression can be an effective gateway for parasites, variation in MHC transcription could influence susceptibility to disease independent of structural variation at the coding region (Ting and Trowsdale 2002). With quantitative real-time reverse transcription PCR (qPCR) we have a tool at hand that allows us to detect such variation on the level of messenger RNA (mRNA). However, although often called for (Sommer 2005; Piertney and Oliver 2006; Spurgin and Richardson 2010), MHC gene expression analyses in ecological and evolutionary studies are still rare. Methodological drawbacks might be one reason for this. Non-model species often lack genomic background information that would facilitate the application of new techniques originally developed on laboratory models (Ouborg and Vriezen 2007). For example, qPCR uses so called reference genes that are believed to show a constant mRNA level in every sampled cell as internal standards for relative quantification purposes (Huggett et al. 2005). Potential reference genes have to be carefully validated before use inasmuch as an incorrect use could lead to erroneous results (Tricarico et al. 2002; Dheda et al. 2005). But for most non-model organisms, there is no sequence information on those potential reference genes hampering the application of this technique. However, from an evolutionary perspective it is essential to study antagonistic coevolution in a system that can be regarded as ‘normal’ (Friberg et al. 2010), because classical model species might be poor reflections of wild animals facing the changing and challenging conditions of their natural environment (Feder and Mitchell-Olds 2003).

Study Species

The two wild study species chosen for these studies have both been in focus in long-term ecological and conservation studies in our research group (Musolf et al. 2004; Meyer-Lucht and Sommer 2005, 2009; Püttker et al. 2006, 2007, 2008).

The yellow-necked mouse, *A. flavicollis* is a murid rodent belonging to the same subfamily Murinae like domestic mice (Michaux et al. 2002). It has a distribution throughout Central and Eastern Europe inhabiting deciduous and mixed forests, is predominantly nocturnal and mainly granivor (Bergstedt 1965; Marsh and Harris 2000). Its marginal areas of range in the North are defined by Southern England and Southern Scandinavia, in the South by the Balkan and Northern Turkey, the Apennines and Pyrenees and in the West by Eastern France (Michaux et al. 2004). In the field *A. flavicollis* can be easily mistaken for *A. sylvaticus*, its closely related sister-species that shows very similar ecological demands. *A. sylvaticus* occurs often sympatric or even syntopic with *A. flavicollis* (Michaux et al. 2001, 2004). The ubiquitous European *Apodemus spp.* has a well described parasite fauna. Comparative studies on the parasite communities of sympatric *Apodemus* populations demonstrated that both species show a great overlap in their intestinal helminth community, not to say they are identical (Lewis 1987; Klimpel et al. 2007). In most *Apodemus* spp. populations the intestinal helminth community is dominated by the most prevalent and high abundant *Heligmosomoides polygyrus* (Abu-Madi et al. 2000; Goüy de Bellocq et al. 2003). *H. polygyrus* has only recently been taxonomically separated from *H. bakeri* (Cable et al. 2006; Behnke and Harris 2010), a well-studied parasitic model organism in domestic mice (for review see Behnke et al. 2009b). Thus the *Apodemus* spp. ~ *H. polygyrus* system is appealing for wildlife studies in infection biology, as the close relatedness of both taxa to the *Mus musculus* ~ *H. bakeri* laboratory model system allows transfer of knowledge and to test predictions in the 'real world' that were previously made in the laboratory (Ferrari et al. 2004, 2009; Behnke et al. 2005, 2009a; Jackson et al. 2009). The MHC II DRB immune gene has been characterised (Musolf et al. 2004) and it was demonstrated that the individual genetic make-up of the MHC II DRB gene is associated with susceptibility to helminth infection (Meyer-Lucht and Sommer 2005) as well as the population wide MHC II DRB diversity is associated with overall infection intensity of helminths (Meyer-Lucht and Sommer 2009).

In contrast to *A. flavicollis* the pallid Atlantic Forest rat, *Deolomys sublineatus* is sensitive to human impact. It was chosen because of its sensitivity to habitat fragmentation and potential accompanying effects on its immune gene diversity and might be an ideal candidate for further immune-ecological studies. *D. sublineatus* is a South-American cricetine rodent belonging to the subfamily Sigmodontinae (D'Elía 2003). It weights up to 75g, is -as far as known- mainly nocturnal and terrestrial (Püttker et al. 2006) and restricted to native vegetation cover of the Atlantic Forest of the

Mata Atlantica (Püttker et al. 2008). It seems to be unable to use the altered habitat matrix around the forest fragments (Umetsu and Pardini 2007) and Pardini et al. (2005) showed that *D. sublineatus* reacts sensitively to habitat fragmentation and decreases in abundance and density in small and isolated forest remnants. There is almost no information about the parasite community or the pathogens of *D. sublineatus*. Previous studies distinguished six different helminth morphotypes (Püttker et al. 2007) and five flea species (de Moraes et al. 2003). Furthermore, infections with the protozoa *Babesia* sp. and the bacteria *Haemobartonella* sp. have been reported (Silva et al. 2007).

Objectives

The overall conception of the study was to include the aspect of MHC expression into infection studies in non-model species using qPCR to complement conventional approaches focusing on the functional importance of structural sequence variance in host-pathogen coevolutionary selection processes. I trapped wild *A. flavicollis* in a single forest in Northern Germany and collected *D. sublineatus* in the Brazilian Coastal rain forest. I transferred knowledge and modified methods from well-established laboratory model systems to facilitate MHC expression studies in wildlife species. I established qPCR assays for both species that allowed to measure transcriptional levels of immune genes such as MHC or interleukins. My overall aim was to understand whether the expression of immune relevant genes is an additional target of co-evolutionary selection processes.

The specific aims of the studies were to answer the following questions:

1. Are quantitative measurements of immune gene expression feasible in in-situ wildlife studies? Therefore
    - potential reference genes in liver and spleen samples of the two study species were tested in their use as reference genes in qPCR experiments,
    - intron-spanning primer systems for MHC expression analyses were designed and tested,
    - laboratory protocols for qPCR experiments in liver and spleen samples of the study species were established.

2. What is the potential role of gene expression in the antagonistic interplay between parasite and hosts' immune system? Specifically I wanted to know whether
    - MHC gene expression is correlated to parasite infection,
    - MHC gene expression is associated with MHC sequence polymorphism.

3. Which selection mechanisms contribute to the observed MHC diversity pattern? Therefore, I focussed on

    - signs of selection in the past,
    - selective advantages of specific MHC alleles,
    - effects of temporal changes in parasite community on MHC allele frequencies

Article Summaries

Summary Article 1

Jan Axtner & Simone Sommer

Validation of internal reference genes for quantitative real-time PCR in a non-model organism, the yellow-necked mouse, *Apodemus flavicollis*

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Quantitative real-time reverse transcription PCR (qPCR) is a well-established method to quantify levels of messenger RNAs. Reference genes are used as internal standards to normalise mRNA transcription levels and allow a direct comparison between samples. So far, most studies using a qPCR approach have been carried out using samples either from cell cultures or from common model species kept under laboratory conditions, but investigations in non-model organisms are rare. However, only field studies in free-ranging populations can reveal the effects of natural selection on the expression levels of functional important genes. In order to test the feasibility of gene expression studies in wildlife samples I identified and validated eight potential reference genes that were originally developed for domestic mice and applied them to liver samples of wild yellow-necked mice, *Apodemus flavicollis*. Reference gene validation is an essential and crucial step in qPCR as the use of inappropriate internal standards can give erroneous results and thus lead to misinterpretation of data. This is especially true for in-situ samples on wildlife species, as there are often many confounding factors that can influence results and that are hard to control for.

I used *A. flavicollis* due to its close relatedness to the domestic mice as a well-established laboratory model. I tested 15 commercial primer sets that were originally designed for domestic mice and tested them on 14 *A. flavicollis* samples. Only eight of those, namely succinate dehydrogenase complex (*Sdha*), ribosomal protein L13a (*Rpl13a*), phosphoglycerate kinase 1 (*Pgk1*), calnexin (*Canx*), β-actin (*Actb*) and ubiquitin (*Ubc*) performed well and could be validated in terms of constancy of expression. I confirmed identity of all validated genes by sequencing and published the results on GenBank.

In wildlife studies often many confounding factors can have an effect on the transcription of reference genes. In our case a high heterogeneity in the samples exists regarding the age or physiological stage of the sampled animal. Thus I expected and did observe a high variation of expression levels in my samples. To assess expression stability of reference genes I used three different programmes each focusing on different approaches and compared the results (geNorm, Vandesompele et al. 2002; NormFinder, Andersen et al. 2004; BestKeeper, Pfaffl et al. 2004). All three programmes showed little differences in their results and a Kendall's W test showed very high concordance of the gained rankings of suitable reference genes. Mean ranking ordered from the

most to the least stable gene expression was *Rps18*, *Sdha*, *Canx*, *Pgk1*, *Actg1*, *Ubc*, *Rpl13a* and *Actb*. There is no gene, whatsoever with an absolute constant expression in every cell, therefore it is recommended to use more than one reference gene (Bustin 2002; Bustin et al. 2005) and calculate a normalization factor for relative quantification purposes (Vandesompele et al. 2002; Pfaffl et al. 2004). As it is inappropriate to use a too high number of reference genes geNorm tries to calculate an optimal number of reference genes to be included in a normalization factor. In my case geNorm suggested using the five most stable genes for normalization mRNA levels of target genes.
I demonstrated in this study that relative quantification via real-time PCR is feasible in samples from wild life animals. This offers possibilities to test hypotheses and findings that were gained in laboratory models in 'real-life' experiments and studies with wildlife species facing the constantly changing environment under which they evolved.

Authors' contribution:

I performed sample collection, establishment of laboratory protocols, acquisition, analyses as well as interpretation of the data and drafting of the manuscript; Simone Sommer initiated and supervised the research and revised the manuscript.

Summary Article 2

Alexandra Weyrich, Jan Axtner & Simone Sommer

Selection and validation of reference genes for real-time RT-PCR studies in the non-model species *Delomys sublineatus*, an endemic Brazilian rodent

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In order to study the effects of environmental changes, selection and related processes in wildlife species, we established a qPCR assay for liver samples of *Delomys sublineatus*. This species has been used before as an indicator species in environmental studies regarding its sensitivity to habitat fragmentation (Pardini et al. 2005; Püttker et al. 2007, 2008; Umetsu and Pardini 2007) and its genetic diversity (Sommer et al. unpublished data). Like in *A. flavicollis* we tested primer sets designed for amplification of reference genes in domestic mice and validated the expression stability of these genes. Out of nine tested primer sets only five performed well and could be used for validation, whereas the others produced no or more than one PCR product. The validated genes were γ-actin 1 (Actg1), succinate dehydrogenase complex (*Sdha*), β-actin (*Actb*), calnexin (*Canx*) and phosphoglycerate kinase 1 (*Pgk1*) and their identity was verified by sequencing of the PCR product. We analysed twelve samples and the results were analysed using three different programmes, each using a different approach (geNorm, Vandesompele et al. 2002; NormFinder, Andersen et al. 2004; BestKeeper, Pfaffl et al. 2004) and then compared. All three programmes demonstrated congruently that *Sdha* had the most stable gene expression, whereas *Actb* and *Actg1* showed high variation. *Canx* and *Pgk1* were differently ranked by the three programmes. According to geNorm the variation was lowest when the four most stable genes are included in the normalization factor.

We were able to establish a real-time PCR assay in order to measure levels of gene transcripts in a non-model organism. *D. sublineatus* inhabits the tropical coastal forest of Brazil, the Mata Atlântica, and thus it is exposed to different selective forces than the focus species of my previous study, the central European *A. flavicollis*. Both species might give us valuable insights into how evolutionary processes might act on the transcriptomic level of genes.

Authors' contribution:

Alexandra Weyrich performed acquisition, analyses and interpretation of the laboratory data as well as drafting of the manuscript. I accomplished sample collection, establishment of laboratory protocols, helped analysing the data and revised the manuscript. Simone Sommer initiated and supervised the research and revised the manuscript.

Summary Article 3

Nina Schwensow, Jan Axtner & Simone Sommer

Are associations of immune gene expression, body condition and parasite burden detectable in nature? A case study in an endemic rodent from the Brazilian Atlantic Forest

Infection, Genetics and Evolution 2011, 11 (23-30)
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Natural populations are permanently exposed to pathogens and parasites that influence survival, condition and fecundity of their hosts and thus represent important evolutionary forces. They are in a constant challenge with the host's immune system that tries to protect the host or at least tries to limit the damage that is caused by infection. Our present knowledge of these interactions is almost exclusively based on laboratory model systems or from studies on humans. This is not surprising as in-situ studies on wildlife species are often influenced by uncontrollable confounding parameters that make it difficult to test theoretical expectations. However, only natural systems reflect the evolutionary reality under which these mechanisms evolved and are therefore important to study. In this work we employed qPCR to measure transcript levels of immune genes in association with body mass index (BMI) and individual helminth burden of wild *D. sublineatus*. In total we discriminated seven nematode morphotype and one cestode morphotypes. All individuals showed infection with the strongyle nematode morphotype N1, which occurred in 39% as a single infection. For qPCR purposes eight potential reference genes for qPCR were tested on twelve spleen samples and six of those genes were validated for expression stability. Validated genes were γ-actin 1 (*Actg1*), β-actin (*Actb2*), ribosomal protein L13a (*Rpl13a*), calnexin (*Canx*) and phosphoglycerate kinase 1 (*Pgk1*). The latter showed high variation in mRNA transcript levels and was excluded for the calculation of the normalization factor. The expression levels of MHC class I and class II *DRB*, as well as interleukin (*Il*) 2, *Il4*, *Il10* and *Tgfb* were measured in 24 spleen samples and analysed in a principle component analysis (PCA). The first two components explained most of the variation and all genes seem to be influenced. *Il10* and *Tgfb* behaved antagonistically to MHC class II as expected. The results show a combination of inflammatory and anti-inflammatory components that are typical representatives of classical T helper cell 2 responses. The associations of cytokine expression levels with different helminths and helminth species richness were rather complex. The cytokine *Il2* and *Il4* were positively associated with the BMI, which might indicate a direct link between the inflammatory aspects of an immune reaction and individual host condition.

This study is one of the few studies aiming to confirm the typical patterns of immune gene expression we know from laboratory models in wildlife species. The data suggest functional associations between regulatory cytokines and parasite burden. Since in nature, adaptation to the omnipresent parasites may be more important than individual defence components (as investigated

in most laboratory studies) further studies in free-ranging mammals are required to understand the evolutionary significance of immune gene expression or modulation in relation to parasite pressure under natural selection conditions.

Authors' contribution:

Nina Schwensow performed acquisition, analyses and interpretation of the laboratory data as well as drafting of the manuscript. I accomplished sample collection, establishment of laboratory protocols, helped analyse the data and revised the manuscript. Simone Sommer initiated and supervised the research and revised the manuscript.

Summary Article 4

Jan Axtner & Simone Sommer

Heligmosomoides polygyrus infection is associated with lower MHC class II gene expression in *Apodemus flavicollis*: Indication for immune suppression?

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MHC genes are in the first defence line of the adaptive immune system of vertebrates recognizing foreign antigens and triggering the adequate immune response. Parasites that are able to interfere with MHC expression can evade immune reaction as it has been suggested for micro-parasites (LeibundGut-Landmann et al. 2004). Furthermore they might be able to promote other bystander infections. *Heligmosomoides polygyrus*, a gut dwelling parasitic nematode in *Appodemus* spp. is known to manipulate its host immune defence in a systemic way resulting in a lowered responsiveness of the innate immune system (Jackson et al. 2009) and a pronounced pattern of bystander infections in its natural host (Behnke et al. 2009a). My intention was to test whether *H. polygyrus* infection is associated with systemic MHC gene expression under natural conditions and if *H. polygyrus* infection influences co-infection patterns with other helminth species. I employed qPCR to measure messenger RNA levels of MHC class II *DRB* gene, transforming growth factor b (*Tgfb*) and interleukin 10 (*Il10*) in liver samples of wild *A. flavicollis*. Prevalence and infection intensity with *H. polygyrus* was rather small compared to other studies (Klimpel et al. 2007; Behnke et al. 2009a). More than half of the infected animals showed low intensities of one or two nematodes limiting the significance of the data as high infection intensities are expected to have a higher impact. Nevertheless linear models demonstrated that *H. polygyrus* infection was negatively associated with hepatic MHC gene expression and this association was highly significant. Furthermore linear models showed that hepatic cytokine expression was not associated with parasite burden. I therefore suggest that the observed systemic effect on MHC expression might be a 'spill-over' reaction of regulatory cytokines that are produced by T_{reg} cells at the site of infection (Ince et al. 2009). Due to its immunosuppressive competence I expected *H. polygyrus* infection to be positively associated with bystander infections. Contrary to my expectations heligmosomoid infection was negatively associated to cestode infestations and I could not find any association to other nematode parasites. In fact, rather the opposite was the case: *H. polygyrus* as well as *Syphacia stroma* prevalence tended to be negatively associated with cestode infection although both not Bonferroni significant. A possible explanation might be that cestodes outcompete both nematodes in the small intestine, whereas the caecum dwelling *Trichuris muris* is not afflicted by cestode infestations, as it inhabits another gut compartment. *T. muris* and *S. stroma* occurred less

in single infections than expected, suggesting that they might depend on previous infections by other parasites.

H. polygyrus has been demonstrated to influence the innate immune system in wild hosts (Jackson et al. 2009) and interference with the adaptive arm of the immune system might be a possible explanation for our results. An overall systemic reduced MHC expression should result in less activated T helper cells that also other parasites might benefit from. This might serve as an immune evasion strategy of *H. polygyrus* as it has been suggested for microparasites that are capable of MHC suppression (LeibundGut-Landmann et al. 2004). My study further demonstrates that in addition to the effects of the nucleic variance of specific MHC class II alleles on helminth susceptibility (Meyer-Lucht and Sommer 2005, 2009) the transcriptions levels of immune relevant genes should also be considered in order to disentangle the complex evolutionary interactions between host and parasite.

Authors' contribution:

I performed sample collection, establishment of laboratory protocols, acquisition, analyses and interpretation of the data as well as drafting of the manuscript; Simone Sommer initiated and supervised the research and revised the manuscript.

Summary Article 5

Jan Axtner & Simone Sommer

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

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Classical MHC genes are a paradigm for genetic diversity and in most species they show a tremendous allelic diversity. Most wildlife studies focus on the variability of MHC genes based on genomic DNA without considering if the alleles found are expressed and thus functional. They mainly focus on short sequence fragments that are supposed to hold most of the variation like the second exon of the MHC class II DRB gene. In this study I analysed a 365bp long fragment spanning from the first into the third exon based on mRNA transcripts. I therefore was able to confirm functionality of alleles that had been previously described (Musolf et al. 2004; Meyer-Lucht and Sommer 2005, 2009) and characterised additional alleles for *A. flavicollis*. In total I identified 33 different nucleotide sequences coding for 29 different amino acid sequences in 69 individuals from a single population. According to neutral theory the nucleotide sequences showed clear signs of positive selection in the past as the rate of non-synonymous substitutions exceeded that of the synonymous by far. This positive selection was evidently restricted to certain sites whereas other areas where notably conserved. Two third of these sites under positive selection were congruent with the antigen binding sites transferred from the human *DR*1 molecule (Brown et al. 1993). As expected these sites were restricted to the second exon, whereas the amplified parts of the first and the third exon were highly conserved. 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. For example the allele *Apfl-DRB**03 rose in frequency, whereas *Apfl-DRB**26 and *Apfl-DRB**23 fell in frequency. Alterations in the parasite community and thus changing selective pressures might be the driving force behind these changes. In fact parasite prevalence did change within those four years and might have led to changing directional selection pressures favouring different alleles at different times. For example, whereas the prevalence of the trichurid nematode *T. muris* increased it decreased for *Aonchotheca* sp..

The allele *Apfl-DRB**23 indicated a pleiotropic effect with resistance to infections by the oxyorid nematode *Syphacia stroma* and at the same time 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.

My 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 on-going studies, wildlife species with low MHC allelic diversity would be desirable, so that measurements of allele-specific expression might be feasible.

Authors' contribution:

I performed sample collection, establishment of laboratory protocols, acquisition, analyses and interpretation of the data as well as drafting of the manuscript; Simone Sommer initiated and supervised the research and revised the manuscript.

Articles

Article 1

Validation of internal reference genes for quantitative real-time PCR in a non-model organism, the yellow-necked mouse, *Apodemus flavicollis*

Jan Axtner and Simone Sommer, BMC Research Notes 2009, 2:294
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Abstract

Background: Reference genes are used as internal standards to normalize mRNA abundance in quantitative real-time PCR and thereby allow a direct comparison between samples. So far most of these expression studies used human or classical laboratory model species whereas studies on nonmodel organism under in-situ conditions are quite rare. However, only studies in free-ranging populations can reveal the effects of natural selection on the expression levels of functional important genes. In order to test the feasibility of gene expression studies in wildlife samples we transferred and validated potential reference genes that were developed for lab mice (*Mus musculus*) to samples of wild yellow-necked mice, *Apodemus flavicollis*. The stability and suitability of eight potential reference genes was accessed by the programs BestKeeper, NormFinder and geNorm.

Findings: Although the three programs used different algorithms the ranking order of reference genes was significantly concordant and geNorm differed in only one, NormFinder in two positions compared to BestKeeper. The genes ordered by their mean rank from the most to the least stable gene were: Rps18, Sdha, Canx, Actg1, Pgk1, Ubc, Rpl13a and Actb. Analyses of the normalization factor revealed best results when the five most stable genes were included for normalization.

Discussion: We established a SYBR green qPCR assay for liver samples of wild *A. flavicollis* and conclude that five genes should be used for appropriate normalization. Our study provides the basis to investigate differential expression of genes under selection under natural selection conditions in liver samples of *A. flavicollis*. This approach might also be applicable to other non-model organisms.

Background

Quantitative real-time RT PCR (qPCR) has become a tool with a broad spectrum of use in molecular biology [1]. By quantifying mRNA levels it allows valuable insights into the variation of gene expression between certain individuals or different treatment groups. The most common practice in qPCR is the relative measurement of the expression of a gene of interest after normalization to an internal reference gene. These formerly called house-keeping genes were thought to be constantly expressed in every cell or every tissue and were supposed to be neither up nor down regulated. This assumption has proven false by a growing number of studies [2-4]. All genes seem to be regulated under some conditions and there seems to be no universal reference gene with a constant expression in all tissues [5-9]. But still the relative quantification against an internal reference gene is the most accurate way to detect expression differences especially in low copy mRNA because it controls for artificial variation, e.g. due to

differences in the amount of sample, RNA extraction or reverse transcription efficiency [10]. Thus, a careful validation of the usefulness of potential reference genes is highly recommended [1, 6, 10-15] but not always applied [16]. So far gene expression studies and therefore also reference gene validations are mainly limited to human or classical laboratory organisms as non-model species often suffer from the lack of background information available. For example the real-time PCR primer data base RTPrimerDB [17] includes 5319 primer sets for animals and humans, whereof 3992 were designed for humans followed by 805 for mice (*Mus musculus*) and 454 for rats (*Rattus norvegicus*) commonly used in labs. But particularly non-model species are of great interest to evolutionary genetics or ecologists as classical model species might be poor reflections of wildlife which face the constantly changing and challenging conditions of their natural environment [18]. Focusing just on model species could mean working on the expense of ecological and evolutionary realism and insitu studies on wild populations are required to account for natural selection conditions. In this study we established a SYBR green qPCR assay for liver samples obtained from wild caught *Apodemus flavicollis*. The yellow-necked mouse is a common European murid in deciduous and mixed forests. It belongs to the subfamily Murinae [19] and has been subject to a broad range of genetic, ecological, evolutionary and parasitological studies [20-25]. Especially host-parasite interactions are of special interest in this species as this species serve as one of the main reservoir for vector-borne diseases agents (e.g. *Salmonella* spp., Borreliosis or Hanta virus infections) in Central Europe [25]. The results of our study are the prerequisite to investigate the adaptive variance of expression levels of immune genes, specifically major histocompatibility complex class II genes, in relation to individual pathogen burden to test the hypothesis that in a natural environment not only structural sequence variation but also differential expression of adaptive genes is under selection. Therefore, we validated eight potential reference genes from a panel of primer sets that were originally designed for *Mus musculus* and tested their application for relative gene expression analysis in *A. flavicollis*.

Methods

Sample collection

We live trapped wild yellow necked mice (*Apodemus flavicollis*) in 2007/08 in a deciduous forest about 35 km north-east of Hamburg, Germany. Mice were anesthetized by inhalation of isoflurane (Forene©) and then sacrificed immediately by cervical dislocation at the trapping site. Liver samples were taken and stored in RNA-Later (Sigma), kept at 4°C for 24 h and then frozen at -20°C until further treatment.

RNA extraction and cDNA synthesis

Thirty mg liver tissue of 14 animals were placed in tubes with 500 µl of QIAzol lyses reagent (Qiagen) with 1.4 mm ceramic beads. Tissue was disrupted in a homogenizer (Precellys, Bertin Technologies) (2 × 10 s at 5000 rpm) and total RNA was extracted following the QIAzol lyses reagent protocol and dissolved in 87.5 µl of water. A DNA digestion with DNase I (RNase-free DNase Kit, Qiagen) and a subsequent clean-up via RNeasy spin columns (Qiagen) according to the manufacturer's protocol was done. Total RNA was finally eluted in 60 µl of water and its amount and purity was assessed with the Nanodrop 1000 (Thermo Scientific) three times and averaged. Two µg of total RNA were reverse transcribed with Oligo-dT18 primers (5 µM). Reverse transcription was run in triplicates of 40 µl using the SensiMix two step kit (Quantace) according to the manufacturer's protocol. All RT-triplicates were mixed and the copied cDNA was diluted 1:16 prior qPCR with aqua dest.

Primer selection

We chose six rodents primer sets out of the RT Primer data base because they potentially amplified reference genes with similar length and identical annealing temperature T_a. We also tested nine intron spanning primer sets out of the commercially available Mouse Normalisation Gene Panel (Quantace) (Table 1). All these potential reference gene primer sets were originally designed for the model organism *Mus musculus* and we applied them to our nonmodel organism *A. flavicollis*.

Quantitative real-time RT PCR

SYBR green qPCR was performed with SensiMix two step kit (Quantace) in a 25 µl volume on a Rotor Gene 3000 (Corbett Research). All qPCR reactions were run in triplicates with a no-template control to check for contaminations. Each tube contained 4 µl of cDNA template, 12.5 µl SensiMix dT (Quantace), 0.5 µl SYBR Green solution, 0.5 µl primer (50 µM) and 7.5 µl dH2O. The qPCR conditions were 10 min at 95°C and 45 cycles of each 95° for 15 s, 55°C for 20 s and 72°C for 20 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 individual amplification rate E for every single reaction tube was determined by the 'comparative quantification' function (Corbett Software 6.1.81) to avoid inter-run variation. E is defined as the average increase of fluorescence in the raw data for five cycles following the 'Takeoff' value. This Takeoff value is specified as the time at which the second derivative of the raw data is at 20% of its maximum (Corbett Software 6.1.81). This point marks the end of the background noise and indicates the transition into the exponential phase of the reaction. E was averaged for each gene out of the three replicates in each run. To normalize the raw data the individual background fluorescence from cycle one to the Takeoff value was averaged and all data points of a sample were divided by this average background level ('Dynamic Tube' function, Corbett Software 6.1.81). Individual threshold cycle values (Ct-values) were obtained by setting a threshold manually at 0.01 of the normalized fluorescence ignoring the first five cycles. The Ct-values for a gene were averaged for the three replicates in each run. We calculated the expression of each gene arbitrarily as $Q = E^{-Ct}$. Note that Q is not the real amount of DNA copies $N_t = N_0 * E^t$ to a time point t but rather the fluorescence that is measured proportional to N_t. As we set a certain fluorescence threshold. we set $N_{Y,Ct_Y} = N_{X,Ct_X}$.

With the known E and the Ct-value the ratio between two genes depends only upon their start amount of cDNA N_0.

Determination of reference gene expression stability

The stability of the selected reference genes was determined by BestKeeper [21], NormFinder [18] and geNorm [12]. Concordance between their different ranking orders was tested with Kendall's W implemented in SPSS 16.0.2. BestKeeper ranks the reference genes by the variation of their Ct-values. The gene with the lowest standard deviation ($SD_{Ct\text{-}value}$) is proposed to be the most suitable reference gene. Like BestKeeper, we excluded every gene showing a $SD_{Ct\text{-}value}$ that would result in a variation of the starting material by the factor two. But unlike BestKeeper, we calculated this SD-threshold for each gene based on its known over-all run average E:

$$SD_{threshold} = \frac{\ln 2}{\ln E}$$

NormFinder [18] instead uses a model based approach to analyse the variance in the expression data. It allows for intra- and intergroup variation which makes it more robust against co-expressed genes. In this experiment it was not necessary to distinguish between intra- and intergroup variation as we had only one group of samples. NormFinder calculates a stability value for each gene and the gene with the lowest value is supposed to be the most stable out of the tested set of genes. GeNorm [12] bases on the simple assumption that expression of two ideal reference genes will always have the same ratio among samples regardless of the experimental conditions before the real-time PCR. The ratio between two genes (Y and X) in a sample is $\frac{Qy}{Qx} = \frac{E_Y^{-Ct_Y}}{E_X^{-Ct_X}}$. The average expression stability value M for each gene is calculated using the expression data. M is the average pairwise variation of a gene compared with each of the other potential reference genes in one sample. The average M of all genes together is then calculated by stepwise exclusion of the least stable gene until the two most stable genes of the set remain that can not be ranked any further. GeNorm also allows estimating the optimal number of reference genes which should be used for normalization. It calculates the normalization factor (NF) based on the geometric mean of the expression of more than one reference gene. The more reference genes included in this NF the less possible

outliers account. On the other hand using to many genes might include unstable reference genes making it less accurate. GeNorm calculates the NF_n for the two most stable reference genes based on the geometric mean of the expression data and then the NF_{n+1} with the next most stable gene. To determine how many genes should be used for accurate normalization the pairwise variation $V_{n/n+1}$ was determined out of two sequential normalization factors (NF_n and NF_{n+1}).

All research reported in this manuscript adhered to the legal requirements of Germany were and complied with the protocols approved by the responsible state office for Agriculture, Environment and Rural Areas of Schleswig-Holstein (Referenz No: LANU 315/5327.74.1.6).

Results and discussion

Potential reference genes

All 15 tested reference gene primer sets were originally designed for Mus musculus (Table 1). It turned out that none of the six primer sets from the RTPrimer data base [17] nor the primers for the reference gene B2 m of the Mouse Normalisation Gene Panel (Quantace) did amplify a product in the related non-model species *Apodemus flavicollis*. Transferring primer sets from closely related organisms limits the set of genes that are tested and might reduce the chance to find a good internal reference as the possible choice depends on the set and number of genes that were used. However, eight intron spanning primer sets of the Mouse Normalisation Gene Panel (Quantace) performed well in *A. flavicollis*, which still is a comparable number to other validation studies [9, 26-28]. They amplified conserved parts of the succinate dehydrogenase complex (*Sdha*), γ-actin (*Actg1*), ribosomal protein S18 (*Rps18*), ribosomal protein L13a (*Rpl13a*), phosphoglycerate kinase 1 (*Pgk1*), calnexin (*Canx*), β-actin (*Actb*) and ubiquitin C (*Ubc*). Further functions and accession numbers are provided in Table 1. As the sequences of the commercial primer sets were unknown we applied molecular cloning and subsequent sequence analysis using the vector primers T7 and M13 to confirm amplicon identity. The GenBank accession numbers are provided in Table 2. All gene identities could be confirmed but *Rpl13a* turned out to be not intron spanning.

Table 1: Names, function, database ID and annealing temperature (Ta) of the tested primer sets.

Abbreviation	Gene	Function	Accession Number	T_a[°C]
Actb-1	actin, beta	cytoskeletal structural protein	2848[#]	60°C
Actb-2	actin, beta	involved in cell motility, structure and integrity	ensmusg00000029580[+]	55°C
Actg1	actin, gamma, cytoplasmic1	cytoskeletal structural protein	ensmusg00000062825[+]	55°C
B2 m-1	beta-2 microglobulin	cytoskeletal protein involved in cell locomotion	3584[#]	60°C
B2 m-2	beta-2 microglobulin	cytoskeletal protein involved in cell locomotion	ensmusg00000060802[+]	55°C
Canx	calnexin	protein folding and quality control in the endoplasmic reticulum	ensmusg00000020368[+]	55°C
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	carbohydrate metabolism	3244[#]	60°C
Hprt1	hypoxanthine guanine phosphoribosyl transferase 1	metabolic salvage of purines in mammals	50[#]	55°C
Pgk1	phosphoglycerate kinase 1	transferase enzyme in the glycolysis	ensmusg00000062070[+]	55°C
Sdha	succinate dehydrogenase complex, subunit A	tricarboxylic acid cycle	ensmusg00000021577[+]	55°C
Rpl13a	ribosomal protein L13A	member of ribosome protein	enst00000270634[+]	55°C
Rplp0	ribosomal protein, large, P0	member of ribosome protein	2861[#]	60°C
Rps18	ribosomal protein S18	member of ribosome protein	ensmusg00000008668[+]	55°C
Tuba1a	tubulin, alpha 1A	structural protein	1484[#]	58°C
Ubc	Ubiquitin C	protein degradation	ensmusg00000008348[+]	55°C

The eight reference genes that performed well in A. flavicollis liver samples are marked in bold.
[#] RTPrimerDB: http://medgen.ugent.be/rtprimerdb
[+] Ensembl Project: http://www.ensembl.org/index.html; Primer of Gene Normalization Panel

Table 2: Descriptive statistics of the tested reference genes.

	Rps18	*Sdha*	*Canx*	*Pgk1*	*Actg1*	*Ubc*	*Rpl13a*	*Actb*
GenBank ID	GU188049	GU188053	GU188051	GU188052	GU188050	GU188054	GU188056	GU188055
$AM_{amplification\ rate}$	1.86	1.88	1.83	1.85	1.88	1.86	1.85	1.82
$CV_{amplification\ rate}$	0.06	0.05	0.05	0.05	0.06	0.05	0.05	0.05
$GM_{Ct\text{-}value}$	14.62	15.37	16.63	15.72	16.83	15.72	26.05	16.05
$AM_{Ct\text{-}value}$	14.64	15.42	16.68	15.78	16.88	15.79	26.10	16.16
$CV_{Ct\text{-}value}$	0.04	0.07	0.07	0.07	0.07	0.08	0.06	0.09
$Minimum_{Ct\text{-}value}$	13.86	13.90	14.65	13.42	24.94	12.87	24.30	13.64
$Maximum_{Ct\text{-}value}$	16.74	17.68	19.61	18.62	19.68	18.26	28.87	20.50
$SD_{Ct\text{-}value}$	0.65	1.09	1.09	1.10	1.11	1.26	1.45	1.49
SD-threshold	1.12	1.10	1.15	1.13	1.10	1.12	1.13	1.16
Minimum[x-fold]	-1.60	-2.53	-3.30	-4.10	-3.29	-5.90	-2.94	-4.24
Maximum[x-fold]	3.71	4.27	6.10	5.93	6.00	4.84	5.65	14.49
SD[±-fold]	1.50	1.99	1.99	2.00	2.01	2.21	2.49	2.56

Arithmetic mean (AM), geometric mean (GM), coefficient of variance (CV) and standard deviation (SD) of the amplification rate E and the Ct-values for every potential reference gene. The genes are ordered by their $SD_{Ct\text{-}value}$. Genes that showed a $SD_{Ct\text{-}value}$ smaller than the SD-threshold are considered to be suitable reference genes and marked in bold. The last three rows show the maximum and minimum values of the over- and underexpression of a gene in relation to its calculated geometric mean (displayed as x-fold ratio) as well as the standard deviation (calculated with BestKeeper).

Sequencing revealed that the commercial primer set for *RPL13a* did amplify part of the small nuclear RNA (sno RNA) *U35* that is situated in the sixth intron of *Rpl13a* and part of the seventh exon of *Rpl13a*.

Amplification rate

The average arithmetic mean (AM) of the amplification rate E ranged from 1.82 for *Actb* to 1.88 for *Actg1* (Table 2). The coefficient of variance (CV) expresses the variance of the amplification rate between the different qPCR runs. It was 0.05 for all reference genes except for *Actg1* and *Rps18* (0.06) (Table 2). The lowest Ct -value recorded was 12.87 cycles and the highest was 28.87 cycles. The difference in the Ct -values between the genes within a run ranged from 9.83 cycles to 14.81 cycles (Table 2).

Identification of optimal reference genes

All our analyses on the stability of the references genes using the different algorithms showed consistent results with only slight differences in the ranking order (Table 3). A Kendall's W test showed a very high concordance of gained orders (Kendall's W = 0.958, ^{2} = 20.108, df = 7, p < 0.01). The resulting mean rank order of the genes from low to high variation was *Rps18, Sdha, Canx, Actg1, Pgk1, Ubc, Rpl13a* and *Actb*.

BestKeeper analysis

The software BestKeeper ranked *all* genes by their Ct-value variance (low to high): *Rps18, Sdha, Canx, Pgk1, Actg1, Ubc, Rpl13a* and *Actb* (Table 2). It considers all genes showing a variation in their amount of starting material by the factor two or more as unstable [14]. In an ideal PCR reaction with an amplification rate of two (100% reaction efficiency) this would be any gene whose Ct-values show a standard deviation $SD_{Ct\text{-}value}$ > 1, which is used as default by BestKeeper. Hibbeler et al. [8] already ruled out that the default setting of BestKeeper might be a too strict rule and limits its use to a very restricted experimental setup. In invivo samples, it is difficult to achieve a $SD_{Ct\text{-}value}$ < 1 as whole-tissue biopsies usually represent a composition of different cell types and show therefore a higher variation [29]. Additionally in biological samples the reaction efficiency is rarely 100% [13]. We therefore adjusted the SD threshold for each gene to its specific efficiency. As a consequence we made BestKeeper more applicable but still rejected every gene whose $SD_{Ct\text{-}value}$ indicated a variation in the starting template by the factor two. According to our study the first four genes could be considered as stable reference genes as the $SD_{Ct\text{-}value}$ was lower than their individual SD-threshold whereas the other genes were considered as unstable (Table 2).

NormFinder analysis

The ranking of the computer program NormFinder [12] is not based on the Ct-values but on the expression values. Compared to the BestKeeper ranking only two changes at the first and the sixth position occurred: *Sdha* (<0.382, Fig. 1) changed place with *Rps18* <0.427) and was the most stable gene while Rpl13a (<0.734) changed place with *Ubc* (>0.771) and became the sixth most stable gene. However, the five most stable genes differ only by just 0.084 points in their stability values, while the difference among the last three genes is more than three times larger than this (Fig. 1).

geNorm analysis

The program geNorm [6] ranks the potential reference genes due to their average pairwise variation in expression of one gene compared to each other gene of the set. It is independent of inter-run variability or different reverse transcription RT efficiencies. Only one change occurred compared to the ranking of BestKeeper: *Canx* becomes together with *Rps18* one of the two most stable genes, which cannot be further ranked ($M_{Canx/Rps18}$ = 0.73) (Fig. 1). Whereas geNorm is susceptible to identify co-regulated genes as optimal reference genes as they would show a constant ratio, NormFinder and BestKeeper do not suffer from this problem. As all three softwares produce consistent results we assume that the potential problem of coregulated genes does not apply to our data.

Number of reference genes

The use of just a single reference gene may result in a more than 6-fold erroneous normalization [6] and it is therefore recommended to use more than one reference gene [1,30] and calculate a normalization factor (NF) [6, 14]. As Vandesompele et al. [6] pointed out it is a trade off between accuracy and feasibility, but it seems

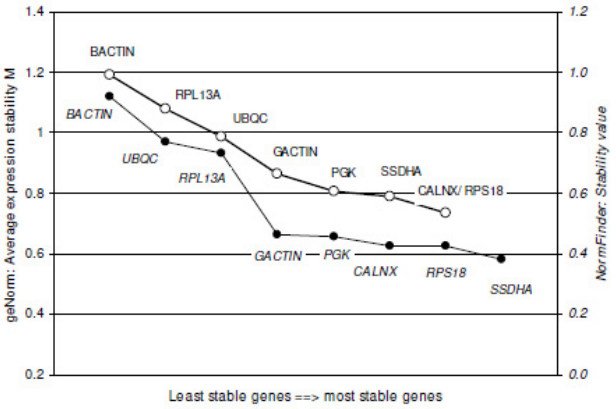

Figure 1. Gene expression stability values of the eight potential reference genes. The stability values on the right axis were calculated with NormFinder [12] (black circles) and the average expression stability values M (white circles) on the left axis were calculated with geNorm [6] after stepwise exclusion of the least stable gene. Genes are plotted from the least to the most stable expressed genes.

inappropriate if the number of reference genes exceeds the number of genes of interest by far. To find the optimal number of reference genes for normalization geNorm calculates whether the stepwise inclusion of a less stable gene into the normalization factor NF_n affects the variance $V_{n/n+1}$ compared NF_{n+1} (Fig. 2). We observed the lowest Variation $V_{n/n+1}$ between inclusion of the fourth and fifth most stable reference gene ($V_{4/5}$ = 0.164) (Fig. 2). A high $V_{n/n+1}$ means that the inclusion of the next gene had a big effect and it still should be included into the calculation of an accurate NF. $V_{4/5}$ = 0.164 is a bit higher than the cut off value of 0.15 suggested by Vandesompele et al. [6]. But this is an empirical value and should not be taken as a too strict cut off value, as it is already suggested by the geNorm manual itself. Although *Actg1* was refused as a reference gene by BestKeeper analysis we would suggest

Table 3: Ranking order of the reference genes obtained by the three used algorithms implemented in BestKeeper, NormFinder and geNorm.

	Rps18	*Sdha*	*Canx*	*Pgk1*	*Actg1*	*Ubc*	*Rpl13a*	*Actb*
BestKeeper	1	2	3	4	5	6	7	8
NormFinder	2	1	3	4	5	7	6	8
geNorm	1.5	3	1.5	4	5	6	7	8
Mean rank	1.5	2	2.5	4	5	6.3	6.7	8

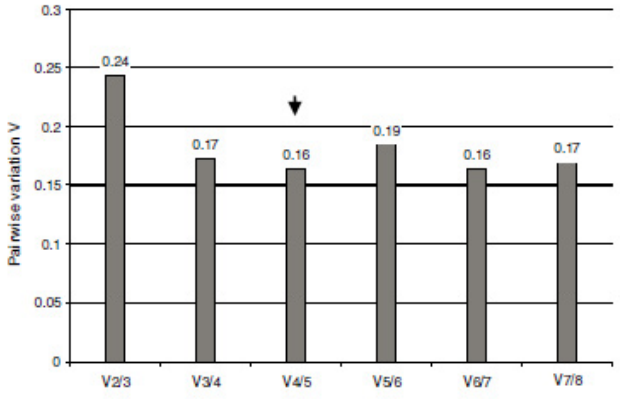

Figure 2. Pairwise variation $V_{n/n+1}$ between the normalizing factors NF_n and NF_{n+1}. The variation $V_{n/n+1}$ between NF_n and NF_{n+1} was calculated with geNorm to determine the optimal number of reference genes that should be used for normalization. The empirical cut-off value 0.15 defined by Vandensompele et al. [6] is marked by a thick line. The lowest variability is marked with an arrow.

to use the first five reference genes *Rps18, Canx, Sdha, Pgk1* and *Actg1* for calculating a NF in *A. flavicollis*, as *Actg1* only slightly missed the SD-threshold. This is further supported by the results of NormFinder as we observed a clear increase of the stability value between the fifth and the sixth most stable gene. This increase is more then three times as high as the over-all difference between the first and the fifth gene. This shows that the first five genes are much more similar in expression stability than the last three ones.

Conclusions

Although we expected higher expression variability due to more heterogeneity in terms of age or physiological stagesin our samples we could show that relative quantification via real-time PCR is feasible in samples from wild caught animals. The five genes *Rps18*, *Canx, Sdha, Pgk1* and *Actg1* were most stable and should allow an appropriate normalization factor for accurate measurement. We hope that our study will encourage other researchers to apply qPCR in eco-genomic studies on other wildlife species.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JA performed the sample collection, data acquisition, analysis and interpretation as well as drafting the manuscript. SS was responsible for the overall study design, supervised the study and helped to draft the manuscript. Both authors read and approved the final manuscript.

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Article 2

Selection and validation of reference genes for real-time RT-PCR studies in the non-model species *Delomys sublineatus*, an endemic Brazilian rodent

Alexandra Weyrich, Jan Axtner and Simone Sommer, Biochemical and Biophysical Research Communications 2010, 392 (145-149)
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Abstract

Quantitative real-time RT-PCR (qRT-PCR) is a sensitive technique for gene expression analysis. A critical factor for creating reliable data in relative quantification is the normalization of the expression data of genes of interest. Therefore the needed normalization factor is calculated out of the expression data of co-amplified genes that are stable expressed in the certain sample material, the so-called reference genes. In this study, we demonstrate the important process of validating potential reference genes using a nonmodel species. As there are almost no sequences known of the Pallid Atlantic Forest Rat (Delomys sublineatus), a rodent used as indicator species in conservation studies of the endangered Brazilian rainforest, suitable primer sets are more problematic to find than in model species. Out of nine tested primer sets designed for the fully sequenced Mus musculus, five could be used for the establishment of a proper running SYBR-Green assay and validation of their constant expression. qRT-PCR results of 12 cDNAs of Delomys livers were analyzed with three different validation software programs: BestKeeper, NormFinder and geNorm. Our approach showed that out of the five (Sdha, Canx, Pgk1, Actb and Actg1) potential reference genes, the first four should be used for accurate normalization in further relative quantification analyses. Transferring data from close-by model organisms makes high sensitive real-time RT-PCR applicable even to free-ranging non-model organisms. Our approach might be suitable for other non-model organisms.

Introduction

The understanding of cellular genetic processes requires both, the knowledge on the genome as well as the transcriptome level. Genomic DNA mainly gives information about evolutionary changes and mutations due to which functional and phenotypical effects can be hypothesized. However to prove those hypothesis its transcripts, the messenger RNA (mRNA), needs to be taken into account to ensure functional importance of the genes of interest. The level of gene activity depends on several environmental factors such as temperature, nutrition resources, seasonality, diseases and many others [1, 2]. The capability of individuals to adapt to repeatedly constantly changing impacts is based on the ability to shift the expression of distinct genes [2]. To study expression changes and compare mRNA levels of single genes, 'quantitative real-time RT-PCR (qRT-PCR)' offers the most sensitive method by measuring the quantity of amplicons in real-time after reverse transcription (RT) of mRNA to complementary DNA (cDNA) [3–5]. Highly expressed genes with a greater amount of initial template show an earlier detectable fluorescence signal above the background level than lower expressed genes. Potential variances between different qRT-PCR runs are normalized by forming the ratio of a reference gene and a gene of interest ('relative quantification') which thus allows the comparison of generated data. Frequently used reference

genes (formerly called 'house-keeping genes') are involved in fundamental metabolic processes or structural mechanisms and a constant expression has been assumed. However, evidence is accumulating that the latter is not a general fact [6–8] and an instability of expression in reference genes were observed among different tissues, treated versus non-treated samples [9] and/or closely related species [10, 11]. Therefore, trusting in normalization with a single reference gene assumed to be stable could easily lead to erroneous results [10, 12]. Thus, the validation of the expression stability of reference genes before starting sample analysis of certain genes of interest is fundamental.

So far most expression studies were carried out in humans and laboratory mice, providing a huge list of reference genes for those organisms and the primer sequences needed for expression analysis. Those are listed in databases like RTPrimerDB (http://www.rtprimerdb.org) [13] and the Primer Bank database (http://pga.mgh.harvard.edu/primerbank/) [14,15]. This is not the case in non-model organisms. For wildlife species that are not or only partially sequenced it is difficult to find suitable primer sets and to establish a proper running qRT-PCR. However, the effects of environmental changes, natural selection and related processes can only be investigated in free ranging populations under natural selection conditions. To address those questions small rodents are very suitable due to their short generation time and the possible achievement of great sample size.

We chose the Pallid Atlantic Forest Rat, *Delomys sublineatus* (Thomas, 1903) which is an endemic species in the Brazilian coastal rain forest Mata Atlantica (Voss, 1993), and is used as an indicator species in conservation studies [16–18]. *D. sublineatus* belongs to the superfamily Muridae, the family Cricetidae and the subfamily Sigmodontinae (Engel, 1998) [19]. In this study, we demonstrate the validation process of several reference genes in liver samples of D. sublineatus by a SYBR-Green real-time PCR approach. Calculations were done by the three excel applications: BestKeeper, NormFinder and geNorm.

Methods

Sample collection

Delomys sublineatus was live-trapped in the Brazilian coastal Atlantic Forest in 2008 with the permission of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA (permission 11573-2). Pit-falls were set in four adjacent woodland fragments of private property in the landscape around Tapirai, State of São Paulo, Brazil. Non-focus species, pregnant or lactating females and non-adult individuals were released near capture site. Twenty-four individuals were collected and anesthetized by inhalation of diethyl-ether and then sacrificed immediately by cervical dislocation. Liver samples were taken and stored in RNA-Later (Sigma–Aldrich Chemie GmbH, Bad Homburg, Germany), kept at 4 °C for 24 h and then frozen at -20 °C until further treatment. Twelve randomly chosen individuals were included in the present validation study.

RNA isolation and cDNA synthesis

We placed 30 mg of liver tissue together with 58 ceramic beads in 500 ll QIAzol lysis reagent in a 1.5 ml tube. Samples were homogenized by shaking the samples twice at 5000 rpm for 10 s in a tissue homogeniser (Precellys, Bertin Technologies, Montigny-le-Bretonneux, France). For total RNA isolation we followed the QIAzol lysis buffer protocol (Qiagen, Hilden, Germany). Total RNA was resolved in RNase-free water and digested with DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) for 20 min at 37 °C. The enzymatic reaction was stopped by adding 16 ll 25 mM EDTA pH 8.0, followed by an incubation for 10 min at 75 °C. RNA was purified by RNAeasy Kit (Qiagen, Hilden, Germany). Its purity and concentration was measured with the NanoDrop 1000 (Thermo Scientific, Karlsruhe, Germany). cDNA synthesis was performed using the Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) including 2 lg of total RNA, using anchored Oligo (dT)18 as well as random hexamer primers.

Initial selection of reference genes

The possible use of commercially available PCR primer sets (Mouse Normalization Gene Panel, Quantace, Berlin, Germany) for nine reference genes (*Rpl13a, Rps18, Actg1, Sdha, B2m, Ubc, Actb, Canx* and *Pgk1*) designed for *Mus*

musculus was tested in D. sublineatus. Two microliters cDNA (dilution 1:20) were mixed with 14.3 µl 10x Incubation Mix T. Pol with MgCl (MP Biomedical, Eschwege, Germany), 1.4 µl 2.5 mM/each dNTPs (MP Biomedical, Eschwege, Germany), 0.3 µl 5 U/µl Taq Polymerase (MP Biomedical, Eschwege, Germany) and 1 µl 50 µM of each primer mixture (Quantace, Berlin, Germany), respectively. Annealing was proceeded with a temperature of 55 °C and the PCR run for 35 amplification cycles. Five microliters of each PCR product was afterwards mixed with 1 ll loading dye including GelRed™ Nucleic Acid Gel Stain (Biotium, Cologne, Germany) and run on a 1.5% agarose gel (PeqLab Biotechnologies GmbH) using a low range (GeneRuler™ Low Range DNA Ladder; Fermentas GmbH, St. Leon-Rot, Germany) and a 100 bp marker (100 bp DNA ladder; Solis Biodyne, Tartu, Estland) for size determination.

Real-time RT-PCR

We performed real-time PCR following the instructions of the SensiMix Kit (Quantace, Berlin, Germany) with a final volume of 25 µl per reaction. The system includes SYBR Green I as the detecting fluorochrome for signal measurements. Runs were performed on a Rotor Gene 3000 (Qiagen, Hilden, Germany) and running conditions followed the manual of the Mouse Normalization Gene Panel (Quantace, Berlin, Germany), starting with the enzyme activation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 20 s. At the end of each run a melting curve was performed starting at 50 °C up to 99 °C with an increase of 1 °C per 5 s. Each sample was run in triplicates, as well as a non-template control (NTC) without cDNA to check for primer dimer and contaminants. To check for reproducibility each sample was run twice.

Analysis

For each sample the mean amplification rate (E, efficiency values) and the mean individual threshold cycle (Ct) values were obtained by the Rotor Gene Software (ver. 6.1, Corbett Research). The 'Comparative Quantification' feature in the Rotor Gene Software calculates the amplification rate (E) by averaging the increase in fluorescence (raw data) for five cycles starting at the 'Takeoff point' when 20% of the maximum fluorescence increase (2nd derivate of raw data) is reached and thus rises above background levels. Unlike 'Standard Normalization' procedures using the background values of only the first five cycles, the 'Dynamic Tube Normalization' feature in the 'Quantitation analysis' averages the level of background for all data points until the 'Takeoff point' of each run and sample. This method is more precise, because the first five cycles may not represent the background values prior to the ampli- fication start. Background correction is then performed automatically by dividing all data points of the sample by this value. For calculating the Ct -values the fluorescence threshold was manually set to 0.01 in the linear fluorescence scale, whereby we ignored the first five cycles due to possible signal variations at the beginning of each reaction. The Ct -value represents the number of cycles until the amplification curve of each sample is crossing the threshold. If the three replicates of a gene and sample give a standard deviation SD ≥ 0.4 the most deviating replicate was excluded. We calculated the 'relative quantities (Q)' by using the mean amplification rate (E) of each gene and the mean Ct -values of each gene and cDNA sample by setting $Q = E^{Ct}$. Three Microsoft Excel based software applications BestKeeper [20], NormFinder [12] and geNorm [21] were applied to analyse the expression stability of the potential reference genes (RGs) based on three different mathematical algorithms. BestKeeper [20] software ranks the reference genes according to their variation in their Ct -values per gene assuming an efficiency of 100% and thus an amplification rate of 2.0 [11]. Therefore we calculated the mean Ct -values of the three replicates of each gene and sample. Instable genes show a standard deviation SD > 1.0 resulting into a variability of the factor two of the starting mRNA amount. The gene with the lowest SD is proposed to be the most stable. NormFinder [12] defines stable gene expression by the variation of the expression values (Q). It calculates a stability value for each gene, whereby the lowest value defines the most stable gene. geNorm [21] is based upon the principle that two genes show the same expression ratio throughout all samples if they are constantly expressed. Therefore the pairwise variations of each RG with all other RGs are determined, resulting in a value defined as the stability value M. The gene with the highest M value is determined as least stable. To calculate the average M value of all RGs together the least stable (with the lowest M value) gets

stepwise excluded, until the two most stable genes remain. In addition geNorm gives the option to determine the number of genes to be used for accurate normalization of target genes. Therefore a normalization factor (NF) is calculated based on the geometric mean of theexpression values. By gradually adding the next less stable gene, an increasing variation between NF_n and NF_{n+1} indicates decreasing stability. This gene will thus be excluded in further analyses.

Results and discussion

RNA quality control

The 260 nm/ 280 nm ratio of the 12 extracted RNAs was photometrically measured and ranged from 2.04 to 2.14 which indicating the absence of protein and the purity of the RNA, an important factor in qRT-PCR [22].

Selection of candidate reference genes

Six of nine primer pairs that were originally designed for M. musculus reference genes (Table 1) performed well on the cDNA liver samples of the Brazilian rodent, *D. sublineatus* and produced single, specific amplicons (Fig. 1). The primers of ribosomal protein *Rpl13* (gene 1) and beta-2 microglobulin (B2m; gene 5) repeatedly did not amplify a PCR fragment, whereas the primers for Ubiquitin C (*Ubc*; gene 6) amplified more than one product (Fig. 1).

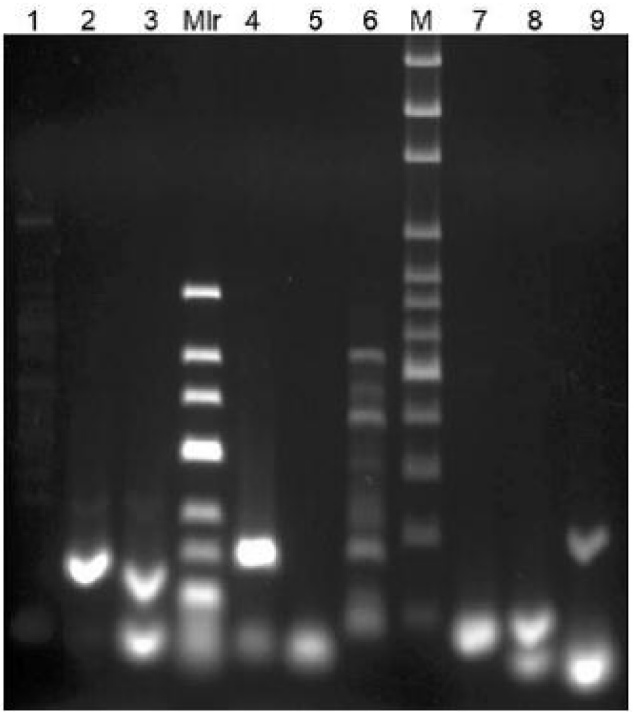

Figure 1. The performance of primer sets designed for *M. musculus* were checked on *Delomys sublineatus* cDNA by PCR. Reference genes are loaded as follows: (1) Rpl13a, (2) *Rps18*, (3) *Actg1*, (4) *Sdha*, (5) *B2m*, (6) *Ubc*, (7) *Actb*, (8) *Canx* and (9) *Pgk1* (Table 1). Two DNA ladders were loaded: Mlr the GeneRuler™ Low Range DNA Ladder (Fermentas; lengths: 700, 500, 400, 300, 200, 150, 100, 75, 50 and 25 bp) and M the 100 bp DNA ladder (Solis Biodyne; lengths: 3000, 2000, 1500, 1000, 800, 700, 600, 500, 400, 300, 200 and 100 bp).

qRT -PCRs of candidate reference genes

Rps18, Actg1, Sdha, Actb, Canx and *Pgk1* were verified by sequencing and BLAST results proved high similarity to the origin sequence derived from *M. musculus*. Those potential reference genes were further investigated in qRT-PCR experiments. In *Rps18*, melting curve analyses revealed a

Table 1: Detailed information of the tested reference genes.

	Abbreviation	Gene	Function	Ensemble ID	GenBank Acc. No.	Product length (bp)]
1	*Rpl13a*	Ribosomal protein L13A	Ribosome protein family	ENST 00000270634	not sequenced	109
2	*Rps18*	Ribosomal protein S18	Ribosome protein family	ENSMUG 00000008668	GU361631	139
3	***Actg1***	Gamma-actin, cytoplasmic	Cytoskeletal structure protein	ENSMUG 00000062825	GU361630	122
4	***Sdha***	Succinate dehydrogenase complex, subunit A	Tricarboxylic acid cycle, electron transport	ENSMUG 00000021577	GU361627	152
5	*B2m*	Beta-2 microglobulin	Immune response, cytoskeletal protein	ENSMUG 00000060802	not sequenced	193
6	*Ubc*	Ubiquitin C	Protein degradation	ENSMUG 00000008348	not sequenced	80
7	***Actb***	Beta-actin	Cell motion, structure and integrity	ENSMUG 00000029580	GU361629	83
8	***Canx***	Calnexin	Protein folding, quality control in the endoplasmic reticulum	ENSMUG 00000020368	GU361628	84
9	***Pgk1***	Phosphoglycerate kinase 1	Transferase in glycolysis, phosphorylation	ENSMUG 00000062070	GU361632	191

Suitable reference genes finally used for validation in D. sublineatus are marked in bold.

second peak indicating an unspecific by-product which was thus not further used. Contrarily, in the five genes *Actg1, Sdha, Actb, Canx* and *Pgk1* the specificity of primer amplification was confirmed by clear single peaks. The qRT-PCRs were highly efficient and the amplification rates ranged from 1.88 (*Actb* run 2) to 1.98 (*Actg1* run 1) in their arithmetic means (Table 2). To ensure the technical reproducibility of qRT-PCRs each run was performed twice (run 1 and 2) and the resulting independent data sets were compared by the three software programs used for the analyses of the expression stability (Table 3). The lowest Ct -values (12.14) were observed in run 1 and 2 for *Actg1*, and the highest in run 1 (22.39) and run 2 (21.70) for *Pgk1* (Table 2).

Table 3: Ranking of reference genes by their stability values after statistical analysis with the three different software packages BestKeeper, NormFinder and geNorm.

	Actg1	*Sdha*	*Actb*	*Canx*	*Pgk1*
BetsKeeper					
Run 1 and 2	5	1	4	2	3
NormFinder					
Run 1 and 2	5	1	4	3	2
geNorm					
Run 1	5	1.5	4	3	1.5
Run 2	5	1.5	4	1.5	3

Results of validation programs

The BestKeeper [20] software: both runs (run 1 and 2) showed identical rankings with slight differences in their Ct -value variation (Table 3). RGs were ranked in the following order: *Sdha, Canx, Pgk1, Actb* and *Actg1*. In both qRT-PCR runs, gamma-actin (*Actg1*) was the only gene with a standard deviation slightly higher than one and thus defined as unstable (Table 2). All other reference genes were considered as stable expressed in liver cells of *D. sublineatus* by BestKeeper. The stability values calculated by NormFinder [12] led to the following ranking: *Sdha, Pgk1, Canx, Actb* and *Actg1* (Fig. 2). The gene listing starts with the most stable gene defined by the lowest stability value. Therefore NormFinder, as BestKeeper before, determined the succinate dehydrogenase (*Sdha*) to be the most stable gene and gamma-actin (*Actg1*) to be the least stable. Only *PGK* and Calnexin, having similar expression values, changed their ranking order in comparison to BestKeeper. As in BestKeeper, beta-actin occupies the 4th place (Table 3). geNorm is ranking the reference genes with the stability factor calculated by the pairwise variation of the expression values [21]. Again, it resulted in Sdha as

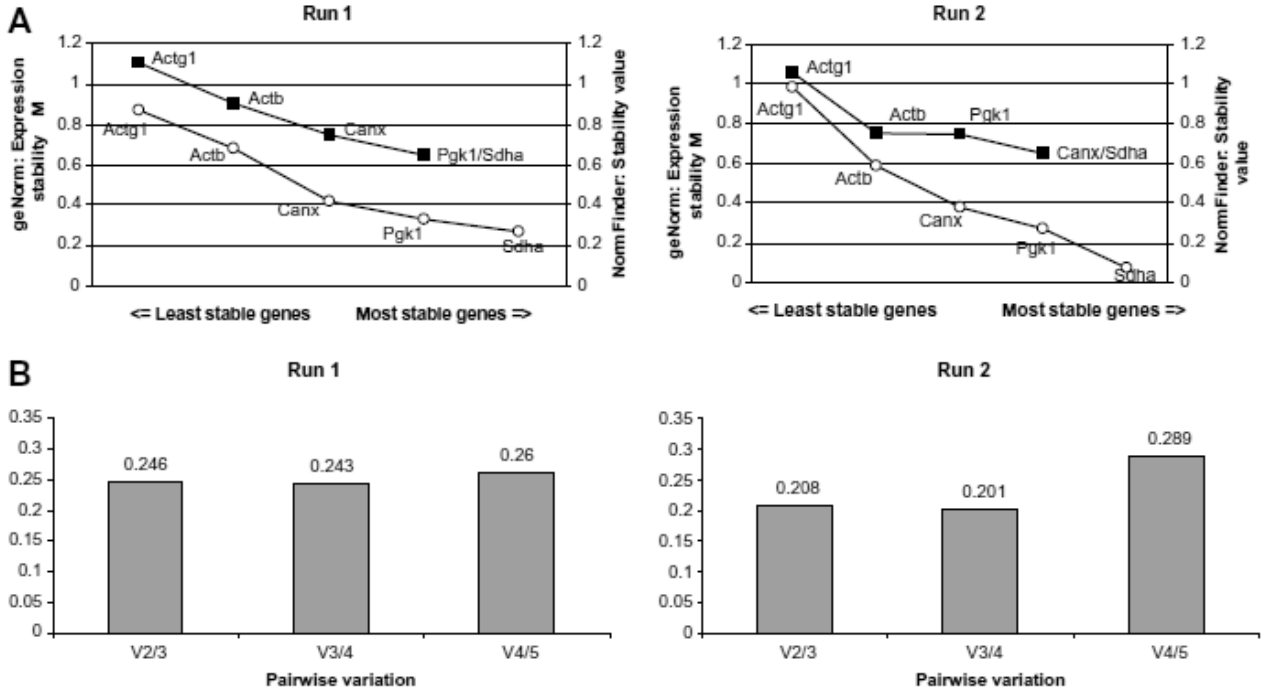

Figure 2: Gene expression stability values of geNorm (Expression stability M, black squares) and NormFinder (Stability value, white circles) (A) and the determination of optimalnumber of control genes for normalization by pairwise variation results generated by geNorm (B) of run 1 and 2.

one of the two most stable genes (Fig. 2A). It occurs that the two genes *Pgk1* and *Canx* show similar expression values and switch their ranks comparing the first and the second qRT-PCR run. In the first run the stability value of *Sdha* and *Pgk1* was $M_{Sdha/Pgk1}$ = 0.650 and in the second run Sdha together with *Canx* gave a value of $M_{Sdha/Canx}$ = 0.546. They are followed by *Actb* and again *Actg1* as the least stable gene tested (Table 3). Due to the pairwise variation method, geNorm is independent of inter-run variability, but susceptible for co-regulated genes that would always result in the same ratio. To avoid this, the use of more than one validation software using different algorithms is recommended.

Number of reference genes for normalization

Erroneous results can easily be created by utilizing only a single reference gene [10, 12, 21]. To find the optimal number of RGs to be used for relative quantification geNorm provides an additional function using pairwise variation$V_{n/n+1}$. By adding each gene in the calculation of the normalization factor (NF_n) in order to their increasingM values (decreasing stability) the effect of each RG included is reflected by a changing variance $V_{n/n+1}$ of the NF_{n+1} (Fig. 2B). In both runs the lowest variation was seen between the inclusion of the third and the fourth most stable reference gene (run 1: $V_{3/4}$ = 0.243, run 2: $V_{3/4}$ = 0.201). The inclusion of gamma-actin as the least stable gene increases the variance and should therefore not be included in the calculation of a normalization factor. As conclusion, in all three software programs Sdha clearly emerged to be the most stable and Actg1 to be the least stable reference gene. The four genes *Sdha, Canx, Pgk1* and *Actb* were proven to be stable expressed in the liver of *D. sublineatus* (Table 3) and will be used for the calculation of a normalization factor, allowing valid relative quantification of gene expression. Hence in this study, we successfully determined stable expressed reference genes in a non-model species. We show that transferring modern molecular techniques to free-ranging non-model organism is possible and opens the field to ask new scientific questions.

Competing interests

The authors declare no competing interests. Non-financial competing interests exist.

Acknowledgments

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Article 3

Are associations of immune gene expression, body condition and parasite burden detectable in nature? A case study in an endemic rodent from the Brazilian Atlantic Forest

Nina Schwensow, Jan Axtner, Simone Sommer, Infection, Genetics and Evolution 2011, 11 (23-30)
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Abstract

Host–parasite co-evolutionary processes are the most important drivers shaping the host's immune system. During successful host immune responses to helminthic infections, usually a balanced cascade of different immune genes like MHC, T helper cell 1 and 2 (Th1 and Th2) cytokines is expressed. This information comes largely from human or laboratory studies. The situation under which the immune system has evolved, however, is more complicated and natural variation need to be included to provide a more complete picture of co-evolutionary processes. We employed quantitative real-time PCR (qPCR) to explore associations of immune gene expression, body mass index (BMI) and helminth burden in a wild population of a non-model rodent (*Delomys sublineatus*). Our study shows that a typical Th2 response with a combination of inflammatory and anti-inflammatory components is detectable also under natural conditions. Complex associations of the expression levels of *Tgfb*, IL-10, IL-4 and IL-2 with different parasites and with the number of different helminth infections, respectively, were detected. A positive association of the body mass index with the expression of IL-2 and IL-4may indicate a link between host condition and the inflammatory part of an immune reaction. Our study shows for the first time that despite several potentially confounding parameters naturally present in a wildlife study, typical patterns of immune gene expression are detectable and influence helminth burden. Thus, in addition to structural variance of immune-relevant genes their expression might reflect host–parasite coevolutionary processes.

Introduction

Under natural conditions individuals are permanently exposed to different parasites. Especially intestinal helminths can reach high prevalence levels in their host populations and represent important evolutionary forces influencing the condition, fecundity, survival and thus fitness of individuals (reviewed by Albon et al., 2002; Degen, 2006; Pedersen and Greives, 2008). To prevent or limit the damage caused by helminths, hosts have evolved a sophisticated defence system. The recognition of antigens is an important step during the initiation of the immune response. T cells, which induce the subsequent steps cannot directly recognize antigens but require the presentation of processed antigens bound to major histocompatibility complex (MHC) molecules. MHC class I molecules are expressed on virtually all somatic cells and present antigens derived from intracellular pathogens. MHC class II molecules are expressed by relatively few cell types as dendritic cells, macrophages and B cells (Carvalho et al., 2009). They process and present extracellular antigens as those derived from helminths. Despite the high diversity of helminths, immune responses of mammalian hosts have some characteristic features in common. Generally, helminths induce a typical CD4+ T helper cell type 2 (Th2) cytokine response that result in eosinophilia, goblet and mucosal mast cell hyperplasia and the production of non-complement fixing antibodies

(reviewed by Gause et al., 2003; Perrigoue et al., 2008). Cytokines involved in a Th2 response are interleukin 4 (*IL4*), *IL5*, *IL6*, and *IL10* which mediates CD4+ T cell dependent host-protective effects. T helper type 1 (Th1) pathways are usually impaired by a strong Th2 response. Th1 cells produce interferon-gamma, lymphotoxin and IL2. The latter induces T cell proliferation, including that of CD4+ cells (reviewed by Parkin and Cohen, 2001). The expression of regulatory cytokines like transforming growth factor b (*Tgfb*) and *IL10* has also been found to be an important feature in the course of parasitic infections because immunological down-modulation during infection can protect the host from pathological outcomes of the infection (Finney et al., 2007). There is evidence that, among other important factors, the degree of expression level of MHC and cytokine genes plays an important role in the immune response and clearance of a helminthic infection because it differs between resistant and susceptible hosts (Pernthaner et al., 2005; Vallance et al., 1999). As part of their adaptation to a parasitic life-cycle, parasites have evolved mechanisms to overcome the host's immune response. For example, parasites may modulate the immune gene expression to reduce inflammation and expulsion (Maizels et al., 2004). Host–parasite interaction is a product of an old and dynamic coevolutionary confrontation in nature. However, our present understanding of the patterns of immune gene expression derived almost exclusively from laboratory models or from human medical studies. Studies that investigate immune gene expression under natural conditions are largely missing. This is probably due to the circumstance that tests of theoretical expectations are difficult under natural conditions because of many confounding parameters. In wild animals usually neither the time point of infection nor the presence of secondary infections or age are known. Also anthropogenic inference, environmental and climatic conditions may influence the course and success of an immune response (Apanius, 1998). However, these are stressors under which the immune system has evolved and natural variation need to be included to provide a more complete picture of host–parasite coevolutionary processes and mechanisms. In favour of a more ecologically appropriate non-classical model we chose the Brazilian rodent *Delomys sublineatus* to investigate immune gene expression of spleen tissue under natural conditions. The spleen is an efficient lymphoid organ for the immune system that monitors the blood. Both, innate and adaptive immune responses can be efficiently mounted (Mebius and Kraal, 2005). The spleen is highly vascular and composed of white and red pulp. The white pulp consists of T cell rich and B cell rich regions. B cell rich follicles bind antigen and proliferate and differentiate into antibody-secreting plasma cells that produce stimulus-specific immunoglobulin. Macrophages are present in the red pulp (summarized by Wluka and Olszewski, 2006). Employing quantitative real-time PCR (qPCR) we first aimed to investigate whether in helminth infected wild rodents similar expression patterns of MHC genes (class I and class II *DRB*) and cytokine genes (*IL2*, *IL4*, *IL10* and *Tgfb*) are detectable as described for laboratory rats or house mice. We established and validated reference genes for the normalization of our target genes. We expected to find evidence for similar interactions of these stimulatory and regulatory cytokines in nature as described from experimental studies. Second, we were interested whether associations of the immune gene expression level with intestinal helminth burden are detectable. Parasites have the potential to reduce the fitness of their hosts severely and thus exert a selective pressure on the host to mount an effective immune response. If differential expression of MHC and cytokine genes in the spleen is of evolutionary importance we expected to find evidence for an associated variation of intestinal parasite burden under natural conditions. Third, we aimed to evaluate the importance of the body condition for immune gene expression. A higher expression rate of certain immune genes may be more effective with respect to parasite expulsion but on the other hand may cause costs and thus might be influenced by the condition of the host (Hanssen et al., 2004; Lochmiller and Deerenberg, 2000; Martin et al., 2003; McKean et al., 2008; Schmid-Hempel and Ebert, 2003). To the best of our knowledge, this is the first study that explores associations of immune gene expression, body mass index (BMI) and helminth burden in a wild population of a non-model organism. Immunological analyses of wild populations will increase our understanding of how vertebrate immune systems respond to 'natural' levels of exposure to diverse infections. This is a central question in host–parasite coevolution tackling different disciplines such as immunology,

parasitology and evolutionary biology.

Materials and methods

Sample collection

Life trapping with pit-falls was performed in 2008 in the Brazilian coastal Atlantic Forest near Tapirai, State of São Paulo, Brazil with the permission of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis 90 – IBAMA (permission 11573-2) as described in detail elsewhere (Weyrich et al., 2010). We collected 24 adult and non-pregnant individuals and anesthetized them by diethyl-ether inhalation and then sacrificed them immediately by cervical dislocation. Complete spleens were taken and stored in RNAlater (Sigma–Aldrich Chemie GmbH, Bad Homburg, Germany) and kept at 4 °C for 24 h and then frozen at -24 °C until further treatment.

RNA isolation and reverse transcription

The whole spleen (up to 150 mg) was placed in a 2 ml tube containing 1000 ml Quiazol lysis reagent. The tissue was disrupted in a homogenizer (Precellis) using 1.4 mm ceramic beads (6000 rpm, 3x 20 s). Thereafter, we splitted the homogenate in two 1.5 ml tubes and used them as technical replicates for each individual. We proceeded with the RNA isolation according to the QIAzol lysis buffer protocol (Qiagen). Total RNA was dissolved in 87.5 µl RNAse-free water, followed by a DNA digestion (RNAsefree DNAse Kit, Quiagen) and a subsequent clean-up using RNeasy spin columms (Quiagen). Finally, total RNA was eluted in 200 µl of water.Wemeasured for each sample three times the concentration and purity of the RNA using a Nanodrop 1000 (Thermo Scientific) and averaged the values. Independent reverse transcriptions of each technical replicate were performed. We applied 2 µg of total RNA using Oligo-dT18 primers and the Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturers' protocol. The cDNA was diluted 1:4 prior qPCR with aqua dest.

Quantitative real-time RT-PCR

SYBR green qPCR was performed with the SensiMix two step kit (Quantance) on a Rotor Gene 3000 (Corbett Research). All qPCRs were run in a 15 µl volume. Each tube contained 5.4 µl aqua dest, 7.5 µl SensiMix dT (Quantace), 0.3 µl SYBR Green Solution, 0.3 µl primer mix (20 mM) and 1.5 µl template cDNA. The qPCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles of each 95 °C for 10 s, 53 °C for 15 s and 72 °C for 20 s. Melting curve analysis was performed from 72 °C to 95 °C in 0.5 °C steps. We performed the qPCR runs for each of the two cDNAs (technical replicates) of an individual in triplicates and always included a no-template control. We determined the amplification rate E for every single reaction using the 'comparative quantification' feature (Corbett Software 6.1.18). In this calculation the average increase in fluorescence during five cycles, starting at the 'takeoff point', is measured. The 'takeoff point' is reached when 20% of the maximum fluorescence increase (second derivate of the raw data) is present. This marks the point when the fluorescence is higher than the background and the exponential phase starts. We used the 'dynamic tube normalization' feature in the 'quantitation analysis' as correction for background noise (Corbett Software 6.1.18) by averaging the level of background for all data points of each sample until the 'takeoff point' is reached. Then all data points of each sample are automatically divided by this value. We averaged E for the triplicates. Mean individual quantitation cycle values (Cq-values) from the triplicates were obtained setting the threshold manually at 0.01. We calculated the expression Q of each gene as $Q = E^{-Cq}$. Due to the exponential nature of the function we log-transformed Q to make the data distribution more symmetric. Thus, we attributed equal weight to conditions with overexpression or underexpression and diminished the influence of outlier values (Willems et al., 2008). We used the mean of the two technical replicates (each with triplicates per run) for each individual for subsequent calculations.

Selection of reference genes

We tested eight commercially available, intron-spanning primer sets for the genes b-actin (*Actb2*), cytoplasmatic g-actin (*Actg1*), ribosomal protein S 18 (*Rps18*), succinate dehydrogenase complex subunit A (Sdha), phosphoglycerate kinase 1 (*Pgk1*), ubiquitin C (*Ubc*), ribosomal protein L13A (*Rpl13a*) and calnexin (*Canx*) designed for *Mus musculus* (Quantace Mouse Normalization Gene Panel) for D. sublineatus splenic cDNA. Six of these primer sets performed well (*Actb2, Rps18, Actg1, Sdha, Pgk1* and *Canx*). Validation was

carried out using the programs NormFinder (Andersen et al., 2004) and GeNorm (Vandesompele et al., 2002). GeNorm software (Vandesompele et al., 2002) calculates the gene expression stability (M) for a tested reference gene as the average pairwise variation in expression of one gene compared to each of the other tested genes. Stepwise exclusion of the gene with the lowest expression stability allows ranking of the tested genes according to their expression stability. GeNorm also estimates the optimal number of reference genes for the normalization (Vandesompele et al., 2002). NormFinder uses a model-based approach to analyse the variance of expression data. It calculates a stability value and the gene with the lowest values is considered as the most stable. The reference gene validation was performed on a subsample of 12 individuals.

Primer design for target gene amplification

To avoid the amplification of genomic DNA we used intron spanning primers for our target genes MHC class I, MHC class II DRB, IL2, IL4, IL10 and *Tgfb*. Primer sequences for MHC DRB, IL-2, IL-4 were designed based on an alignment with Cricetidae sequences (Accession numbers: AY247760, AF398549, EU729351, DQ446203, SF421390, AF046213). Primers for MHC class I were originally designed for felid species but proofed to amplify successfully in several other mammal species, too (accession numbers GU971407–14, Castro-Prieto et al., unpublished data). The MHC class II DRB forward primer was JS1 (Schad et al., 2004), the reverse primer was designed based on *D. sublineatus* sequences available in our lab. Primer sequences for IL-10 (RTPrimerID: 140) and *Tgfb* (RTPrimerID: 148) were taken from the RTPrimerDB and were originally designed for *Mus musculus*. Sequences are available under the accession numbers XY (Supplementary data).

Parasite burden

We used faecal egg counts to assess the gastrointestinal parasite burden. For the faecal egg counts we applied a modification of the McMaster flotation technique (Gordon and Whitlock, 1939) using a flotation-dilution of potassium iodite which enhances the detectability of eggs due to its high specific weight (Meyer-Lucht and Sommer, 2005). Helminth eggs were assigned to morphotypes based on size and morphological characteristics. Photographs of all morphotypes were taken for later taxonomic classification.

Statistical analysis

We used principle component analyses (PCA) to analyse the gene expression and the parasitological data, respectively. The goal of PCA is to extract important information from a table and to express it in new orthogonal variables (summarized by Abdi and Williams, 2010). To investigate possible associations between the expression of immune genes and parasite burden we used coinertia analysis. Co-inertia links two independent multivariate analyses, i.e. the principle component analyses of the expression matrix and the parasitological matrix. Co-inertia is robust to correlation between variables, can be used with all types of variables (Dray et al., 2003). In the parasitological matrix we included the number of different helminth infections (NHI) per individual as measure for the parasite richness. We measured the correlation between the gene expression data and parasitological data using the Rv-coefficient. A random permutation test (the array rows were permutated 1000 times and compared to the observed value) was used to evaluate the statistical significance of Rv. These methods are implemented in the ade4TkGUI package for R. To explore associations between body condition and the expression rate of immune genes, we used linear regression. Body mass index (BMI) was used as measure for the condition of an individual. We used the PCA scores of the largest component of the expression data as a composite variable derived as the sum of the products of individual observations and variable coefficients which is a standard data reduction technique (Jackson et al., 2009). The significance of the largest first component of the PCA was assessed by a randomisation test on the eigenvalue (Jackson et al., 2004b). All calculations were performed in R (R_Development_Core_Team, 2009) or in SPSS 16.0 (SPSS Inc., Chicago, IL, USA), respectively.

Results

Validation of stability and optimal number of reference genes

Validation was carried using two different programs. In NormFinder (Andersen et al., 2004), *Pgk1* had the highest value (NormFinder stability value = 1.110) and was thus the least stable one. The second least stable one was

Canx (0.676). All other genes had much lower stability values lying closely together between 0.382 (*Actg1*) and 0.511 (*Actb2*). The results from our GeNorm analysis were similar to the NormFinder results. Again *Pgk1* was found to be the least stable potential reference gene (average M = 1.22) followed by *Canx* (average M = 0.97). The most stable genes identified by GeNorm that cannot be further ranked were *Actg1* and *Actb2* (average $M_{Actg1/Actb2}$= 0.600). Since GeNorm also indicated, that the inclusion of *Pgk* increases the variance of the normalization factor we excluded it from further runs and used only the remaining five reference genes. For each individual the geometric mean of these reference genes was used to normalize the target gene expression.

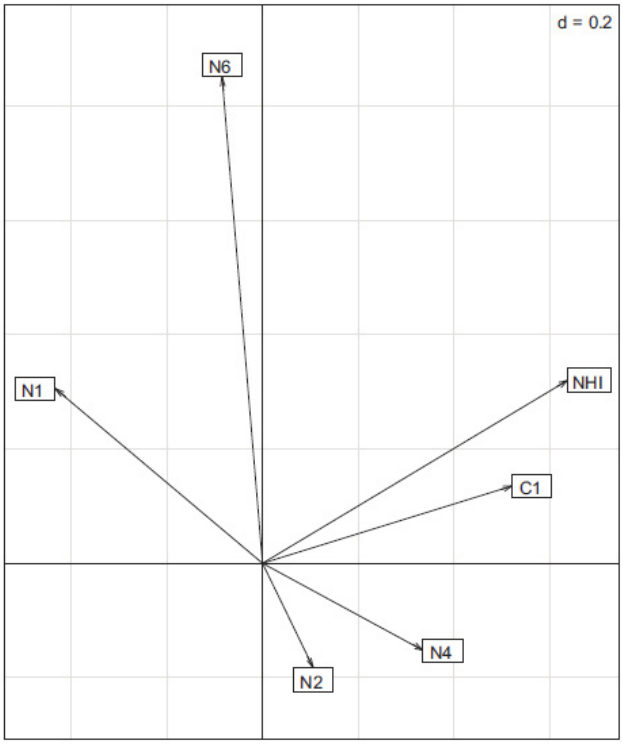

Figure 1: Principle component analyses of the parasite data. Included are the faecal egg counts of the nematode egg morphotypes N1, N2, N4 and N6, the cestode egg morphotype C1 as well as the number of different helminth infections (NHI).

Parasite burden

By faecal investigations we discriminated seven different nematode egg morphotypes (named N1–N4 and N6–N7) and one cestode egg morphotype (C1). Some of these egg morphotypes have already been observed in previous studies (unpublished data, Püttker et al., 2008). Since literature on parasites of *D. sublineatus* is missing, no in-depth taxonomic classification was possible. The morphotypes N1 and N6 belong to strongyle nematodes, N2 is a spirurid egg, N4 is an oxyruid egg (most likely *Syphacia* sp.) and C1 was assigned to *Hymenolepis* sp. All individuals were infected with N1 (prevalence 100%). Egg morphotype N6 was found in 39.1% of all individuals and N2, N4 and C1 occurred in 8.7%. N3 and N7 were both found together in only one individual and were excluded from further analysis. The number of different helminth infections (NHI) ranged from one to three. In the faeces of 39.1% of the individuals only N1 was found, in 43.5% we observed two and in 17.4% of the individuals three different egg morphotypes were identified. In the principle component analysis of the parasite data the first two axes accounted for 49.04% of the total variance (F1: 29.69%, F2: 19.37%). The first axis F1 was mainly structured by the number of different helminth infections (NHI) and the egg morphotypes C1 and N1 (Fig. 1). Thereby NHI and C1 were opposed to N1. The second axis F2 was structured by N6. The FECs of N2 and N4 had low factorial values and were thus projected close to the origin. They did not discriminate individuals.

Expression of target genes

The highest mean expression rate was found for MHC class II DRB (mean ln Q_{DRB} = 2.03, SD_{rel} = 0.20) while the mean expression rate of MHC class I was considerably lower (mean ln $Q_{MHC\ class\ I}$ = -1.87, SD_{rel} = 0.423). The mean expression rates of cytokines were much lower compared to MHC expression (mean ln Q_{IL4} = -5.78, SD_{rel} = 0.182; mean ln Q_{IL2} = -6.84, SD_{rel} = 0.139; mean ln Q_{Tgfb} = -7.57, SD_{rel} = 0.125; mean ln Q_{IL10} = -7.72, SD_{rel} = 0.140). Interestingly, the variance in expression was lower in the cytokines compared to the MHC genes, with the highest variance found in MHC class I expression (Fig. 2). In the PCA the first two components explained much of the variance (71.80%). None of the genes was placed close to the origin suggesting that all are influential. The first component accounted for 41% of the variance and all genes were placed in the same direction which indicates

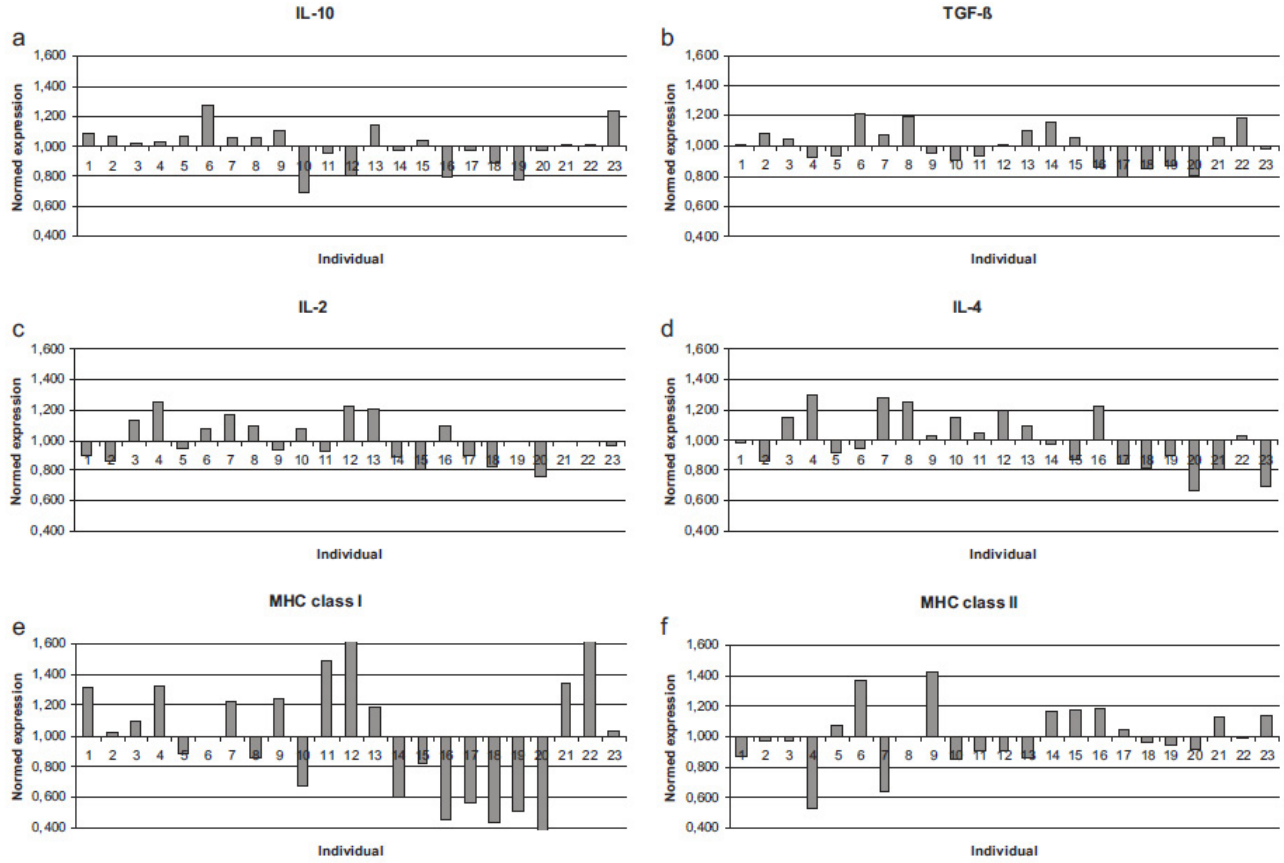

Figure 2: Variance of cytokine and MHC expression. Shown is the expression value of each individual divided by the mean expression value of the respective gene to illustrate an individual's above/below average expression of (a) *IL10*, (b) *Tgfb*, (c) *IL2*, (d) *IL4*, (e) MHC class I and (f) MHC class II.

coexpression (Fig. 3). The contribution of each original variable is indicated by the magnitude of the factorial values. *IL2* and *IL4* had the highest factorial values and thus mainly structured the data on the first component. The second component accounted for 30% of the variance and was mainly structured by individuals three different egg morphotypes were identified. In the principle component analysis of the parasite data the first two axes accounted for 49.04% of the total variance (F1: 29.69%, F2: 19.37%). The first axis F1 was mainly structured by the number of different helminth infections (NHI) and the egg morphotypes C1 and N1 (Fig. 1). Thereby NHI and C1 were opposed to N1. The second axis F2 was structured by N6. The FECs of N2 and N4 had low factorial values and were thus projected close to the origin. They did not discriminate individuals.

Coinertia analysis of parasitological and expression data

The co-inertia coupled the independent results of the two principal component analyses. The first axis accounted for 50.44% and the second for 36.73% of the total inertia. Although we did not find an overall relationship between the gene expression and parasitological matrices (Rv-coefficient = 0.215, simulated P = 0.278) the co-inertia factor analysis still indicated covariance of certain parasites with the expression of immune genes (Fig. 4). Below, we concentrate only on the associations with factorial values >|0.3|. *Tgfb* expression covaries negatively with the number of cestode C1 eggs (factorial value: -0.35). *IL10* expression is positively associated with N4 (factorial value: 0.48) and negatively with N1 (factorial value: -0.40) infection intensity. *IL2* expression is indicated to be negatively associated with the number of helminth infections (factorial value: -0.48) and with N2 infection intensity (factorial value: -0.40). For *IL4* a negative association with the number of helminth infections (factorial value: -0.34) and a positive one with N1 (factorial value: 0.32) was observed. The expression was associated with C1infection (factorial value: 0.3). No associations with parasite burden were indicated for MHC class I or MHC class II DRB expression, respectively.

Influence of BMI on expression rate

To test whether the expression rate is influenced by the condition we performed a linear regression. As dependent variable we used the scores from the significant first component (PC1) of the PCA which was mainly structured by the cytokines *IL2* and *IL4*. There was a significant positive correlation between PC1 (and thus the expression of *IL2* and *IL4*) and the body mass index (linear regression, $R^2 = 0.193$, P = 0.036, Fig. 5).

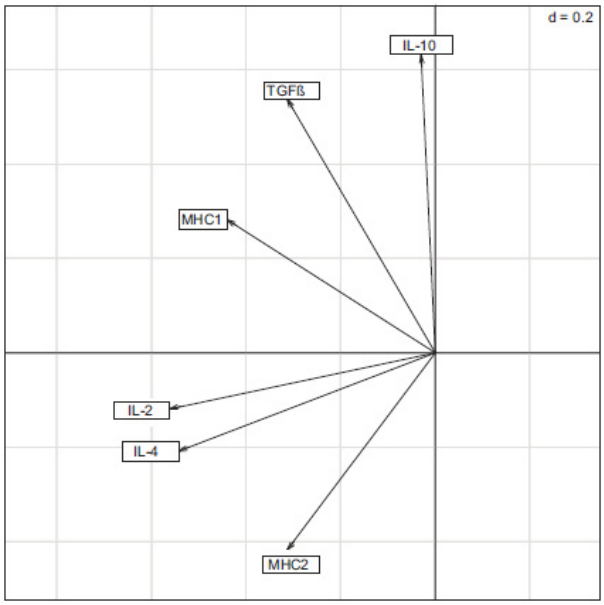

Figure 3: Principle component analyses of the expression data. Shown are the first two components. On the first axis *IL4* and *IL2* had the highest factorial values, on the second component *IL10* and *Tgfb*.

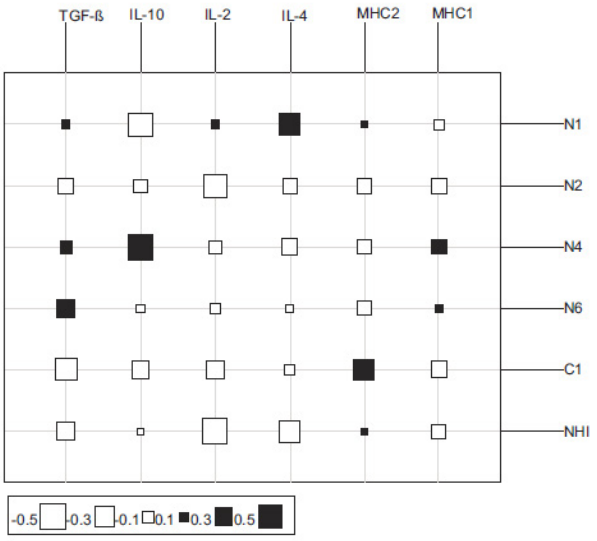

Figure 4: Indicated covariance of factors of the principle component analyses illustrated by square sizes. The size of the squares is proportional to the sum of the cross products between faecal egg counts of the gastrointestinal parasites and expression variables of the immune relevant genes. Black squares indicate positive and white squares negative associations.

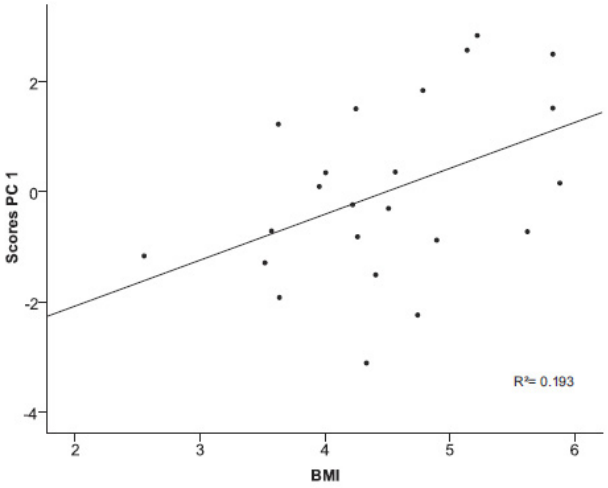

Figure 5: Positive association of BMI and PC1 scores of the expression data. PC1 is mainly structured by the cytokines *IL2* and *IL4*.

Discussion

Wild living animals are permanently confronted with different parasite species and the immune system and expression rate of immune genes is thought to be under parasite driven selection. In this studywe aimed to investigate immune gene expression froman evolutionary biologists' point of view.Weexploredwhether we find comparable expression patterns of cytokines as observed in experimental laboratory studies and whether under natural conditions immune gene expression is associated with intestinal parasite burden, which would be a precondition for natural selection. We were also interested whether the condition of an individual has an influence on the expression rate of immune genes.

Is in wild, naturally infected rodents a similar immune gene expression detectable as observed under laboratory conditions?

We measured the expression rate of six immune genes. Since all individuals were infected we did not have an uninfected 'control' group to evaluate to which extent immune genes are up-regulated during the course of an

infection. The PCA of the gene expression data of several immune genes revealed that all investigated genes structured the data, indicating important roles for all of them. This is not surprising given the complexity of immune responses. Our PCA is in congruence of what could be expected during an immune reaction against helminth parasites, namely a typical Th2 response. Although different parasites (or parasitic antigens) induce slightly different immune responses, it has been suggested that the host immune system has only a limited ability to distinguish among different nematode parasites (Finkelman et al., 2004). Reciprocal coevolution may have shaped the interaction between the host's immune system and helminthic parasites (Jackson et al., 2008). This may have led to the evolution of a more or less stereotypic Th2 response which is somewhat protective against most intestinal parasites. Functional resistance against particular species may thus depend on different subsets of mediators and effectors within this cascade. On the first component, our data were mainly structured by *IL2* and *IL4* expression. They had the highest factorial values of the same sign, indicating a positive association. An upregulation of the Th1 cytokine *IL2* suggests some level of increased T cell activation as *IL2* is typically considered as a T cell growth factor (Kindt et al., 2007). Thus, an increased level of *IL2* production may be also important for the expression of the Th2 cytokine *IL4*. There is evidence, that *IL4* production is dependent from *IL2* which acts as co-stimulatory agent (Yagi et al., 2006). To develop an effective host-protective response against helminth parasites, the differentiation of *IL4* producing T cells is an important step. *IL4* can mediate directly worm expulsion and is required for the amplification of Th2 cells (reviewed by Finkelman et al., 2004; Gause et al., 2003). However, even if the Th2 response has a limited effect on the parasite burden itself, it exerts an enormous influence on the survival of the host because Th2 cytokines modulate excessive inflammation. This prevents a runaway pathology (reviewed by Maizels and Yazdanbakhsh, 2003). The importance of immune modulation is also indicated by our data since the second component of the PCA is mainly structured by the expression of the cytokines *IL10* and *Tgfb* and, placed in opposite direction and to a lesser degree, MHC DRB expression. *IL10* can be produced by many different myeloid and lymphoid cells such as Th2 cells, macrophages, dendritic cells and regulatory T (Treg) cells. It inhibits the expression of pro-inflammatory cytokines, chemokines and co-stimulatory molecules and, interestingly, also MHC class II expression. This may be also reflected by our PCA as MHC class II *DRB* expression was indicated as negatively associated with *IL10* and *Tgfb* expression. *Tgfb* is an antiinflammatory cytokine that is often co-expressed with IL-10 and there is evidence for a cooperative interaction of these two cytokines (Beiting et al., 2007; Couper et al., 2008).

Is there an association between immune gene expression and parasite burden under natural conditions?

In our natural population of *D. sublineatus*, 100% of the investigated specimens were infected with at least one nematode species (N1). This is comparable to the observations of Püttker et al. (2008) who recorded a prevalence of 97% in this species. In 39% of the individuals N1 was the only infection, all others carried additional infections. We found seven different helminth egg morphotypes in total in our study population, six of which were nematodes. The infection rate was considerably high but this was also the case in other small mammals investigated in the same region (Meyer-Lucht, 2009;Püttker et al., 2008) and is possibly a feature of the Brazilian Atlantic Forest. A high parasite prevalence, however, potentially represents an increased selective pressure on the expression of immune genes. Under these circumstances, we would expect associations to parasite burden also under natural conditions. Indeed, our analyses indicated some associations; however, due to the complexity of immune reactions and confounding parameters, the coinertia could not provide significant covariance. However, most of the indicated relationships are in congruence with what is known from studies on immune responses against helminths under laboratory conditions. In contrast to the situation in most laboratory studies, the majority of naturally acquired nematodes affecting humans/ livestock form long-lasting chronic infections and re-infections are not unusual. Chronic infections typically evoke Th2 responses, normally in combination with an anti-inflammatory component (reviewed by Behnke et al., 2009). While the Th2 response reflects the recognition of worms, the down-regulatory component prevents the host from deleterious effects of

the inflammation but may also reflect the parasites' adaptation to immune evasion (reviewed by Díaz and Allen, 2007). Chronic infections can be assumed to predominate also in our study population, at least for omniprevalent nematode N1. The morphology of the eggs and adult worms of nematode N1 resemble *Heligmosomoides* sp., a common parasite of laboratory house mice, which forms longlasting infections in rodent model systems. We assume that N1 may be closely related to *Heligmosomoides* sp. and thus immunologically similar. Our analyses indicated a positive association of nematode N1 infection intensity with *IL4* expression level and a negative association with *IL10* expression level. This may be concordant with the pattern found in laboratory study conducted by Finney et al. (2007). They observed expression level changes during the time course of an infection with *Heligmosomoides polygyrus*: *IL10* expression in mesenteric lymph node cells was elevated but after a peak on day 28 it decreased again. *IL4* expression in mesenteric lymph nodes increased and was still clearly elevated at day 70. Assuming chronic infections with N1, a similar general pattern may be reflected by our data. However, Finney et al. (2007) reported a decrease of *IL4* in the spleen (but not in mesenteric lymph nodes) after day 21 which is not in congruence with our findings. However, sixty-one percent of our studied specimen harboured more than one helminth species and relatively little is known about the effects of such the immune responses in natural multi-species systems. Gastrointestinal nematodes of different species have been shown to be associated with different host responses. For laboratory *Mus musculus*, for example, contrasting immune responses have been described for *H. polygyrus* and *Trichuris muris*. While in the former a highly polarized Th2 response correlates with protection in the lab, during *T. muris* challenge different mouse strains develop different responses and show thus a different degree of susceptibility (reviewed by Gause et al., 2003). Therefore, it seems highly probable that helminth species-specific effects could produce significant heterogeneity in the immune responses of natural populations exposed to multi-species helminth infections. Effects of co-infection with two helminthic parasites (*Ascaris lumbricoides* and *Trichuris trichuria*) on the expression of cytokines but also on re-infections, for example, have been reported in humans (Jackson et al., 2004a). In gastrointestinal helminth infections, multi-species interactions with the host immune system are complex and may also be related to past responsiveness of host individuals. Interestingly, our data also indicated effects of the number of different helminth infections on the expression rate. The association was negative, i.e. individuals with higher NHI expressed the pro-inflammatory cytokines *IL2* and *IL4* to a lower degree. This may indicate a general down regulation in chronic infections. It has been described, that different helminth species can secrete cystatin-like proteins that induce *IL10* production. Cystatin is a protease inhibitor which interferes with two classes of protease during the MHC class II pathway and blocks the presentation of exogenous peptides by MHC molecules. Parasite derived cystein reduces the expression of HLA-*DR* by human monocytes and induces *IL10* production (Dainichi et al., 2001; reviewed by Harnett and Harnett, 2006; Schönemeyer et al., 2001). For example, *IL10* has been found to be elevated in responses to T. muris although it is not generally elevated in responses to trichostrongylids. In Schistosoma mansoni resistant mice, a higher level of *IL10* and *Tgfb* can be observed, indicating regulatory mechanisms that insure a balanced immune response (Maizels and Yazdanbakhsh, 2003). Also the negative association of our egg counts of the spirurid N2 with *IL2* and the positive association of N4 (possibly Syphacia sp.) with *IL10* may indicate down regulatory mechanisms although the prevalence of both, N2 and N4, was very low and thus inferences must be drawn cautiously. The same is true for the negative association of the cestode C1 (Hymonolepis sp.) with the regulatory cytokine *Tgfb*. From laboratory studies it is known, that at least *Hymenolepis diminuta* has a rather immunosuppressive effect with elevated rates of *IL10*, for example (Hunter et al., 2005). Why our data indicate the opposite remains elusive but may be also attributed to the too low prevalence in our population. In contrast to others, we did not find evidence for associations of parasite burden with MHC class II *DRB* or MHC class I expression. Wegner et al. (2007) found an elevated MHC II expression in stickleback susceptible to eye-fluke infection than in resistant individuals. They conclude that this may be explained by the finding that a higher MHC expression is associated with potential costs in terms of higher levels of oxidative stress (Kurtz et al., 2006). It is interesting to note,

that the MHC *DRB* is the gene with the highest expression rate in all individuals while the variance was only slightly elevated compared to the much lower expressed cytokine genes. MHC class I, in contrast, was the most variable gene, with a high variance between the individuals. MHC class I molecules bind and present intracellular derived antigens, therefore, an elevated expression level might be expected during viral infections. We have no information on secondary viral infections from the animals investigated here but the high variance suggests that MHC class I expression might be a target for natural selection in the wild. MHC DRB expression structured also partly the second component of our PCA, although much less than *IL10*/ *Tgfb*. This may indicate that MHC *DRB* expression is potentially negatively associated with *IL10*/*Tgfb* expression. However, we could not detect such association in the coinertia analysis. Another explanation may be that elevated MHC expression in response to helminth parasites is not detectable in the spleen, but rather in the intestines, the tissue that is in direct contact with the parasites. A comparison of the expression levels in different tissues in response to parasites would be desirable to elucidate this.

Immune gene expression and body condition

Our analyses showed a positive association of BMI with the first component from our PCA. The first component was mainly structured by the expression of *IL2* and *IL4* and may thus represent the inflammatory part of an immune reaction. Immune reactions are thought to cause energetic costs. Elevated metabolic rates after immune challenges have been observed in different mammal and bird species and reductions in body fat have been found to be correlated with impaired immunity in different species of seasonal rodents (reviewed by Demas, 2004). A correlation of a good body condition with the ability to mount a stronger immune response may be indicated by our data. It has also been suggested that immunity costs may become only obvious under stressful conditions (reviewed by Schulenburg et al., 2009) which may not be present in the relatively large and undisturbed forest habitat of our study population. Further studies that address possible associations of body condition and the costs of immune reactions under natural conditions are needed.

Conclusions

For the first time, our data indicate that also under natural conditions classical patterns of immune gene expression are detectable despite several potentially confounding parameters. Our data further suggest functional associations between the expression level of Th2 stimulatory and anti-inflammatory cytokines, respectively, and parasite burden. Since in nature adaptation to the omnipresent parasites may be more important than individual defence components (as investigated in most laboratory studies) further studies in free-ranging mammals are required to understand the evolutionary significance of immune gene expression or modulation in relation to parasite pressure under natural selection conditions.

Ethical statement

All research reported in this manuscript adhered to the legal requirements of Germany and Brazil and complied with the protocols approved by the appropriate institutional Animal Care and Use Committee (Bundesministerium fu¨ r Naturschutz (BfN), Germany; Instituto Brasileiro do meio ambiente e dos recursos naturais revovaveis (IBAMA), Brazil.

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Supplementary data

Supplementary data associated with this article can be found, in the appendix at end of this document.

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Article 4

Heligmosomoides polygyrus infection is associated with lower MHC class II gene expression in *Apodemus flavicollis*: Indication for immune suppression?

Jan Axtner, Simone Sommer, Infection, Genetics and Evolution 2011, 11 (2063–2071)
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Abstract

Due to their key role in recognizing foreign antigens and triggering the subsequent immune response the genes of the major histocompatibility complex (MHC) provide a potential target for parasites to attack in order to evade detection and expulsion from the host. A diminished MHC gene expression results in less activated T cells and might serve as a gateway for pathogens and parasites. Some parasites are suspected to be immune suppressors and promote co-infections of other parasites even in other parts of the body. In our study we found indications that the gut dwelling nematode Heligmosomoides polygyrus might exert a systemic immunosuppressive effect in yellow-necked mice (*Apodemus flavicollis*). The amount of hepatic MHC class II DRB gene RNA transcripts in infected mice was negatively associated with infection intensity with *H. polygyrus*. The hepatic expression of immunosuppressive cytokines, such as transforming growth factor b and interleukin 10 was not associated with *H. polygyrus* infection. We did not find direct positive associations of *H. polygyrus* with other helminth species. But the prevalence and infection intensity of the nematodes *Syphacia stroma* and Trichuris muris were higher in multiple infected individuals. Furthermore, our data indicated antagonistic effects in the helminth community of *A. flavicollis* as cestode infection correlated negatively with *H. polygyrus* and helminth species richness. Our study shows that expression analyses of immune relevant genes can also be performed in wildlife, opening new aspects and possibilities for future ecological and evolutionary research.

Introduction

Parasites are omnipresent in wild mammalian populations and often share a long evolutionary history with their particular hosts, on which they can cause strong selection by having impacts upon their fitness and survival (Anderson and May, 1979; May and Anderson, 1979). Our understanding of the mechanisms that are involved in host–parasite coevolutionary processes is primarily based on laboratory rodents that live under non-natural pathogen-free and stress-free conditions (Jackson et al., 2009). But laboratory models are simplified models of reality and might be poor images of the wildlife that faces the constantly changing and challenging conditions of natural environments. Focusing just on laboratory models could mean working at the expense of ecological and evolutionary realism (Feder and Mitchell-Olds, 2003). Furthermore, most interaction studies have focused on single parasite species and their host disregarding interspecific interactions between co-infesting parasites or the cumulative effect of co-infections (Bordes and Morand, 2009a). However, the occurrence of such effects and associations has been demonstrated in model organisms (Graham, 2002, 2008) and wildlife species (Salvador et al., 2011). Multiple simultaneous parasite infections are the norm in nature (Behnke, 2008; Bordes and Morand, 2009b) and might exert a different pressure upon host immunity than single parasite infections (Bordes and Morand, 2009a)

as the selection might be bidirectional. In-situ studies on naturally infected wild populations that transfer and validate knowledge gained in the laboratories might give new insights into how the immune system functions in its natural context (Bradley and Jackson, 2008; Jackson et al., 2009). Helminth parasites comprise the nematodes, trematodes and cestodes. Despite their unrelatedness they provoke a stereotypical modified T helper cell 2 response and can provoke fatal diseases in their hosts (Moreau and Chauvin, 2010). For this reason helminths have often been used as health indicators in ecological and co-evolutionary studies and to explain variability in the pattern of expression of immune relevant genes in the host (e.g. Axtner and Sommer, 2007; Lenz et al., 2009; Schwensow et al., 2011). Genetic variability can be crucial to resistance of populations against parasites and pathogens (Altizer et al., 2003). The most diverse genes in vertebrates are the immune related genes of the major histocompatibility complex (MHC) that act at the interface of infection detection and immune defence (Piertney and Oliver, 2006). The so called 'classical' MHC genes code for membrane glycoproteins that bind to and present foreign antigens to T cells and thereby trigger an immune response (Hughes and Yeager, 1998). MHC genes can be divided in two classes, the class I molecules are constitutively expressed in almost every somatic cell, whereas class II glycoproteins are generally restricted to a subset of antigen presenting cells, such as dendritic cells, B cells or macrophages (Piertney and Oliver, 2006). Some microparasites like Chlamydia, Mycobacterium spp. or Toxoplasma directly influence the expression of MHC class II genes (Wojciechowski et al., 1999; Zhong et al., 1999; Noss et al., 2000; Lüder et al., 2003; Pai et al., 2003) by avoiding immune cell activation and T cell initiation (LeibundGut-Landmann et al., 2004). A reduced MHC expression is advantageous for the parasite as it increases the probability of establishing a constant infection. The probable mechanism used by parasites to lower MHC expression is to interfere with the expression of the class II transactivator CIITA, which controls and orchestrates the interaction of various promoters involved in MHC expression (Muhlethaler-Mottet et al., 1997; Harton and Ting, 2000; Ting and Trowsdale, 2002; LeibundGut-Landmann et al., 2004). Also, some macroparasites like helminths are known to manipulate the mammalian immune defence to evade immune reactions and avoid expulsion from their host (reviewed in Maizels et al., 2004). For example, heligmosomoid nematodes are suspected to act in an immunosuppressive way (Jenkins and Behnke, 1977; Behnke et al., 1978, 1983). The outcome of this is an attenuation of deleterious inflammations (Bazzone et al., 2008) and pronounced patterns of bystander infections in their natural hosts like Apodemus spp. (Behnke et al., 2009; Jackson et al., 2009). This immune suppression seems to be systemic rather than limited to the site of infestation as it affects different parts of the host's body (Jenkins and Behnke, 1977; Su et al., 2005; Wilson et al., 2005; Bazzone et al., 2008). Potential agents of heligmosomoids that influence the host's immunity are excretory– secretory antigens that induce regulatory T (Treg) cells via the transforming growth factor b (Tgfb) signalling pathway (Grainger et al., 2010). These induced Treg cells provoke elevated levels of the immunosuppressive cytokines Tgfb and interleukin 10 (Il10) (Metwali et al., 2006; Finney et al., 2007) which in turn reduce MHC class II presentation in antigen presenting cells (Knolle et al., 1998; Romieu-Mourez et al., 2007). Although there is ample evidence of an association between MHC class II alleles and varying levels of susceptibility to helminths (Sommer, 2005; Goüy de Bellocq et al., 2008), only few studies have investigated whether helminths might be able to interfere with MHC class II expression using laboratory models (e.g. Silva et al., 2006; Perrigoue et al., 2009) and are almost missing in wildlife (Schwensow et al., 2011). To address this gap, we chose wild-caught yellow necked mouse (Apodemus flavicollis) as our non-classical model organism. TheMHC class II DRB gene of A.flavicollis have been characterised (Musolf et al., 2004) and its alleles show manifold associations to nematode susceptibility (Meyer-Lucht and Sommer, 2005, 2009); and Heligmosomoides polygyrus is a ubiquitous helminth in this host species (Ferrari et al., 2004, 2009; Klimpel et al., 2007). We employed quantitative real-time PCR (qPCR) to measure messenger RNA levels of MHCclass II DRB gene, Tgfb and Il10 in liver samples of wild A. flavicollis. Due to its immunological function of mediating tolerance to antigens but also immune responses to antigens entering the body from the gastrointestinal tract (Selmi et al., 2007; Tiegs and Lohse, 2009), the liver comprises a large reservoir of antigen

presenting cells (Racanelli and Rehermann, 2006; Nemeth et al., 2009) making it an ideal target to study systemic effects of H. polygyrus infection on MHC class II expression. Furthermore, liver resident Treg cells have been demonstrated to mediate a systemic antigen specific immune tolerance by a hepatic pathway in transgenic mice (Lüth et al., 2008). Our aim was to investigate whether heligmosomoid infections are associated with immune gene expression under natural conditions and if a H. polygyrus infection influence co-infection patterns. This study contributes to close the gap between our understanding of immune regulation in laboratory models and its application to ecological studies in free-ranging mammal populations naturally confronted by multiple parasite species.

Methods

Sample collection

We trapped yellow-necked mice (Apodemus flavicollis) of a single population using Sherman live traps (7.7 cm x 7.7 cm x 30.5 cm) in a deciduous forest approximately 35 km North–East of the city of Hamburg, Germany. Animals were anesthetized at the trapping site using isoflurane and then killed immediately by cervical dislocation. Only adult animals with a body weight above 16 g (Jüdes, 1979) that were neither pregnant nor lactating were sampled. Liver samples were stored in RNA-Later (Sigma) and kept at 4 °C for 24 h and were subsequently frozen at -20 °C until RNA extraction. The intestinal tract was removed and stored in 75% ethanol until parasitological screening. The stomachs and intestines of all animals were screened for helminth infestations under a microscope. Nematodes were assigned to the genus level at least, whereas all cestodes were pooled as they could not be further distinguished. Infection intensity was assessed as number of adult worms per individual. All research reported in this manuscript was carried out in accordance with the legal requirements of Germany and complied with the protocols approved by the state office for Agriculture, Environment and Rural Areas of Schleswig–Holstein (Reference No.: LANU 315/5327.74.1.6).

RNA extraction and cDNA synthesis

The whole liver was homogenised (2 x 10 s at 5000 rpm, Precellys, Bertin Technologies) in QIAzol lyses reagent (Qiagen). From each homogenate we placed 0.5 ml in two 1.5 ml tubes and treated each of these aliquots separately as independent replicates of a sample (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. Genomic DNA was digested with DNase I (RNase-free DNase Kit, Qiagen) followed by a clean-up using RNeasy spin columns (Qiagen) according to the manufacturer's protocol. Extracted total RNA was finally eluted in 100 µl of water; concentration and purity were assessed three times by a Nanodrop 1000 (Thermo Scientific) and averaged. RNA quality was checked on agarose gels by electrophoresis. Two micrograms of total RNA were reverse transcribed with Oligo-dT18 primers (5 µM). Reverse transcription was run in a 20 ll reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. Complementary DNA (cDNA) was diluted 1:3 prior to quantitative real-time RT PCR (qPCR) with water.

Quantitative real-time RT PCR

Quantitative PCR was performed for the three target genes (MHC-DRB and the regulatory cytokines *Il10* and *Tgfb*, Supplement 1) and for the four reference genes (ribosomal protein S18, calnexin, cytoplasmic actin gamma 1, phosphoglycerate kinase 1). Details on reference gene validation and reference gene primers are given elsewhere (Axtner and Sommer, 2009). We used intron-spanning primers to avoid the amplification of genomic DNA contaminants. We used the SensiMix SYBR No-ROX Kit (Quantace) in a 25 µl volume on a Rotor Gene 3000 (Corbett Research). To avoid interrun variation and to control RT differences we analysed the A and B replicates of a sample in the same qPCR run. All reactions were run in triplicates (in total six reactions per individual) with a no-template control to check for contaminations. Each reaction tube contained 2.5 µl of cDNA template, 12.5 µl SensiMix™ SYBR No-ROX (Quantace), 0.5 µl primer (20 µM) and 9.5 µl dH2O. The qPCR conditions were 10 min at 95 °C and 45 cycles of each 95° for 15 s, 55 °C for 15 s and 72 °C for 15 s. To confirm that only a single amplicon was present and to check for the absence of primerdimers, a melting curve analysis was performed from 65° to 95 °C in 0.5 °C steps lasting 5 s. The run-specific PCR efficiency for each gene was calculated using the program

LinRegPCR 11.6 (Ramakers et al., 2003; Ruijter et al., 2009). The individual quantitation cycle (Cq) was defined by the time-point when the individual fluorescence reached a threshold of 0.5 of the log normalised fluorescence. We then calculated the individual expression value $Q = E^{-Cq}$, where E is the mean amplification rate of each gene in each run. Data were inspected visually for outliers and runs repeated as necessary. The arithmetic mean Q of each target gene (MHC-*DRB*, *Il10* and *Tgfb*) was calculated from the triplicate repeats and normalised to the geometric mean of the four reference genes (Vandesompele et al., 2002). These normalised relative gene expression levels were log transformed to lower the influence of extreme values and to attribute equal weights to conditions of over- or under-expression (Willems et al., 2008). The ratio of A and B replicates was calculated and data points were accepted only when values were between 0.85 and 1.15 to ensure the reproducibility of our results. In case A and B replicates showed inconsistent results we repeated reverse transcription and qPCR for the sample. If we were unable to obtain congruent results after three attempts we excluded the sample from the analyses.

Data analysis

Helminth prevalence, mean and median of infection intensity and their 95% confidence intervals (CI) were calculated by bootstrapping (20,000 repeats) using Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005). Uninfected individuals were excluded from the calculation of mean and median. Due to the discrete nature of the data, it was not always possible to construct exactly the 95% confidence limits. In such cases, the shortest interval with a 95% confidence level was chosen. If the occurrences of two parasites are independent from one another, the multiplied prevalence of both should give the frequency of their co-occurrence (f_{exp}). Thus we compared the fexp of co-occurrence of two parasites with the observed frequency (f_{obs}). In the case of avoidance we expect: $f_{exp} > f_{obs}$; in the case of independence: $f_{exp} = f_{obs}$; and in the case of co-occurrence it should be $f_{exp} < f_{obs}$. The odds ratio between the prevalence of two parasites was calculated and tested for deviance from one with a two-sided Fisher's exact test using a Bonferroni corrected level significance. To assess if infection with one parasite may facilitate the infection with others, we tested whether the prevalence and infection intensity of certain parasites was higher in multiple infected individuals. The expected prevalence was calculated out of the observed percentage of single, double or triple infections multiplied by the number of total infection of most common nematodes. We compared the observed versus the expected prevalence within the different levels of helminth species richness with an exact multinomial test. A Spearman rank correlation matrix based on all hosts was used to test for associations between infection intensities of co-infecting parasites as well as with helminth species richness. When we calculated the correlation between a certain helminth species and helminth species richness we subtracted one from the helminth species richness for the samples infected with the particular parasite. In order to investigate the relationship between gene expression of MHC, *IL10* and *Tgfb* and parasite load, linear models were built using the infection intensities of helminths as predictor variables. To avoid over-parameterization we included only the most common helminths, i.e. those showing a prevalence of more than 20% in the model. First we calculated the maximal model including all K predictors without interaction terms. Deviance of the residuals from a normal distribution was tested with a Shapiro–Wilks test. To find the best explanatory model for our gene expression data all 2^K possible models out of all subsets of predictors were computed (Mac Nally, 2000). The amount of deviance explained by each model (R^2) was calculated and Akaike's second-order information criterion (AICc) for small sample sizes was used as a goodness- of-fit parameter. The difference ΔAICc between the AICc of each model, the minimum AICc and the AICc weights (R-script by K. McGarigal, pers. comm.) were calculated. The AICc weight represents the normalized relative likelihood of a model. It is calculated by $e^{(-0.5*\Delta AICc)}$ and this value is normalized to the sum of the relative likelihoods of all models. It can be interpreted as the probability that a certain model is the best model, given the data and the set of candidate models (Wagenmakers and Farrell, 2004). The contribution of each predictor to the total explained variance in our linear models was calculated for all possible subsets of predictors

Table 1: Prevalence and infection intensity (maximum, mean and median) of gastrointestinal parasites as well as their 95% confidence intervals (CI) in *A. flavicollis* (N= 64). / CI could not be calculated due to insufficient data. For comparison, the prevalence and mean infection intensity of other study populations of *A. sylvaticus* (A, B, C from Behnke et al. 2009) and *A. flavicollis* (D from Klimpel et al. 2007) are shown.

	Prevalence		Infection Intensity					A		B		C		D	
		CI_{Prev}	Max.	Mean	CI_{Mean}	Median	CI_{Median}	Prev	Inf Int	Prev	Inf Int	Prev	Inf Int	Prev	Inf Int
Heligmosomoides polygyrus	54.7	42.2 - 66.6	44	4.3	2.7 - 8.7	2	1 - 3	54.9	15.7	77.7	11.4	73	14.4	85.2	23.9
Syphacia stroma	43.8	31.9 - 56.3	265	23.3	9.9 - 59.4	4	2 - 8	30.7	16.7	50.6	23.6	6.1	0.8	3.7	12
Trichuris muris	28.1	18.2 - 40.6	7	2.2	1.5 - 3.2	1	1 - 2	0.2	<0.01	14.2	0.55	0.3	0.05	/	/
Cestode	21.9	13.1 - 33.5	6	2.9	1.9 - 4.4	2.5	1 - 3	0.4	/	3.7	/	0	/	14.3	/
Mastophorus sp.	5.8	2.0 - 14.3	1	1	/	1	/								
Aonchotheca sp.	4.7	1.3 - 13.1	4	2.3	1 - 3.3	2	/								

by hierarchical partitioning (Chevan and Sutherland, 1991; Walsh and Mac Nally, 2008). Additionally we applied stepwise back model selection according to Crawley (2007) as it is used for predictive models and checked whether the results are consistent with our exhaustive search (Mac Nally, 2000). All statistical analysis were performed in R (ver. 2.10.1, R Development Core Team 2009), SPSS (ver. 16.0.2, 2008 SPSS Inc., an IBM Company) or in Excel.

Results

Parasite load in A. flavicollis

For seven out of 71 wild caught *A. flavicollis* we were not able to get congruent results for all three genes of interest so that we decided to exclude them from our analyses. In the remaining 64 specimen five different nematodes were identified: *Trichuris muris* (Trichuridae) and *Aonchotheca* sp. (Capillaridae) both of the order Rhabditida, *Mastophorus* sp. (Spirocercidae, Spiruridea), *Syphacia stroma* (Oxyuridae, Oxyurida) and *H. polygyrus* (Heligmosomoidae, Strongylida). Cestodes could not be distinguished to the family level and were treated as one group. *Mastophorus* sp. was exclusively found in the stomach, whereas *H. polygyrus*, *Aonchotheca* sp. and cestodes infestations were restricted to the small intestine. *S. stroma* occurred mainly in the small intestine and was found occasionally in the colon. *T. muris* infection was limited to the caecum and transition to the colon. The highest prevalence showed *H. polygyrus* and *S. stroma*, both with more than 40%, and *T. muris*, which was found in 28% of the mice (Table 1). Seven rodents (~11%) showed no helminth infestations. Infestations with a single helminth morphotype (N = 23) were as common as with two morphotypes (N = 23, both ~36%), whereas three different morphotypes occurred in 17% (N = 11) of the investigated specimens. The infection intensity of the most common helminths showed an even distribution, only *S. stroma* showed a highly skewed distribution as the mean was substantially bigger than the median (Table 1). This indicates that some animals showed very heavy infections whereas the majority had very low infection intensities. We focused our subsequent analyses upon the four most common helminths. Contrary to expectations of a pronounced co-occurrence, *H. polygyrus* as well as *S. stroma* infections seemed to be negatively associated with cestode infestation as co-infections with cestodes occurred less frequently than expected (Table 2). Calculating the odds for cestode co-infection revealed that the odds ratio were smaller than one for *H. polygyrus* ($OR_{H.\ polygyrus}$ = 0.25, two-sided Fisher's exact test p = 0.035) and for *S. stroma* ($OR_{S.stroma}$ = 0.28, two-sided Fisher's exact test p = 0.072). It was positive between *H. polygyrus* and *S. stroma* (OR = 3.05, two-sided Fisher's exact test p = 0.021) but all associations were not Bonferroni significant ($\alpha_{Bonferroni}$ =

Table 2: Expected (f_{exp}), observed frequencies (f_{obs}) and their differences (diff) of single infections as well as co-occurence of the four most common gut dwelling helminths in *A. flavicollis*. The expected frequencies were calculated by multiplying the frequency of single infections with their observed prevalence. The expected frequencies for co-occurrence were calculated by multiplying the individual observed prevalences.

	H. polygyrus			***S. stroma***			***T. muris***			**Cestode**		
	f_{exp}	**f_{obs}**	**diff**	**f_{exp}**	**f_{obs}**	**diff**	**f_{exp}**	**f_{obs}**	**diff**	**f_{exp}**	**f_{obs}**	**diff**
H. polygyrus	0.20	0.16	-0.04									
S. stroma	0.24	0.27	+0.03	0.16	0.09	-0.07						
T. muris	0.15	0.17	+0.02	0.12	0.13	+0.01	0.10	0.03	-0.07			
Cestode	0.12	0.06	-0.06	0.10	0.05	-0.05	0.06	0.08	+0.02	0.08	0.06	-0.02

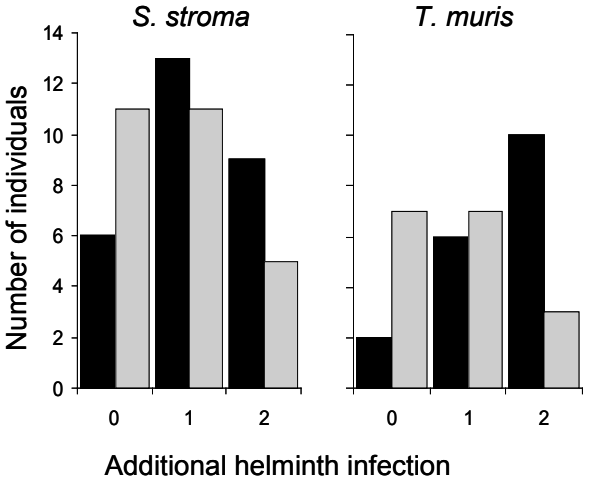

Figure 1: Influence of additional helminth infections upon the number of infected individuals with *S. stroma* and *T. muris*. Observed (black bars) and expected (grey bars) number of infected animals is plotted in relation to individual helminth species richness.

0.0083). *S. stroma* and *T. muris* occurred less frequently as single infections (Table 2). Multiple infected mice seemed to show a higher infection rate with *T. muris* and *S. stroma* than expected (Fig. 1). An exact multinomial test as a goodness of fit test showed that the observed difference is significant for *T. muris* ($p_{T.muris}$ = 0.0007) but not for *S. stroma* ($p_{S.stroma}$ = 0.0619). Only the infection intensity with cestodes was negatively correlated with helminth species richness (Table 3). The infection intensities of the parasite species were not correlated to each other except for *H. polygyrus*, which showed again a weak negative correlation with cestode infestations (Table 3). Since this was not Bonferroni-significant, both predictors (*H. polygyrus*, cestode) were included in subsequent models, whose predictors are supposed to be independent from each other.

Table 1: Correlation matrix of the infection intensity by gut dwelling helminths. Displayed are Spearman's correlation coefficient (C) and the level of significance (p). The Bonferroni corrected level of significance is $\alpha_{Bonferroni}$= 0.002.

		S. stroma	***T. muris***	**Cestode**	***Aonchotheca sp.***	***Mastophorus sp.***	**Helminth richness**
H. polygyrus	**C**	0.055	0.135	-0.253	-0.085	-0.177	0.071
	p	0.667	0.286	0.044	0.506	0.161	0.577
S. stroma	**C**		-0.072	-0.238	0.108	-0.064	0.060
	p		0.571	0.059	0.395	0.617	0.636
T. muris	**C**			0.102	0.005	-0.035	0.108
	p			0.423	0.968	0.781	0.395
Cestode	**C**				-0.116	0.029	-0.271
	p				0.360	0.820	0.030
Aonchotheca sp.	**C**					-0.057	-0.059
	p					0.653	0.642
Mastophorus sp.	**C**						-0.151
	p						0.234

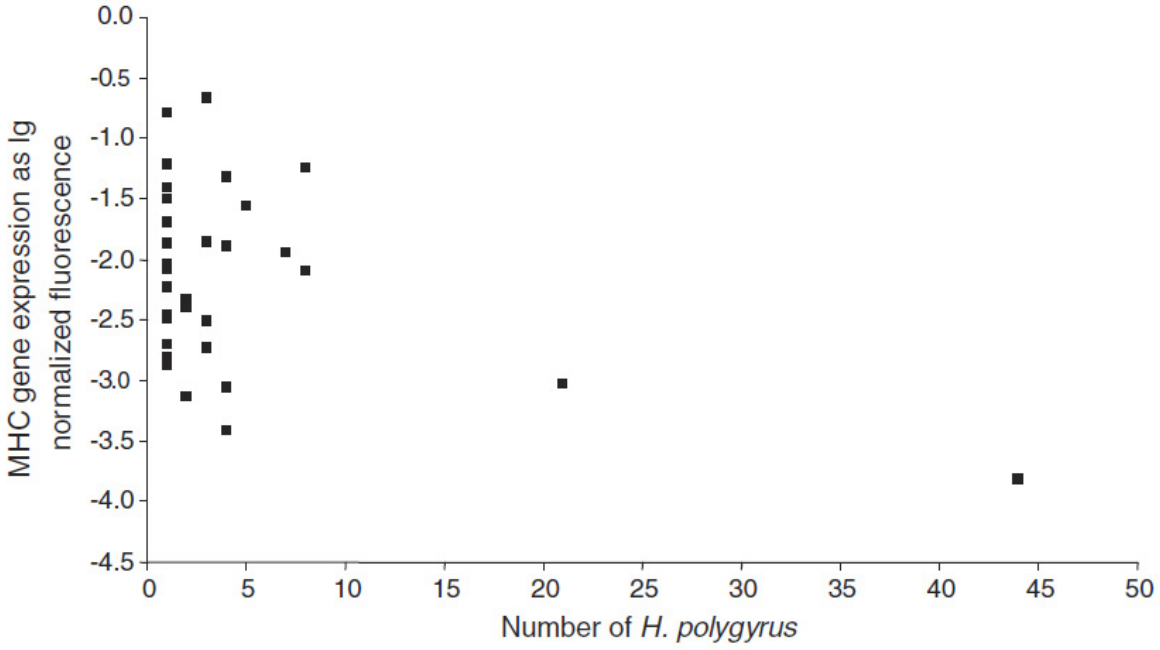

Figure 2: Observed MHC-DRB gene expression versus the individual burden with *H. polygyrus*.

Expression levels of MHC, Il10 and Tgfb and association to parasite load

All gene expression data showed no deviance from a normal distribution (Shapiro–Wilks test, data not shown). MHC-*DRB* was expressed a hundred times more than the cytokines *Il10* and *Tgfb*. Its relative standard deviation (rSD_{MHC}: 0.33) was more than twice as big as those of *Il10* (rSD*Il10*: 0.16) and Tgfb (rSD_{Tgfb}: 0.13). The maximal model explained more than 15% of the MHC *DRB* variance and contained only one significant predictor, *H. polygyrus* (Fig. 2). For *Il10* the samples L53, L68, L55 and L31 had to be excluded from the dataset as inspection of the residual plots showed that their leverage upon the model was too strong (data not shown). For *Tgfb* the sample L68 had to be excluded for the same reason (data not shown). For *Il10* and *Tgfb* the maximal model explained 8.6% and 2.3% of the variance, respectively. For both cytokines the maximal model included no significant predictor (Table 4). In order to find the most parsimonious linear model explaining the gene expression data we calculated all 15 possible models (Table 5). Regarding the MHC gene expression, the best model with the lowest Akaikes second-order criterion (AICc) contained only *H. polygyrus* as a predictor and explained 9.4% of the variance. Its likelihood was 64 times that of the model with the highest AICc and 3.6 times that of the maximal model, which always had the best fit.

Table 4: Maximal linear models explaining the effects of helminth infection intensity on MHC-*DRB*, *Il10* and *Tgfb* expression. All parasites having a prevalence >20% were included as predictors. Displayed are the Estimate (regression coefficient), SE standard error, t-value of the Estimate, p (significance of the Estimate) and R^2 (proportion of explained deviance).

Maximal model	Predictor	Estimate	SE	t-value	P
MHC	Intercept	-2.030	0.114	-17.86	<0.001
	H. polygyrus	-0.041	0.014	-2.86	0.006
	T. muris	0.093	0.064	1.45	0.152
R^2: 0.153	Cestode	-0.075	0.054	-1.39	0.171
	S. stroma	-0.001	0.002	-0.41	0.685
Il10	Intercept	-7.229	0.186	-38.83	<0.001
	Cestode	-0.175	0.112	-1.57	0.123
	S. stroma	0.005	0.004	1.42	0.160
R^2: 0.086	*T. muris*	0.103	0.105	0.98	0.332
	H. polygyrus	-0.066	0.082	-0.80	0.426
Tgfb	Intercept	-6.745	0.151	-44.74	<0.001
	H. polygyrus	0.028	0.037	0.73	0.470
	T. muris	0.057	0.087	0.66	0.513
R^2: 0.023	Cestode	0.024	0.072	0.33	0.742
	S. stroma	0.001	0.003	0.22	0.829

Stepwise reduction of the maximal model led to the same model as the exhaustive search in the case of MHC (Table 6). Analyses did not reveal any significant effect of helminth infection on neither *Il10*, nor *Tgfb* gene

expression. For *Il10* and *Tgfb* no model was better than the null model showing that cytokine expression in the liver is not associated to and could not be explained by helminth infection intensity. Hierarchical partitioning demonstrated that *H. polygyrus* infection intensity in the small intestine accounted for almost 70% of the explainable variance of MHC gene expression in the liver (Fig. 3).

Table 5: Explanatory model search: all possible subsets of linear models explaining the effects of infection intensity by helminths on hepatic MHC-*DRB*, *Il10* and *Tgfb* expression are shown. Models are ordered top-down by their Akaike's second-order information criterion (AICc). Displayed are the R^2 (proportion of explained deviance), Δ AICc (difference between model AICc and the minimum AICc) and the AICc weights (normalized likelihood of a model).

	Linear term	R^2	AICc	Δ AICc	AICc weights
MHC-*DRB*	*H. polygyrus*	0.094	137.568	0.000	0.194
	T. muris + *H. polygyrus*	0.124	137.621	0.053	0.188
	T. muris + *H. polygyrus* + Cestode	0.150	137.929	0.361	0.162
	H. polygyrus + Cestode	0.118	138.048	0.480	0.152
	S. stroma + *H. polygyrus*	0.097	139.550	1.982	0.072
	T. muris + *S. stroma* + *H. polygyrus*	0.125	139.798	2.230	0.063
	S. stroma + *H. polygyrus* + Cestode	0.123	139.992	2.424	0.058
	T. muris + *S. stroma* + *H. polygyrus* + Cestode	0.153	140.105	2.537	0.054
	T. muris	0.016	142.839	5.271	0.014
	Cestode	0.014	143.000	5.432	0.013
	S. stroma	0.005	143.574	6.006	0.010
	T. muris + Cestode	0.031	144.094	6.526	0.007
	S. stroma + Cestode	0.020	144.799	7.231	0.005
	S. stroma + T. Muris	0.019	144.833	7.265	0.005
	S. stroma + T. Muris + Cestode	0.035	146.082	8.514	0.003
Il10	Cestode	0.037	178.725	0.000	0.163
	S. stroma	0.035	178.862	0.137	0.152
	S. stroma + Cestode	0.066	179.081	0.356	0.136
	T. muris + Cestode	0.042	180.589	1.864	0.064
	T. muris + *S. stroma*	0.041	180.692	1.967	0.061
	H. polygyrus + Cestode	0.040	180.714	1.989	0.060
	T. muris	0.003	180.765	2.040	0.059
	T. muris + *S. stroma* + Cestode	0.075	180.818	2.093	0.057
	H. polygyrus	0.001	180.920	2.195	0.054
	S. stroma + *H. polygyrus*	0.035	181.025	2.300	0.051
	S. stroma + *H. polygyrus* + Cestode	0.070	181.154	2.429	0.048
	T. muris + *H. polygyrus* + Cestode	0.052	182.290	3.565	0.027
	S. stroma + *T. muris* + *H. polygyrus* + Cestode	0.086	182.504	3.779	0.025
	S. stroma + *T. muris* + *H. polygyrus*	0.045	182.738	4.013	0.022
	T. muris + *H. polygyrus*	0.006	182.806	4.081	0.021
Tgfb	*H. polygyrus*	0.012	168.103	0.000	0.169
	T. muris	0.012	168.130	0.027	0.166
	Cestode	0.001	168.818	0.715	0.118
	S. stroma	0.000	168.860	0.757	0.116
	T. muris + *H. polygyrus*	0.019	169.837	1.734	0.071
	H. polygyrus + Cestode	0.014	170.172	2.069	0.060
	S. stroma + *H. polygyrus*	0.012	170.293	2.190	0.056
	T. muris + Cestode	0.012	170.295	2.192	0.056
	T. muris + *S. stroma*	0.012	170.299	2.196	0.056
	S. stroma + Cestode	0.001	171.011	2.908	0.039
	T. muris + *H. polygyrus* + Cestode	0.021	172.013	3.910	0.024
	S. stroma + *T. muris* + *H. polygyrus*	0.020	172.080	3.977	0.023
	S. stroma + *H. polygyrus* + Cestode	0.015	172.430	4.327	0.019
	S. stroma + *T. muris* + Cestode	0.013	172.534	4.431	0.019
	S. stroma + *T. muris* + *H. polygyrus* + Cestode	0.022	174.325	6.222	0.008

Table 6: Best parsimonious model explaining MHC gene expression. Stepwise reduction of the maximal model led to the minimal adequate model including only *H. polygyrus* as single predictor. Displayed are the Estimate (regression coefficient), SE standard error, t-value of the Estimate and p (significance of the Estimate).

	Predictor	Estimate	SE	t-value	p
R^2: 0.094	Intercept	-2.054	0.092	-22.33	<0.001
	H. polygyrus	-0.036	0.014	-2.54	**0.014**

Discussion

Does Heligmosomoides infection influence systemic MHC expression?

Due to their fundamental role in the immune system MHC genes are one of the key candidates that might be attacked by mechanisms of heligmosomoid systemic immune suppression. The expression level of MHC genes is known to affect both arms of immunity, the adaptive and the innate (LeibundGut-Landmann et al., 2004; Frei et al., 2010). A recent study demonstrated that MHC class II deficient mice showed reduced responsiveness to toll like receptor stimuli (Frei et al., 2010) and innate receptors are known to be altered by feed-back from the adaptive immune system (Friberg et al., 2010). This suggests that MHC class II molecules in addition to their fundamental role in adaptive immunity are also involved in building up adequate innate immunity. Jackson et al. (2009) demonstrated in wild wood mice (*Apodemus sylvaticus*) that heligmosomoid infected mice had lower toll like receptor responsiveness and thus a lower innate immune system responsiveness. Recent studies have indicated that the susceptibility to helminth infection in *A. flavicollis* is associated with specific alleles of MHC class II genes (Meyer-Lucht and Sommer, 2005, 2009). We detected a negative association between *H. polygyrus* infection intensity and MHC class II *DRB* gene expression in the liver. This may indicate that *H. polygyrus* influences its host's immune system by lowering the systemic expression of MHC genes under a natural infectious regime. MHC class II molecules are responsible for the binding of helminth derived antigens and present them to naïve CD4+ T helper cells, which trigger the subsequent immune response. Thus *H. polygyrus* might have the same strategy and evade detection by the immune system as has been suggested for microparasites (LeibundGut-Landmann et al., 2004). At least they might attenuate the immune response, as the lower level of mRNA transcripts of the MHC class II genes is tantamount to less activated T cells (Perrigoue et al., 2009). Given the complex regulatory mechanisms in the immune system it is not surprising that many other confounding factors seem to influence MHC gene expression in a wildlife study. Life history characteristics like age (Herrero et al., 2002) or genetic components like differences in the promoter region (Mitchison and Roes, 2002) are known to influence MHC expression and are sometimes difficult to control for in an in situ study. Though helminth infection could explain only 15% of the variance in MHC gene expression, *H. polygyrus* itself contributed 9% and the effect of nematode infection was significant. Due to limited sample size we had to do multiple testing of the gene expression with the same data set risking the production of a second-order error. Yet our estimated p-value of 0.014 was still lower than the corrected level of significance ($\alpha_{Bonferroni}$ = 0.016).

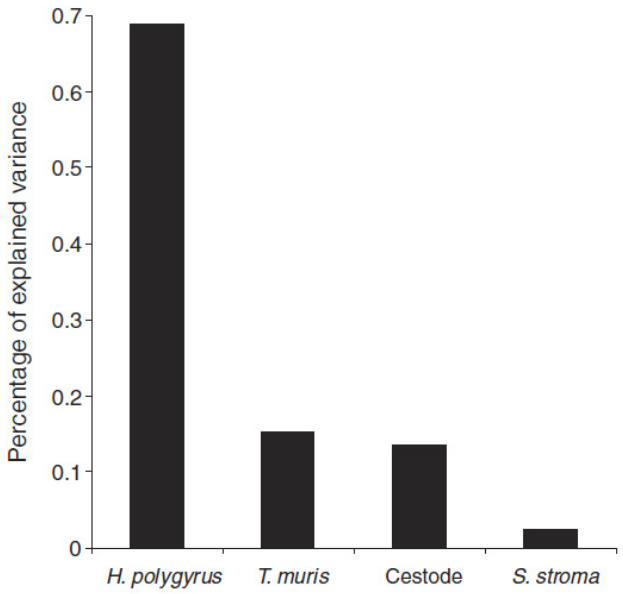

Figure 3: Contribution of each helminth predictor to the total explained variance in linear models explaining the MHC gene expression. The bars show the percentage of the independent effects of each helminth predictor on MHC gene expression, calculated from hierarchical partitioning.

Potential pathways of MHC suppression

It is still unknown by what mechanism *H. polygyrus* affects MHC class II DRB mRNA levels. *H. polygyrus* infection is associated with elevated levels of mucosal *Il10* and *Tgfb* produced by T_{reg} cells (Ince et al., 2009). Both cytokines are known to down regulate CIITA (Harton and Ting, 2000; Ting and Trowsdale, 2002) and thus MHC class II expression (Romieu-Mourez et al., 2007; Couper et al., 2008). The amount of blood that passes the hepatic portal vein every minute, coming from the digestive system is 30% of the total blood (Wick et al., 2002). Hence, cytokines could be easily transported from the site of infection to the liver. The systemic immunosuppression could be a spill-over reaction of these cytokines and according to our results would not be associated with hepatic cytokine levels. Excretory–secretory antigens could also (Grainger et al., 2010). Liver resident T_{reg} cells have been demonstrated to mediate a systemic antigen specific immune tolerance in transgenic mice (Lüth et al., 2008). A reduced expression of MHC after exposure to excretory–secretory antigens has been observed in cultured dendritic cells (Segura et al., 2007). As we did not detect an association between helminth infection and neither hepatic *Il10* nor *Tgfb* expression, we consider this explanation unlikely.

Do immune gene expression differences alter the host susceptibility to helminths?

An other explanation for the observed association would be that a lowered MHC gene expression facilitates *H. polygyrus* infection. In that case our measured hepatic MHC expression would reflect individual differences in the overall MHC expression between the sampled individuals and these differences would also occur in the draining lymph nodes adjoining the site of infection resulting in a lowered immune response. Expression differences for different MHC alleles are explained by heritable nucleotide differences in the promoter regions (Díaz et al., 2005, Mitchison and Roes, 2002). These polymorphisms in the promoter region show even indices of balancing selection in primates (Loisel et al., 2006) demonstrating the link between MHC gene expression and parasite infection. This does not necessarily require a direct influence of hepatic MHC expression upon intestinal helminths infections, because MHC class II gene expression is normally induced in immune competent cells at the site of infestation whereas hepatic MHC expression happens downstream from the site of infection.

Does infection with Heligmosomoides influence co-infection?

Other parasites might benefit from a systemic reduction of MHC class II expression that might be induced by *H. polygyrus*. The nematodes of this genus have been suspected to be immunosuppressive for a long time, as experimental co-infection studies revealed a delayed expulsion of other helminth species (Jenkins, 1975; Jenkins and Behnke, 1977; Behnke et al., 1978, 1984) or increased susceptibility to microparasites during concurrent *Heligmosomoides* spp. infections (Chen et al., 2005, 2006; Su et al., 2005). In their natural hosts heligmosomoid nematodes can show a pronounced pattern of co-infection with other parasite species (Behnke et al., 2005, 2009; Jackson et al., 2009). In our present data we did not observe a pronounced co-occurrence of parasite species with *H. polygyrus*. In fact, rather the opposite was the case: *H. polygyrus* as well as *S. stroma* prevalence tended to be negatively associated with cestode infection although both not Bonferroni significant. A possible explanation might be that cestodes outcompete both nematodes in the small intestine, whereas the caecum dwelling *T. muris* is not afflicted by cestode infestations, as it inhabits another gut compartment. This is further supported as cestode infection intensity is negatively correlated to helminth species richness, although again not Bonferroni significant.

Study limitations

Compared to other studies the observed prevalence was moderate. For example Behnke et al. (2009) found a mean prevalence of 68.5% in three study populations of *A. sylvatica*, the closely related sister species of *A. flavicollis*, and Klimpel et al. (2007) even 85.2% for *H. polygyrus* in wild *A. flavicollis*. Compared to our data, also the infection intensity was two to five times higher in the mentioned studies of Behnke et al. (2009) and Klimpel et al. (2007). More than half of our infected animals showed very low intensities of one or two nematodes limiting the significance of our data as high infection intensities are expected to have a higher impact on MHC expression. Thus also possible effects on concomitant infections are hard to detect. Our study demonstrates the problems of in

situ studies where it is hard to control for the number of confounding factors and the outcome of a study is not always predictable. Nevertheless, we believe it demonstrates that it is possible to apply new laboratory methods also to wild species and that these attempts facilitate new questions in the emerging field of ecoimmunology.

Conclusions

Our results show a weak negative association between *H. polygyrus* infection and hepatic MHC gene expression in wild hosts. *H. polygyrus* has been demonstrated to influence the innate immune system in wild hosts (Jackson et al., 2009) and interference with the adaptive arm of the immune system by lowering the expression MHC class II genes might be a possible explanation for our results. This might serve as an immune evasion strategy of *H. polygyrus* as it has been suggested for microparasites that are capable of MHC suppression (LeibundGut-Landmann et al., 2004). Our study further revealed that in addition to the effects of the structural variance of specific MHC class II alleles on helminth susceptibility (Meyer-Lucht and Sommer, 2005, 2009) the expression levels of immune relevant genes should also be considered in order to disentangle the complex interactions of host– parasite co-evolutionary processes. Future studies will focus on the effects of MHC allele-specific expression levels on the infection intensities by multiple parasites.

Ethical statement

All research reported in this manuscript accorded to the legal requirements of Germany and were 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).

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Supplementary data

Supplementary data associated with this article can be found, in the appendix at end of this document.

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Article 5

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

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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 pleiotropic effect with resistance to infections by the oxyorid 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.

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 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 mechansms such as alternative mating and maternal-foetal interactions have been suggested as alternative or complementary mechanisms maintain 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, *A. 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 363bp 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 35km North-East of the city of Hamburg, Northern Germany. Only adult animals with a body mass over 16g (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 24h 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 (2x 10s at 5000rpm, Precellys, Bertin Technologies) in QIAzol lyses reagent (Qiagen) with 1.4mm ceramic beads. From each homogenate, we placed 0.5ml in two 1.5ml 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 87.5µl 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 100µl 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. 2µg of total RNA was reverse transcribed with Oligo-dT_{18} primers (5µM) in a 20µl 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 (218bp, Supplemental Figure 1) according to Meyer-Lucht & Sommer (2005). The PCRs were performed in a 20µl 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). Two 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 15s at 37°C and the reaction was stopped by heating up to 85°C for 15min. Two µl of sequencing buffer, as well as 1 µl of BigDye v3.1 (Applied Biosystems) and 1mM primer was added to the sequencing reaction. Cycle sequencing was done for 20 cycles consisting of 96°C for 10s, 52°C for 10s and 60°C for 90s. The sequencing products were cleaned-up using BigDye XTerminator Purification Kit (Applied Biosystems) and subsequently analysed on a 3130xl 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 365bp 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 172bp 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 2.5µl of cDNA template, 12.5µl SensiMix™ SYBR® No-ROX (Bioline), 0.5µl primer (20µM) and 9.5µl dH_2O. The qPCR conditions were 10min at 95°C and 45 cycles of each 95°C for 15s, 55°C for 15s and 72°C for 15s. Melting curve analysis was performed from 65° to 95°C in 0.5°C steps each lasting 5s 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 & 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 & 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 (365bp). Substitutions rates were also analysed within and outside the antigen binding sites (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 & 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 out 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 by 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 intensitiesvaried 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 by taking ear tissue and faeces samples (Meyer-Lucht and Sommer 2005). The parasite burden of all those 22 samples was assed via faecal egg counts and all could be were genotyped based on gDNA. In 2008, we collected 71 individuals but only 69 could be genotyped based on cDNA and gDNA and were included in the following analyses. No family units were collected (microsatellite data not shown).

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.

	Prevalence		
Species	**2004**	**2008**	**p**
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

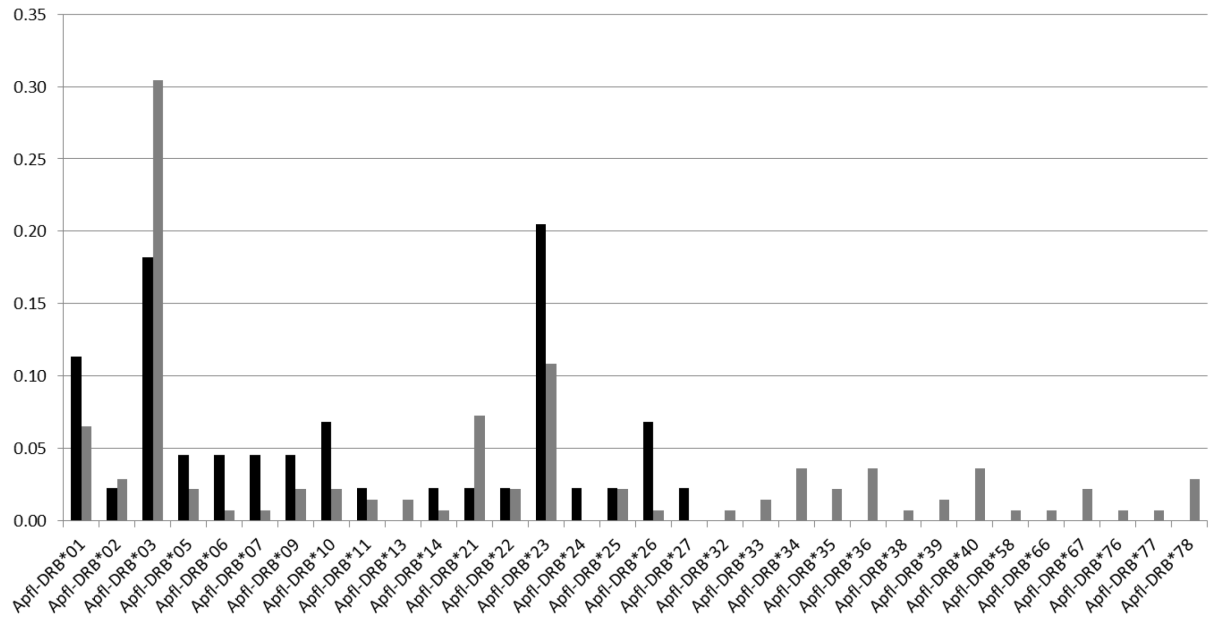

Figure 1: Temporal change of relative MHC allele frequencies between 2004 (22 individuals, black bars) and 2008 (69 individuals, grey bars).

Changes in parasite load between 2004 and 2008

All five parasite species observed in 2004 (Meyer-Lucht & 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 *Trichuris muris* (Trichuridae) and *Aonchotheca* sp. (Capillaridae) both belonging to the order Rhabditida, *Mastophorus* sp. (Spirocercidae, Spiruridea), *Syphacia 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 ± 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).

MHC sequence variability and selection pattern

The genotyping of the second exon (217bp) 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 (365bp), 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 ± 5).

Forty-seven different genotypes were observed (average frequency: 1.47 ± 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 χ^2= 154.43, df= 2; p<0.001). CODEML identified 16 positively selected sites (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 (Figure 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 two years (p= 0.005). Changes of more than five per cent between 2004 and 2008 were detected in *Apfl-DRB**03 (+12%), as well as for *Apfl-DRB**23 (-10%) and *Apfl-DRB**26 (-6%).

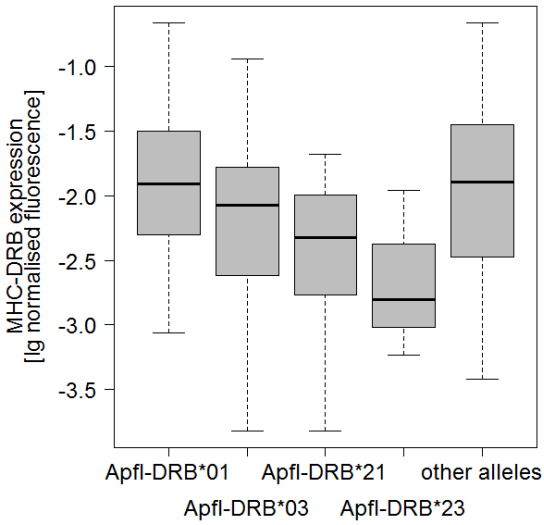

Figure 2: MHC gene expressions of the most common alleles (> 0.05 relative frequency). Indicated are normalized log transformed values.

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 oxyorid 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= 0.007).

Table 2: Effects of specific MHC alleles on the prevalence (presence/absence) of parasitic infections. The odds ratios (OR), their 95% confidence interval (CI) and the corresponding P-values are shown. All results did not reach Bonferroni significance of α= 0.003.

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

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 The estimates denotes the effect of of the particular factor on the dependent variable.

***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		

*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. 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.

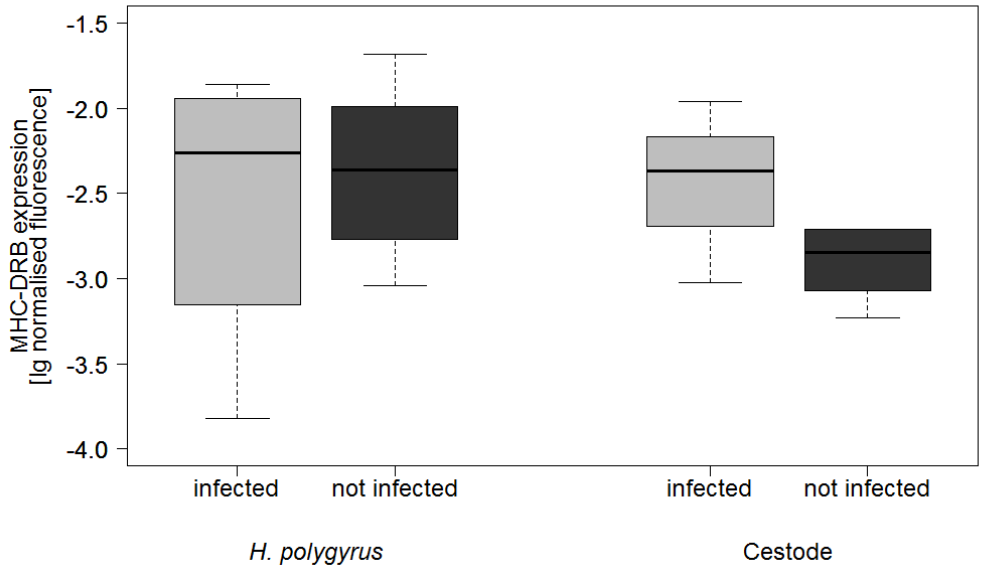

Figure 3: MHC DRB gene expression in individuals carrying the allele Apfl-*DRB**21 or Apfl-*DRB**23 in relation to the prevalence by *H. polygyrus* and cestodes, respectively.

Thus we excluded them and restricted our MHC expression analyses to 63 animals. MHC *DRB* gene expression showed considerable variation (Figure 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, χ^2= 2, df= 2, p= 0.3679). 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, $p_{Apfl\text{-}DRB*23}$ = 0.302; Figure 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; Figure 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; Figure 4A) or in their total helminth burden (W= 210, p= 0.904; Figure 4B). They also showed no differences in their expression levels (W= 124, p= 0.1178, Figure 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 ($mean_{n1}$= 0.113, $mean_{nx}$= 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 $\alpha_{Bonferroni}$= 0.01, $roh_{H.\ polygyrus}$= 0.026, p= 0.035; $roh_{S.\ stroma}$= -0.0114, p= 0.926; $roh_{T.\ muris}$= -0.1373, p= 0.261; $roh_{cestodes}$=

0.0120; p= 0.871, Figure 5A) as well as on the total infection intensity (roh_{total}= -0.021, p= 0.862, Figure 5A).

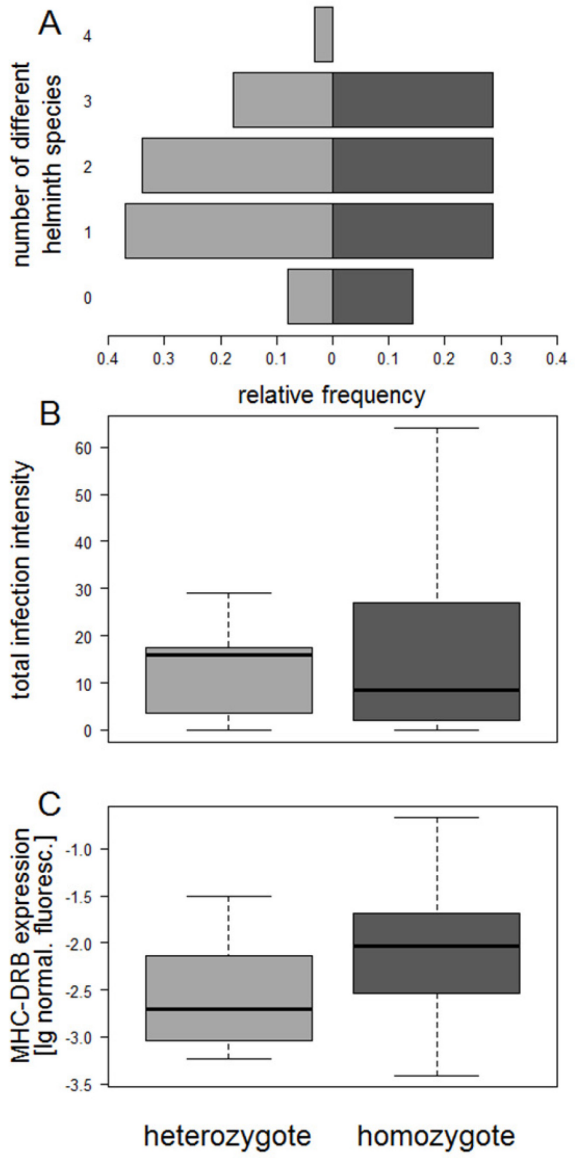

Figure 4: **A)** Number of different helminth infections, **B)** total infection intensity and **C)** MHC genotype expression in homo- and heterozygote individuals. No difference between homo- and heterozygote individuals were observed.

Also the MHC gene expression did not correlate with the parasite burden (Spearman rank correlation, roh = -0.1069, p = 0.4045, Figure 5b) and the amino acid distance (Pearson correlation, t= 1.665, df= 61, p= 0.101, Figure 5c). We repeated all analyses by calculating the genetic distance of PSS sites only. No significant differences were observed (data not shown).

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 (363bp) 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 positive selected sites (PSS) were located within the second exon and were congruent with or in close vicinity to ABS of the human *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

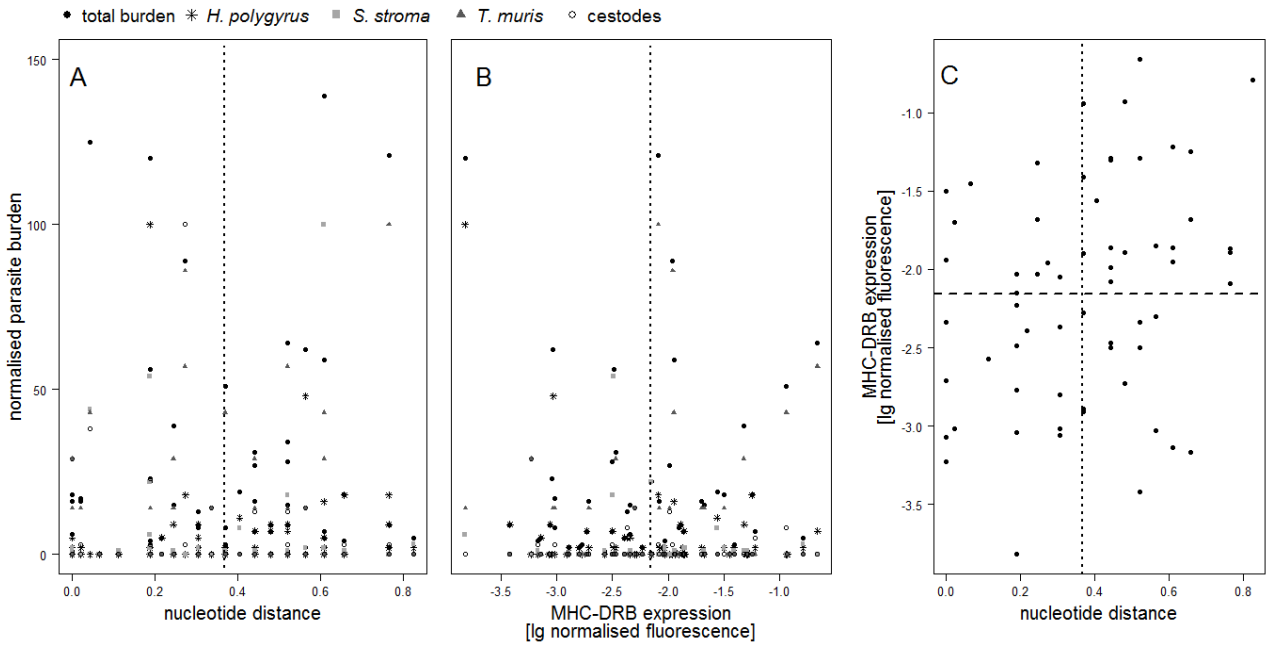

Figure 5: Normalised infection intensity of the four most common helminth species as well as total infection intensity in relation to **A)** amino acid distance and **B)** MHC class II *DRB* gene expression. **C)** MHC class II *DRB* gene expression in relation to amino acid distance. No significant 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. Total burden is indicated by a filled circle, cestode burden by an open circle, *H. polygyrus* burden by a star, *S. stroma* burden by a square and *T. muris* burden by a triangle.

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 five 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.

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Supplementary data

Supplementary data associated with this article can be found, in the appendix at end of this document.

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Discussion

My dissertation studies have focused on different levels and aspects of the interplay between parasites and their vertebrate host's immune genes under natural selection conditions. The work represented in this thesis clearly demonstrates that it is possible to measure MHC-expression even in non-model organisms. We used highly sensitive real-time PCR and established assays on in-situ samples of *A. flavicollis* and *D. sublineatus*. We carefully validated potential reference genes used for normalization of the target gene subject to the analyses. A careful validation of the expression stability of candidate genes is necessary prior to every real-time PCR experiment using relative quantification as instable references genes would produce erroneous results for the target gene (Thellin et al. 1999; Vandesompele et al. 2002; Dheda et al. 2005; Huggett et al. 2005). Early breakthrough studies on wildlife MHC gene expression used single reference genes and did non validate the used reference genes (Bowen et al. 2006, 2007). Thus, it is hard to tell how robust their findings were. Nevertheless, they were the first attempts into this direction and compared to studies on established laboratory model organisms these in situ studies often had to deal with a lack of available background information on their study species, e.g. on potential reference genes. The rapid development in modern genomics research might help to solve this problem in the future.

We were able to establish qPCR essays on liver and spleen tissue for *A. flavicollis* and *D. sublineatus*, so we could measure the level of immune gene transcriptions. MHC gene expression and also its variance was always higher than those of cytokines. Our results were in congruence with expectations for immune reactions as a reponse to helminth infection. Interestingly helminths evoke a very stereotypic Th2 response in vertebrates although they belong to different taxonomic groups (Díaz and Allen 2007; Jackson et al. 2008; Harris and Gause 2011). It has been suggested that the vertebrate immune system has only a limited ability to distinguish among different parasites (Finkelman et al. 2004). On the other hand, convergent evolution of those groups might be an explanation (Díaz and Allen 2007). Helminth groups date back hundreds of millions of years and so does their interaction with the adaptive immune system of vertebrates and reciprocal co-adaptation might have shaped the characteristics of both (Jackson et al. 2008). A typical Th2 reaction is orchestrated by cytokines including *Il4*, *Il5*, *Il10* and *Il13* produced by T cells and characterized by activation of alternative macrophages, basophils and mast cells, eosinophil maturation and Immunoglobolin E production (Díaz and Allen 2007; Jackson et al. 2008). We could demonstrate that helminth infection in free living South-American sigmodontine *D. sublineatus* induces a typical Th2 response with increasing *Il4* levels associated with increased strongyle nematode infection. There has been a long debate whether Th2 responses are an evolutionary adequate defensive response against the worm or if they are the result of immune manipulation by the parasite (Díaz and Allen 2007). In fact, a host has two adequate answers to infection that we have to differentiate as they both might produce different evolutionary outcomes (Råberg et al. 2009). One is resistance

which protects the host at the expense of the parasite, affecting its prevalence. This imposes strong selective forces on the parasite to overcome the host's immune reaction which in turn selects for an improved resistance in the host, leading to an antagonistic coevolution of both antagonists. On the contrary, tolerance only minimizes the negative effects of infection on the host without negative consequences for the parasite, thus limiting selective forces on the parasite (Råberg et al. 2009). Many helminthic parasites are in general well tolerated and show often high prevalence within their host populations (Maizels 2005). However, Th2 responses are often effective against gastrointestinal nematode infection, thus reflecting resistance (Maizels et al. 2004; Finkelman et al. 2004). The stereotypic Th2 response might be an evolutionary appropriate response for both, controlling helminths and repairing damage they have caused. Nevertheless, many helminths are able to tune the true Th2 response and add an anti-inflammatory component by elevated *Il10* and *Tgfb* levels as part of an immune evasion strategy (Díaz and Allen 2007). This modified Th2 response allows for a certain tolerance by the host and leads to a chronic infestation of the parasite. In *D. sublineatus,* we found no significant association of splenic expression of the two major immune regulators *Il10* and *Tgfb* with helminth infection, as we would have expected (Maizels and Yazdanbakhsh 2003; Maizels et al. 2004). The only potential associations we observed remain disputable, since they were based on only two individuals infected with cestodes or *Syphacia* sp..

Also no significant associations between expression of specific MHC alleles and parasite infection could be observed. The extraordinary high allelic diversity in *A. flavicollis* made it impossible, especially as it was only possible to measure genotypic MHC expression levels with SYBR green. This clearly limits the explanatory power of my model/study and demonstrates the risks and limitations of pure in situ studies.

I could, however, demonstrate a negative correlation between hepatic MHC class II expression and thus likewise systemic MHC expression with *H. polygyrus* infection intensity in *A. flavicollis*. A lower level of mRNA transcripts of the MHC class II genes is tantamount to less activated T cells (Perrigoue et al. 2009) and might be the cause for higher *H. polygyrus* infection intensity. Differences in MHC expression might also be explained by heritage when they are caused by nucleotide differences in the promoter regions as it was found in some species (Mitchison and Roes 2002; Díaz et al. 2005; Loisel et al. 2006; Wegner et al. 2006). Nevertheless, due to linkage disequilibrium they would be related to certain alleles or at least clusters of alleles, which was not the case in *A. flavicollis*.

I rather assume that *H. polygyrus* interferes with MHC gene expression to evade recognition and the subsequent immune response, as has already been suggested for some micro-parasites (LeibundGut-Landmann et al. 2004). In this case, a low MHC expression would be the result rather than the cause of higher *H. polygyrus* infections. This nematode has been suspected to act as animmunosuppressent for many years (Jenkins and Behnke 1977; Behnke et al. 1978, 1983) and there is ample evidence of its immune-manipulative abilities (Bazzone et al. 2008; Behnke et al.

2009a; Jackson et al. 2009). *H. polygyrus* might be able to attenuate the immune response and if this holds true, it would also imply that MHC binding motifs become less important in *H. polygyrus* related infection, making *H. polygyrus* not a classical model for MHC - parasite coevolution. Contradicting this hypothesis are the associations or at least strong tendencies between MHC alleles and *H. polygyrus* Meyer-Lucht and Sommer (2005) and I have found in the present study. A potential immunosuppression by *H. polygyrus* might also serve as a door opener for other parasites. In fact, several wildlife studies have shown a pronounced pattern of co-infestations with other parasite species for this nematode (Behnke et al. 2005, 2009a; Jackson et al. 2009). In infection trials concomitant *Heligmosomoides* sp. infections allow *T. muris* to establish chronic infestations, whereas it is rapidly expelled by the hosts Th2 response in the absence of *Heligmosomoides* (Jenkins and Behnke 1977; Behnke et al. 1984). I did not observe a direct correlation between both nematodes, but *T. muris* occurred more often in multiple infections than alone, whereas *H. polygyrus* was unaffected by concomitant infestations.

Although there is strong indirect evidence for the immunosuppressive abilities of *H. polygyrus*, it is as yet unclear how it accomplishes this. Its infection is associated with elevated mucosal levels of *Il10* and *Tgfb* produced by regulatory T (T_{reg}) cells (Ince et al. 2009). Both cytokines down-regulate CIITA, the main regulator of MHC expression (Harton and Ting 2000; Ting and Trowsdale 2002). In our study, neither elevated levels of *Il10* or *Tgfb* in the spleen of *D. sublineatus* nor in the liver of *A. flavicollis were* related to strongyle nematode infection, although it is known that liver resident T_{reg} cells can also mediate tolerance (Lüth et al. 2008). Thus, it seems more likely that a potential systemic MHC suppression is mediated by blood. Potential effectors might be cytokines produced by a spill-over function of T_{reg} cells resident at the site of infestation or other excretory-secretory antigens produced by the helminth itself (Grainger et al. 2010).

In *A. flavicollis* I found ample evidence for historical as well as ongoing selection on the MHC class II *DRB* gene. It exhibits an excessive degree of diversity of the MHC class II DRB gene and unlike in previous studies, my results are based on mRNA and not nuclear DNA, thus I can also infer functionality of all sequences. Furthermore, I sequenced a longer segment of the gene spanning from exon one through exon three which allowed me to demonstrate that nucleic diversity is clearly restricted to the second exon. This finding is perfectly in line with expectations (Hughes and Nei 1988, 1989), but has never been confirmed by other MHC studies on wildlife species, although they often focus a priori on this region. While the polymorphic areas of the so called antigen binding region are responsible for the large repertoire of binding motifs, the conserved parts of the mature protein ensure structural stability. In *A. flavicollis,* all the polymorphic key residues that are characterized by a huge excess of non-synonymous substitutions are without exception located within the second exon. These key residues or positively selected sites were congruent or in close vicinity to those forming the human antigen binding groove in the DR molecule (Brown et al. 1993).

Their high rate of non-synonymous substitutions is regarded as a strong indicator for an adaptive evolutionary process and is assumed to be the product of positive selection over an evolutionary timescale (Parham and Otha 1996).
However, on-going selection processes in contemporary generations are much harder to prove (Piertney and Oliver 2006) and there is often only indirect evidence. Besides positive selection, I found further indirect signs for the on-going antagonistic interplay between MHC genes and parasites in *A. flavicollis*. First, positive and negative associations of specific alleles with parasitic helminths indicate the important role of MHC in parasite resistance. One MHC allele was associated with reduced prevalence of the oxyorid nematode *S. stroma* and higher infection intensity with cestodes. Such pleitropic effects have also been demonstrated in a South-African rodent (Froeschke and Sommer 2012) and might indicate a trade-off between both parasites for the host. In my results, the MHC allele *Apfl-DRB**23 showed a strong tendency of being associated with higher *H. polygyrus* infection intensity. The same allele was found to be protective against strongyle nematode infection in a former study (Meyer-Lucht and Sommer 2005). This allele might have lost its protective abilities, which could indicate frequency dependent selection. On the other hand, we cannot rule out that this might be the effect of local adaptations in the parasite community, as the study of Meyer-Lucht and Sommer was sampled over several different populations.

Contemporary selection is also reflected in changes in allele frequencies in rather short time scales. I could demonstrate such changes in allele frequencies as well as parasite prevalence within a few years in a population. Onlya few MHC studies have looked at different time scales so far, but some have demonstrated that these changes can happen in a rather short time or even in consecutive generations (Westerdahl 2004; Charbonnel and Pemberton 2005; Eizaguirre et al. 2012). These changes might indicate fluctuating selection, which has been suggested to be a major driving force in maintaining MHC diversity (Hedrick et al. 1987; Hill et al. 1991).

Conclusion

The vertebrates' immune genes show an exceptional high degree of polymorphism that might be crucial for the vitality of wildlife populations and has been studied extensively in many vertebrate taxa and species to date (e.g. Axtner and Sommer 2007; Miller et al. 2011; Savage and Zamudio 2011; Eizaguirre et al. 2012). Whereas previous evolutionary studies on the interaction between parasites and host MHC have focussed mainly on structural genetic diversity, I have extended the focus in my dissertation to test if quantitative measurement of MHC gene expression levels is possible in wild animals and to determine its role in host–parasite relationships.
The studies in this thesis demonstrate that valid procedures to quantify expression of immune relevant genes are also feasible in non-model wildlife organsims. In addition to structural MHC diversity also MHC gene expression should be considered to obtain a more complete picture on

host-pathogen coevolutionary selection processes. This is especially true if parasites are able to interfere with systemic MHC expression. In this case advantageous or disadvantageous effects of allelic binding motifs are abated and host-parasite co-evolutionary processes might not only be reflected by the structural variance of MHC alleles. Nevertheless I also found ample evidence for the importance of structural variance in MHC genes reflected by high allelic diversity, strong indices of selection in the past and contemporary associations to parasite infection and recents shifts in allelic frequencies. The studies indicated that in addition to structural sequence variance also MHC gene expression might play an important role in antagonistic coevolution though the interplay is not clear yet. The study suggested that it depends on the specific parasite species involved as some parasite species are able to interfere with the expression pattern of immune relevant genes fascilating the gateway for further parasite infections. Furthermore heritable expression differences of MHC alleles are known, but their effect on susceptibility to parasites is still unclear and leaves an open field for future studies.

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Appendix

Supplement Article 3

Supplement Table 1: Primer sequences for cytokine and MHC genes used in the qPCR

Gene	Important functions	Sequence	Fragment length [1)]
Il2	T- and B-cell proliferation, NK cell activation	forward 5' CTTTCCCTTGGAAGATGCTG 3' reverse 5' TGGTGCTTTGACAGAAGGTG 3'	118bp
Il4	Th2-cell differentiation, isotope switch to IgE	forward 5' CGGAGAAGGAACTCATCTGC 3' reverse 5' CGTGGATTCATTCACAGTGC 3'	133bp
Il10	mediates regulatory functions, anti-inflammatory	forward 5' GGTTGCCAAGCCTTATCGGA 3' reverse 5' ACCTGCTCCACTGCCTTGCT 3'	141bp
Tgfb	inhibits T-cell proliferation and effector functions, anti-inflammatory	forward 5' TGACGTCACTGGAGTTGTACGG 3' reverse 5' GGTTCATGTCATGGATGGTGC 3'	127bp
MHC class I	presents intracellular derived antigens	forward 5' CCTGAGGTATTTCTACACCGC 3' reverse 5' CTGGAAAACGGGAAGGAGAC 3'	473bp
MHC class II	presents extracellular derived antigens	forward 5' GAGTGTCATTTTGAGAACGGA 3' reverse 5' AGAGCAGACCAGGAGGTTGTG 3'	248bp

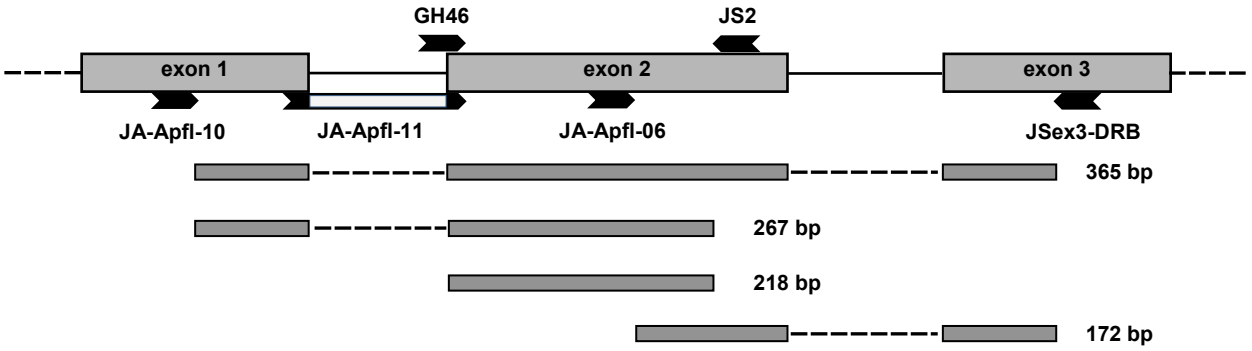
[1)] Fragment length without primer sequences

Supplement Article 4

Supplement 1: Quantitative real-time PCR primers for the amplification of fragments of mRNA transcripts.

Genes	Primer sequences	Length	Exons
MHC-*DRB*	forward 5' GCGGTGACCGAGCTGGG 3' reverse 5' CACAACCTCCTGGTCTGCTCT 3'	172bp	2 to 3
Il10	forward 5' GGTTGCCAAGCCTTATCGGA 3' reverse 5' ACCTGCTCCACTGCCTTGCT 3'	152bp	3 to 4
Tgfb	forward 5' TGACGTCACTGGAGTTGTACGG 3' reverse 5' GCACCATCCATGACATGAACC 3'	127bp	3 to 5

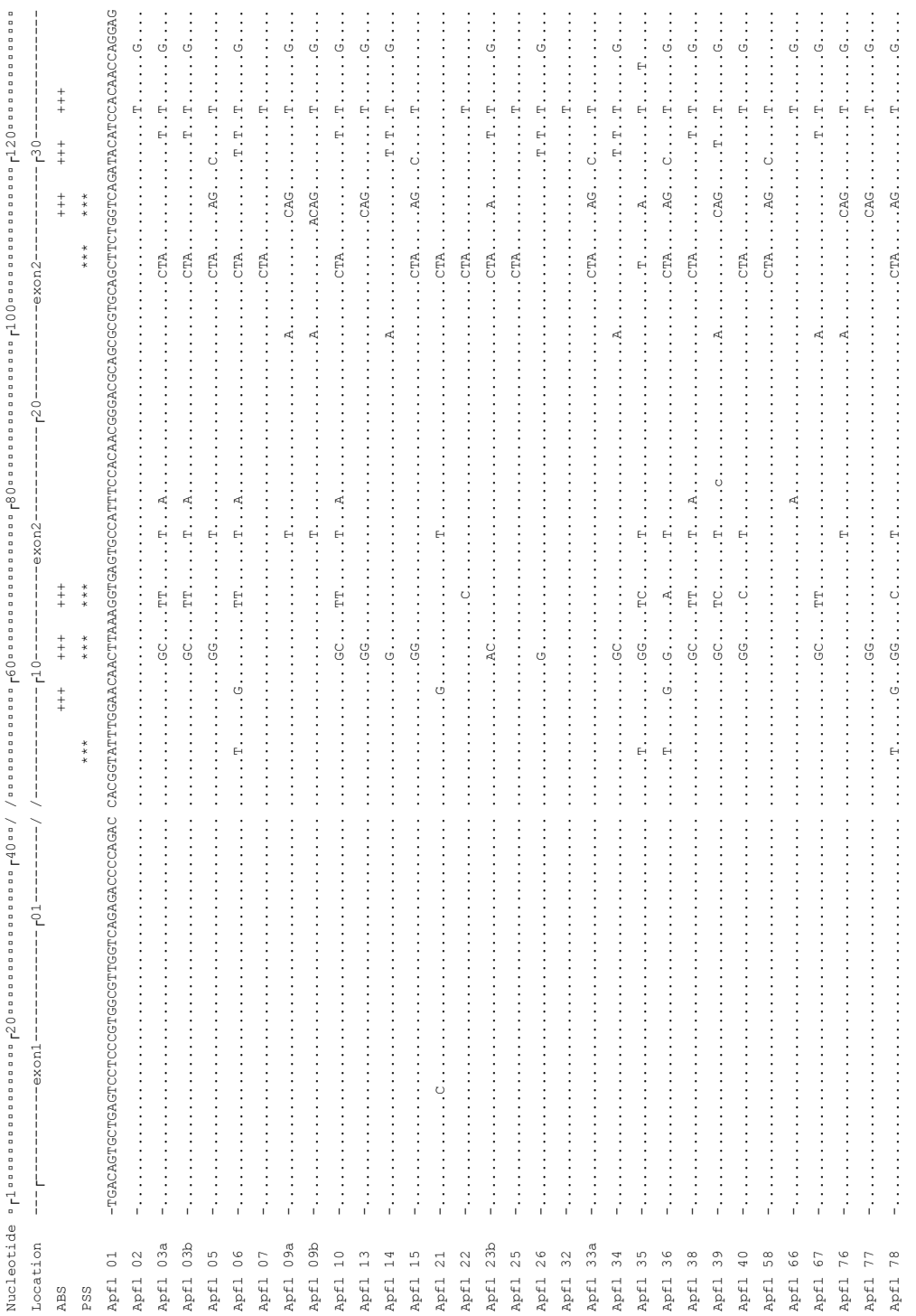
Supplement Article 5

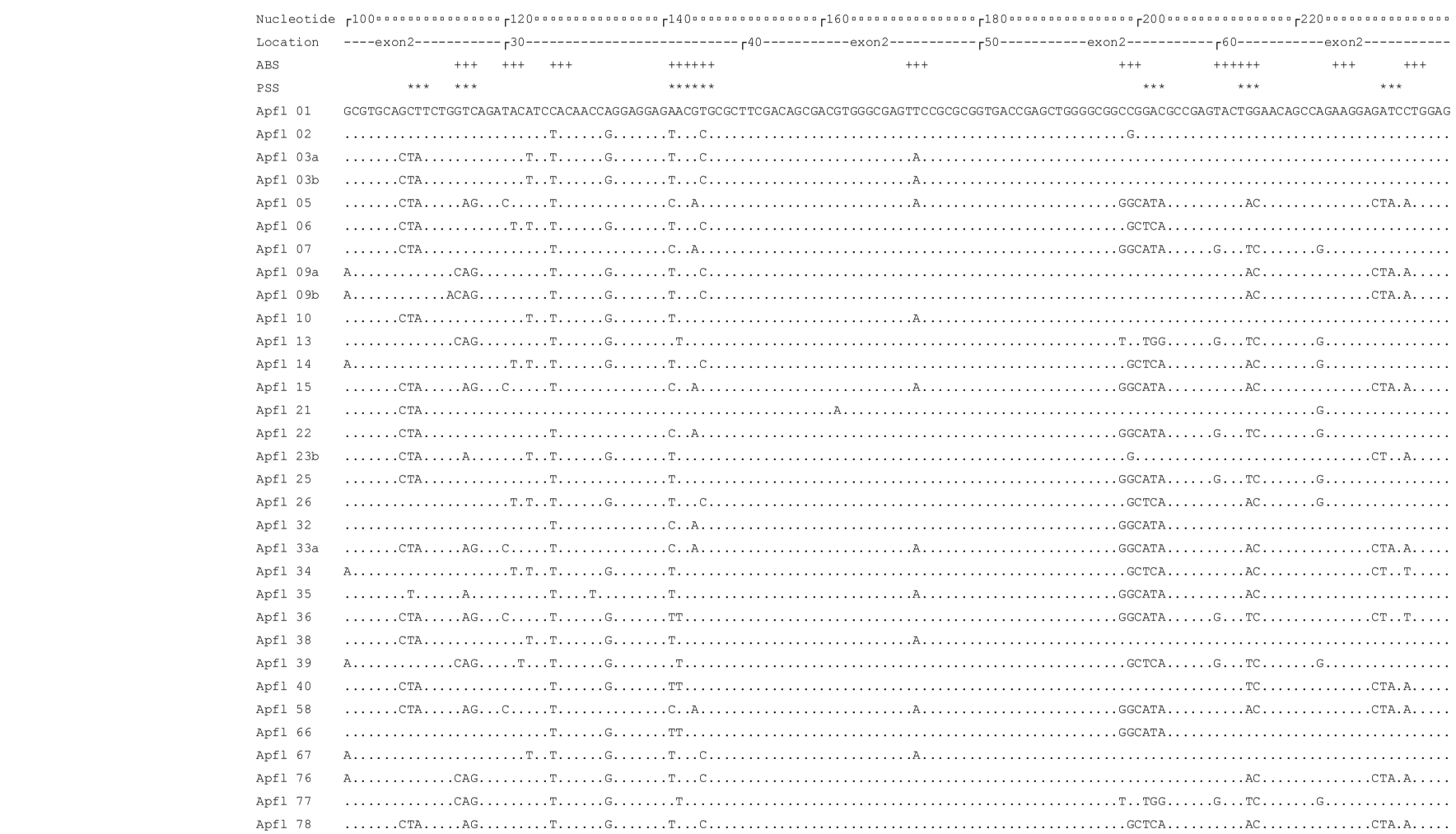


Genes	Sequence (all in 5' -3' direction)	Reference
JA-Apfl-06	GCGGTGACCGAGCTGGG	this study
JA-Apfl-10	GGCAGCTGTGGTCCTGATGC	this study
JA-Apfl-11	GTCAGAGACCCCAGACCACG	this study
Gh46	CCGGATCCTTCGTGTCCCCACAGCACG	Ehrlich and Bugawan 1990
JS2	GCAGACACAACTACGGGATC	Schad *et al.* 2004
JSex3-DRB	AGAGCAGACCAGGAGGTTGTG	Schad *et al.* 2011

Supplement Figure 1: Location and sequences of the different primers used in PCRs for MHC genotyping from gDNA and cDNA, as well as for MHC expression analysis. Arrows indicate the direction of forward and reverse primers and the amplified fragment length is shown. JA-Apfl-11 is spanning the border between the first and the second exon.

Supplement Figure 2: Alignment of 33 MHC DRB alleles (365bp) detected in A. flavicollis sampled in 2008. In the first line the location of the exons 1-3 is shown and the codon numbers are given according to the enumeration based on the mature human DR1 protein (Brown et al. 1993). The second line indicates the antigen-binding sites (ABS) according to the human sequence (Brown et al. 1993) and the third line the species-specific positively selected sites (PSS) identified by CODEML. Dots indicate no nucleotide change to the top sequence. / / exon borders.




Nucleotide ┌100▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌120▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌140▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌160▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌180▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌200▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌220▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫
Location ----exon2-----------┌30----------------------------┌40-----------exon2-----------┌50-----------exon2-----------┌60-----------exon2-----------
ABS +++ +++ +++ ++++++ +++ +++ ++++++ +++ +++
PSS *** *** ****** *** *** ***
Apfl 01 GCGTGCAGCTTCTGGTCAGATACATCCACAACCAGGAGGAGAACGTGCGCTTCGACAGCGACGTGGGCGAGTTCCGCGCGGTGACCGAGCTGGGGCGGCCGGACGCCGAGTACTGGAACAGCCAGAAGGAGATCCTGGAG
Apfl 02 .....T......G.......T...C.....G.....
Apfl 03a .......CTA.....T..T......G.......T...C.....A.....
Apfl 03b .......CTA.....T..T......G.......T...C.....A.....
Apfl 05 .......CTA.....AG...C.....T.....C..A.....A.....GGCATA.....AC.....CTA.A.....
Apfl 06 .......CTA.....T.T..T......G.......T...C.....GCTCA.....
Apfl 07 .......CTA.....T.....C..A.....GGCATA.....G...TC.....G.....
Apfl 09a A.....CAG.....T......G.......T...C.....AC.....CTA.A.....
Apfl 09b A.....ACAG.....T......G.......T...C.....AC.....CTA.A.....
Apfl 10 .......CTA.....T..T......G.......T.....A.....
Apfl 13 .....CAG.....T......G.....T.....T..TGG.....G...TC.....G.....
Apfl 14 A.....T.T..T......G.......T...C.....GCTCA.....AC.....G.....
Apfl 15 .......CTA.....AG...C.....T.....C..A.....A.....GGCATA.....AC.....CTA.A.....
Apfl 21 .......CTA.....A.....G.....
Apfl 22 .......CTA.....T.....C..A.....GGCATA.....G...TC.....G.....
Apfl 23b .......CTA.....A.....T..T......G.......T.....G.....CT..A.....
Apfl 25 .......CTA.....T.....T.....GGCATA.....G...TC.....G.....
Apfl 26 .....T.T..T......G.......T...C.....GCTCA.....AC.....G.....
Apfl 32 .....T.....C..A.....GGCATA.....
Apfl 33a .......CTA.....AG...C.....T.....C..A.....A.....GGCATA.....AC.....CTA.A.....
Apfl 34 A.....T.T..T......G.......T.....GCTCA.....AC.....CT..T.....
Apfl 35 .....T.....A.....T....T.....T.....A.....GGCATA.....AC.....
Apfl 36 .......CTA.....AG...C.....T.....G.....TT.....GGCATA.....G...TC.....CT..T.....
Apfl 38 .......CTA.....T..T......G.......T.....A.....
Apfl 39 A.....CAG.....T...T......G.....T.....GCTCA.....G...TC.....G.....
Apfl 40 .......CTA.....T.....G.....TT.....TC.....CTA.A.....
Apfl 58 .......CTA.....AG...C.....T.....C..A.....A.....GGCATA.....AC.....CTA.A.....
Apfl 66 .....T.....G.....TT.....GGCATA.....
Apfl 67 A.....T..T......G.......T...C.....A.....
Apfl 76 A.....CAG.....T......G.......T...C.....AC.....CTA.A.....
Apfl 77 .....CAG.....T......G.....T.....T..TGG.....G...TC.....G.....
Apfl 78 .......CTA.....AG.....T......G.......T...C.....GCTCA.....AC.....CTA.A.....

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Nucleotide ┌240▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌260▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌280▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌300▫▫▫▫▫▫▫▫▫▫▫▫/ /▫▫▫┌320▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌340▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫▫┌360▫▫
Location   ┌70----------exon2----------┌80----------exon2----------┌90------------/ /-------------┌100----------exon3----------┌110--
ABS        ++++++      +++         +++      ++++++      ++++++
PSS           ***                  ***                  ******   ***                  * **
Apfl 01    CAGAAGCGGGCCGAGGTGGACACGGTGTGCAGACACAACTACGAGATCTTAGATCGCTTCCTAGTGTCGCGGAAAG CTGAGCCCATGGTGACTGTGTACCCCACAAAGACGCAGCCCCTGGAACAC
Apfl 02    .............................................GG...T...G...........C......... ..............................................
Apfl 03a   .G...........................................T...G...........C......... ..............................................
Apfl 03b   .G...........................................C...G...........C......... ..............................................
Apfl 05    .G...........................................C...G...........C......... ..............................................
Apfl 06    .G...........................................C...G...........C......... ..............................................
Apfl 07    .............................................G...CT....C.........C......... ..............................................
Apfl 09a   ...CT........C..........TAC..................G...CT....C.........C......... ..............................................
Apfl 09b   ...CT........C..........TAC..................G...CT....C.........C......... ..............................................
Apfl 10    .G...........................................T...G...........C......... ..............................................
Apfl 13    ...CT........C..........TAC..................GG...T...G...........C......... ..............................................
Apfl 14    ...CT.......................................G...........C......... ..............................................
Apfl 15    .............................................C...G...........C......... ..............................................
Apfl 21    .G...........................................C...G..................... T............................................
Apfl 22    .............................................G...CT....C.........C......... ..............................................
Apfl 23b   ........................TAC.....................................C......... T............................................
Apfl 25    .............................................G...CT....C.........C......... ..............................................
Apfl 26    ...CT.......................................G...........C......... ..............................................
Apfl 32    ........................TAC..................G...CT....C.........C......... ..............................................
Apfl 33a   ....C........C.............................G...CT....C.........C......... ..............................................
Apfl 34    ...CT........C..........TAC..................GG...T...G.................... ..............................................
Apfl 35    G.TGC........C..........TAC..................G...CT....C.........C......... ..............................................
Apfl 36    ...CT........C..........TAC..................G...CT....C.........C......... ..............................................
Apfl 38    .G...........C.....................................T...G...........C......... ..............................................
Apfl 39    ...CT........C..........TAC..................GG...T...G...........C......... ..............................................
Apfl 40    ...CT.......................................CT....C.........C......... ..............................................
Apfl 58    .G...........C..........TAC.....................C...G...........C......... ..............................................
Apfl 66    ........................C..................G...CT....C..................... ..............................................
Apfl 67    .G...........................................T...G...........C......... ..............................................
Apfl 76    ...CT........C..........TAC..................G...CT....C.........C......... T............................................
Apfl 77    ...CT........C..........TAC.....................................C......... ..............................................
Apfl 78    ....C........C..........TAC......T...................G...........C......... ..............................................
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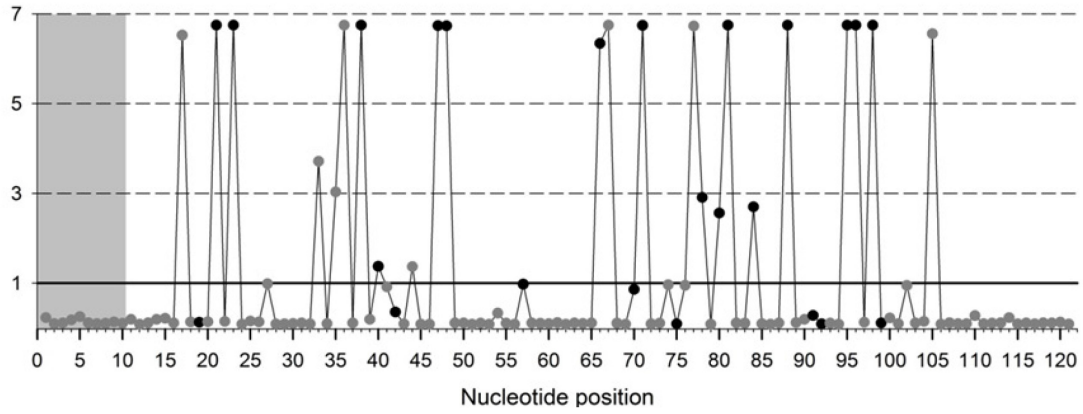
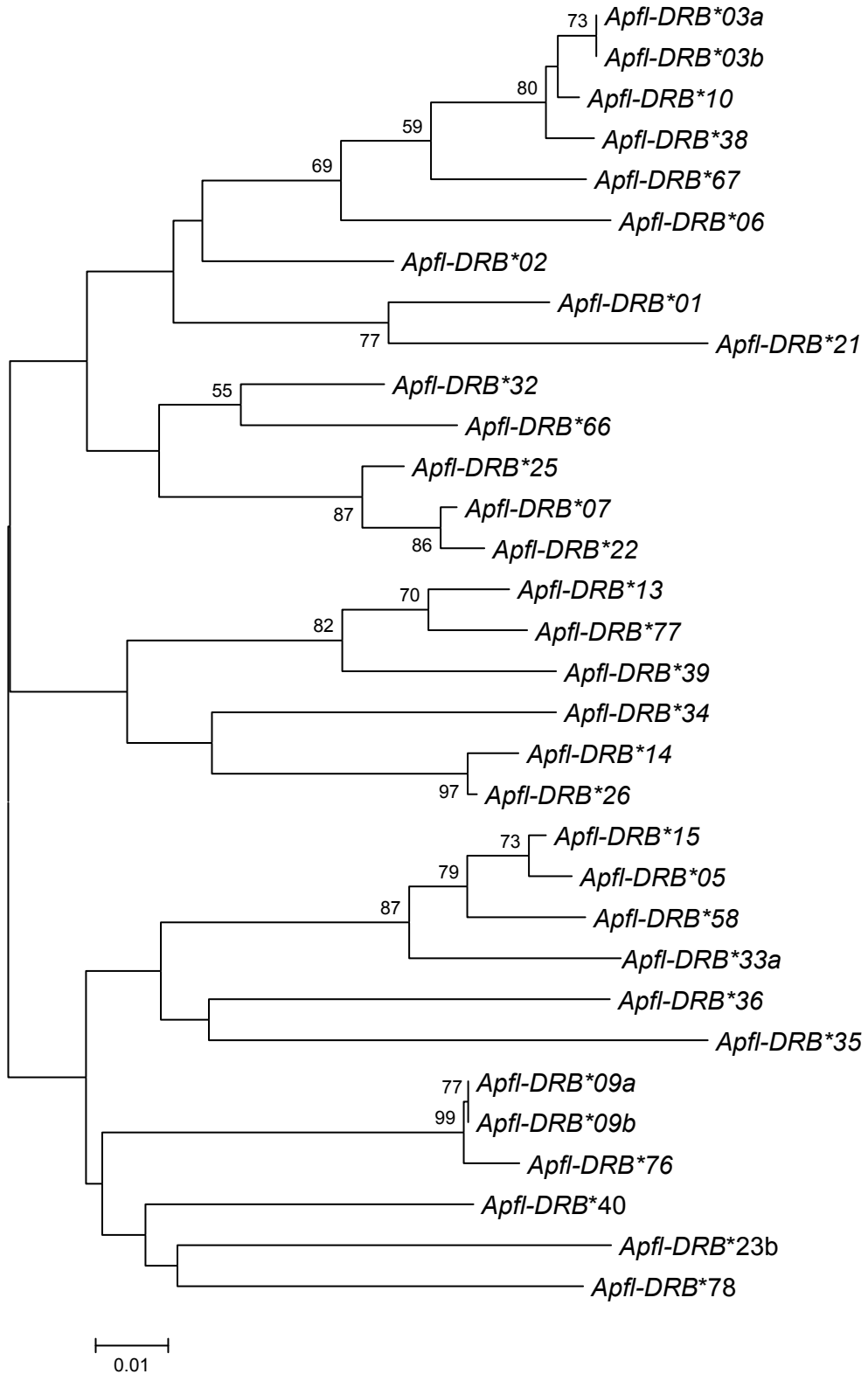


Supplement Figure 3: Ratio (ω) of non-synonymous versus synonymous substitutions in MHC DRB exon1 to exon3 (365bp). The second exon exerts from codon position 15 to position 105. All sites having a ω > 5 were significant according to CODEML analyses except for position 65. Black dots mark the residues whose side chains are included in the antigen binding area of the human DR gene (Brown *et al.* 1993). The shaded area is not part of the mature protein.



Supplement Figure 4: Neighbour joining tree of the observed MHC DRB alleles in *A. flavicollis* collected in 2008 based on the amino acid distance. Bootstrap values > 50 are displayed (1000 replicates).

Supplement Table 2: Estimates of non-synonymous (d_N) and synonymous (d_S) substitutions (± standard error, SE) and their ratio ω analysed over all 121 codon positions and separately for the antigen binding sites (ABS) as well as for the non-ABS (inferred from human DR1 molecule (Brown *et al.* 1993)).

	N	d_N	SE (d_N)	d_S	SE (d_S)	ω	P
all	121	0.086	± 0.017	0.031	± 0.011	2.76	< 0.001
ABS	24	0.346	± 0.075	0.111	± 0.050	3.10	< 0.003
non-ABS	97	0.034	± 0.010	0.014	± 0.009	2.35	0.057

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