Does variability matter? Major histocompatibility complex (MHC) variation and its associations to parasitism in natural small mammal populations

Ph.D. Thesis
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Does variability matter? Major histocompatibility complex (MHC) variation and its associations to parasitism in natural small mammal populations

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Preface

In my Ph.D. thesis I have studied adaptive genetic variation at the major histocompatibility complex (MHC) in different small mammal populations and assessed its relevance in pathogen resistance in the natural environment. My thesis comprises three article manuscripts, which can be read independently. The first article is published, while the remaining two are submitted to international peer-reviewed scientific journals. Because all articles include co-authors, they are written in the first person plural. Each article summary contains a declaration of the authors' individual inputs to highlight my own independent contributions.

Summary

The adaptive evolutionary potential of a species or population to cope with omnipresent environmental challenges is based on its genetic variation. Variability at immune genes, such as the major histocompatibility complex (MHC) genes, is assumed to be a very powerful and effective tool to keep pace with diverse and rapidly evolving pathogens. In my thesis, I studied natural levels of variation at the MHC genes, which have a key role in immune defence, and parasite burden in different small mammal species. I assessed the importance of MHC variation for parasite burden in small mammal populations in their natural environment. To understand the processes shaping different patterns of MHC variation I focused on evidence of selection through pathogens upon the host. Further, I addressed the issue of low MHC diversity in populations or species, which could potentially arise as a result from habitat fragmentation and isolation.

Despite their key role in the mammalian evolution the marsupial MHC has been rarely investigated. Studies on primarily captive or laboratory bred individuals indicated very little or even no polymorphism at the marsupial MHC class II genes. However, natural levels of marsupial MHC diversity and selection are unknown to date as studies on wild populations are virtually absent. I investigated MHC II variation in two Neotropical marsupial species endemic to the threatened Brazilian Atlantic Forest (*Gracilinanus microtarsus, Marmosops incanus*) to test whether the predicted low marsupial MHC class II polymorphism proves to be true under natural conditions. For the first time in marsupials I confirmed characteristics of MHC selection that were so far only known from eutherian mammals, birds, and fish: Positive selection on specific codon sites, recombination, and trans-species polymorphism. Beyond that, the two marsupial species revealed considerable differences in their MHC class II diversity. Diversity was rather low in *M. incanus* but tenfold higher in *G. microtarsus*, disproving the predicted general low marsupial MHC class II variation.

As pathogens are believed to be very powerful drivers of MHC diversity, I studied parasite burden in both host species to understand the reasons for the remarkable differences in MHC diversity. In both marsupial species specific MHC class II variants were associated to either high or low parasite load highlighting the importance of the marsupial MHC class II in pathogen defence. I developed two alternative scenarios with regard to MHC variation, parasite load, and parasite diversity. In the '*evolutionary equilibrium*' scenario I assumed the species with low MHC diversity, *M. incanus*, to be under relaxed pathogenic selection and expected low parasite diversity. Alternatively, low MHC diversity

could be the result of a recent loss of genetic variation by means of a genetic bottleneck event. Under this '*unbalanced situation*' scenario, I assumed a high parasite burden in *M. incanus* due to a lack of resistance alleles. Parasitological results clearly reject the first scenario and point to the second scenario, as *M. incanus* is distinctly higher parasitised but parasite diversity is relatively equal compared to *G. microtarsus.* Hence, I suggest that the parasite load in *M. incanus* is rather the consequence than the cause for its low MHC diversity.

MHC variation and its associations to parasite burden have been typically studied within single populations but MHC variation between populations was rarely taken into account. To gain scientific insight on this issue, I chose a common European rodent species. In the yellow necked mouse (*Apodemus flavicollis*), I investigated the effects of genetic diversity on parasite load not on the individual but on the population level. I included populations, which possess different levels of variation at the MHC as well as at neutrally evolving genetic markers (microsatellites). I was able to show that mouse populations with a high MHC allele diversity are better armed against high parasite burdens highlighting the significance of adaptive genetic diversity in the field of conservation genetics. An individual itself will not directly benefit from its population's large MHC allele pool in terms of parasite resistance. But confronted with the multitude of pathogens present in the wild a population with a large MHC allele reservoir is more likely to possess individuals with resistance alleles. These results deepen our understanding of the complex causes and processes of evolutionary adaptations between hosts and pathogens.

Zusammenfassung

In einer sich ständig verändernden Umwelt ist es unverzichtbar, sich fortwährend zu verändern und anzupassen. Dabei gründet sich das Anpassungsvermögen oder das evolutionäre Potential einer Art auf ihre genetische Variabilität. In der Krankheitsabwehr ist die Variabilität der Immungene ein besonders wichtiges und effektives Instrument, weil Pathogene sehr vielfältig sind und schnell evolvieren. Im Rahmen meiner Doktorarbeit habe ich mich mit der Variabilität des Immungen-Komplexes MHC (major histocompatibility complex) beschäftigt, der eine Schlüsselrolle in der Immunabwehr bei Vertebraten einnimmt. Anhand verschiedener Arten und Populationen von Kleinsäugern habe ich den Einfluss der MHC Vielfalt auf den Parasitenbefall unter natürlichen Bedingungen untersucht. Dabei interessierte mich insbesondere das Vorkommen geringer MHC Variabilität in Populationen, das möglicherweise eine Folge von Lebensraum-fragmentierung und Isolation ist.

Obwohl Beuteltiere eine zentrale Rolle in der Evolution der Säugetiere spielen, ist über ihren MHC bislang nur sehr wenig bekannt. Einige Studien befassten sich mit Labor- oder Zootieren, und deuteten auf geringe oder sogar gar keine Variation im MHC Klasse II bei Beuteltieren hin. Allerdings gab es bislang nahezu keine Studien an frei lebenden Beuteltieren, deshalb war bislang ein natürliches Ausmaß der MHC Variabilität unbekannt. Anhand von zwei endemischen neotropischen Beuteltieren aus dem brasilianischen Küstenregenwald (*Gracilinanus microtarsus, Marmosops incanus*) habe ich überprüft, ob sich diese geringe MHC Vielfalt unter natürlichen Freilandbedingungen bestätigt. Erstmals konnte ich zeigen, dass der MHC II bei Beuteltieren charakteristische Merkmale positiver Selektion aufweist, die bisher nur von placentalen Säugern, Vögeln und Fischen bekannt waren: Positive Selektion auf spezifischen Aminosäurepositionen, Rekombination und Trans-Species-Polymorphismus. Darüber hinaus unterschieden sich die beiden Beuteltierarten beträchtlich in ihrer MHC II Variabilität. Während *M. incanus* sich als relativ wenig divers erwies, zeigte *G. microtarsus* eine zehnmal höhere Vielfalt und widerlegt damit die generelle Gültigkeit der ursprünglich angenommenen geringen MHC II Variabilität bei Beuteltieren.

Um diese beachtlichen Diversitätsunterschiede zwischen den beiden Arten zu erklären, habe ich die Parasitenbelastung untersucht. Bei beiden Arten konnte ich nachweisen, dass bestimmte MHC Varianten mit entweder hoher oder niedriger Parasitenbelastung verknüpft waren. Solche Assoziationen spiegeln Pathogen-vermittelte

Selektion wider, untermauern die Funktionalität des MHC Klasse II bei Beuteltieren und weisen auf dieselbe Bedeutsamkeit des MHC wie bei placentalen Säuger, Vögeln und Fischen hin. Ich entwickelte zwei alternative evolutionäre Szenarien, unter denen eine geringe MHC Variabilität denkbar ist. Im Szenario des '*evolutionären Gleichgewichts*' ist geringe MHC Variabilität die Folge eines verminderten Selektionsdruckes durch wenige Parasiten, sodass eine geringe Parasitendiversität zu erwarten ist. Alternativ könnte eine geringe MHC Variabilität aber auch Folge eines kürzlich erlittenen Verlustes an genetischer Variabilität sein, beispielsweise durch ein Flaschenhalsereignis. Unter diesem Szenario des '*Ungleichgewichts'* wäre bei *M. incanus* im Falle eines potentiellen Verlustes von Resistenzallelen eine starke Parasitenbelastung zu erwarten. Die parasitologischen Ergebnisse widersprechen dem ersten und deuten eher auf das zweite Szenario. *M. incanus* war deutlich stärker parasitiert als *G. microtarsus*, wohingegen die Parasitendiversität bei beiden Arten ungefähr gleich war. Die hohe Parasitenbelastung bei *M. incanus* ist offenbar weniger der Auslöser als vielmehr eine Folge seiner geringen MHC Vielfalt zu sein.

Üblicherweise werden sowohl die Variabilität des MHC als auch seine Verknüpfung mit Parasitenbelastung innerhalb von einzelnen Populationen untersucht, nur selten wird die Variation zwischen Populationen in Betracht gezogen. Um Erkenntnisse auf dieser Ebene zu gewinnen, habe ich den Zusammenhang zwischen genetischer Vielfalt und Parasitenbelastung nicht auf der Ebene des Individuums, sondern auf der Populationsebene anhand der europäischen Gelbhalsmaus (*Apodemus flavicollis*) erforscht. Dabei wurden Populationen mit unterschiedlicher genetischer Variabilität am MHC und an neutralen genetischen Markern (Mikrosatelliten) betrachtet. Ich konnte nachweisen, dass Populationen, die über ein großes Spektrum verschiedener MHC Allele verfügen, besser gegen starke Parasitenbelastung gewappnet sind als Populationen mit einer geringen Anzahl MHC Allele. In einer MHC-diversen Population ist die Gegenwart von Individuen mit Resistenzallelen deutlich wahrscheinlicher, und damit die Überlebenswahrscheinlichkeit der Population. Diese Ergebnisse erweitern und vertiefen unsere Erkenntnisse zu die komplexen evolutionären Vorgängen und Mechanismen zwischen Wirt und Parasit in ihrem fortwährenden Wettstreit.

Introduction

Genetic Variation, Pathogens, and Selection

In a continuously changing world genetic variation is crucial to cope with omnipresent environmental challenges (Frankham *et al.* 2002). The polymorphic variety of traits in a population or a species is the essential base for evolutionary adaptation processes. Genetic variation confers the flexibility to keep pace in the evolutionary race and to avoid dropping behind competing organisms (the 'Red Queen' hypothesis, Van Valen 1973). The ultimate source of all genetic variation is random and spontaneous mutation, which is thereupon shaped by the dynamic forces of natural selection. In the long term, natural selection creates an adaptive evolutionary potential in a species or population that enables it to buffer and respond to further environmental challenges (Altizer *et al.* 2003; Frankham *et al.* 2002).

Pathogens[1] are universal evolutionary forces that have the potential to drive rapid changes in the genetic composition of their hosts (Altizer *et al.* 2003; Haldane 1949; Spielman *et al.* 2004). They are able to control host populations in size and demography analogue to the impact of predators or resource limitation (Anderson & May 1979). Further, they are extremely rapidly evolving, so that in terms of pathogen defence genetic variation is of particular importance (Altizer *et al.* 2003; O'Brien & Evermann 1988). The resistance to pathogens is expected to be increased in a genetically diverse population (Frankham *et al.* 2002). High levels of genetic diversity enhance the probability of at least some protective alleles to be present in the host population and hence allow dealing with coevolving pathogens (Hedrick 2001; Liersch & Schmid-Hempel 1998; O'Brien & Evermann 1988). In contrast, populations that have lost genetic diversity, e.g. due to inbreeding and genetic drift, are expected to suffer more seriously from diseases than those with high genetic variation (Frankham *et al.* 2002). In genetically uniform host populations pathogens may spread easily and quickly because most hosts share the same resistance genotype (Meagher 1999; Spielman *et al.* 2004). Moreover, in inbred populations recessive deleterious mutations are more likely to be expressed due to an increased proportion of homozygotes (Keller & Waller 2002). A number of studies empirically support this scenario by showing associations between low levels of genetic variation and increased pathogen

[1] I use the terms 'pathogen' and 'parasite' synonymously, and include both microparasites (viruses, fungi, bacteria, protozoa) and macroparasites (helminths, arthropods), which can cause a reduction in host fitness (Anderson & May 1979).

susceptibility (e.g. Acevedo-Whitehouse *et al.* 2003, 2006; Cassinello *et al.* 2001; Coltman *et al.* 1999; Liersch & Schmid-Hempel 1998; Meagher 1999; Spielman *et al.* 2004), reduced immune function (Reid *et al.* 2003; Sanjayan *et al.* 1996; Whiteman *et al.* 2006), and severe disease progression (Acevedo-Whitehouse *et al.* 2005; Dorman *et al.* 2004).

The variation at a specific genetic marker may affect the host's fitness in three ways: generally (genome-wide), by linkage, or directly (summarised in Hansson & Westerberg 2002). Neutrally evolving genetic markers, such a microsatellites, are commonly applied in population genetic studies to asses the genetic diversity. Significant associations between microsatellite heterozygosity and fitness related traits have been reported for several species (e.g. Acevedo-Whitehouse *et al.* 2003; Amos *et al.* 2001; Coltman *et al.* 1999; Slate *et al.* 2000). These correlations are explained by either general or local effects: heterozygosity at a set of markers might reflect genome-wide genetic variation or the markers could be positioned in local chromosomal vicinity and therefore in linkage disequilibrium to fitness relevant loci. However, direct influence of genetic variation on fitness is reflected by another set of genetic markers: only coding markers subjected to natural selection are capable to picture evolutionary relevant and adaptive processes (Sommer 2005).

Major Histocompatibility Complex

In the context of pathogen defence variation at the immune genes of the major histocompatibility complex (MHC) may be especially important. The MHC is a multigene family that controls immunological self/non-self recognition and plays a central role in the adaptive immune response of vertebrates. MHC molecules bind peptide fragments that derive from pathogens and display them on the cell surface for recognition by appropriate T-cells (Klein 1986; Murphy *et al.* 2008). Only the combination of an antigen and a MHC molecule can be recognised by the T-cell receptors. The antigens can derive from pathogens from the inside of cells, such as viruses, or from pathogens or their products internalised by endocytosis through immunocompetent cells, such as bacteria or helminths. Mainly, there are two classes of MHC molecules - class I and class II - which differ in both their structure and expression pattern in the tissues (Murphy *et al.* 2008). MHC class I molecules are expressed on the surface of all nucleated somatic cells and present mainly intracellular derived antigens. MHC class II molecules, on the other hand, are only expressed on specialised antigen-presenting cells like B-cells and macrophages and present predominantly extracellular derived antigens like bacteria or helminths (Hughes &

Yeager 1998b; Klein & Horejsi 1997). The genes of the MHC show an extraordinarily high polymorphism in most vertebrates studied to date (Bernatchez & Landry 2003; Piertney & Oliver 2006; Sommer 2005). This high variability is especially pronounced in the regions that are directly involved in the antigen binding process, the so-called peptide binding region or antigen recognition site (Hughes & Nei 1988, 1989). The leading, non-exclusive hypotheses to explain MHC polymorphism are (a) selection driven by the necessity to recognise a wide array of pathogens and (b) disassortative mating preferences (Apanius *et al.* 1997; Penn & Potts 1999). The high MHC diversity is maintained by forms of balancing selection, i.e. selection that retains alleles in relatively intermediate frequencies, resulting in polymorphism (Apanius *et al.* 1997; Hedrick 1994; Klein 1986; Sommer 2005). In the mode of pathogen-driven selection, heterozygotes could have a selective advantage over homozygote individuals due to their larger number of MHC variants to recognise and combat parasite antigens (‘heterozygote advantage’, Doherty & Zinkernagel 1975). Pathogen-driven selection could also act via the advantage of specific MHC alleles, which could be temporarily protective depending on their frequency in the population ('frequency-dependent selection', Takahata & Nei 1990). In this scenario, escaping the common host alleles is the primary goal of parasite evolution. This gives a disadvantage to common host alleles and an advantage to rare alleles, which then will rise in frequency on their part (May & Anderson 1990). Moreover, selection may be dynamic and vary over time and space and thereby continuously result in polymorphism (Hedrick 2002; Meyer & Thompson 2001). Studies in wild populations detected associations between MHC variants and high or low parasite loads and thus mirror the classical pattern of pathogen-driven selection acting on the MHC (e.g. Axtner & Sommer 2007; Deter *et al.* 2008; Froeschke & Sommer 2005; Harf & Sommer 2005; Meyer-Lucht & Sommer 2005; Paterson *et al.* 1998; Schad *et al.* 2005). Their key function in the immune response and their characteristic extraordinary polymorphism place the genes of the MHC among the best markers to study molecular adaptation (Sommer 2005) as genetic diversity at the MHC is directly related to a species’ survival (Frankham *et al.* 2002).

In theory, in a long-term shared evolutionary relationship a diverse pathogen array will induce high MHC variation in the host species or population, whereas low pathogenic diversity will relax the selection pressure on the MHC and result in lower variation. This holds true in the state of an evolutionary equilibrium among host and pathogen. Studies on different human populations worldwide (Prugnolle *et al.* 2005), stickleback populations from different habitats (Wegner *et al.* 2003a), and different rodent species (Goüy de Bellocq *et al.* 2008) confirmed these relations empirically: all studies revealed higher MHC variation in populations or species, respectively, exposed to a high pathogen diversity compared to

such confronted with fewer pathogens. On the other hand, pathogens do exhibit the potential to cause serious population declines once host genetic diversity is lost. In such unbalanced situations, pathogens may even pose a severe extinction risk on small populations that have lost their ability to buffer challenges (Altizer *et al.* 2003, 2007; McCallum & Dobson 1995; Woodroffe 1999). A prominent example is the recent case of the contagious tumour (devil facial tumour disease), which is currently placing the Tasmanian devil (*Sarcophilus harrisii*) under threat of extinction (Siddle *et al.* 2007a). Presumably a monomorphism at the MHC class I is accounting for the easy spread of this disease, as a result of a bottleneck event (Siddle *et al.* 2007a, b).

There is broad knowledge on the naturally high levels of MHC class II diversity in populations of eutherian mammals, birds, and fish. In marsupials, in contrast, it was suggested that very little polymorphism if not indeed monomorphism at the MHC class II was characteristic (Stone *et al.* 1996, 1998). This assumption was based on experiments within marsupial cellular immunology (Infante *et al.* 1991; Stone *et al.* 1996, 1998) and directly in immunogenetic studies that revealed very low levels of variation in a few captive or laboratory bred individuals (Lam *et al.* 2001; McKenzie & Cooper 1994; Schneider *et al.* 1991; Siddle *et al.* 2007a, b; Stone *et al.* 1999). Considering the extraordinary polymorphism in the majority of vertebrate species, is it possible that this low marsupial MHC II polymorphism really holds true for natural populations?

Helminth Parasitism

Gastrointestinal helminths are well suited candidates to study pathogen-driven selection on the host and are considered as suitable indicators of the host's health status. Gastrointestinal helminths are parasitic worms comprising nematodes, cestodes, trematodes, and acanthocephalans (Hudson & Dobson 1995) that inhabit the host's gastrointestinal tract and release reproductive units into their host's faeces. They have significant impact on the energetic demands, fitness and mortality in both, livestock (Coyne & Smith 1994) as well as wild host species (Albon *et al.* 2002; Gulland 1992; Stien *et al.* 2002; Tompkins & Begon 1999). Also, in small mammal populations helminths are widespread, abundant, and have a strong effect on survival and fecundity (Georgiev *et al.* 2006; Morand *et al.* 2006). The host immune activity rarely completely eliminates helminth infections but controls and maintains them at a low level of infection (Weil *et al.* 2006). Thus, their impact on the host is usually not temporary but rather long-lasting. Helminth burden was investigated by counting the worm eggs in the host's faeces using a

modification of the non-invasive McMaster flotation technique (Gordon & Whitlock 1939; Meyer-Lucht & Sommer 2005). Faecal egg counts (number of eggs per gram faeces) reflect the overall worm burden as comparisons between host dissections and faecal egg counts revealed correlations between the number of nematode eggs counted and the number of adult worms inhabiting the guts (Froeschke & Sommer, in prep.; Seivwright *et al.* 2004; Stear *et al.* 1995). Because of the non-invasive sampling procedure faecal egg counts are a widely used approach in field studies and studies on rare or threatened species (e.g. Cassinello *et al.* 2001; Coltman *et al.* 1999; Ferrari *et al.* 2004; Froeschke & Sommer 2005; Harf & Sommer 2005; Meyer-Lucht & Sommer 2005; Schad *et al.* 2005; Schwensow *et al.* 2007; Seivwright *et al.* 2004).

Study Aims

The overall aim of my study was to describe the adaptive genetic variation at the immune gene complex MHC and to assess the importance of different levels of MHC variation for parasite burden in the natural environment. To understand the processes shaping different patterns of MHC variation I focused on selection through pathogens upon the host in different small mammal species and populations. Especially, I addressed the issue of low MHC diversity in populations or species, which could potentially arise as a result from habitat fragmentation and isolation.

I studied two species of Neotropical marsupials, which are endemic from the Atlantic forest (*Gracilinanus microtarsus* WAGNER, 1842 and *Marmosops incanus* LUND, 1840). To test whether the predicted low levels of marsupial MHC II diversity are of general validity, I examined MHC II variation in these two species under natural conditions. For the first time in marsupials I studied characteristics of MHC selection that were so far only known from eutherian mammals, birds, and fish. By comparison between these two species I included the effects of different ecological demands as *M. incanus* is assumed to be more sensitive to habitat fragmentation compared to *G. microtarsus*. I investigated the pathogen burden with gastrointestinal parasites to explain the different patterns of MHC variation I found in the two mouse opossum species. I developed and tested two alternative evolutionary scenarios with regard to MHC variation, helminth load, and helminth diversity. Moreover, I examined functional associations between individual MHC variants and parasite load to demonstrate the functional importance of MHC class II in marsupials.

For another aspect of my thesis, I chose a European rodent species, the yellow necked mouse (*Apodemus flavicollis* MELCHIOR, 1834). It is one of the few small mammal

species in which the MHC class II DRB was not found to be duplicated but instead had a single locus (Meyer-Lucht & Sommer 2005; Musolf *et al.* 2004). This feature allows more accurate analyses and interpretations, for instance, heterozygosity calculations. I analysed the effects of population-wide genetic diversity on parasite load. For this purpose I included mouse populations, which possess different levels of variation at the MHC class II as well as at neutrally evolving genetic markers (microsatellites). I tested the prediction that host populations with high MHC diversity are better armed against parasite infections than populations with low MHC diversity.

This thesis shall increase the understanding of the importance of MHC diversity in natural mammal populations and draw conclusions on the causes and consequences of diverse patterns of MHC variation. The key questions can be phrased as follows:

1. Are the predicted general low levels of marsupial MHC class II polymorphism confirmed in wild marsupial populations under natural conditions?

2. Are there characteristic signs of positive selection on the marsupial MHC class II emphasising its functional importance?

3. Is there indication for pathogen-driven selection on the marsupial MHC class II? Are there functional associations between certain MHC variants and pathogen load?

4. Do the studied marsupial species differ in their burden with gastrointestinal helminths?

5. What may be the causes and consequences of low MHC diversity?

6. Are there effects of population-wide genetic diversity on the parasite load? If so, which genetic indicators are influential?

Study Framework and Study Species

The German-Brazilian Research Project

This study is part of the cooperation project 'BioCAPSP - Biodiversity conservation in fragmented landscapes at the Atlantic Plateau of São Paulo', which forms part of the German-Brazilian scientific joint program 'Science and Technology for the Mata Atlântica' funded by the German Ministry of Science and Education (BMBF) and the Brazilian National Council for Scientific and Technological Development (CNPq). In an interdisciplinary approach the project aims to develop strategies and action plans for conservation, sustainable management and use of the residual Brazilian Atlantic Forest. The scientific results should contribute to improve the efficiency of biodiversity protection in the Mata Atlântica. Within this project, comparative studies on the ecology, immunogenetics, and parasitology are conducted in small mammal populations inhabiting fragmented landscapes.

The coastal Atlantic Forest of Brazil (Mata Atlântica) is one of the most threatened biomes in the world (Myers *et al.* 2000). At the same time, it still harbours an extraordinary concentration of endemic species including more than 8000 species of vascular plants, amphibians, reptiles, birds, and mammals. It has been classified as one of the five most important biodiversity hotspots for conservation priorities (Myers *et al.* 2000). The Mata Atlântica once ranged almost continuously along the Brazilian coast covering more than 1.5 million km^2, while today only 12% of its original extent remains (Ribeiro *et al.* 2009; SOS Mata Atlântica *et al.* 2008). Direct causes for the massive habitat loss are human agricultural activities and overexploitation of forest resources throughout the last few centuries (Dean 1995). Along with ongoing habitat loss the remaining forest patches of the Atlantic Forest are continuously being fragmented and degraded (Tabarelli *et al.* 2004, 2005; Teixeira *et al.* 2009). In the course of forest fragmentation populations from the residual habitat islands are becoming increasingly restricted and isolated. After migration between fragments is reduced or entirely lost, the isolated populations might be limited to a small effective population size. The loss of genetic diversity through inbreeding or genetic drift becomes very probable in such populations (Saccheri *et al.* 1998).

Study Species

The Marsupials *Gracilinanus microtarsus* and *Marmosops incanus*

In my study I focussed on the two Neotropical marsupial species *Gracilinanus microtarsus* (Brazilian gracile mouse opossum, Fig. 1) and *Marmosops incanus* (gray slender mouse opossum, Fig. 2). Both species are endemic to the Brazilian Atlantic Forest and belong to the largest American family of marsupials, the Didelphidae. *G. microtarsus* is smaller (females ø 24.2 g, males ø 29.3 g) than *M. incanus* (females ø 32.5 g, males ø 41.7 g) and mainly arboreal using the canopy (Vieira & Monteiro-Filho 2003). *M. incanus* more frequently uses the understorey and the forest ground (Cunha & Vieira 2002). Like all didelphid marsupials, both species are nocturnal and solitary (Caceres 2004). They are omnivorous (Fonseca & Kierulff 1989; Martins & Bonato 2004) and show a remarkable mating behaviour, which is very unique for mammals but also known for Australian marsupials: Semelparity (Lorini *et al.* 1994; Martins *et al.* 2006). Males in both species contribute to only one mating season with multiple copulations and die afterwards due to stress. Females can survive and reproduce for a second or a third season.

Figure 1: *Gracilinanus microtarsus,* the Brazilian gracile mouse opossum

Figure 2: *Marmosops incanus*, the gray slender mouse opossum (Photo: Thomas Püttker)

Ecological studies revealed that *M. incanus* may be more sensitive to habitat fragmentation than *G. microtarsus*. *M. incanus* is restricted to native vegetation forest, prefers undisturbed forest with dense canopy cover and is less abundant in small and isolated fragments. In contrast, *G. microtarsus* does

not seem to be strongly affected by fragmentation of the forest habitat: It is not less abundant in small or isolated fragments, even prefers vegetation of disturbed forest and has even been captured in eucalyptus plantations (Pardini *et al.* 2005; Püttker *et al.* 2008; Umetsu & Pardini 2007).

The Rodent *Apodemus flavicollis*

Apodemus flavicollis (yellow necked mouse, Fig. 3) belongs to the family Muridae and is very common in European deciduous and mixed forests (Bergstedt 1965; Jüdes 1979). It is omnivorous and predominantly active at dawn and night (Niethammer 1978). It is closely related to common laboratory model organisms like the house mouse (*Mus musculus*) or the black rat (*Rattus rattus*). As most of our current knowledge on immunogenetic mechanisms comes from laboratory studies on these organisms, *A. flavicollis* is a promising candidate species to study immunogenetic processes in natural populations. Moreover, together with its sister species *A. sylvaticus,* the yellow necked mouse is a very well investigated wildlife murid model in terms of parasitology (e.g. Abu-Madi *et al.* 2000; Behnke *et al.* 1999, 2001a, 2005; Brown *et al.* 1994; Ferrari *et al.* 2004; Klimpel *et al.* 2007; Montgomery & Montgomery 1988).

Figure 3: *Apodemus flavicollis*, the yellow necked mouse

In this species I found populations, which differ in their genetic variation, which was a necessary prerequisite to study the effects of population-wide genetic diversity. Although common species like the yellow necked mouse are usually little relevant in research questions in the field of conservation genetics, they are of course also affected by isolation of their habitat and associated loss of genetic diversity. Thus, they represent helpful models to investigate causes and consequences of different levels of genetic diversity.

Article Summaries

Article 1

YVONNE MEYER-LUCHT, CELINE OTTEN, THOMAS PÜTTKER AND SIMONE SOMMER

Selection, diversity and evolutionary patterns of the MHC class II DAB in free-ranging Neotropical marsupials

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Marsupials hold a major position in the mammalian evolution yet the marsupial MHC has been scarcely investigated. Studies on primarily captive or laboratory bred individuals indicated very little or even no variation in the marsupial MHC class II genes. To date, natural levels of marsupial MHC diversity and selection are unknown as studies on wild populations are virtually absent.

Here, I investigated MHC class II variation in two species of free-ranging Neotropical mouse opossums, *Gracilinanus microtarsus* and *Marmosops incanus.* I tested whether the predicted low marsupial MHC class II polymorphism proves true under natural conditions. Further, I studied typical features of selection on the MHC known from eutherian mammals, birds, and fish and traced the evolutionary history of MHC lineages within the group of marsupials.

In 54 *G. microtarsus* and 56 *M. incanus* individuals I examined a 195 bp fragment of the MHC class II DAB gene. This fragment codes for a large part of the ß1 domain and is analogous to that part of the human DR1 molecule, which carries the majority of antigen binding sites (ABS) (Brown *et al.* 1993). For MHC genotyping single strand conformation polymorphism (SSCP) was used in *M. incanus* and molecular cloning in *G. microtarsus*. In both species, the resulting MHC-DAB sequences were tested for positive selection on specific codon sites by applying the program CODEML integrated in PAML 3.15 (Yang 1997). The occurence of recombination events was tested by applying the programs GENECONV (Sawyer 1999) and LDHAT (McVean *et al.* 2002). Further, I constructed a phylogenetic tree from the MHC-DAB sequences including DAB sequences from all additional marsupials studied so far.

G. microtarsus displayed high MHC class II variation, both within and among individuals, with 47 MHC-DAB sequences and high levels of sequence divergence at a

minimum of four loci. In the following I refer to these sequences as 'alleles' although they derived from different loci. Positively selected codon sites were identified of which most were congruent with human antigen binding positions. In contrast, MHC class II diversity in *M. incanus* was rather low (five times reduced) with only eight alleles at a minimum of two loci. Again, positive selection was identified on specific codon sites, all congruent with human ABS and with positively selected sites observed in *G. microtarsus*. In both marsupial species recombination events were confirmed. Intralocus recombination is known to play an important role in the generation of the large allelic polymorphism at MHC loci (Hughes & Yeager 1998a; Parham & Ohta 1996; Richman *et al.* 2003). This was not shown in marsupials before. In a phylogenetic comparison the alleles of *M. incanus* interspersed within the alleles of *G. microtarsus* with four alleles even being shared between species, documenting the presence of trans-species polymorphism (Klein *et al.* 1998). Trans-species polymorphism describes the retention of alleles and allelic lineages across speciation events. It is a typical feature of MHC evolution and an indicator of balancing selection acting in a genetic system (Bernatchez & Landry 2003; Klein 1987; Klein *et al.* 1998).

This study revealed extensive MHC class II polymorphism in a natural marsupial population contrary to previous assumptions. *G. microtarsus* is the first marsupial that shows high MHC class II polymorphism. Furthermore, for the first time in marsupials I confirmed the presence of three characteristic features of MHC selection: positively selected codon sites, recombination and trans-species polymorphism. Beyond that, the two investigated marsupial species revealed considerable differences in their MHC class II diversity, which remain to be explained in future analyses. This is the first study on natural MHC diversity in non-model, free-ranging Neotropical marsupials.

Authors' contributions:

Samples were collected by T. Püttker and me. I designed the primers, carried out the MHC genotyping of G. microtarsus, all statistical analyses, interpretation of the data, and writing of the manuscript. C. Otten performed the MHC genotyping of M. incanus. S. Sommer conceived the study design, initiated its key collaborations, supervised the research, and revised the manuscript.

Article 2

YVONNE MEYER-LUCHT, CELINE OTTEN, THOMAS PÜTTKER, RENATA PARDINI, JEAN PAUL METZGER AND SIMONE SOMMER

Variety matters: adaptive genetic diversity and parasite load in two mouse opossums from the Brazilian Atlantic forest

Conservation Genetics (in review)

Pathogens are believed to be very powerful driving forces of MHC diversity. Here I studied the parasite burden with gastrointestinal helminths in *G. microtarsus* and *M. incanus* to understand the reasons for the distinct differences in MHC diversity, which were revealed in the previous article. I developed two scenarios on parasite burden and MHC diversity to explain the observed patterns:

(1) Low MHC diversity could result from a selection pressure by low parasite diversity. In a long-term coevolution between pathogens and hosts, numerous pathogens will have selected for high MHC polymorphism in the host population. The presence of only few pathogens, on the other hand, will cause low MHC polymorphism in a population (*'Evolutionary equilibrium'* scenario). Assuming this scenario, I expected relative high parasite diversity in the MHC diverse species, *G. microtarsus*, and relative low parasite diversity in the species with low MHC diversity, *M. incanus*.

(2) Low MHC diversity could also be a consequence of a recent loss in genetic diversity due to a bottleneck event. The species with low MHC diversity could have lost resistance alleles or other important parts of its adaptive evolutionary potential ('*Unbalanced situation*' scenario). In such genetically homogeneous populations the spread of pathogens is facilitated. Assuming this scenario, I expected higher parasite loads in terms of prevalence and infection intensity in the species with low MHC diversity, *M. incanus,* than in the MHC diverse species, *G. microtarsus*.

The number of individuals was doubled from the previous work to 102 in *G. microtarsus* and 123 in *M. incanus*, to further verify the differences in MHC class II allelic diversity between the two species. For each individual the parasite burden with gastrointestinal helminths was assessed non-invasively by faecal egg counts. Parasite load was measured as the number of different helminth infections per individual, helminth

prevalence and intensity of infection in an individual. I also recorded parasite diversity, i.e. the number of different helminth morphotypes per species. To investigate the functional importance of the MHC class II in marsupials, I studied the effects of the individual MHC class II constitution - besides environmental and biotic factors - on the parasite load by calculating generalised linear models (GLMs).

Parasite diversity did not markedly differ between the two host species, with thirteen helminth morphotypes in *M. incanus* and eleven in *G. microtarsus*. But I detected clear differences in the parasite load: *M. incanus* showed low MHC DAB diversity and high parasite load, whereas *G. microtarsus* revealed a tenfold higher population wide MHC DAB diversity and lower parasite burden. These results support the second scenario of an unbalanced situation. However, one should keep in mind that low levels of MHC class II diversity were suggested to be a general marsupial characteristic. It therefore might be that not the low diversity in *M. incanus* but the high diversity in *G. microtarsus* is an exception to the rule. The question whether or not low MHC class II diversity is a phylogenetic characteristic of marsupials will not be resolved until substantial knowledge on the marsupial MHC in natural populations is gathered. In both marsupial species specific MHC class II variants were associated to either high or low parasite load, highlighting the importance of the marsupial MHC class II in defence against gastrointestinal helminths.

Authors' contributions:

Samples were collected by T. Püttker and me. C. Otten performed the MHC genotyping of M. incanus and participated in parasitological investigations. I carried out the primer design, the MHC genotyping of G. microtarsus, performed all statistical calculations, interpreted the data, and wrote the manuscript. R. Pardini and J. P. Metzger provided data on the landscape structure of the research area and together with S. Sommer initiated the collaboration project. S. Sommer conceived the study, supervised the research, and revised the manuscript. All co-authors were involved in very fruitful discussions of the results.

Article 3

YVONNE MEYER-LUCHT AND SIMONE SOMMER

Number of MHC alleles is related to parasite load in natural populations of yellow necked mice (*Apodemus flavicollis*)

Evolutionary Ecology Research (in review)

In this study, I investigated the influence of genetic diversity on parasite load not on the individual but on the population level. I predicted that populations with high MHC class II diversity are better protected against parasite infections than populations with low MHC class II diversity. Consequently, I expected a lower number of infected individuals and lower mean infection intensities in MHC diverse populations. In a population with reduced MHC variation, specific resistance alleles could have been lost leading to a diminished adaptive evolutionary potential. This prediction should be valid if low MHC diversity does not follow from low pathogen pressure in a long-term coevolution but from a recent loss of genetic diversity through, for instance, population isolation and fragmentation effects.

To test this prediction, I sampled eight populations of the yellow necked mouse (*Apodemus flavicollis*), a common rodent in European deciduous and mixed forest habitats. Approximately 20 individuals per population were live-trapped in eight capture sites in and around the city of Hamburg, Northern Germany. Five sites were located in public urban parks and three sites in deciduous forests in rural areas. The trapping sites were subjected to different levels of urban influence, i.e. the degree of isolation and disturbance. I roughly described these differences by using the variable 'distance to the city centre' of Hamburg (1.2 to 34 km from the city centre). I surveyed genetic diversity at the MHC class II DRB gene as well as neutrally evolving genetic markers (microsatellites). Mice were genotyped at a 217 bp fragment of the MHC II DRB exon 2, which corresponds to the part of the human DR1 molecule that harbours the majority of ABS (Brown *et al.* 1993). Six previously described microsatellite markers were used to assess the effects of general or genome-wide genetic diversity. To characterise MHC diversity I used the variables 'observed heterozygosity' and 'allelic richness', the latter corrected for sample size by a rarefaction index (Fstat, Goudet 2001). To describe microsatellite variation I used the mean microsatellite MLH (multilocus heterozygosity, Coltman *et al.* 1999) and mean microsatellite d^2 (difference in repeat units, Coulson *et al.* 1998). I analysed the gastrointestinal

nematode load by faecal egg counts and tested for the effects of these four variables of population-wide genetic variation on nematode burden by means of GLMs.

MHC diversity, neutral genetic diversity, nematode prevalence and nematode infection intensity varied between mice populations. Neutral genetic diversity did not reveal an influence on the nematode load. Regarding the coding MHC marker, I could not find an effect of heterozygosity on nematode load. However, allelic richness in a population had a significant effect on nematode load. A high number of different MHC alleles in a population decreased the nematode prevalence and mean nematode infection intensity in a population. The differences in MHC diversity between the study populations are likely caused by isolation effects in the urban parks, as I detected an association between MHC allelic richness with the distance to the city centre. The host populations with low MHC diversity probably experienced a recent loss of genetic diversity.

This study supports the hypothesis that populations with a high MHC allele diversity are better armed against high parasite burdens. A population with a large MHC allele reservoir is more likely to possess resistance alleles to the multitude of pathogens present in the wild.

Authors' contributions:

The study was conceived and planned by S. Sommer and me. I collected the samples, carried out the MHC and microsatellite analyses as well as the parasitological investigations, performed the statistics, interpreted the data, and wrote the manuscript. S. Sommer supervised the research and revised the manuscript.

Discussion

Variation at immune genes is a very powerful and effective way to keep pace with diverse and rapidly evolving pathogens (Altizer *et al.* 2003; Charbonnel *et al.* 2006). In my thesis, I studied natural levels of variation at the immune gene complex MHC, which is among the best studied immune defence gene families, and parasite burden in different small mammal populations. I observed considerable differences in MHC variability between the populations and species under study and aimed to understand potential causes and consequences of high and low levels of immune gene variation for parasite burden in the natural environment.

In three articles I documented MHC class II variation and demonstrated associations to parasite defence. On the one hand, I entered new territory by studying MHC diversity in two species of free-ranging Neotropical marsupials (*G. microtarsus*, *M. incanus*), which are virtually unknown to immunogenetics. I characterised evolutionary mechanisms of MHC selection and proved the functional relevance of the marsupial MHC class II. In order to explain the MHC diversity differences between the two marsupial species I conceived two scenarios regarding parasite load and diversity under which low MHC diversity could occur. On the other hand I focussed on the rodent *A. flavicollis*, which is a very well characterised European wildlife murid regarding parasite fauna and immunogenetics. In individual based studies, the MHC was well studied in this species (Meyer-Lucht & Sommer 2005; Musolf *et al.* 2004), as was the strong impact of gastrointestinal helminths (Ferrari *et al.* 2004, 2009; Klimpel *et al.* 2007). Based on this knowledge, *A. flavicollis* is perfectly suited to study the effects of genetic diversity on the population level. I was able to show that mouse populations with a high number of different MHC alleles exhibit lower infection rates and intensities than populations with a low number of MHC alleles.

The Marsupial MHC Class II

Past experiments on the immunobiology of marsupials revealed radical differences in the immune response between marsupials and eutherian mammals. Despite a normal rejection of allogenic skin transplants, which is a sign of MHC class I polymorphism (Stone *et al.* 1997), there was almost no response in mixed lymphocytes cultures in several marsupial species (Infante *et al.* 1991; Stone *et al.* 1996, 1998; Wilkinson *et al.* 1992). The mixed lymphocyte culture response is a measure of T-cell function and highly dependent

on polymorphism at MHC class II loci. A lack of polymorphism at the MHC class II loci in marsupials was inferred from these observations. In fact, immunogenetic analyses reported very low MHC class II variation in several marsupial species (Lam *et al.* 2001; McKenzie & Cooper 1994; Schneider *et al.* 1991; Siddle *et al.* 2007a, b; Stone *et al.* 1999). However, most of these investigations were performed in laboratory or otherwise captive breeds, or even single individuals. The only study in a wild marsupial population by then was conducted on the Tasmanian devil (*Sarcophilus harrisii*, Siddle *et al.* 2007a, b) and revealed very low variation at the MHC class II. However, this species is not the best example for a natural marsupial population under undisturbed conditions, as it seems to be monomorphic at the MHC class I (Siddle *et al.* 2007a, b).

In view of this lack of knowledge I investigated whether the low levels of marsupial MHC class II polymorphism are a general attribute of marsupials and thus also present under natural conditions. Studying the two Neotropical marsupial species *G. microtarsus* and *M. incanus* revealed opposing results. In contrast to the quoted investigations, I was able to show high MHC class II diversity for the first time in a wild marsupial under natural conditions: *G. microtarsus* is highly polymorphic at the MHC class II, with 75 MHC-DAB alleles in 102 individuals, high levels of sequence divergence, and a minimum of four MHC DRB loci. This mode of diversification through locus duplication often occurs in immune genes, whereby the number of duplications may even vary between individuals (Doxiadis *et al.* 2000). Duplication provides a way of retaining, by conservation of one duplicate, the currently useful function of the encoded protein, whilst the twin is liberated to mutate and possibly acquires a novel function (Charbonnel *et al.* 2006). The dimension of allelic polymorphism in *G. microtarsus* is very similar to levels of MHC II diversity described in numerous wild eutherian species (summarised in Bernatchez & Landry 2003; O'Brien & Yuhki 1999; Piertney & Oliver 2006; Sommer 2005). It is clear evidence against the assumed general low MHC class II diversity in marsupials. On the other hand, the second marsupial species revealed a tenfold lower MHC II diversity. With eight MHC-DAB alleles in 123 individuals at a minimum of two loci and very low genotypic variation *M. incanus* is perfectly concordant with the supposed general low MHC class II diversity in marsupials. In both species, I confirmed evolutionary mechanisms for generating and maintaining MHC diversity: I identified positive selection on specific amino acid sites, recombination events, and trans-species polymorphism between the two species. All three mechanisms are well known features of positive selection at MHC loci of eutherian mammals, birds, and fish (Klein 1987; Richman *et al.* 2003; Wong *et al.* 2004) and underline the functional relevance of the MHC class II in marsupials.

Very recently and shortly after publication of my first study on this subject (Meyer-Lucht *et al.* 2008), two other studies on marsupial MHC class II have been published based on free-ranging populations or larger samples sizes. Considerable MHC class II diversity was reported in the Australian brushtail possum (*Trichosurus vulpecula*, Holland *et al.* 2008a, b) as well as in the tammar wallaby (*Macropus eugenii*, Cheng *et al.* 2009). It now becomes apparent that the high level of MHC class II polymorphism in *G. microtarsus* is not a singular case in marsupials. In fact, if high MHC class II polymorphism in marsupials was rather the rule than the exception, then the low MHC class II variation in *M. incanus* remains to be explained. It may be possible that in *M. incanus* genetic diversity was lost due to inbreeding or a bottleneck. But as long as research on the marsupial MHC still lacks comprehensive knowledge from natural populations, the question whether or not low MHC class II diversity is a phylogenetic characteristic of marsupials is not sufficiently answered. Obviously, this topic is not solved yet and should be further addressed.

Beyond the evolutionary traces of positive selection on the marsupial MHC class II, the data indicate pathogen-driven selection in both marsupial species. I was able to identify associations between specific MHC variants and the individual parasite load. Both beneficial and disadvantageous MHC variants were found, which reduced or increased parasite burden, respectively. The presence of MHC alleles associated with parasite infection is a classical indicator of pathogen-driven selection (Apanius *et al.* 1997; Sommer 2005) and was detected in several studies (e.g. Froeschke and Sommer 2005; Harf and Sommer 2005; Langefors et al. 2001; Lohm et al. 2002; Meyer-Lucht and Sommer 2005; Schad et al. 2005; Schwaiger et al. 1995; Westerdahl et al. 2005). In the marsupials, a higher number of different MHC variants was not protective against helminth infections, which would correspond in a broader sense to the hypothesis of heterozygote advantage (Doherty & Zinkernagel 1975). Although there were no RNA samples and consequently no information on MHC expression available, the genomic level mirrors the capacity of all possibly expressible MHC variants, independent of temporal variation in expression. It became apparent that - beside environmental factors like season and spatial variation - the host genetics affect the individual parasite load in marsupials.

Helminth Parasitism in *G. microtarsus* and *M. incanus*

In search of an explanation for the different levels of MHC class II diversity in *G. microtarsus* and *M. incanus* I investigated the species' loads with gastrointestinal helminths. In terms of parasite diversity the two marsupial species did not feature obvious

differences, with thirteen helminth morphotypes in *M. incanus* and eleven in *G. microtarsus*. The helminth fauna in both species widely overlapped: six parasite morphotypes were shared, two of them were frequent ones. In contrast, the differences in parasite prevalence and infection intensity were very obvious: more individuals were infected (100%) and mean infection intensity was almost double in *M. incanus* than in *G. microtarsus*.

It is now well recognised that resistance and susceptibility to helminths infections in mammals is under genetic control (see summaries in Charbonnel *et al.* 2006; Wakelin *et al.* 2002). But as parasitism is a multifactorial phenomenon, these conspicuous differences in parasite load between the marsupial species are probably additionally influenced by a number of other factors. For example, helminth diversity and abundance in wild rodents is strongly influenced by seasonal and spatial variation as well as host age and sex (Abu-Madi *et al.* 2000; Behnke 2008; Behnke *et al.* 2001b). In the comparison between species, there are even more varying factors. To name only a few, parasite load can be influenced by differences in the social system, habitat use, or physiological mechanisms (reviewed in Weil *et al.* 2006). In terms of the two study species, differences in the microhabitat use may e influential. *G. microtarsus* is mainly arboreal and uses the canopy, while *M. incanus* uses both the understorey and the forest ground (Cunha & Vieira 2002; Vieira & Monteiro-Filho 2003). Thus, the terrestrial locomotion of *M. incanus* might increase the exposure to soil borne parasites or such of faecal-oral transmission (Nunn *et al.* 2003).

Faecal egg counts revealed to be an ideal form of parasitological examination because dissection of a large numbers of hosts is avoided, which would not be feasible in wild species from a threatened biome. A potential inaccuracy may arise from the morphotype classification, e.g. especially in the group of strongyle nematodes the eggs resemble each other and can only be separated by size (Sloss *et al.* 1994), so that two or more helminth species could have been recorded as one morphotype. This means that parasite diversity in this study was rather under- than overestimated. However, the non-invasive procedure of faecal egg counts was evaluated to provide accurate estimates of helminth parasitism (Seivwright *et al.* 2004; Stear *et al.* 1995).

No matter what the reasons for the differences are: the host species do differ in their load with gastrointestinal helminths. *M. incanus*, the species with low MHC class II diversity shows high infection rates and intensities, whereas *G. microtasus*, the species with a tenfold higher MHC class II diversity, shows lower infection rates and intensities.

Low MHC Class II Diversity in *M. incanus*

Although the genes of the MHC are the most polymorphic loci known for vertebrates (Apanius *et al.* 1997; Hedrick 1994; Klein 1986), species with low levels of MHC variation are reported occasionally. Thereby, reduced MHC polymorphism might be a consequence of a monogamous mating system combined with limited gene flow and limited gene pool (Sommer *et al.* 2002). But low levels of MHC diversity must not necessarily be problematic. Some species thrive well and show no signs of severe diseases despite their low MHC variation or even monomorphism, like moose (*Alces alces*) or mountain goats (*Oreamnos americanus*) (Mainguy *et al.* 2007; Mikko & Anderson 1995). Northern ungulate species tend to have a lower MHC diversity compared to those at lower latitudes, presumably because they are exposed to fewer pathogens and under a lower pathogenic selection pressure (Mainguy *et al.* 2007; Van den Bussche *et al.* 1999, 2002). Studies comparing populations of the same species exposed to different parasite arrays supported the strong dependence of host MHC diversity on the pathogen diversity. Populations exposed to a more diverse pathogen regime exhibited a higher MHC diversity than those exposed to fewer pathogens (Prugnolle *et al.* 2005; Wegner *et al.* 2003b). And more recently, in different rodent species a positive correlation was shown between a species' MHC allelic diversity and its helminth diversity (Goüy de Bellocq *et al.* 2008).

On the other hand, some species lack MHC variation and are particularly susceptible to infectious diseases and parasites. In these cases a genetic bottleneck is assumed to account for the low MHC variation, whereby resistance alleles or other important parts of the evolutionary potential of a species could have been lost. Famous mammalian examples are the giant panda (*Ailuropoda melanoleuca*) (Wan *et al.* 2006) or the Tasmanian devil (*S. harrisii*), which is under the threat of extinction by a fatal contagious tumour disease that is attributed to a severely depleted MHC class I (Siddle *et al.* 2007a, b).

For *M. incanus* I conceived two scenarios under which low MHC diversity could occur, including parasite load and parasite diversity. *M. incanus* could be under relaxed pathogenic selection pressure and therefore I predicted low parasite diversity under the '*evolutionary equilibrium*' scenario. Alternatively, low MHC diversity could be the result of a recent loss of genetic variation by means of a genetic bottleneck event. Under this '*unbalanced situation*' scenario, I assumed a high parasite burden in *M. incanus* due to a lack of resistance alleles. Parasitological results clearly reject the first scenario and point to the second scenario, as *M. incanus* is distinctly higher parasitised but parasite diversity is relatively equal compared to *G. microtarsus.* Hence, I suggest that the parasite load in *M. incanus* is rather the consequence than the cause for its low MHC diversity. *M. incanus*

may be in an unbalanced situation through a recent loss of genetic diversity. The high parasite burden may results from its low genotypic variation because genetic homogeneity facilitates the spreading of pathogens through a population (Meagher 1999).

Based on a number of ecological studies it was suggested that *M. incanus* is more sensitive to habitat fragmentation than *G. microtarsus* (Pardini *et al.* 2005; Püttker *et al.* 2008; Umetsu & Pardini 2007). Such ecological restrictions might be responsible for a reduced migration between suitable habitat fragments, which in turn could lead to isolation and loss of genetic variation as a consequence of genetic drift. Indeed, ongoing studies indicate that gene flow between forest fragments measured by microsatellites is reduced in the study area compared to another study region with a higher amount of remaining forest (Fernandes, pers. com.). Also, calculations of immigrating individuals based on mark-recapture studies show a lower number of immigrants in the study area compared to the other study region with a higher amount of remaining forest habitat (Püttker *et al.*, in prep.). But within the study area, no effect of the forest patch size on the number of MHC alleles was detectable for *M. incanus*, and very few alleles were also detected in the control site. Thus, fragmentation sensitivity is not sufficient to explain the observed low MHC diversity in *M. incanus.* Moreover, there is no indication for a recent population bottleneck.

Genetic Diversity and Parasite Load on the Population Level

The focus of conservation genetic studies primarily lies on small populations of endangered species, while common wild species are usually of minor interest. However, isolation and habitat fragmentation do affect populations of common species as well. Through past bottleneck events they could have experienced a severe loss of genetic diversity although often not visible as an obvious population decline (Hitching & Beebee 1997). I studied the importance of genetic diversity for parasite burden in eight populations of the yellow necked mouse, which is a very common European rodent species. I discovered a significant effect of the population-wide allelic richness at the MHC on parasite resistance. Mice populations with a large number of MHC alleles displayed relatively low parasite loads. In contrast, I did not find indication for the influence of neutral, genome-wide genetic diversity or MHC heterozygosity in a population on parasite burden.

Very few studies so far have investigated MHC variation and its associations to parasite burden not within single populations but between different populations (but see the above cited studies by Prugnolle *et al.* 2005 and Wegner *et al.* 2003). In contrast to these studies, I did not compare populations from different habitats with different levels of

pathogen diversity. I investigated mouse populations from similar habitats within the same geographic region that featured similar parasite communities and diversities, because my aim was to focus exclusively on the impact of genetic variation and to minimise the variation of additional factors. In the case of my study, the populations differed in their MHC diversity probably because of isolation effects in the investigated park areas, as indicated by an association between MHC allelic richness and the distance to the city center. Likewise, Hirota *et al.* (2004) demonstrated for *A. speciosus,* the large Japanese field mouse, that in urban areas migration is inhibited leading to a loss of gene diversity in urban populations.

Constantly challenged by different infectious agents acting simultaneously in the wild, populations with a large MHC allele pool are more likely to harbour individuals that possess protective alleles for each of the different infections. Individuals within genetically variable populations differ widely in their response to pathogens and thereby complicate the spreading of pathogens (Frankham *et al.* 2002; Meagher 1999). In contrast, with reduced diversity at the MHC in small populations and a lack of different resistance alleles, a pathogen capable of infecting one individual becomes capable of infecting all.

There was no indication for an effect of population-wide MHC heterozygosity or neutral genetic diversity on parasite burden. However, the low number of eight populations leads to low statistical power to detect significant effects. Although not deducible from these data, MHC heterozygosity or neutral genetic variation in a population might play a major role in parasite burden as well. Therefore, I would rather like to point out that this fact fortifies the positive result of a significant association between MHC allelic richness and parasite resistance because it was detectable despite the low statistical power.

Conclusion

In contrast to the prediction that marsupials lack polymorphism at the MHC class II, I detected high levels of diversity in *G. microtarsus*, a free-ranging Neotropical marsupial. Furthermore, I confirmed for the first time in marsupials the presence of three characteristic features common at MHC loci of eutherian mammals, birds, and fish: positive selection on specific sites, recombination, and trans-specific polymorphism. In both marsupial species it became apparent that the MHC class II is functionally important in disease defence against gastrointestinal helminths. Beyond that, the two investigated marsupials revealed interesting differences in their MHC class II diversity because diversity in *M. incanus* was very low. These differences could not be explained by different pathogen selection

pressures. It seems that in *M. incanus* a high parasite load is rather the consequence than the cause for its low MHC diversity. Fragmentation sensitivity might play a role explaining the low MHC diversity in *M. incanus* but for a distinct conclusion on this aspect a more comprehensive and deeper survey of the genetic diversity in *M. incanus* is required. The question whether or not low MHC class II diversity is a phylogenetic characteristic of marsupials will not be resolved until substantial knowledge on the marsupial MHC in natural populations is gathered and more marsupial species are characterised.

MHC variation and its associations to parasite burden have been typically studied within single populations but rarely MHC variation between populations was taken into account. I was able to show that mouse populations with a high MHC allele diversity are better armed against high parasite burdens highlighting the significance of adaptive genetic diversity in the field of conservation genetics. An individual itself will not directly benefit from its population's large MHC allele pool in terms of parasite resistance but confronted by the multitude of pathogens present in the wild a population with a large MHC allele reservoir is more likely to possess resistance alleles and cope with coevolving pathogens.

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Appendix

Article 1:

Selection, diversity and evolutionary patterns of the MHC class II DAB in free-ranging Neotropical marsupials

Selection, diversity and evolutionary patterns of the MHC class II DAB in free-ranging Neotropical marsupials

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Abstract

Background

Research on the genetic architecture and diversity of the MHC has focused mainly on eutherian mammals, birds and fish. So far, studies on model marsupials used in laboratory investigations indicated very little or even no variation in MHC class II genes. However, natural levels of diversity and selection are unknown in marsupials as studies on wild populations are virtually absent. We used two endemic South American mouse opossums, *Gracilinanus microtarsus* and *Marmosops incanus*, to investigate characteristic features of MHC selection. This study is the first investigation of MHC selection in free-ranging Neotropical marsupials. In addition, the evolutionary history of MHC lineages within the group of marsupials was examined.

Results

G. microtarsus showed extensive levels of MHC diversity within and among individuals as 47 MHC-DAB alleles and high levels of sequence divergence were detected at a minimum of four loci. Positively selected codon sites were identified, of which most were congruent with human antigen binding sites. The diversity in *M. incanus* was rather low with only eight observed alleles at presumably two loci. However, these alleles also revealed high sequence divergence. Again, positive selection was identified on specific codon sites, all congruent with human ABS and with positively selected sites observed in *G. microtarsus*. In a phylogenetic comparison alleles of *M. incanus* interspersed widely within alleles of *G. microtarsus* with four alleles being present in both species.

Conclusions

Our investigations revealed extensive MHC class II polymorphism in a natural marsupial population, contrary to previous assumptions. Furthermore, our study confirms for the first time in marsupials the presence of three characteristic features common at MHC loci of eutherian mammals, birds and fish: large allelic sequence divergence, positive selection on specific sites and trans-specific polymorphism.

Background

The vertebrate immune system possesses two very efficient tools to ward off constantly evolving pathogens, the innate and the adaptive immune system. As a part of the adaptive immune system the molecules of the major histocompatibility complex (MHC) recognize antigens, present them to T-lymphocytes and thereby initiate an immune response [1]. The need to recognize a wide range of pathogens drives an adaptive polymorphism in the MHC, which indeed contains the most variable functional genes in vertebrates [2, 3]. Therefore, the MHC constitutes a powerful model to study processes, causes and consequences of selection on a molecular level [3-5].

The MHC is a multigene family that codes for cell-surface glycoproteins. These molecules are key receptors for the presentation of peptide fragments deriving from pathogens. MHC class I molecules mainly correspond to intracellular pathogens and are expressed on the surface of all nucleated somatic cells. MHC class II genes are predominantly involved in the defence against extracellular pathogens and are expressed only on specialized antigen-presenting cells, such as B cells and macrophages [6]. The polymorphism at the MHC class I and II genes is especially pronounced in the codons that are directly involved in antigen binding, the so-called antigen binding sites (ABS) [7, 8].

Research on the genetic architecture and diversity of the MHC has focused mainly on eutherian mammals, birds and fish. From this broad range of studies it has become apparent that the extreme allelic diversity found at class I and II loci is characteristic for the MHC [4]. This unusually high level of polymorphism found at the MHC of most vertebrate species is assumed to be maintained by means of balancing selection. It is supposedly driven by pathogens through heterozygote advantage and/or frequency-dependent selection [3, 5, 9-11] as well as mechanisms linked to reproduction, such as disassortative mating or pre-natal selection [12-14]. Balancing selection is reflected in an increased rate of non-synonymous over synonymous substitutions and an elevated rate of recombination events relative to neutral expectations as well as in retaining certain alleles longer than expected under a neutral model [7, 8, 15, 16].

The latter phenomenon has been described as trans-species polymorphism [17], where certain MHC alleles or allelic lineages are found in related species indicating that they are older than the speciation event and passed on from the ancestral to the descendant species. Long-lasting trans-species polymorphism occurs only in genetic systems under balancing selection and is a typical mode of evolution in the MHC [18].

In contrast to the great attention being paid to the MHC of eutherian mammals, birds and fish, the diverse group of marsupials has been poorly investigated so far. Earlier studies on marsupial cellular immunology reported dramatic differences in the immune response between marsupials and eutherians and predicted little or no polymorphism at the MHC class II genes [19-21]. Only recently has the complete MHC region been mapped for the first marsupial (*Monodelphis domestica*) [22, 23]. Belov et al. [24] presented in a phylogenetic review that the marsupial MHC class II ß genes cluster in two groups clearly separated from the eutherian ß gene families, and therefore constitute non-orthologous loci. Recently, a third lineage has been reported [22]. The marsupial and eutherian lineages appear to have maintained different MHC class II ß gene clusters after duplication events early in the mammalian evolution. The authors recommended using the nomenclature DAB, DBB and DCB for the marsupial ß gene families [22, 24].

At present, molecular research on the marsupial MHC class II is restricted mainly to captive or laboratory bred individuals. So far five marsupial species have had their MHC class II ß genes examined. Regarding the Australian marsupials, Schneider and co-workers [25] investigated one red-necked wallaby (*Macropus rufogriseus*) from a zoo and detected two DAB alleles, one of them a transcribed pseudogene, and one DBB allele. A study on the MHC of captive bred tammar wallabies (*Macropus eugenii*) using RFLP suggested at least 12 MHC class II ß loci in tammars, but variation between individuals appeared to be significantly reduced compared with most eutherians [26]. In *Trichosurus vulpecula*, the brushtail possum, five DAB alleles were discovered, again from a single individual [27]. Siddle et al. [28] isolated six MHC II DAB alleles from one Tasmanian devil (*Sarcophilus harrisii*), and very recently a study on wild 26 individual *S. harrisii* revealed no additional alleles [29]. In the sole South American marsupial to have its MHC studied, *M. domestica* (gray short-tailed opossum), only a single DAB sequence was identified from five individuals [30], and two DBB and one DCB sequences in a genome sequencing project [22].

Levels of natural diversity and processes of selection on the marsupial MHC are largely unknown, as studies on wildlife population of marsupials virtually do not exist [but see 29]. Yet, marsupials represent an important milestone in the mammalian evolution. Based on genetic data the separation of marsupials from eutherians took place some 120 to 100 million years ago [31]. Tracing back the evolution of the adaptive immune system in mammals may allow fundamental insights to its origin and function.

The marsupials of South and Central America are a widespread group comprising 76 recent species [31]. There are species adapted for dry habitats or cool regions at high

altitudes of the Andes, but the majority of American marsupials are rainforest dwellers [31]. As representatives of free-ranging marsupials under natural condition, we chose two species of mouse opossums that belong to the largest family of American marsupials, the Didelphidae: *Gracilinanus microtarsus* (Brazilian gracile mouse opossum, Wagner 1842) and *Marmosops incanus* (Gray slender mouse opossum, Lund 1840). These two species are endemic to the highly threatened coastal Atlantic forest of South America [32, 33] and differ in their sensitivity to habitat fragmentation [34, 35], one of the major threats to the coastal Atlantic forest. By studying these two species we aimed to (1) investigate MHC selection processes for the first time in free-ranging populations of marsupials, and (2) test whether the predicted low levels of polymorphism at MHC class II in marsupials are to be confirmed under natural conditions. Moreover, in a phylogenetic comparison we explored (3) the evolutionary history (trans-species evolution) of MHC lineages within marsupials comprising *G. microtarsus*, *M. incanus* and additional marsupial taxa.

To the best of our knowledge this is the first study on natural levels of MHC diversity in non-model, free-ranging Neotropical marsupials.

Results

MHC-DAB diversity in *G. microtarsus* and *M. incanus*

The MHC-DAB genes of 54 Brazilian gracile mouse opossums (*G. microtarsus*) and 56 Gray slender mouse opossums (*M. incanus*) were genotyped. Following the suggestion of Klein et al. [36], the DAB alleles of *G. microtarsus* and *M. incanus* were denominated as *Grmi*-DAB and *Main*-DAB, respectively. A BLAST search revealed >80% identity of all identified *Grmi*-DAB and *Main*-DAB alleles to MHC II ß genes of the gray short-tailed opossum (*M. domestica*), the tammar wallaby (*M. eugenii*), the brushtail possum (*T. vulpecula*) or eutherian DRB sequences. The alleles discovered in this study were published in GenBank under accession No's EU350142 - EU350149 and EU350150 - EU350196.

G. microtarsus displayed a high degree of MHC-DAB diversity, both within and among individuals (Table 1, Fig. 1). A total of 47 distinct *Grmi*-DAB alleles were discovered through cloning and sequencing of fifteen recombinant clones per individual (Fig. 1). The average number of alleles per individual was 4.6 ± 1.7, and ranged from one to seven; thus we report a minimum of four DAB loci in *G. microtarsus*. The alleles *Grmi*-DAB*04 and *Grmi*-DAB*17 contained stop codons, and one allele (*Grmi*-DAB*37) carried a deletion of two nucleotides that lead to a frameshift. These three alleles were classified as

pseudogenes and excluded from further analyses. In the remaining 44 nucleotide sequences, 93 out of 195 positions were variable (Table 1). The alleles differed in one to 46 nucleotide positions, with an average of 28.2 ± 2.6. On the amino acid level 44 out of 65 positions were polymorphic. Two nucleotide sequences translated to the same amino acid sequence (*Grmi*-DAB*01a and *Grmi*-DAB*01b). Except for these two, alleles differed in one to 26 positions, with an average of 15.9 ± 2.1. The most common allele *Grmi*-DAB*01a was detected in all but three individuals (frequency= 0.944), followed by *Grmi*-DAB*02 and *Grmi*-DAB*09 (both 0.278). The remaining *Grmi*-DAB alleles occurred in one to twelve individuals (Fig.1).

Table 1: MHC-DAB diversity in *G. microtarsus* and *M. incanus.* The number of DAB alleles (+ sequences including a stop codon or a frameshift) in the samples (N) and the inferred minimum number of DAB loci are displayed for both species, as well as variable positions and the mean number of differences between alleles (Ø differences).

Species	N	DAB alleles	DAB loci	Nucleotide sequence		Amino acid sequence	
				variable positions	Ø differences	variable positions	Ø differences
G. microtarsus	54	44 (+3)	≥ 4	93/195 (47.7%)	28.2 ± 2.6 (14.5%)	44/65 (67.7%)	15.9 ± 2.1 (24.5%)
M. incanus	56	8	≥ 2	66/195 (33.8%)	25.6 ± 2.7 (13.1%)	30/65 (46.2%)	14.1 ± 2.2 (21.7%)

Unlike *G. microtarsus*, the number of different DAB alleles found in *M. incanus* was low (Table 1). A total of eight *Main*-DAB alleles were assigned via SSCP and cloning from 56 individuals (Fig. 1). The number of alleles within an individual was on average 3.0 ± 0.6, with a range from two to four alleles. Hence in *M. incanus* the DAB locus is at least duplicated. Nucleotide divergence between the *Main*-DAB alleles was high. In eight *Main*-DAB alleles, 66 from 195 nucleotide positions were variable and no indels were detected. Alleles differed amongst themselves by two to 45 nucleotide positions and in 25.6 ± 2.7 on average. Each nucleotide sequence translated to a unique amino acid sequence. 30 out of 65 amino acid positions were polymorphic. Alleles varied in one to 24 positions, averaging in 14.1 ± 2.2 substituted amino acids. The most common allele *Main*-DAB*01 was present in a frequency of 0.875, followed by *Main*-DAB*02 (0.750) and *Main*-DAB*03/4 (0.732). The remaining alleles occurred in ≤ 12 individuals (Fig. 1).

Position		22	32	42	52	62	72	82
Human ABS		* *	* **	*	* **	* * **	* * * *	* **
Grmi-DAB*01a	*0.944*	EHVRHVDRYF	YNREEYVRFD	SNVGVFEAVT	ELGRPDSEYW	NSQKEILEQM	RAEVNSVCRH	NYEIL
Grmi-DAB*01b	*0.056*	....	....	....	....	....	....	.....
Grmi-DAB*02	*0.278*	.D........	....	....	....	....	....	.....
Grmi-DAB*03	*0.093*	...QY.K.H.	.....F....	....	.....SA...	..L.DY...R	..Q.DNY...	...VF
Grmi-DAB*04#	*0.222*	...QY.E*HI	.....F....	.....Y....	.....SA...	..L.DY..DR	..S.DNY...	.....
Grmi-DAB*05	*0.037*	...QFLE.HI	.....FMH.H	.....Y....	.....IA..Y	....DY.D.K	..A.DNY.L.	...VS
Grmi-DAB*06	*0.111*	...QY.N...	..G.......	....	......A...	..L.DY...K	..R.DNY...	.....
Grmi-DAB*07	*0.148*	...QF.E..I	T.G..N....	.....YK...	.R..Q.A...	....DY...K	..R.DTF...	...VS
Grmi-DAB*08	*0.204*	...QF.E.HI	......L...	.....Y....	.....IA..L	.....R..YA	..A.DTF...	...GS
Grmi-DAB*09	*0.278*	...QY.K.H.	.....F....	....	.....IA.D.	....DY...R	..Q.DNY...	...VF
Grmi-DAB*10	*0.130*	...QF.E.HI	T.G..T....	.K...Y....	.R..Q.AD..	.....L..SR	..S.DNY.G.	...GS
Grmi-DAB*11	*0.037*	...QY.E.HI	T.G..N....	.K...Y....	.R..Q.A.D.	.....L..SR	..A.DNY...	...VS
Grmi-DAB*12	*0.111*	...QY.E.HI	T.G..N....	.K...Y....	.R..Q.A.D.	........SR	..A.DNY...	...VS
Grmi-DAB*13	*0.148*	...QF.E.H.	.....F....	.....Y....	......AD..	.........K	..R.DNY...	...VS
Grmi-DAB*14	*0.037*	....	.....H....	....	........N.	....	....	S....
Grmi-DAB*15	*0.130*	...QF.K.HI	....	.....Y....	.....SA..Y	.....R..YA	..A.DTF...	...GS
Grmi-DAB*16	*0.185*	...QF.E.RI	T.G..N....	.....Y....	......AD..	........GA	..A.DNY...	...VF
Grmi-DAB*17#	*0.148*	...QF.E.RI	T.G..N....	.....Y....	......AD..	.......*GA	..A.DNY...	...VF
Grmi-DAB*18	*0.204*	...QF.E.HI	T.G..N....	.....Y....	......AD..	.....L..DR	..Q.DT....	..KGF
Grmi-DAB*19	*0.148*	....Y.E...	....	....	.....SA.KL	.....L..DR	..S.DNY..P	...VS
Grmi-DAB*20	*0.111*	....Y.N...	......L...	....	.....IA.D.	....DY...K	..R.DNY...	...VS
Grmi-DAB*21	*0.019*	....Y.N...	......L...	....	.....IA..L	....DY...K	..R.DNY...	...VS
Grmi-DAB*22	*0.093*	...QY.E.RI	.....N....	.....Y....	.....SA...	.....L..DR	..S.DNY.G.	...VF
Grmi-DAB*23	*0.019*	.D..F...H.	.....N....	....	.......D..	........DI	..Q.......	..KG.
Grmi-DAB*24	*0.019*	..AQ.....I	....	....	......A...	....	....DT....	..K.V
Grmi-DAB*25	*0.037*	....Y.E...	.....L....	.....Y....	.....EA...	........DA	..S.DTF...	...VS
Grmi-DAB*26	*0.019*	....F.Q.HI	.....F....	....	....Q.A.H.	........DK	..A.DTY...	...VF
Grmi-DAB*27	*0.037*	...QY.E.RI	.........H	.K...Y....	.....SAD..	........DA	..S.DTF...	...VF
Grmi-DAB*28	*0.019*	........H.	....	....	....	........H.	......F...	.....
Grmi-DAB*29	*0.037*	....F.Q.HI	.....FL...	....	.R..Q.A.H.	........DE	..A.DTY...	...VF
Grmi-DAB*30	*0.056*	...QF.E.HI	.....FMH.H	.....Y....	.....IA..Y	....DY..SR	..V.DTG...	...VS
Grmi-DAB*31	*0.019*	...QFLE.H.	....	.....Y..L.	.....SAK..	..L..R..HA	..A.DTF...	...VS
Grmi-DAB*32	*0.037*	...QY.E.HI	T.G..T....	.K...Y....	.R..Q.AD..	.....L..DR	..S.DNY...	...GS
Grmi-DAB*33	*0.019*	...QFL....	..G.......	.....Y....	......A...	.....R..DA	..A.DTY...	...VS
Grmi-DAB*34	*0.019*	....L.G.FI	....	....A.....	.....SV.D.	....DY..NT	..G.DRF.GN	...SS
Grmi-DAB*35	*0.037*	....	.....F....	....	....	....	....	.....
Grmi-DAB*36	*0.037*	....	......M...	....	....	....	....	.....
Grmi-DAB*37#	*0.019*	....	....	.......V..	....	....	.....A....	...S-
Grmi-DAB*38	*0.056*	...QF.E.H.	..G..F....	.....Y...M	......AD..	.....R..DA	..A.DTY...	...VS
Grmi-DAB*39	*0.019*	...QFL....	..G...L...	.....Y....	.....SAD..	........DA	..A.DTF...	...VS
Grmi-DAB*40	*0.037*	...QF.E.H.	....	.....Y....	......AD..	........GA	..A.DNY...	...VS
Grmi-DAB*41	0.037	...QF.E.RI	T.GA.N....	....	....Q.AD..	........RR	..A.DTF...	...GS
Grmi-DAB*42	*0.019*	...QY.E.RI	T.GA.N....	.....Y....	....Q.AD..	....DY..DR	..A.DTF...	...GS
Grmi-DAB*43	*0.019*	....Y.N...	.....F....	....	.....IA.D.	....DY...K	..R.DNY...	...VS
Grmi-DAB*44	*0.019*	...QL.....	.....F....	....	.....IA.D.	....DY...R	..Q.DTY...	...VS
Grmi-DAB*45	*0.019*	...QL.....	..G.......	....	....	....	....	.....
Grmi-DAB*46	*0.056*	...QFL....	..G..T....	....	......A...	.....R...K	..S.DTF...	...VS
Main-DAB*01	*0.875*	EHVRLVGRFI	YNREEYVRFD	SNVGVFEAVT	ELGRPSVEDW	NSQKDYLENT	RAGVDRFCGN	NYESS
Main-DAB*02	*0.750*	....F.Q.H.	.....FL...	....	.R..QDA.H.	....EI..DE	..A..TY.RH	...VF
Main-DAB*03 }	*0.732*	....F.Q.H.	.....F....	....	.R..QDA.H.	....EI..DE	..A..TY.RH	...VF
Main-DAB*04 }		....F.Q.H.	.....F....	....	....QDA.H.	....EI..DK	..A..TY.RH	...VF
Main-DAB*05	*0.214*	....F.Q.Y.	T.G..N....	....	....QDA.H.	....EI..DE	..A..TY.RH	...VF
Main-DAB*06	*0.107*	....F.Q.Y.	T?G..N....	....	.R..QDA.H.	....EI..RL	L.A..TY.RH	...VF
Main-DAB*07	*0.143*	....H.D.YF	....	....	.....DS.Y.	....EI..QM	..E.NSV.RH	...IL
Main-DAB*08	*0.143*	...QF.E.H.	T.G..T....	.K...Y....	.R..QDADY.	....EL..SR	..S..NY..H	...G.

Figure 1: Alignment of amino acid sequences of *Gracilinanus microtarsus* MHC-DAB alleles (*Grmi*-DAB) and *Marmosops incanus* MHC-DAB alleles (*Main*-DAB). Allele frequencies are given in italic, dots represent sequence identity with allele *Grmi*-DAB*01a and # indicates a pseudogene. The bracket combining *Main*-DAB*03 and *Main*-DAB*04 signifies that these two alleles revealed identical SSCP patterns and the frequency value is therefore composed from both alleles. Numeration is according to the human DR1 molecule and asterisks indicate the human antigen binding sites (ABS) defined by [37].

Test for positive selection

The marsupial MHC-DAB was suggested to be not orthologous to the eutherian DRB [24], therefore we did not assume an *a priori* concordance with the antigen binding sites from the human HLA class II molecule DR1 [37, 38]. Accordingly, the d_N/d_S ratio, which is commonly applied to test for balancing selection [7, 8], was calculated for the whole sequence and not separately for the ABS and non-ABS. There was no excess of non-synonymous over synonymous substitutions for the whole sequence in both species (Table 2). However, in alignment with the human DR1 molecule, 17 (89%) positions in *G. microtarsus* and 15 (79%) positions in *M. incanus* out of the 19 predicted ABS revealed variation (Fig. 1).

Positive selection on specific codon sites was detected using the maximum likelihood method CODEML implemented in PAML3.15 [39]. Two pairs of models were applied: M1a versus M2a, and M7 versus M8. The pair M1a - M2a has limitations in the presence of recombination, while M7 - M8 is robust against the effects of recombination [40, but see 41]. The models M2a and M8 that allow for positive selection fitted our data significantly better than the null hypothesis models M1a and M7 (Table 2). In *G. microtarsus*, both models M2a and M8 identified nine sites under positive selection, which were detected by Bayesian analysis (Table 2). Six of these are congruent with ABS from the human DR1 molecule [37], two are located directly next to an ABS, and another one at a distance of two amino acids from an ABS. For *M. incanus* only three positions were found to be under positive selection (Table 2). One position was detected by both models M2a and M8, and the two other positions were each detected by only one of the models. All three sites are congruent with human ABS.

Test for gene conversion and recombination

Within the 44 MHC-DAB alleles of *G. microtarsus* the GENECONV analyses [42] detected no fragment significantly involved in gene conversion events in a global comparison (based on the whole alignment), but two fragments in the eight alleles of *M. incanus*. In pairwise tests, 98 fragments in *G. microtarsus* and two additional fragments in *M. incanus* were discovered but did not withstand corrections for multiple comparisons. However, in both species the numbers of pairwise internal fragments exceeded the random-assumption of 5% (*G. microtarsus*: 98 out of 946 (= 10.3%); *M. incanus*: four out of 28 comparisons (= 14.3%)) suggesting the occurrence of gene conversion in both taxa.

Table 2: Test for positive selection and identification of positively selected sites. The rates of non-synonymous (d_N) and synonymous (d_S) substitutions were calculated for the entire sequence. In the analyses of positively selected sites, twice the difference in likelihood $2(L_b-L_a)$ between the models was compared with a χ^2-distribution (df = 2) to assess significance [100]. x represents sites under positive selection identified at the >95% confidence level. Sites in bold are antigen binding sites (ABS) according to the human DR1 molecule [37], distance (dist.) to human ABS corresponds to the amino acid distance between identified site and the nearest human ABS.

Species	Substitution rate d_N	Substitution rate d_S	$\omega = d_N/d_S$	Model [100]	$2(L_b-L_a)$	p	Positively selected sites 26	37	57	60	70	71	74	77	86
G. microtarsus	0.159	0.178	0.893	M1 - M2a	63.46	<0.001	x	x	x	x	x	x	x	x	x
	(±0.028)	(±0.039)	n.s.	M7 - M8	56.01	<0.001	x	x	x	x	x	x	x	x	x
				Dist. to human ABS			2	0	1	0	0	0	0	1	0
M. incanus	0.143	0.183	0.781	M1 - M2a	13.32	<0.005		x				x			
	(±0.026)	(±0.047)	n.s.	M7 - M8	11.50	<0.005					x	x			
				Dist. to human ABS				0			0	0			

Population recombination was estimated using the software LDhat [43]. For *G. microtarsus,* the population recombination rate was high (ρ = 26) and deviated significantly from the null hypothesis (ρ = 0) in the likelihood permutation test. Here, the presence of recombination was confirmed while in *M. incanus* population recombination rate was quite low (ρ = 3) and, hence, the null hypothesis was not rejected.

The Hudson four-gamete test [44] implemented in DnaSP [45] revealed 21 and nine recombination events (R_M) in *G. microtarsus* and *M. incanus*, respectively. These values indicate the minimum number of recombination events in the history of the samples.

Evolutionary pattern of the MHC-DAB lineages

To characterise the phylogenetic relationship of the marsupial DAB alleles found in this study a neighbour joining tree was constructed. It included the marsupial DAB, DBB and DCB loci, the prototherian DZB [46], representatives from the eutherian ß gene families DOB, DPB, DQB and DRB as well as bird, reptile and anuran MHC sequences (Fig. 2). All MHC II ß alleles discovered in this study clustered with published DAB sequences from other marsupial species (Fig. 2). The eight alleles of *M. incanus* did not form a monophyletic clade but were widely interspersed within the groups of *Grmi*-DAB alleles. Moreover, four *Main*-DAB alleles were found to be identical to alleles of *G. microtarsus,* at least for the 195 investigated base pairs. The identical allele pairs were: *Main*-DAB*02 and *Grmi*-DAB*29, *Main*-DAB*04 and *Grmi*-DAB*26, *Main*-DAB*07 and *Grmi*-DAB*01a, and *Main*-DAB*08 and *Grmi*-DAB*10. The Neotropical marsupials formed a sister group to the Australian marsupials, but without bootstrap support (Fig. 2). The entire DAB lineage was held together with a bootstrap support of 65, and separated from the other two known marsupial lineages DBB and DCB as well as from the eutherian and prototherian MHC II ß gene lineages. However, the topology of ß gene lineages was not supported by high bootstrap values.

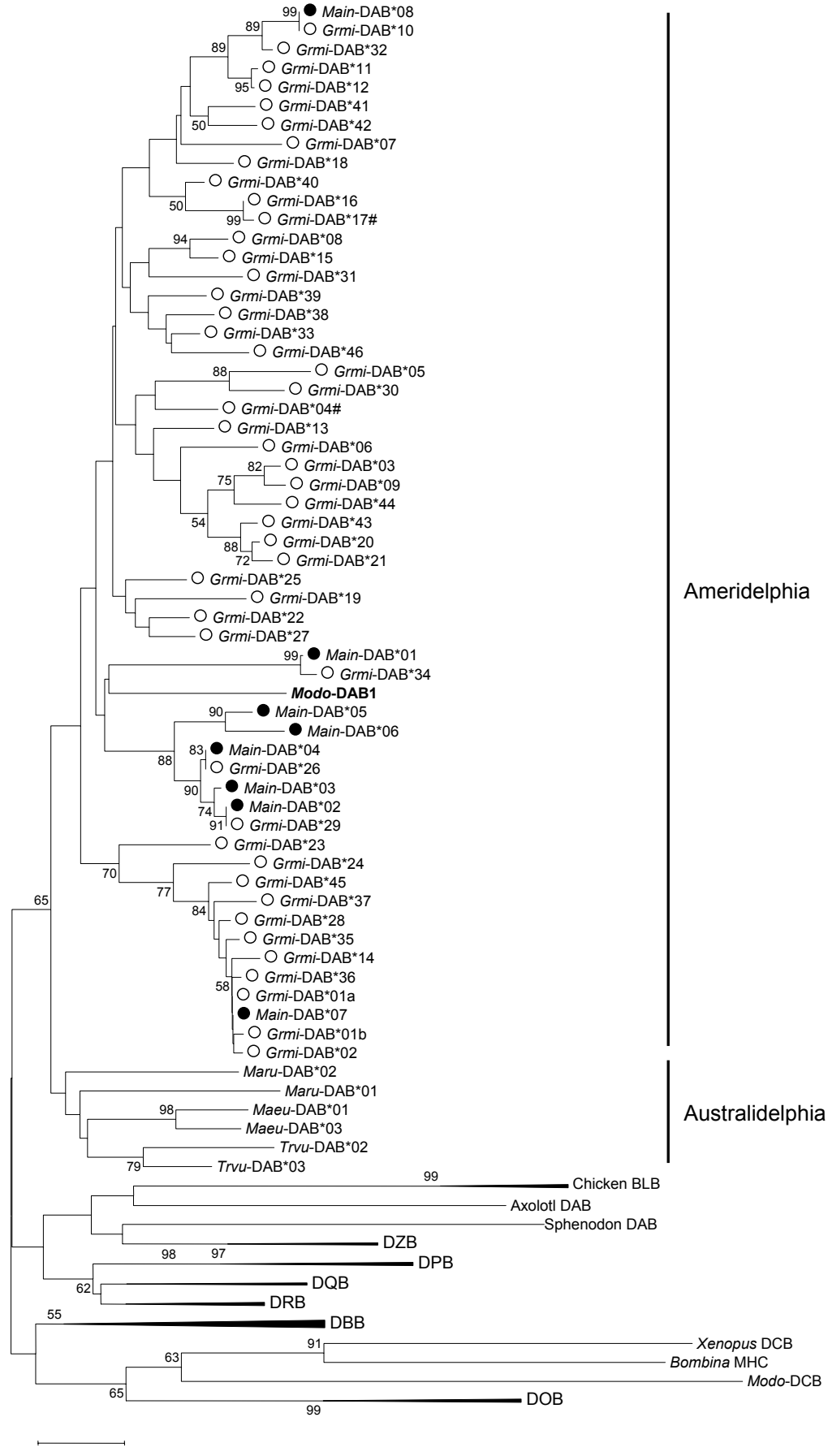
Main-DAB*08
Grmi-DAB*10
Grmi-DAB*32
Grmi-DAB*11
Grmi-DAB*12
Grmi-DAB*41
Grmi-DAB*42
Grmi-DAB*07
Grmi-DAB*18
Grmi-DAB*40
Grmi-DAB*16
Grmi-DAB*17#
Grmi-DAB*08
Grmi-DAB*15
Grmi-DAB*31
Grmi-DAB*39
Grmi-DAB*38
Grmi-DAB*33
Grmi-DAB*46
Grmi-DAB*05
Grmi-DAB*30
Grmi-DAB*04#
Grmi-DAB*13
Grmi-DAB*06
Grmi-DAB*03
Grmi-DAB*09
Grmi-DAB*44
Grmi-DAB*43
Grmi-DAB*20
Grmi-DAB*21
Grmi-DAB*25
Grmi-DAB*19
Grmi-DAB*22
Grmi-DAB*27
Main-DAB*01
Grmi-DAB*34
Modo-DAB1
Main-DAB*05
Main-DAB*06
Main-DAB*04
Grmi-DAB*26
Main-DAB*03
Main-DAB*02
Grmi-DAB*29
Grmi-DAB*23
Grmi-DAB*24
Grmi-DAB*45
Grmi-DAB*37
Grmi-DAB*28
Grmi-DAB*35
Grmi-DAB*14
Grmi-DAB*36
Grmi-DAB*01a
Main-DAB*07
Grmi-DAB*01b
Grmi-DAB*02
Maru-DAB*02
Maru-DAB*01
Maeu-DAB*01
Maeu-DAB*03
Trvu-DAB*02
Trvu-DAB*03
Ameridelphia
Australidelphia
Chicken BLB
Axolotl DAB
Sphenodon DAB
DZB
DPB
DQB
DRB
DBB
Xenopus DCB
Bombina MHC
Modo-DCB
DOB
0.05

Figure 2: Phylogenetic tree of the marsupial DAB locus in relation to the other MHC II ß gene families. The loci DOB, DPB, DQB, DRB are eutherian MHC class II ß gene families, DZB is the prototherian family, and DBB and DCB are additional marsupial ß gene families. Some branches have been compressed for a better overview. The tree was constructed with the neighbour joining method (Kimura-2-parameter), bootstrap values >50 are indicated (1,000 replications). The scale bar represents genetic distance in nucleotide substitution per site. # following an allele's name indicates a pseudogene. *Grmi = Gracilinanus microtarsus*, open circle, *Main = Marmosops incanus*, filled circle; *Modo = Monodelphis domestica*, in bold; *Maeu = Macropus eugenii*, *Maru = Macopus rufogriseus*, *Trvu = Trichosurus vulpecula.* Chicken = *Gallus gallus,* Axolotl = *Ambystoma mexicanum,* Xenopus = Xenopus laevis, *Bombina = Bombina bombina* and *Sphenodon= Sphenodon punctatus.*

Discussion

Dramatic differences in the immune response between marsupials and eutherian mammals have been reported from earlier studies on marsupial immunology. For instance, *M. domestica* shows virtually no mixed lymphocyte response, which is a measure of T-cell function and highly dependent on MHC class II polymorphism [19-21]. The authors inferred that T-cell receptors are atypical and there is little or no polymorphism at the MHC class II genes in this species. A recent study by Siddle et al. [29] revealed a severely depleted MHC class I diversity in wild Tasmanian devils. The depleted MHC is probably accounting for the easy spread of the devil facial tumour disease, a contagious tumour that puts Tasmanian devils currently under the threat of extinction. In the present study, we investigated characteristic features of the MHC in free-ranging populations of Neotropical marsupials and tested whether the low levels of polymorphism at MHC class II in marsupials can be confirmed under natural selection conditions as inferred by earlier studies. Three different mechanisms for generating and maintaining MHC diversity were studied: positive selection, recombination and trans-species polymorphism.

In both study species (*Marmosops incanus*, *Gracilinanus microtarsus*) patterns of positive selection acting on the DAB loci were found by maximum likelihood analyses. Power and accuracy of the maximum likelihood method for detecting positive selection has been evaluated as strong by Wong and co-workers [47]. In these analyses ω was estimated not for the entire sequence, but separately for each codon site resulting in several significantly positively selected sites being detected [39]. There were nine positively selected sites in *G. microtarsus*; the human DR molecule contains 19 ABS in the corresponding segment [37]. Two ABS positions (65 and 68) out of those 19 human ABS were completely invariable in *G. microtarsus* suggesting that they are not functionally

involved in antigen binding. In *M. incanus* only three positively selected sites were detected with two different models. We assume that only three positively selected sites were detected due to the low sample size of eight *Main*-DAB alleles. However, all of these three sites were congruent with human ABS and with positively selected sites in *G. microtarsus* emphasising structural similarities of sites probably involved in antigen binding throughout evolutionary lineages. This approach has recently been applied in MHC studies on wild populations of chamois [48, 49], African mole-rats [50], voles [51], primates [52] and several salmonid species [53] to identify species-specific positively selected sites. All studies revealed high congruence of positively selected sites with the human ABS.

Intralocus recombination plays an important role in the generation of the large allelic polymorphism at the MHC [15, 54, 55]. Supporting results from free-ranging populations were provided by a number of studies [48, 52, 53, 56, 57]. In our study, we also found evidence for gene conversion and recombination in the history of the MHC-DAB alleles of the two marsupials although the tests we applied detected uneven numbers of events and probabilities. The program GENECONV was evaluated as having one of the highest probabilities of correctly inferring gene conversion events [58]. However, under extensive recombination the power of GENECONV might be reduced [51]. Richman et al. [15] showed that LDhat yields reliable estimates for the population recombination rate even when the amounts of both recombination and mutation are large, as is the case under balancing selection. The minimum number of recombination events calculated by DnaSP [45] showed a similar tendency as LDhat. The ambiguity in our results might be based on the presence of multiple loci in our data. We cannot discriminate between intra- and interlocus recombination as both mechanisms might interfere with each other in our dataset but it is assumed that interlocus recombination plays a minor role in MHC evolution of mammals [54].

Another indicator of balancing selection acting in a genetic system is trans-species polymorphism [18]. This non-neutral retention of alleles and lineages, across even multiple speciation events, is a typical feature of MHC evolution [3, 17, 18]. Patterns of this mode of evolution were described in a wide range of taxa including rodents [59-61], salmonids [53, 62], ungulates [48, 63], carnivores [64, 65] and primates [66]. In our study we found MHC-DAB lineages shared between both species. But as we have shown that recombination is likely to have played a significant role in generating DAB diversity, conclusions about potential trans-species polymorphism should be treated cautiously. Recombination may have strong effects on phylogenetic inferences. For instance, it will cause allelic lineages appearing to have diverged over a longer time period than they actually did whereas the

polymorphism as a whole is younger. This effect makes trans-species polymorphism more apparent [67]. Not only did we find shared lineages of MHC-DAB but also pairs of identical alleles, which constitute a more conclusive evidence of trans-species polymorphism. Trans-species sharing of MHC class II alleles between species of the same genus was documented in some studies on primates [68, 69] but this phenomenon is rarely observed across genera. One striking example is the extensive MHC-DRB allele sharing between families of Malagasy lemurs [70].

In this study we are faced with multiple MHC-DAB loci, at least two in *M. incanus* and a minimum of four in *G. microtarsus.* A common problem in MHC-studies are difficulties in assigning alleles to their respective locus [51, 71-73], which limits the significance of analyses on selection patterns. Different loci may be subjected to different selection pressures and processes and in a strict sense they cannot be combined in calculations on positively selected sites, trans-species polymorphism and sequence diversity. However, interlocus allelic exchange is known to occur at MHC loci [71], emphasizing its functional coherence. Also gene duplication is an important mechanism to generate polymorphism at the MHC, which is known, for instance, from rhesus macaques or Californian sea lions [74, 75]. Despite this lack of knowledge, our study describing features of marsupial MHC diversity from natural populations is worthwhile even without locus assignment because it revealed results contradicting previous claims of low MHC class II variability in marsupials.

Our data on 54 free-ranging *G. microtarsus* disprove the previous assumption of very limited polymorphism at the MHC class II. *G. microtarsus* shows extensive polymorphism in the number of alleles within an individual and within the total sample as well as in the genetic distance among alleles. This level of variation is typical for classical MHC loci in studies on natural populations of numerous eutherian mammals. The evaluation of large genetic distance among the alleles should, however, be treated with caution, because we cannot discriminate between intra- and interlocus distances. At the current state of this work, tissue samples providing sufficient amounts of RNA are not available and thus we have no information on expression patterns of MHC class II in the investigated species.

In *G. microtarsus* one to seven alleles per individual were detected via cloning and sequencing. In preparation for this study, extensive sequencing revealed that 15 sequenced clones per individual provide a good estimate for allelic diversity [see Additional File 1]. Our results may still represent an underestimation of individual allelic diversity because the number of alleles detected per individual is correlated with the number of clones sequenced. However, we do not assume that low numbers of detected *Grmi*-DAB alleles in some individuals result from deficient sequencing, but from the presence of null

alleles and/or a varying number of MHC-DAB loci in *G. microtarsus.* In individuals with one to three *Grmi*-DAB alleles some loci may have been missed by our primers or they simply do not exist. In some species the number of MHC class II loci varies between populations or even individuals of the same population. In cichlid fishes, for instance, the number of MHC class II B loci varies between haplotypes and individuals, ranging from one to thirteen loci [76]. In voles Bryja et al. [51] found that individuals differ in the number of DQA loci, showing either alleles from one or from two loci. Also for humans and other primates the number of DRB genes present per haplotype can vary, it ranges from one to four in humans and from two to seven in the rhesus macaque [75, 77].

M. incanus shows a similar dimension of MHC II diversity at the individual level, with each individual carrying 2-4 DAB alleles. In contrast, the number of eight MHC-DAB alleles in the total sample of 56 individuals is low (more than five times reduced compared to *G. microtarsus*) resulting in similar genotypes throughout the sample. Each individual revealed two to four *Main*-DAB alleles, which is in full concordance with the assumption of two loci in this species. Still the presence of null alleles cannot be excluded.

In general, low MHC variation in a population can be explained by relaxed selection pressure through pathogens and/or genetic drift in small populations, e.g. through a bottleneck [17, 78] or by the mating system [79]. Diminished MHC diversity is demonstrated, for instance, in fin whales (*Balaenoptera physlaus*), sei whales (*B. borealis)*, Northern elephant seals (*Mirounga angustirostris*) [78, 80], Malagasy giant jumping rats (*Hypogeomys antimena*) [79], moose (*Alces alces*) [81, 82], common hamsters (*Cricetus cricetus*) [83] and in Scandinavian beavers (*Castor fiber*) [71].

The two marsupial species live sympatricly in the Atlantic rainforest of Brazil. They inhabit the same type of habitat, although *M. incanus* frequently uses the forest ground and the lower strata [84, 85], while *G. microtarsus* is mainly arboreal and uses the canopy [86]. It was shown that both species differ in their microhabitat preferences and in their sensitivity to forest fragmentation [34, 35]. Fragmentation does not negatively affect *G. microtarsus'* abundances, and it prefers more disturbed forest habitats. *M. incanus,* on the other hand, responds sensitively to forest fragmentation with decreasing abundances in small and isolated areas and increased abundance in old and less disturbed forests [34, 35]. Males of both species are at least partially semelparous, meaning that they contribute to only one breeding season with multiple copulations and die afterwards due to severe physiological stress [87, 88]. The females may survive and reproduce again, often in two or exceptionally in three consecutive seasons.

Hence, a reduced exposure to pathogens due to a monogamous mating system or a different environment can be excluded as an explanation of the low MHC diversity in *M. incanus* compared to *G. microtarsus*. Moreover, *M. incanus* should harbour more parasite infections compared to *G. microtarsus* because the mode of terrestrial locomotion increases the risk of parasitic infections through greater exposure to soil borne or faecal-orally transmitted parasites [89]. In fact Püttker et al. [90] reported from the same study populations a much higher nematode prevalence in *M. incanus* than in *G. microtarsus*. We assume that the explanation for the reduced MHC diversity in *M. incanus* might be a genetic bottleneck in the investigated population and that the diminished MHC diversity reflects a genome-wide loss of diversity. This explanation is supported by the fact that *M. incanus* is sensitive to habitat fragmentation, which probably led to reduced migration and isolation of the study population. *G. microtarsus* on the other hand is ecologically more flexible and non sensitive to fragmentation. Probably migration processes are less or not restricted and therefore MHC diversity is high. The pattern of low MHC diversity in *M. incanus* resembles several examples from eutherian mammals that revealed low MHC variation in populations after a genetic bottleneck [summarized in 5].

In future analyses we aim to combine parasitological and genetic data on adaptive and overall neutral variability to investigate the role of forest fragmentation in shaping the different patterns of MHC diversity in the two marsupials. Lastly, expression analyses are required to shed light onto the functional differences in the MHC constitution of these two species.

Conclusion

Despite the previous assumption that marsupials lack polymorphism at the MHC class II, our study revealed high levels of diversity in a free-ranging population of a South American marsupial. We tested for three different mechanisms that may generate and maintain MHC diversity: positive selection, recombination and trans-species polymorphism. The presence of all three mechanisms was confirmed. Moreover, typical large sequence divergence of the MHC alleles was found in both species. Beyond that, the two investigated marsupial populations revealed considerable differences in their MHC-DAB diversity. The processes leading to these differences will be investigated in future analyses including parasitological data and expression analyses.

Methods

Sampling

54 individuals of the Brazilian gracile mouse opossum (*G. microtarsus*) and 56 individuals of the Gray slender mouse opossum (*M. incanus*) were captured in forest fragments of the Brazilian Atlantic rainforest in the state of São Paulo, about 60 km south-west of the city of São Paulo. Animals were live-trapped and anesthetized (Forene, Abbott GmbH, Wiesbaden, Germany) for 1–2 minutes to allow small tissue samples to be taken from the ear, which were then stored in 70% ethanol. Subsequently, animals were released at their respective trapping location. For a detailed description of the study area, sites and trapping procedures see Püttker et al. [90, 91].

PCR and SSCP

DNA was extracted from ear tissue using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). We examined a 195 bp fragment of the marsupial MHC class II DAB molecule coding for the major part of the ß1 domain. This part contains most of the functionally important antigen binding sites in the ß chain of the human DR1 molecule [37]. The fragment was amplified using primers JS1 (5'-GAG TGT CAT TTC TAC AAC GGG ACG-3') [92] and ML8 (5'- ACG CGC CTG CGC ACT AAG AAG GGC TC -3'). JS1 was previously designed for the mouse lemur *Microcebus murinus* and was successfully applied to several rodent species [71, 93-95]. We designed ML8 for this study based on the DAB sequence of *M. domestica* (*Modo*-DAB1, accession No. AF010497).

PCR was conducted in a total reaction volume of 20µl including 30-100ng DNA, 0.375µM of each primer (Sigma-Aldrich, Steinheim, Germany), 1x reaction buffer (10mM TrisHCl, 50mM KCl, 0.1% Triton X100, 0.2mg/ml BSA), 0.175mM dNTPs and 1 unit of *Taq* polymerase (MPBiomedicals, Heidelberg, Germany). Thermocycling comprised an initial denaturation step at 95°C for 5min, followed by 35 cycles of 30s denaturation at 95°C; 60s annealing at 56°C for *G. microtarsus* or 51°C for *M. incanus,* and 60s extension at 72°C. A final extension step was performed at 72°C for 10min.

M. incanus individuals were genotyped via single strand conformation polymorphism (SSCP) [96]. For denaturation 1-4µl PCR product were mixed with 6µl loading dye (50mM NaOH + 1mM EDTA, Xylencyanol) and heated for 10min at 50°C. The samples were subjected to electrophoresis on a 15% non-denaturing polyacrylamid gel (ETC, Kirchentellinsfurt, Germany). The best separation of MHC alleles was achieved by setting 200V, 10mA, 10W for 20min followed by 3h at 450V, 30mA, 20W (all steps conducted at

constant 12°C). Gels then were fixed and silver stained (Plus One DNA Silver Staining Kit, GE Healthcare, Munich). The single strand patterns were excised from the gel matrix, eluted in 1 x TBE, re-amplified as described above for PCR and prepared for sequencing. Two alleles (*Main*-DAB*03 and *Main*-DAB*04) differed in two nucleotide but revealed identical SSCP patterns. Individuals with this pattern were labelled as *Main*-DAB*03/4 if their band patterns were not sequenced. Due to a large number of MHC alleles per individual in *G. microtarsus* full separation of band patterns could not be accomplished through SSCP. *G. microtarsus* was examined therefore via molecular cloning after initial SSCP trials.

Cloning and Sequencing

Molecular cloning often leads to an increase in misincorporation errors, therefore PCR products for cloning were generated with a proofreading polymerase (Hotstar Hifidelity polymerase, Qiagen, Hilden, Germany). Moreover, in heterogeneous templates (such as MHC genes) PCR amplifications are susceptible to the creation of artificial chimeric molecules from two related sequences [97]. The probability of chimeric molecule formation was reduced by two modifications of the PCR protocol described above: The number of cycles was reduced to 32 and the extension time prolonged to 2:30 min [97]. PCR products were purified (Cycle pure, Peqlab, Erlangen, Germany) and cloned into a pCR®4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen, Karlsruhe, Germany). At the beginning of the study we determined the required number of clones to be sequenced to reflect the individual MHC-diversity by extensive sequencing in two individuals [see Additional File 1]. Fifteen recombinant clones per sample were selected, PCR-amplified using the vector-primers T7 and M13rev, and purified. Sequencing and sequence analysis was carried out on an ABI PRISM 310 Automated Genetic Analyzer (Applied Biosystems, Foster City, Ca, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI).

A clone sequence was accepted as a MHC-DAB allele if one of the following criteria were met: (1) occurrence in two independent PCR reactions, (2) confirmation by SSCP or (3) a minimum of two positions differing from a known allele. Single sequences differing by one nucleotide from a known allele were attributed to the polymerase misincorporation error (declared by the company: 2.3×10^{-6} per base per cycle, under our conditions one misincorporation every 70 molecules). Sequences differing in one position were observed more frequently than explained by this error, but we decided to follow a conservative approach so as to avoid overestimation of allelic diversity.

Sequences that were not confirmed by (1) or (2) were checked visually for PCR-mediated chimeric formations. Sequences composed of more than two fragments were assumed to be true recombination events because a multiple template switching during PCR is highly improbable. However, no PCR-mediated chimeric sequence was detected in our data. The MHC-DAB alleles of *G. microtarsus* and *M. incanus* were denominated as *Grmi*-DAB and *Main*-DAB corresponding to the MHC nomenclature suggested by Klein and co-workers [36].

Test for positive selection

Under balancing selection a relative excess of non-synonymous over synonymous substitutions is expected [7, 8]. We calculated the relative rates of synonymous (d_S) and non-synonymous (d_N) substitutions following the method of Nei and Gojobory [98] with the Jukes-Cantor [99] correction for multiple substitutions implemented in MEGA 3.1. The ratio $\omega = d_N/d_S$ was tested for significant deviation from one using a Z-test.

We used CODEML integrated into the software PAML 3.15 [39] to identify and test for positively selected codon sites ($d_N/d_S > 1$) in the MHC-DAB sequences. This was done separately for the two marsupial species. Based on maximum likelihood procedures the program estimates heterogeneous ω ($=d_N/d_S$) ratios among sites applying different models of codon evolution. The models are used to detect positively selected codon sites, and evaluated using a likelihood-ratio test (LRT). In the LRT two nested models are compared and the better fitting one is detected. Therefore, a model based on the null hypothesis of no selection is applied as well as a more complex model that assumes positive selection. The LRT is a comparison of twice the log-likelihood difference (2[Lb – La]) with a χ^2-distribution with the degrees of freedom equal to the difference in number of estimated parameters between the two models [100]. In this study, the models M1a (nearly neutral), M2a (positive selection), M7 (ß) and M8 (ß + ω) were employed [100]. Null model M1a assumes two site classes in the molecule with $0 < \omega_0 < 1$ and $\omega_1 = 1$ in proportions p_0 and $p_1 = 1 - p_0$. The alternative model M2a incorporates another class of sites with $\omega_2 > 1$ and the proportion p_2 estimated from the data. Null model M7 assumes a beta distribution for ω, not allowing for positive selection ($0 < \omega < 1$). In the alternative model M8 a third class of sites is added, which allows for positive selection ($\omega > 1$) [101]. Anisimova et al. [40] showed that the combination of models M7 and M8 is robust against impact of recombination [41]. In the models M2a and M8 the probabilities for the site classes have been calculated by the Bayes empirical Bayes method (BEB) [101]. A site class with a mean $\omega > 1$ is likely to be under positive selection.

Test for gene conversion and recombination

The program GENECONV version 1.81 was used to detect sequence fragments that were likely to have been subjected to gene conversions. GENECONV detects pairs of sequences that share unusually long stretches of similarity given their overall polymorphism [102]. We used global and pairwise permutation tests (10,000 replicates) for both species separately to assess significance. No mismatches were accepted and p-values were corrected for multiple comparisons. In general, this approach is considered to be a powerful way of inferring recombination events correctly [58], but under extensive recombination as present at some MHC loci the power of GENECONV might be reduced [51].

We further applied the composite-likelihood method after Hudson [103] implemented in the program LDhat [43] to estimate the population recombination rate ($\rho=4N_e r$). The population recombination rate is a product of the crossing over rate per generation, *r*, and the effective population size, N_e. This product can be estimated without prior information on these parameters [43]. LDhat works efficiently for sequences evolving under balancing selection even in the presence of numerous recombination events [104]. In a likelihood permutation test the population recombination estimate ρ was tested for statistical significance against the hypothesis of no recombination ($\rho=0$). The minimum number of recombinant events (R_M) was calculated after Hudson and Kaplan [four-gamete method, 43] using the software DnaSP [45].

Phylogenetic analyses

Sequence alignment and manual revision were performed in MEGA version 3.1 [105]. The same software was applied for the construction of a phylogenetic tree using the neighbour joining method [106], based on Kimura's two-parameter evolutionary distances [107]. In the phylogenetic tree the following Genbank sequences were applied: Marsupialia: *Macropus eugenii*: *Maeu*-DAB1 (AY438042), *Maeu*-DAB3 (AY856412), *Maeu*-DBB1 (AY438038), *Maeu*-DBB2 (AY438039), *Maeu*-DBB4 (AY438041); *Macropus rufogriseus: Maru*-DAB1 (M81624), *Maru*-DAB2 (M81626), *Maru*-DBB1 (M81625); *Monodelphis domestica: Modo*-DAB*01 (AF010497), *Modo*-DBB1 (XM_001369154), *Modo*-DBB2 (XM_001376782), *Modo*-DCB (XM_001376662); *Trichosurus vulpecula: Trvu*-DAB*02 (AF312029), *Trvu*-DAB*03 (AF312030), *Trvu*-DBB (AY271265). Monotremata: *Tachyglossus aculeatus: Taac*-DZB1 (AY288075); *Ornithorhynchus anatinus: Oran*-DZB1 (AY288074). Eutherians: *Aotus nancymaae*: *Aona*-DPB1 (AF486448); *Bos taurus*: *Bota*-

DOB (AB117945); *Canis*: *Cafa*-DQB1 (NM_001014381); *Calu*-DOB (NM_001048127); *Felis catus*: *Feca*-DRB1 (AJ428212); *Homo sapiens: Hosa*-DRB (AM419948), *Hosa*-DPB1 (NM_002121), *Hosa*-DOB (NM_002120); *Macaca fascicularis*: *Mafa*-DPB1 (AB235893). *Sus scrofa*: Susc-DRB (AY135583), Susc-DQB (AY135571). Chicken: GagaBLB1 (NM_001044694), GagaBLB2 (M29763), GagaBLB3 (M26307); *Xenopus* XelaB3 (D13685); *Bombina bombina*: Bobom-Beta1-3 (EF210770), Bobom-5Race-1 (EF210766) and Bobom-3Race-1 (EF210761); *Ambystoma mexicanum*: *Amme*-DAB (AF209115); *Sphenodon punctatus*: *Sppu* DAB (DQ124232).

Authors' contributions

TP and YML collected the field data. YML and CO carried out the molecular genetic analyses and managed the data. Statistical analyses, interpretation of the data and writing of the manuscript were performed by YML. SS conceived the study and the design, initiated its key collaborations and helped in drafting the manuscript. All authors read and approved the final manuscript.

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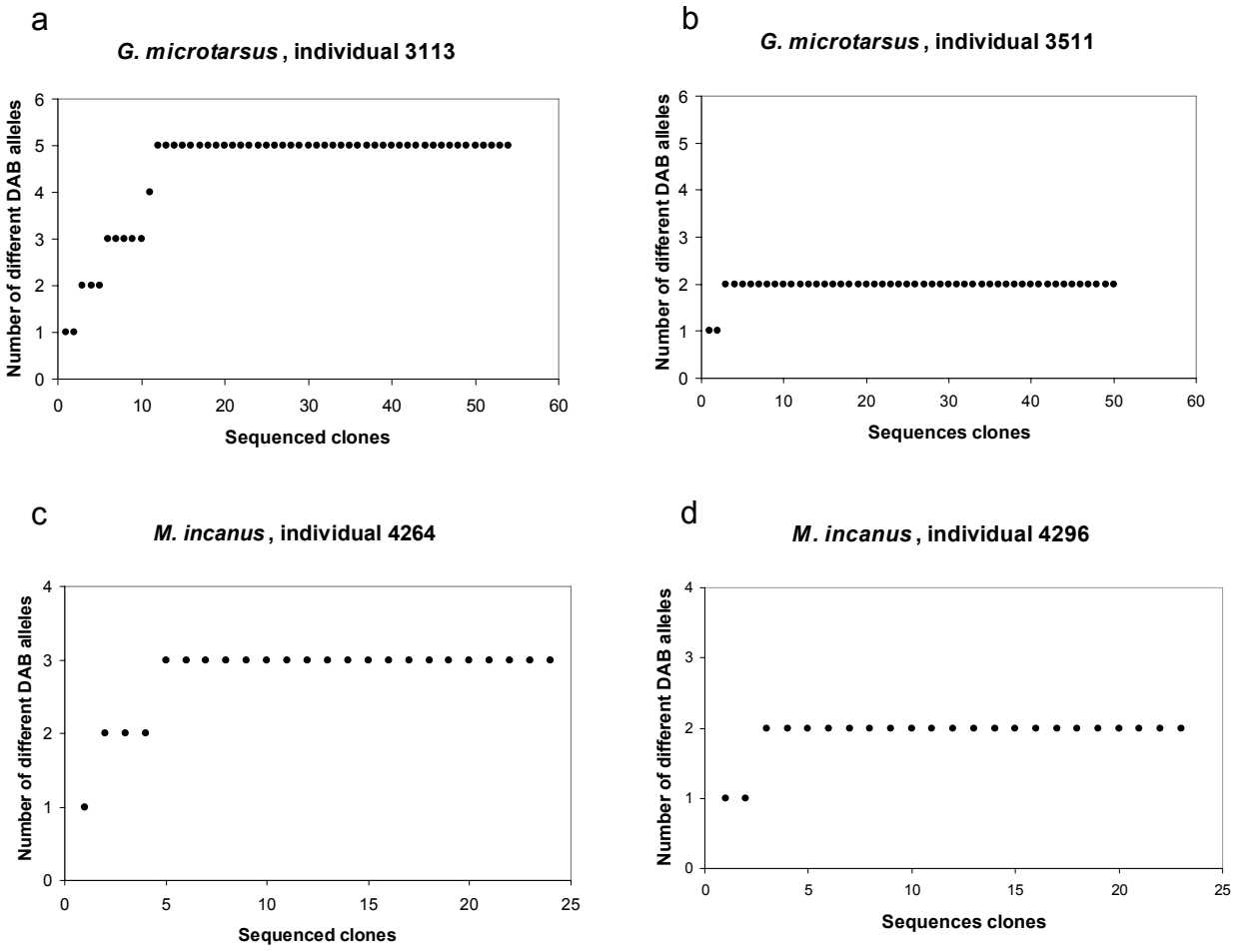
Supplement 1: Relationship between the number of sequenced clones and the number of different MHC-DAB alleles revealed in two individuals of *G. microtarsus* (a, b) and in two individuals of *M. incanus* (c, d).



Article 2:

Variety matters: adaptive genetic diversity and parasite load in two mouse opossums from the Brazilian Atlantic forest

Variety matters: adaptive genetic diversity and parasite load in two mouse opossums from the Brazilian Atlantic forest

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Abstract

The adaptive potential of a species to a changing environment and in disease defence is primarily based on genetic variation. Immune genes, such as genes of the major histocompatibility complex (MHC), may thereby be of particular importance. In marsupials, however, there is very little knowledge about natural levels and functional importance of MHC polymorphism, despite their key role in the mammalian evolution. In a previous study, we discovered remarkable differences in the MHC class II diversity between two species of mouse opossums (*Gracilinanus microtarsus, Marmosops incanus*) from the Brazilian Atlantic forest, which is one of the most endangered hotspots for biodiversity conservation. Since the main forces in generating MHC diversity are assumed to be pathogens, we investigated in this study gastrointestinal parasite burden and functional associations between the individual MHC constitution and parasite load. We tested two contrasting scenarios, which might explain differences in MHC diversity between species. We predicted that a species with low MHC diversity would either be under relaxed selection pressure by low parasite diversity (*'Evolutionary equilibrium'* scenario), or there was a recent loss in MHC diversity leading to a lack of resistance alleles and increased parasite burden (*'Unbalanced situation'* scenario). In both species it became apparent that the MHC class II is functionally important in defence against gastrointestinal helminths, which was shown here for the first time in marsupials. Parasite diversity did not markedly differ between the two host species. However, we did observe considerable differences in the parasite load (parasite prevalence and infection intensity): while *M. incanus* revealed low MHC DAB diversity and high parasite load, *G. microtarsus* showed a tenfold higher population wide MHC DAB diversity and lower parasite burden. These results support the second scenario of an unbalanced situation.

Introduction

Pathogens represent very powerful agents of selection that have the potential to drive rapid changes in the genetic composition of natural host populations. In the coevolutionary host-pathogen interplay they are particularly important in maintaining host genetic variation (Altizer et al. 2003; Altizer et al. 2007; McCallum and Dobson 1995). On the other hand, pathogens do exhibit the potential of triggering serious population declines once host genetic diversity is lost due to inbreeding or genetic drift. In such unbalanced situations, pathogens may even pose a severe extinction risk to small populations that have lost their ability to buffer environmental challenges (Altizer et al. 2003; Altizer et al. 2007; McCallum and Dobson 1995; Woodroffe 1999). A number of studies showed indeed that low levels of genetic diversity are associated with a reduced immune reaction and high pathogen loads (e.g. Liersch & Schmid-Hempel 1998; Coltman *et al.* 1999; Meagher 1999; Cassinello *et al.* 2001; Dorman *et al.* 2004; Spielman *et al.* 2004).

In terms of pathogen defence there is a group of well studied immune genes that plays a critical role in triggering the vertebrate adaptive immune response: the major histocompatibility complex (MHC). The MHC encodes receptor molecules that recognise and bind antigens in order to present them to T-lymphocytes (Klein 1986). While MHC class I molecules mainly present peptides derived from the inside of viral or cancer-infested cells, MHC class II molecules primarily correspond to extracellular pathogens (e.g. bacteria, nematodes, cestodes, Klein and Horejsi 1997). One of the most typical attributes of MHC genes is the enormous polymorphism found in the majority of vertebrates studied to date, generated and maintained by balancing selection (Apanius et al. 1997; Hedrick 1994; Klein 1986; Sommer 2005). Selection processes are thought to be largely driven by the necessity to recognise a wide array of pathogens (Bernatchez and Landry 2003; Hedrick 1994; Piertney and Oliver 2006; Sommer 2005). Thereby, MHC heterozygotes could exhibit a selective advantage over homozygote individuals, because they possess a larger number of MHC variants to recognize and defend parasite antigens (‘heterozygote advantage’, Doherty and Zinkernagel 1975). Or certain MHC alleles could be advantageous, whereby selection is frequency-dependent and varies over time and space (‘rare-allele-advantage’, Hedrick 2002; Slade and McCallum 1992; Takahata and Nei 1990).

In marsupials, the MHC has been poorly investigated so far, although this group represents a milestone in the evolution of mammals. Studies on captive or laboratory bred marsupials indicated low levels of MHC class II diversity, and very limited polymorphism

was assumed to be a general feature of the marsupial MHC class II (Stone et al. 1996; Stone et al. 1998). However, there is a lack of knowledge about natural levels of marsupial MHC class II diversity, aside from our previous study (Meyer-Lucht et al. 2008) and two other recent investigations (Holland et al. 2008b; Holland et al. 2008a; Siddle et al. 2007b). The latter studies reported considerable MHC class II diversity in the Australian brushtail possum (Trichosurus vulpecula, Holland et al. 2008b; Holland et al. 2008a), but no MHC class II variation in Tasmanian devils (Sarcophilus harrisii, Siddle et al. 2007b). Interestingly, our study on free-ranging Neotropical marsupials revealed contradictory patterns of MHC class II diversity in the two investigated species as well. Diversity was high in the Brazilian gracile mouse opossum (*Gracilinanus microtarsus*, Wagner 1842) and congruent with levels of MHC II diversity described in numerous wild eutherian species (Meyer-Lucht et al. 2008). In contrast, MHC II diversity was rather low (five times lower) in the second species under study, the Gray slender mouse opossum (*Marmosops incanus*, Lund 1840).

The two investigated mouse opossum species are endemic to the Brazilian Atlantic forest, which is one of the most threatened biomes in the world (Myers et al. 2000). Once ranging almost continuously along the Brazilian coast and covering more than 1.5 million km^2, the Atlantic forest nowadays comprises only 12% of its original extent (Ribeiro et al. 2009; SOS Mata Atlântica 2008). Nevertheless, it still harbours an extraordinary concentration of endemic species (Myers et al. 2000). Based on these facts the region has been classified as one of the five most important biodiversity hotspots for conservation priorities (Myers et al. 2000). With ongoing expansion of human activities, however, the remaining Atlantic forest patches are continuously being fragmented and degraded (Tabarelli et al. 2004; Tabarelli et al. 2005; Teixeira et al. 2009).

The two study species, *G. microtarsus* and *M. incanus,* feature similar ecological characteristics: they are nocturnal, solitary (Caceres 2004) and feed on a similar omnivorous diet (Fonseca and Kierulff 1989; Martins and Bonato 2004). However, they differ in habitat use as *G. microtarsus* is mainly arboreal while *M. incanus* more frequently uses the understorey and forest ground (Cunha and Vieira 2002; Vieira and Monteiro-Filho 2003). Both species show at least partially a semelparous mating behaviour, which means that individuals contribute to only one mating season and die thereafter (Lorini et al. 1994; Martins et al. 2006).

In view of this apparent similar environmental background the question arises what causes the conspicuous differences in MHC diversity between the two mouse opossum species. Assuming that the main selective forces in generating and maintaining a high

MHC diversity are pathogens (Apanius et al. 1997; Hughes and Yeager 1998), we focussed in this study on the parasite load of the two mouse opossum species, more precisely on the burden with gastrointestinal helminths. We considered two possible scenarios with respect to MHC diversity and parasite burden and predicted the following:

(1) '*Evolutionary equilibrium*' scenario*:* at a state of evolutionary equilibrium between hosts and pathogens, selection through diverse pathogens will have caused high MHC polymorphism in a species or population, whereas low MHC polymorphism indicates the presence of relaxed pathogenic selection pressure. This scenario holds true if hosts and pathogens share a long-term coevolutionary history. This scenario was indeed supported by a comparison of different human populations worldwide (Prugnolle et al. 2005), and a study on stickleback populations from different habitats (Wegner et al. 2003). Both investigations revealed that populations exposed to a more diverse pathogen regime exhibit higher MHC diversity than those exposed to fewer pathogens. And more recently, in a meta-analysis on rodent species and their helminth parasites, Goüy de Bellocq and coworkers (2008) detected likewise a significant positive correlation between MHC allelic diversity in a species and the number of different parasite species, the so called parasite diversity. According to this scenario, we expected to observe relative high parasite diversity in the MHC class II diverse species *G. microtarsus*, and relative low parasite diversity in the species with low MHC class II diversity, *M. incanus*.

(2) '*Unbalanced situation*' scenario: as a contrasting scenario we assumed an unbalanced situation, which might be caused by a recent loss of genetic diversity in the depleted host by means of, for instance, a bottleneck event. The species with low MHC diversity could have lost resistance alleles or other important parts of its adaptive evolutionary potential. Moreover, after a recent loss the host population most likely is genetically more or less homogenous. This fact facilitates an easy spread of pathogens throughout the population, because most individuals share the same resistance genotype (Meagher 1999). According to this second scenario, we expected that the MHC depleted species should face higher parasite loads than the MHC diverse species, at least in terms of individual parasite prevalence and infection intensity. We did not necessarily expect different parasite diversities in the two species because both study species originated from similar habitats, in contrast to the previous studies by Prugnolle et al. (2005), Wegner et al. (2003) and Goüy de Bellocq et al. (2008).

We tested these theoretical assumptions by analysing the patterns of gastrointestinal helminth burden and MHC class II diversity in the two mouse opossum species *G. microtarsus* and *M. incanus*. In addition, we investigated functional associations

between parasite load and individual MHC variation to understand selection processes acting on the marsupial MHC DAB under natural conditions.

Methods

Study area and sampling

The study was carried out in the Brazilian Atlantic forest in the region of Caucaia do Alto, municipalities of Cotia and Ibiúna, State of São Paulo, Brazil. Small mammal trapping was conducted in five forest fragments (sites S1 to S5) within a fragmented landscape with 31% native forest cover and one control site (CS) within an adjacent continuous forest at the Morro Grande Forest Reserve. We used Sherman live traps in a grid of 100 trap locations in 20 m intervals and captured in five trapping sessions per site from July 2003 to March 2005. Within this period, 102 Brazilian gracile mouse opossums (*G. microtarsus*) and 123 Gray slender mouse opossums (*M. incanus*) were trapped. *G. microtarsus* was not captured in forest site S2. From the trapped animals small ear cuts were taken for tissue samples (~5 mm^2) and stored in 70% ethanol. Faeces were collected from the used traps and stored in 96% ethanol. Traps were cleaned prior to re-use. For detailed information on trapping sessions and study sites see Püttker et al. (2006), for further details upon the study area see Pardini et al. (2005).

Molecular analyses

We genotyped the MHC DAB genes of all 102 *G. microtarsus* and 123 *M. incanus* individuals to assess allelic diversity, and thereby doubled the sample size from our previous work (Meyer-Lucht et al. 2008). Genomic DNA from ear tissue samples was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). A 195 bp fragment of the marsupial MHC class II DAB was amplified. This fragment codes for the major part of the ß1 domain of the molecule and contains positively selected amino acid sites that are presumably involved in antigen binding (Meyer-Lucht et al. 2008). In the human DR1 molecule this part comprises a major part of the antigen binding sites as defined by Brown et al. (1993).

We used the forward primer JS1 (Schad et al. 2004) and the reverse primer ML8 (Meyer-Lucht et al. 2008) for PCR amplification. Single stranded conformation polymorphism (SSCP) analyses on 15% polyacrylamide gels were used to genotype *M.*

incanus. SSCP band patterns were assigned to alleles, and exemplary bands were excised and re-amplified. Two *Main*-DAB alleles (*Main*-DAB*03 and *Main*-DAB*04) could not be distinguished on the SSCP patterns, and were therefore handled together as Main-DAB*03/04. The two alleles differed in one synonymous and one non-synonymous nucleotide substitution (Meyer-Lucht et al. 2008).

Due to the large number of alleles per individual *G. microtarsus* was genotyped via molecular cloning and sequencing of fifteen recombinant clones per individual. This number was determined from initial trials on extensive sequencing of recombinant clones in two individuals (Meyer-Lucht et al. 2008). In comparison to SSCP analyses, this procedure probably still induces an underestimation of individual allelic diversity because the number of detected alleles is correlated with the number of sequenced clones. Transformed clones were PCR amplified using the vector primers T7 and M13. Amplification products were sequenced on an ABI PRISM 310 Automated Genetic Analyzer (Applied Biosystems, Foster City, Ca, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI). A precise description of the molecular techniques is given in Meyer-Lucht et al. (2008).

Parasitological examinations

To analyse the degree of gastrointestinal helminth parasitism in *G. microtarsus* and *M. incanus*, presence and number of helminth eggs was non-invasively assessed in the host faecal samples. Faecal egg counts (FEC) are not only informative in terms of prevalence but also appropriate to assess the intensity of infections (Soulsby 1982). These counts reflect the overall worm burden and worm fecundity, which both are influenced by the immune state of the host (Stear et al. 1995; 1997). Due to its non-invasive nature FEC is applicable even to wildlife populations, hence, it is a widely used approach in field studies (e.g. Cassinello et al. 2001; Coltman et al. 1999; Ferrari et al. 2004; Froeschke and Sommer 2005; Harf and Sommer 2005; Meyer-Lucht and Sommer 2005; Schad et al. 2005; Schwensow et al. 2007; Seivwright et al. 2004). Parasite eggs were assigned to morphotypes defined by size and morphological characters, and the family was determined if possible from the egg morphology. To quantify the number of eggs we applied a modification of the McMaster flotation technique (Gordon and Whitlock 1939), which uses a very dense potassium iodide solution to enhance the ability to detect heavy weighed eggs (Meyer-Lucht and Sommer 2005). We used as measures for parasite load the number of different helminth infections per individual (NHI), the helminth prevalence (infected individuals /examined individuals) and the intensity of nematode infection (nematode eggs /g faeces). Due to their extremely low prevalence trematodes were not considered. In

cestodes, numerical variation in egg release can be extremely high; therefore intensity of cestode infection was not included.

Software and data analyses

MEGA 4.0 (Tamura et al. 2007) was used to edit nucleotide sequences manually, to align them, and to calculate amino acid differences between the alleles. As the number of amino acid differences between MHC alleles might not be a good predictor for their functional differences, we grouped MHC alleles according to similar antigen-binding motifs into so called MHC supertypes (Supplement 1). The technique was invented in human vaccine development studies and is supported by the fact that in humans different HLA alleles overlap in their peptide binding specifities (Sette and Sidney 1999; Southwood et al. 1998; Trachtenberg et al. 2003). This technique requires a minimum number of alleles, which was not observed in *M. incanus* (see below). For *G. microtarsus*, we followed a procedure proposed by Doytchinova and Flower (2005), which was successfully applied in a study on a natural primate population by Schwensow et al. (2007). In a first step amino acid sites under positive selection were identified, which are presumably involved in the antigen binding process, using the program CODEML implemented in PAML 3.15 (Yang 1997). For details on the calculations see Meyer-Lucht et al. (2008). In a second step, for each *Grmi*-DAB allele these positively selected amino acid sites were characterized by five *z*-descriptors (Sandberg et al. 1998) based on their lipophilic, steric and electronic properties: *z*1 (hydrophobicity), *z*2 (steric bulk), *z*3 (polarity), *z*4 and *z*5 (electronic effects). For each site these five *z*-values were assigned and a hierarchical cluster was calculated using the Euclidian distance method. The resulting groups (= MHC supertypes) possess similar physicochemical binding features at the putative antigen binding sites. These MHC supertypes were included as predictors in subsequent analyses.

The individuals were trapped in six forest sites of different size. We tested if there was an effect of the forest patch size on immune gene diversity to account for area effects. Therefore, we calculated linear regression analyses of the number of MHC alleles found per forest site in relation to the forest patch size. To compare the parasite loads between the two host species, we based our calculations on the total sample sizes of 102 *G. microtarsus* and 123 *M. incanus* individuals. The number of different helminth infections between the two host species was analysed by the non parametric Mann-Whitney-U-Test. The helminth prevalence in the two hosts was compared by a χ^2-test. The values for nematode infection intensity were log-transformed to account for heterogeneity, and comparison between species was again carried out with the Mann-Whitney-U-Test.

To understand selection processes acting on the marsupial MHC DAB under natural conditions we investigated the relationship between the individual MHC DAB constitution and the parasite load in *G. microtarsus* and *M. incanus*. Therefore we used generalized linear models (GLMs) and included environmental, biotic and genetic predictors to explain different measures of parasite burden. As environmental predictors the categorical variables 'forest site' and 'capture session' were included, biotic predictors comprised the individual body condition (body mass index, BMI) and the sex. BMI was obtained from regressing body mass on body size (length of tibia), and using the residuals from this regression (Schulte-Hostedde et al. 2005). For *G. microtarsus* we used the presence/absence of 13 MHC DAB supertypes that occurred in frequencies >0.05 and <0.95 as genetic predictors. In addition we used the number of different MHC supertypes per individual as a measure for individual MHC diversity. In the case of *M. incanus* we used the presence/absence of the detected MHC DAB alleles, with *Main*-DAB*03 and *Main*-DAB *04 combined to one predictor (see above), and a combination of *Main*-DAB*07 and *Main*-DAB *08 because they always co-occurred. As a measure for individual MHC diversity the number of different MHC DAB alleles per individual was used. To avoid collinearity we tested all predictors for correlations and, if necessary, included them in separate, otherwise identical models. Missing data in the predictors BMI or sex reduced the number of included cases to 96 in *G. microtarsus* and 121 in *M. incanus.*

We fitted models for the number of different helminth infections, the helminth prevalence and the nematode infection intensity (FEC). The models for NHI were calculated with a poisson error distribution and log link function. For prevalence data, logistic regression models were applied with a binomial error distribution and logit link function. For the infection intensity data, we used a quasipoisson error distribution with a log link function, which accounts for overdispersion in these data. We started with the full model including all predictor variables and conducted backward selection to find the minimal adequate model to explain our data. Backward selection was performed by dropping sequentially non significant predictors from the model. The new, less complex model was compared with the previous, more complex model by testing the change in deviance for significance. If the simplification was not associated to a significant increase in deviance, the less complex model was preferred. When all predictors had to be excluded, no model was better in explaining the data than the null model, i.e. there were no effects of any predictor on the response variable. The adjusted R^2 was calculated as 1 - (residual deviance/residual d.f.) / (null model deviance/null model d.f.). Odds ratio estimates were calculated by exponentiation of the coefficient from the logistic regression model. Statistical

tests were performed in R (version 2.7.0, R Development Core Team 2008) and SPSS 16.0, using a threshold of p = 0.05.

Results

MHC DAB diversity

G. microtarsus

The 102 individuals of *G. microtarsus* revealed high allelic diversity at the MHC DAB, with a total of 80 *Grmi*-DAB alleles (Table 1). We have described 47 of these alleles in an earlier study on a smaller sample size (N = 54, Meyer-Lucht et al. 2008, EU350150 - EU350196). The 33 additional *Grmi*-DAB sequences were deposited at GenBank under FJ374838 - FJ374858, FJ374860, FJ374862 - FJ374870. Five groups of nucleotide sequences translated each into one identical amino acid sequence (*Grmi*-DAB*01a, *01b, *01c & *01d; *Grmi*-DAB*10a, *10b, *10c & *10d; *Grmi*-DAB*12a & *12b; *Grmi*-DAB*19a & *19b and *Grmi*-DAB*40a & *40b). Three alleles featured stop codons (*Grmi*-DAB*01e, *04 and *17) and two alleles (*Grmi*-DAB*37 and *49) carried a deletion of two nucleotides leading to a frame shift. These five alleles were regarded as potential pseudogenes and excluded from further calculations. In the remaining 75 *Grmi*-DAB alleles, sequence divergence in terms of variable amino acid sites and number of amino acid differences between alleles was high (Table 1). The number of alleles per individual ranged from one to nine, confirming our previous conclusion of multiple duplicated DAB loci in this species and identifying at least five MHC DAB loci in *G. microtarsus*. The number of different Grmi-DAB alleles on the amino acid level varied per forest site from 21 to 38 (Table 2). We did not detect an effect of the forest patch size on the number of MHC alleles in a forest site (R^2 = 0.466, N = 5, ANOVA, F= 2.623, n.s.).

For further analyses of the relationship between the individual MHC DAB constitution and the parasite load we clustered the 75 *Grmi*-DAB alleles into 17 functional MHC supertypes based on similar antigen-binding motifs (Supplement 1) (Doytchinova and Flower 2005). Supertypes ST11, ST16 and ST17 were not included in subsequent calculation because they occurred in frequencies < 0.05. ST01 was likewise excluded because it was present in all but three individuals (> 0.95) and therefore not informative.

Table 1: Number of different MHC DAB alleles (+ sequences including a stop codon or an indel) in the total samples of *G. microtarsus* and *M. incanus*. Variable amino acid positions (from a total of 65 positions) and the mean number of differences ± standard error between alleles are displayed (Ø differences). N refers to the sample size.

Species	N	DAB alleles	Number of alleles per individual	DAB loci	Amino acid sequences	
					variable positions	Ø differences
G. microtarsus	102	75 (+5)	1 - 9	≥ 5	50/65 (76.9%)	16.2 ± 2.1 (24.9%)
M. incanus	123	8	2 - 4	≥ 2	30/65 (46.2%)	14.1 ± 2.2 (21.7%)

M. incanus

In contrast to the diversity in *G. microtarsus*, only eight different *Main*-DAB alleles were detected in the total sample of 123 *M. incanus* individuals (EU350142 - EU350149, Table 1). All of them were already known from our earlier study (N = 56, Meyer-Lucht et al. 2008). This means, extending the sample size to more than double did not reveal a single new allele. However, average sequence divergence among these eight *Main*-DAB alleles was similar to the divergence in the alleles of *G. microtarsus* (Table 1). Within a single individual two to four *Main*-DAB alleles were identified. In the six forest sites the number of *Main*-DAB alleles varied from five to seven (Table 2). There was no significant effect of the size of the forest site on the number of MHC alleles per forest site (R^2 = 0.033, N = 6, ANOVA, F= 0.137, n.s.).

Parasite load

G. microtarsus

In 102 faecal samples of *G. microtarsus*, we detected eleven distinct helminth egg morphotypes. Eight of these were classified as nematodes, among them six morphotypes belonging to the group of strongyle nematodes. The remaining two nematode morphotypes could not be further assigned. In addition, two different cestode morphotypes from the

family Hymenolepidae were detected. In two individuals of *G. microtarsus* one trematode morphotype was detected with a single egg. This morphotype was excluded from the analyses due to its very low frequency.

Table 2: MHC DAB diversity (number of different MHC DAB alleles per site) and parasite loads differentiated by the forest sites (S1-S5) and control site (CS). In addition, the number of sampled individuals (N) and the size of the forest patch are given. *G. microtarsus* was not captured at S2.

	N	Patch size [ha]	Number of MHC alleles per site	Helminth prevalence [%]	Mean nematode intensity ± standard deviation [log nematode eggs +1]
G. microtarsus					
S1	23	19.54	35	69.6	1.37 ± 1.35
S2	0	14.01	-	-	-
S3	32	29.61	38	78.1	1.72 ± 1.34
S4	23	159.19	31	73.9	1.75 ± 1.45
S5	9	176.03	21	66.7	1.71 ± 1.34
CS	15	5 811.95	25	73.3	1.62 ± 1.42
M. incanus					
S1	21	19.54	5	100.0	3.04 ± 0.45
S2	32	14.01	7	100.0	2.98 ± 0.40
S3	22	29.61	5	100.0	3.27 ± 0.45
S4	17	159.19	7	100.0	2.99 ± 0.35
S5	21	176.03	6	100.0	3.20 ± 0.33
CS	10	5 811.95	5	100.0	3.67 ± 0.37

Individuals carried zero (26.4%), one (49.0%), two (16.7%), three (6.9%) or four (1.0%) helminth infections. The most common parasite morphotype was the strongyle nematode N1, with a prevalence of 38.2% (Fig. 1a), followed in frequency by the nematode N3 (30.4%) and the cestode C1 (22.5%). The remaining morphotypes were rarely represented. They were added to the group 'others' unless they were shared with *M. incanus*. Infection intensity with nematode N1 in *G. microtarsus* was low compared to *M. incanus* (Fig. 1b). Nematode N3 occurred in moderate infection intensities in *G. microtarsus* and was absent in *M. incanus*.

a

M. incanus

In 123 *M. incanus* individuals the faecal samples revealed thirteen distinct helminth egg morphotypes, thus parasite diversity in the species was slightly higher than in *G. microtarsus*. Eleven of these were nematodes, among them five morphotypes of strongyle nematodes, one of the family Trichuridae (*Capillaria* spec.) and one of the family Oxyuridae (*Syphacia* spec.). The remaining four nematode morphotypes could not be assigned to the family level. Moreover, the same two cestode morphotypes were identified as found in *G. microtarsus* (Fig. 1a). *M. incanus* individuals carried one (63.4%), two (31.7%) or three (4.9%) helminth infections. The strongyle nematode N1 was prevalent in all *M. incanus* individuals (Fig. 1a). The second most prevalent parasite was the cestode C1 (13.8%) followed by the nematode N2 (10.6%). The remaining morphotypes occurred sporadically in one to three samples and were merged to 'others', if they were not shared between host species. N1 reached very high infection intensities in *M. incanus*, whereas the other morphotypes occurred in low quantities (Fig. 1b).

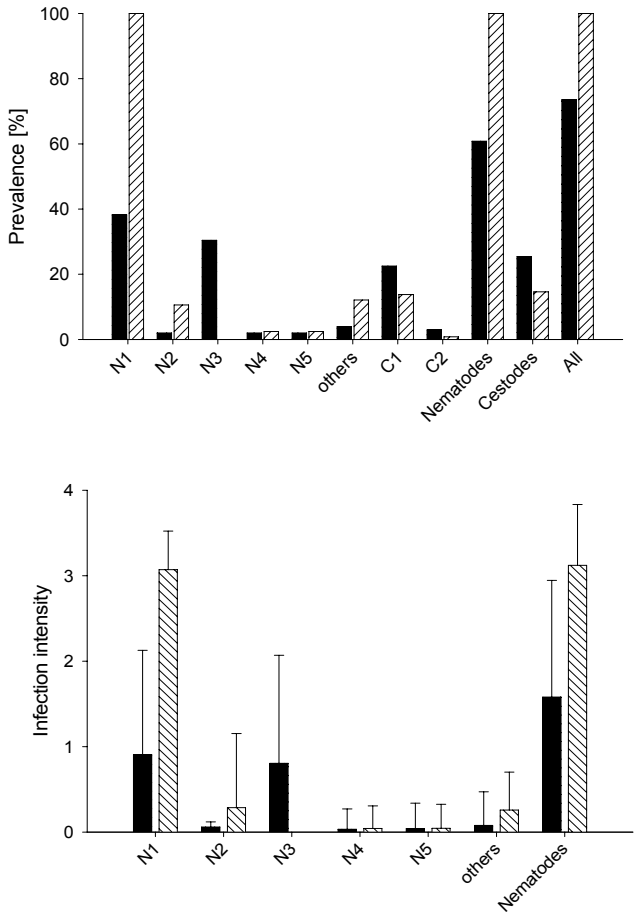

Figure 1: Parasite load with gastrointestinal nematodes and cestodes in *G. microtarsus* (*N* = 102, black bars) and *M. incanus* (*N* = 123, hatched bars). 'Others' represent nematode morphotypes that occurred in low frequencies and were not shared between host species. (a) Prevalence, (b) Intensity of infection (only nematodes). Bars illustrate the mean log-transformed FEC values [log(nematode eggs +1)] and the standard deviation.

The two host species shared six helminth morphotypes, four nematodes (N1, N2, N4, N5) and the two cestodes C1 and C2 (Fig. 1a). The differences in individual parasite load between the two marsupial species were highly significant: the

number of different helminth infections in *M. incanus* individuals was higher than in *G. microtarsus* individuals (Mann-Whitney-U-Test; Z = -3.92; p < 0.001). The helminth prevalence (χ^2 = 55.1, df = 1, p < 0.001) and nematode infection intensity (Mann-Whitney-U-Test; Z = -9.03; p < 0.001) in *M. incanus* exceeded by far the corresponding measures in *G. microtarsus*, too (Fig. 1a & b).

Factors influencing the parasite load

G. microtarsus

In terms of environmental predictors, the capture session explained a substantial part of the variation regarding both the number of different helminth infections and the nematode infection intensity. In these models capture session 5 was associated with increased parasitism, as removing this term from the model led to a significant increase in deviation (NHI: z = 2.59, df = 90, p = 0.009; nematode infection intensity: t = 2.51, df = 91, p = 0.014; Table 3). Neither the BMI nor the sex of the host had a significant influence on the different measures of parasitism in *G. microtarsus*.

Regarding genetic predictors, the number of different helminth infections was influenced by the presence or absence of ST09. The supertype was associated to a lower number of different nematode infections (z = -2.07, df = 90, p = 0.039). In terms of helminth prevalence two MHC supertypes were identified to be influential. The chance of being helminth infected was reduced to an odds ratio (OR) of 0.24 in presence of ST09 (z = -2.20, df = 93, p = 0.028). In the same way, ST14 reduced the helminth infection probability (z = -2.09, df = 93, p = 0.037; OR = 0.18).

M. incanus

No predictor revealed a significant effect on the number of different helminth infections, as no model explained the variance in the data better than the null model. No model could be calculated for helminth prevalence because all individuals were infected. The nematode infection intensity varied between sites in *M. incanus;* site 3 and the control site were associated to increased infection intensities (t = 2.08, df = 114, p = 0.040 and t = 5.72, df = 114, p < 0.001, respectively, Table 3). Again, neither host BMI nor sex had a significant influence on parasitism. In terms of genetic predictors, the presence of allele *Main*-DAB*05 increased the probability of high nematode infection intensities (t = 2.90, df = 114, p = 0.005).

Table 3: Effects of environmental, biotic and genetic predictors on different measures of parasite load in *G. microtarsus* and *M. incanus*, in the most parsimonious generalized linear models. In each host species, models were fitted for the individual number of different helminth infections, helminth prevalence as well as nematode infection intensity. .Predictors with significant effects on the response variables are listed, with the coefficients ± standard errors, * = p <0.05, ** = p <0.01, *** = p <0.001. The values for 'sites' and 'sessions' are in relation to S1 and session 1. *M. incanus*: for the number of helminth infections no model was better than the null model. NA indicates that for this response variable calculations could not be accomplished due to a helminth prevalence of 100%.

Response variable	Adj R^2	Predictor	Coefficient ± Standard error	Test statistic	d.f.	p	effect
G. microtarsus							
Number of helminth infections	0.149	Session 5	0.92 ± 0.35**	z = 2.59	90	0.009	negative
		ST09	-0.88 ± 0.42*	z = -2.07	90	0.039	positive
Helminth prevalence	0.052	ST09	-1.42 ± 0.65*	z = -2.20	93	0.028	positive
		ST14	-1.71 ± 0.82*	z = -2.09	93	0.037	positive
Nematode infection intensity	0.171	Session 5	1.73 ± 0.69*	t = 2.51	91	0.014	negative
M. incanus							
Number of helminth infections	0						
Helminth prevalence	NA						
Nematode infection intensity	0.326	Site 3	0.49 ± 0.24*	t = 2.08	114	0.040	negative
		Control site	1.34 ± 0.23***	t = 5.72	114	<0.001	negative
		Main-DAB*05	0.49 ± 0.17**	t = 2.90	114	0.005	negative

Discussion

In this study we investigated possible causes and consequences of low MHC diversity by analysing gastrointestinal parasite burden and functional associations between the individual MHC constitution and parasite load in two species of mouse opossums. We tested two scenarios under which low MHC diversity could occur. We assumed that the species with low MHC diversity could be under relaxed selection pressure to maintain immune gene diversity and predicted low parasite diversity ('*Evolutionary equilibrium*' scenario*)*. In an alternative scenario ('*Unbalanced situation*' scenario), we assumed that a recent loss in MHC diversity lead to a lack of resistance alleles and predicted an increased parasite burden in the species with low MHC diversity. We found that parasite diversity (the total number of helminth morphotypes) between the two host species was not markedly different. However, considerable differences in the parasite loads (parasite prevalence and infection intensity) between the two species were observed: while *M. incanus* revealed low MHC DAB diversity and high parasite load, *G. microtarsus* showed a tenfold higher population wide MHC DAB diversity and lower parasite burden supporting the second scenario.

MHC DAB diversity in G. microtarsus and M. incanus

One of the most prominent features of the MHC is the enormous variability found in the majority of vertebrates studied to date (Apanius et al. 1997; Hedrick 1994; Klein 1986; Piertney and Oliver 2006; Sommer 2005). The extensive MHC DAB diversity detected in *G. microtarsus* resembles high levels of MHC II diversity that are usually observed in wild populations of eutherians (summarized in Bernatchez and Landry 2003; O'Brien and Yuhki 1999; Piertney and Oliver 2006; Sommer 2005). Moreover, the high number of at least five DAB loci in *G. microtarsus* suggests the presence of a mechanism for generating MHC diversity that is known, for example, from the rhesus macaque (*Macaca mulatta*) and the Californian sea lion (*Zalophus californianus*). In contrast to high levels of allelic variation from a single MHC gene locus, this organisation comprises multiple duplicated MHC loci, each of them with limited variability, and present in variable configurations between individuals (Bowen et al. 2004; Doxiadis et al. 2000). However, for marsupials very limited polymorphism at the MHC class II was assumed to be characteristic, inferred from marsupials used in laboratory investigations (Stone et al. 1996; Stone et al. 1998). Most molecular studies on marsupial MHC indeed revealed low class II variation (e.g. Lam et al. 2001; McKenzie and Cooper 1994; Schneider et al. 1991; Siddle et al. 2007b; Stone et al.

1999). *G. microtarsus* is the first marsupial that shows this explicit MHC class II polymorphism and signs of positive selection (Meyer-Lucht et al. 2008). But it does not appear to be a South American exception, as Holland and colleagues (2008b; 2008a) recently described considerable MHC class II variation in an Australian marsupial species: the brushtail possum (*Trichosurus vulpecula*).

In contrast, limited MHC DAB polymorphism was indeed found in our second study species, *M. incanus*, which is concordant with the supposed general low MHC class II diversity in marsupials. One might argue that the low variation in *M. incanus* might be of methodological origin: the existence of null alleles. Although the occurrence of null alleles in both host species cannot be excluded, we do not assume a major impact on our results for the reasons discussed elsewhere (Meyer-Lucht et al. 2008).

In general, interpretations of low MHC polymorphism in natural populations refer to constraints of the mating system (Sommer 2003), reduced selection pressure by pathogens (Slade 1992) or loss of genetic diversity through inbreeding or genetic drift in restricted populations (Klein 1987; Sommer 2005). While we can exclude constraints of different mating systems in *M. incanus* and *G. microtarsus* because both species share the same partially semelparous mating behaviour (Lorini et al. 1994; Martins et al. 2006), we addressed the explanations of a lowered selection pressure and lost genetic diversity by investigations of the parasite load.

Parasite load in G. microtarsus and M. incanus

Parasite diversity between the host species was not conspicuously different as we detected thirteen helminth morphotypes in *M. incanus* and eleven in *G. microtarsus*. Moreover, the two host species showed a similar fauna of gastrointestinal helminthes: six parasite morphotypes were shared, among them two frequent ones. Parasite prevalence and infection intensity was higher in *M. incanus*, the species featuring low immune gene variation.

Parasitism is a highly complex and dynamic process dependent on a multitude of factors. The differences in the parasite load between the two species may be affected by their different microhabitat preferences. While *G. microtarsus* is mainly arboreal and uses the canopy, the terrestrial locomotion of *M. incanus* might increase its infection risk due to a greater exposure to soil borne parasites or those with faecal-oral transmission (Nunn et al. 2003). It is well known that spatial and temporal environmental variation plays an important role in parasite prevalence and intensity, beside the immune state, sex, and age of the host

(Abu-Madi et al. 2000; Behnke et al. 2001). In our study, the statistical models indicated that environmental factors, such as the forest sites or the capture sessions, influence the parasite load in both species. However, we did not detect a strong influence of the BMI or the sex on parasite load in either species, although there is a well documented association between testosterone and the immune system. Differences in parasite load between sexes are known from a number of studies on vertebrates. Sexually mature male vertebrates are often more susceptible to infection and carry higher parasite burdens, while estrogens even stimulate immunity (reviewed in Poulin 1996; Zuk and McKean 1996). But such sex biased differences in parasite loads might be small and therefore very difficult to detect (Zuk and McKean 1996).

Parasite load and functional associations with host genetics

In both species, our data provide evidence that the parasite load is affected by host genetics. We confirmed functional associations between specific MHC variants and the individual parasite load. Beneficial MHC variants were detected in *G. microtarsus*: the presence of ST09 and ST14 reduced parasite infections. In ST09 this association was due to a single allele (*Grmi*-DAB*16), whereas in ST14 not a single allele, but the whole group accounted for the positive effect (Supplement 1). On the other hand, the allele *Main*-DAB*05 in *M. incanus* appeared to be disadvantageous, as it was identified to increase parasite infection intensity. We do not have information on the expression of the MHC DAB alleles, as RNA samples are not available. However, the genomic level shows a comprehensive pattern of all available MHC variants, independent of temporal variation in expression. The existence of beneficial and disadvantageous MHC variants is explained by dynamic processes between host and parasites. Natural selection will favour pathogens that are not recognized by the most common MHC molecules, so there is selection for rare or new MHC alleles and against common ones in the host population (May & Anderson 1990; Takahata & Nei 1990). These functional associations between MHC variants and high or low parasite loads mirror the classical pattern of pathogen-driven selection acting on the MHC. Beneficial and/or disadvantageous MHC alleles with regard to parasite infections were detected in several studies (e.g. Froeschke and Sommer 2005; Harf and Sommer 2005; Langefors et al. 2001; Lohm et al. 2002; Meyer-Lucht and Sommer 2005; Schad et al. 2005; Schwaiger et al. 1995; Westerdahl et al. 2005). Recent studies also demonstrated associations between certain MHC supertypes and diseases resistance or susceptibility (Schwensow et al. 2007; Trachtenberg et al. 2003). Here, we provide the first

study indicating that pathogen-driven selection acts on the MHC class II of natural marsupial populations.

We did not find indication for a larger individual number of different MHC variants being advantageous in terms of parasite defence, which would correspond in a broader sense to the hypothesis of heterozygote advantage (Doherty and Zinkernagel 1975). According to this hypothesis, heterozygous individuals are in selective advantage over homozygous ones, because they are able to detect a broader range of parasites as a result of a larger number of different MHC molecules and thereby obtain a higher parasite resistance.

Low MHC diversity: evolutionary equilibrium or unbalanced situation?

Parasite diversity in the species with low MHC diversity, *M. incanus*, was not lower but even higher than in *G. microtarsus*. We therefore reject the first scenario predicting a relaxed pathogenic selection pressure accounting for the low MHC diversity in *M. incanus.* On the contrary, this species was distinctly higher parasitized. In the state of evolutionary equilibrium, host species exposed to a diverse array of parasites should harbour a variety of resistance alleles or a repertoire of inducible defences (Altizer et al. 2003). With its numerous MHC DAB alleles *G. microtarsus* seems to be well equipped, but this is not fulfilled in *M. incanus*.

Parasite load in *M. incanus* seems to be rather the consequence than the cause for its low MHC diversity. The explicitly higher helminth prevalence and infection intensities in *M. incanus* could be a result of its genetic homogeneity and low genotypic variation because genetic uniformity facilitates the spreading of pathogens through a population (Meagher 1999). The pattern of a relative low MHC II diversity combined with a relative high parasite load in *M. incanus* might be a sign of an unbalanced situation, i.e. a recent loss of genetic diversity. Thereby, *M. incanus* could have lost resistance alleles or other important parts of its adaptive evolutionary potential.

Some species are known to perform very well despite low MHC variation or even monomorphism and show no signs of severe infectious diseases, e.g. moose (*Alces alces*) or mountain goats (*Oreamnos americanus*) (Mainguy et al. 2007; Mikko and Anderson 1995). However, the pattern of low MHC class II variation and high parasite load in *M. incanus* resembles findings from other prominent species, for instance, the giant panda (*Ailuropoda melanoleuca*), which is particularly susceptible to infectious diseases and parasites. Wan et al. (2006) found only seven MHC DRB alleles in 60 individuals, and

credited this relatively low variation to a genetic bottleneck. Moreover, a recent study on the Tasmanian devil (*Sarcophilus harrisii*) detected a severely depleted MHC class I and ascribed the easy spreading of a contagious tumour (devil facial tumour disease) to this depletion, which is currently putting the Tasmanian devil under threat of extinction (2007a).

Other examples of mammals with extremely low MHC variation due to genetic bottlenecks are, for instance, cheetahs (Acinonyx jubatus, O'Brien et al. 1985), Asian lions (Panthera leo persica, Yuhki and O'Brien 1990) or Przewalski's horses (Equus przewalskii, Hedrick et al. 1999). In these cases, it is assumed that genetic drift was stronger than balancing selection in shaping MHC variation (Sommer 2005). As these species revealed very low levels of neutral genetic variation, too, it cannot be discriminated between effects based on reduced MHC variation and effects attributed to low variation at other fitness relevant loci (Hedrick et al. 1999; O'Brien et al. 1985; Yuhki and O'Brien 1990).

We have no evidence for a population bottleneck in *M. incanus*. Ecological studies, however, indicated that *M. incanus* may be more sensitive to habitat fragmentation than *G. microtarsus*, since *M. incanus* is restricted to patches of native vegetation (Umetsu and Pardini 2007) and prefers old, undisturbed forest with dense canopy cover (Püttker et al. 2008). In small and isolated fragments *M. incanus* decreases in abundance (Pardini et al. 2005). *G. microtarsus,* on the other hand, is not less abundant in small or isolated fragments (Pardini et al. 2005), prefers vegetation of disturbed forest (Püttker et al. 2008) and has even been captured in eucalyptus plantations (Umetsu and Pardini 2007). Ecological constraints might be responsible for a reduced migration between forest fragments in *M. incanus*, leading to lower gene flow and loss of genetic variation as a consequence of genetic drift. However, as only very few alleles were also detected in the control site and no effect of the forest patch size on the number of MHC alleles was visible, fragmentation sensitivity is not sufficient to explain the observed low variation in *M. incanus.*

To further investigate whether a bottleneck event and/or phylogenetic constraints account for the low MHC II variability in *M. incanus,* a deeper survey of the genetic diversity in this species is required, including neutral markers such as microsatellites, as well as more studies on marsupial species. In future studies we aim to compare *M. incanus* populations from small fragments in different landscapes surrounded by 25-35%, 45-55% and 85-100% of forest habitat to discover whether the low level of immune gene variation in *M. incanus* is characteristic for this species or result of a local bottleneck event.

In conclusion, our study revealed a relatively low parasite load in the species with high MHC DAB diversity, whereas very high parasite burdens were detected in the

opossum showing low immune gene diversity. In both species it became apparent that the MHC class II is functionally important in disease defence against gastrointestinal helminths, which has been shown here for the first time in marsupials. The question whether or not low MHC class II diversity is a phylogenetic characteristic of marsupials will not be resolved until substantial knowledge on the marsupial MHC in natural populations is gathered. It may be that the high diversity in *G. microtarsus* and not the low level of diversity in *M. incanus* is an exception to the rule. However, a definite explanation for the remarkable differences in MHC diversity in marsupials needs further investigations. Research on the marsupial MHC has only just begun.

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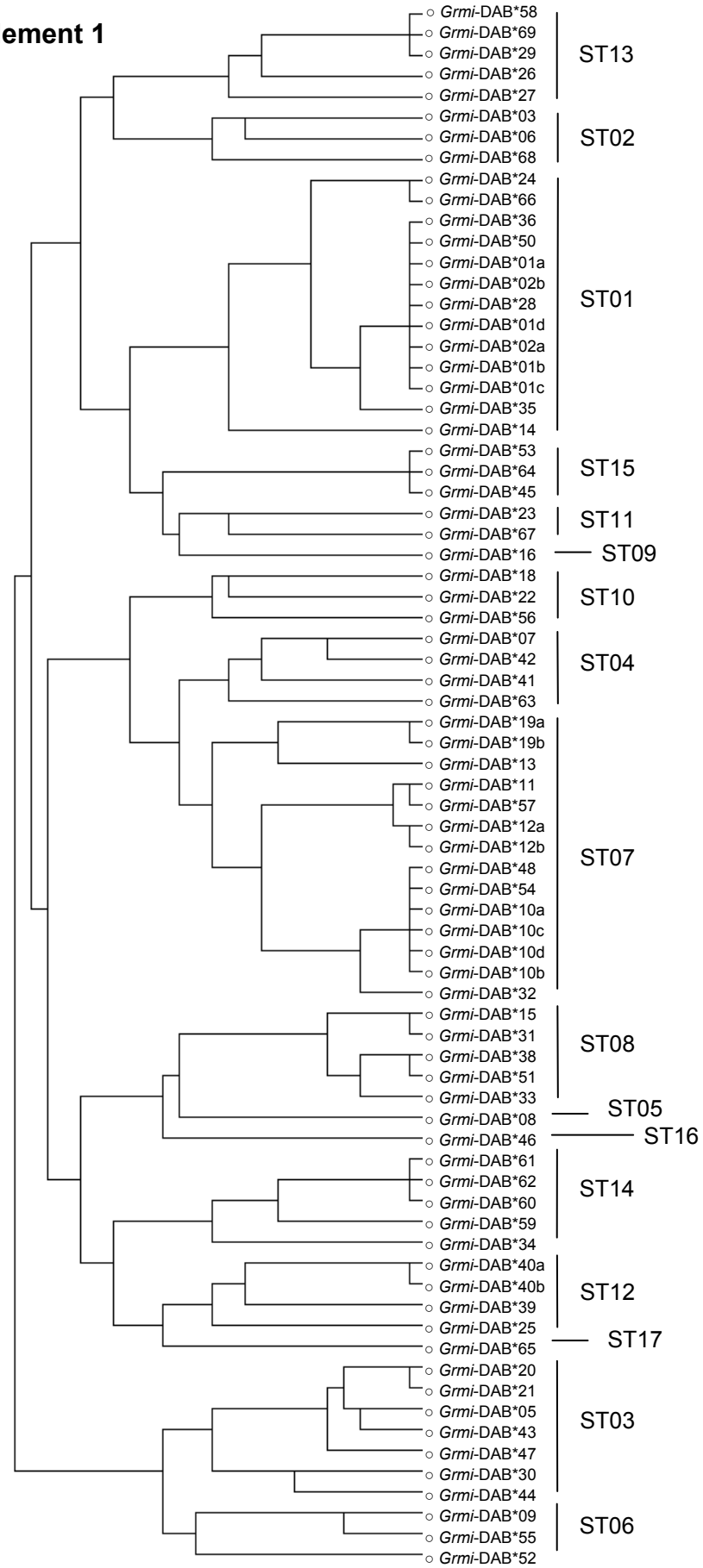
Supplement 1

Hierarchical cluster of the 75 MHC DAB alleles to functional supertypes (ST) based on physicochemical similarities at the positively selected amino acid sites in the mouse opossum *G. microtarsus* (*Grmi* = *Gracilinanus microtarsus*).

Supplement 1



Article 3:

Number of MHC alleles is related to parasite loads in natural populations of yellow necked mice (*Apodemus flavicollis*)

Number of MHC alleles is related to parasite load in natural populations of yellow necked mice (*Apodemus flavicollis*)

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ABSTRACT

Hypothesis: Low levels of major histocompatibility comlex (MHC) variation in a population increase the parasite load and infection intensity.

Organism: Different populations of the yellow necked mouse (*Apodemus flavicollis*), a common rodent in European deciduous and mixed forest habitats

Methods: We assessed genetic diversity at selectively neutral, non coding markers (microsatellites) and adaptive genetic variation at a functionally important part of the immune complex MHC (major histocompatibility complex). We investigated the load with gastrointestinal parasites non-invasively by faecal egg counts and tested the influence of population genetic variation on parasite burden.

Results: Both neutral and adaptive genetic diversity differed between mice populations. We could not detect an effect of neutral genetic diversity on the parasite burden in a population. Heterozygosity at the MHC did not reveal an effect on the parasite burden, either. However, we identified significant effects of the number of different MHC alleles in a population on parasite burden. Mice populations with a large number of different MHC alleles displayed lower parasite loads than those populations with few different MHC alleles.

INTRODUCTION

Genetic variability is of paramount importance for the adaptive potential of a population to a changing environment. In particular, it is assumed to be essential for an efficient immune function and pathogen defence (e.g. Altizer *et al.*, 2003; Hedrick, 2001; O'Brien & Evermann, 1988; Reid *et al.*, 2003). The role of genetic variation in buffering host populations against pathogens was emphasized in a number of studies that revealed associations between low levels of genetic diversity and an increased pathogen susceptibility (e.g. Acevedo-Whitehouse *et al.*, 2003; Acevedo-Whitehouse *et al.*, 2006; Cassinello *et al.*, 2001; Coltman *et al.*, 1999; Liersch & Schmid-Hempel, 1998; Meagher, 1999; Spielman *et al.*, 2004), a reduced immune reaction (Reid *et al.*, 2003; Sanjayan *et al.*, 1996; Whiteman *et al.*, 2006) and a severe progression of diseases (Acevedo-Whitehouse *et al.*, 2005; Dorman *et al.*, 2004). Moreover, pathogens may spread easily and quickly in genetically uniform host populations (Meagher, 1999; Spielman *et al.*, 2004). Reversely, resistance to pathogens is expected to increase with genetic diversity in a population (Hedrick, 2001; Liersch & Schmid-Hempel, 1998; O'Brien & Evermann, 1988) because high levels of genetic diversity in a population enhance the chance of at least some protective alleles being present and allow some individuals of the host population to successfully deal with coevolving pathogens.

Routinely in population genetic studies, neutral genetic variation is measured to assess genetic diversity. Significant associations between individual microsatellite heterozygosity and fitness related traits have been reported in several species (e.g. Acevedo-Whitehouse *et al.*, 2003; Amos *et al.*, 2001; Coltman *et al.*, 1999; Slate *et al.*, 2000). These correlations can be explained by either general or local genetic effects (Hansson & Westerberg, 2002): multilocus heterozygosity at the assessed markers might reflect genome-wide genetic variation or the markers could be in linkage disequilibrium with fitness relevant loci. Markers that directly affect the host's fitness and therefore reflect adaptive genetic variation are, for instance, immune genes, such as the genes of the major histocompatibility complex (MHC). This gene complex plays a key role in the mammalian immune system in terms of pathogen recognition (Klein, 1986). MHC molecules are cell surface glycoproteins that bind antigens and present them to T-lymphocytes in a process essential for the specific immune response. Recent studies in wild populations confirmed that certain MHC alleles are associated with either increased or decreased parasite load (e.g. Axtner & Sommer, 2007; Deter *et al.*, 2008; Froeschke & Sommer, 2005; Harf & Sommer, 2005; Meyer-Lucht & Sommer, 2005; Paterson *et al.*, 1998; Schad *et al.*, 2005).

Typically, the genes of the MHC show an extraordinary high polymorphism, which is supposed to be driven by selection processes through pathogens (Doherty & Zinkernagel, 1975).

Studies have supported that at a state of evolutionary equilibrium between hosts and pathogens, selection through diverse pathogens cause high MHC polymorphism in a species or population, whereas low MHC polymorphism indicates the presence of relaxed pathogenic selection pressure (Goüy de Bellocq *et al.*, 2008; Prugnolle *et al.*, 2005; Wegner *et al.*, 2003). This scenario holds true if hosts and pathogens share a long-term coevolutionary history. However, in a contrasting unbalanced situation, i.e. after a recent loss of genetic diversity through, for instance, fragmentation effects, species with low MHC diversity could have lost resistance alleles or other important parts of its adaptive evolutionary potential. This would facilitate an easy spread of pathogens throughout the population, because most individuals share the same resistance genotype (Meagher, 1999). It has been claimed that the loss of even a single MHC allele may have serious consequences for a population (Hughes, 1991).

Here, we studied eight wild populations of the yellow necked mouse (*Apodemus flavicollis* Melchior 1834). Specifically, we tested the influence of interpopulation genetic variation on parasite burden. We predicted that host populations with high MHC allelic diversity are better armed against parasite infections than populations with low MHC diversity and hence expected less infected individuals and/or lower infection intensities in MHC diverse populations. Adaptive genetic variation at a functionally important part of the immune complex MHC (MHC class II DRB exon 2) was assessed and neutral genetic diversity was measured using microsatellites.

MATERIAL AND METHODS

(a) Sites and sampling

Mice were trapped at eight sites in and around the city of Hamburg, Northern Germany. Sites 1 to 5 represent public parks in urban areas (1.2 to 8.6 km distance to the city centre) with deciduous trees. Sites 6 to 8 were located in deciduous forest in rural areas (16 to 34 km from the city centre). Live-trapping was carried out in 1215 trap nights in the summers of the sampling years 2002 and 2004 using Sherman® traps (3x3.5x9"). Captured animals were anaesthetized for 1-2 minutes by inhalation of isoflurane (Forene®,

Abbott GmbH, Germany), sexed, weighted, measured, and individually marked by ear punches of approximately 3 mm^2. These tissue samples were stored in 70% ethanol and used for subsequent genetic analyses. After the sampling procedure animals were released at their respective trap location. Faeces were collected from each trap and fixed in 4.0 v/v% formaldehyde for examinations of gastrointestinal nematodes. Traps were thoroughly cleaned after each use. Individuals with a body mass of < 16g were classified as juveniles (Jüdes, 1979) and excluded from subsequent analyses to avoid the sampling of family groups.

(b) Genetic analysis

DNA was isolated from ear tissue using the DNeasy™ Tissue Kit (Qiagen®, Hilden, Germany) following the manufacturer's protocol. To assess neutral genetic diversity, we analyzed six non-coding microsatellite markers. The loci CAA2A, GTTC4, GCATD7S (Markova *et al.*, 1998), MSAF3, MSAF8, and MSAF22 (Gockel *et al.*, 1997) were previously described for *A. flavicollis* and were amplified following the authors' protocols. Microsatellite PCR products were genotyped using the CleanGel HyRes Plus System (ETC®, Kirchentellinsfurt, Germany) based on the manufacturer's instructions. 2-5 µl of the PCR product were mixed with 10 µl loading dye (gel buffer, 1 % orange G, 1 % xylene cyanol, 0.2 M EDTA). 6 µl of each sample were loaded on a HyRes gel in a horizontal-cooling electrophoresis system (Amersham Pharmacia, Freiburg, Germany). Best results were achieved at the following conditions: 45 min at 200 V, 35 mA, 8 W followed by 15 min at 350 V, 50 mA, 20 W and 90-150 min at 500 V, 42 mA, 22 W; all steps were performed at constant 12°C. After electrophoresis gels were fixed and silver stained (Plus One DNA Silver Staining Kit, Amersham Pharmacia®, Freiburg, Germany). The visualized patterns were classified to length alleles.

The amplification of the MHC class II DRB exon 2 and single strand conformation polymorphism (SSCP) procedures were described previously (Meyer-Lucht & Sommer, 2005). At least three independent examples of each identified allele were cut from an SSCP gel and re-amplified prior to sequencing. Products were sequenced bi-directionally using a dye terminator sequencing kit (Applied Biosystems, Foster City, CA) with an Applied Biosystems® automated sequencer model 3100 following the manufacturer's instructions. MHC-DRB sequences are available at GenBank (accession numbers AY699757 - AY699771, AY918078 - AY918090).

(c) Parasitological examinations

We focused parasite analyses in *A. flavicollis* on gastrointestinal nematodes due to their high abundance in small mammals (Behnke *et al.*, 1999; Keymer & Dobson, 1987; Montgomery & Montgomery, 1988; Stefancíková *et al.*, 1994) and their impact on fitness attributes as well as mortality in a wide range of livestock and wild animals species (Albon *et al.*, 2002; Gulland, 1992; Stien *et al.*, 2002; Tompkins & Begon, 1999). Parasite loads were investigated by counting the nematode eggs in the animal's faeces using a modification of the non-invasive McMaster flotation technique (Gordon & Whitlock, 1939; Meyer-Lucht & Sommer, 2005). Faecal egg counts (FEC, number of eggs per gram faeces) reflect the overall worm burden and their fecundity, which are both influenced by the immune state of the host (Stear et al. 1995; Stear et al. 1997). FEC is a widely used approach in field studies as a result of the non-invasive procedure (e.g. Cassinello *et al.*, 2001; Coltman *et al.*, 1999; Ferrari *et al.*, 2004; Froeschke & Sommer, 2005; Harf & Sommer, 2005; Meyer-Lucht & Sommer, 2005; Schad *et al.*, 2005; Schwensow *et al.*, 2007; Seivwright *et al.*, 2004). We used the nematode prevalence (infected individuals/ examined individuals) and the mean intensity of nematode infection (log-transformed FEC values) per population as variables for parasitism.

(d) Data analysis

Neutral genetic diversity per population was measured as the mean microsatellite MLH (multilocus heterozygosity, Coltman *et al.*, 1999) and mean microsatellite d^2 (difference in repeat units, averaged over all loci, Coulson *et al.*, 1998). Microsatellite F_{ST} values were calculated using MICROSATELLITE ANALYSER (MSA, Dieringer & Schlötterer, 2003). MHC nucleotide sequences were edited manually and aligned in MEGA 4 (Tamura *et al.*, 2007). MHC genetic diversity was described using the observed heterozygosity and the allelic richness. As the observed number of alleles in a sample is highly dependent on the number of sampled individuals, we calculated the allelic richness corrected for different sample sizes by using a rarefaction index implemented in FSTAT (Goudet, 2001). Thereby, the expected number of alleles in each sub-sample is calculated for the number of individuals present in the smallest sample. Observed and expected heterozygosity as well as F_{ST} values for the MHC marker were calculated by Arlequin 3.0 (Excoffier *et al.*, 2005).

We investigated the effects of genetic diversity for both markers on parasite load by means of generalized linear models (GLMs). Models were fitted for nematode prevalence and mean nematode infection intensity. For prevalence data, logistic regression models

were applied with a binomial error distribution and logit link function. For the log-transformed infection intensity data we used a Gaussian error distribution with an identity link function. Due to the small number of eight populations the four predictors of genetic diversity (microsatellite MLH, microsatellite d^2, MHC heterozygosity and number of MHC alleles) were included in separate models, each model comprising the sampling year (2002, 2004) and one of the genetic predictors. The proportion of deviance explained by each variable (r^2) was assessed by removing this predictor from the model, comparing the change in deviance to the full model deviance and testing it for significance. GLMs were performed in R (version 2.7.0, R Development Core Team, 2008). Figures were generated in Sigma Plot Version 10.0.

RESULTS

Genetic diversity within and among populations

159 individuals from eight populations were genotyped. The study populations differed in their levels of genetic diversity with regard to both genetic markers (Table 1). Out of the four predictors describing genetic diversity mean MLH and mean d^2 were correlated (Spearman's rho = 0.762, N = 8, $P < 0.05$). Regarding the microsatellite markers, there was low variation in the mean MLH values among populations but mean d^2 varied notably from 22.07 (population 3) to 74.17 (population 1). In total, 28 different MHC DRB alleles were found. Thereof two nucleotide sequences translated into the same amino acid sequence and were combined to one allele (*Apfl*-DRB*23a and *Apfl*-DRB*23b). Nucleotide diversity of all *Apfl*-DRB alleles was described in Meyer-Lucht & Sommer (2005). There was a wide range in observed MHC heterozygosity among populations from 0.48 (population 4) to 0.95 (population 8). Corrected allelic richness varied highly as well from 7.56 in population 1 to 14.51 in population 8 (Table 1). Differentiation among populations was highly significant in both types of markers (microsatellites: $F_{ST} = 0.059$, $P < 0.001$; MHC: $F_{ST} = 0.0633$, $P < 0.001$). From the four predictors describing genetic diversity only the corrected MHC allelic richness per population was correlated with the population's geographic distance to the city center (Spearman's rho = 0.952, N = 8, $P < 0.01$).

Table 1: Nematode parasitism and genetic diversity in eight populations of *A. flavicollis* and in the total sample. N = sample size, sd = standard deviation, FEC = fecal egg count, epg = eggs per gram, H_{obs} = observed heterozygosity, H_{exp} = expected heterozygosity according to Hardy-Weinberg. Allelic richness was corrected for the sample size.

		Nematodes		**Microsatellites**		**MHC**		
Population	N	Prevalence [%]	Mean intensity ± sd [FEC, log epg +1]	Mean MLH	Mean d^2	H_{obs} / H_{exp}	Number of alleles	Allelic richness
1	20	80.0	2.01 ± 1.17	0.996	74.17	0.70 / 0.86	8	7.56
2	21	66.7	2.23 ± 1.80	1.043	55.87	0.86 / 0.85	8	7.70
3	20	70.0	1.72 ± 1.32	0.952	22.07	0.90 / 0.87	9	8.52
4	21	52.4	1.13 ± 1.22	0.987	37.50	0.48 / 0.71	11	9.32
5	18	61.1	1.45 ± 1.30	0.934	29.17	0.72 / 0.87	9	8.66
6	16	50.0	1.37 ± 1.46	0.980	25.20	0.94 / 0.89	12	12.00
7	21	71.4	1.88 ± 1.34	1.025	43.62	0.86 / 0.85	11	9.52
8	22	45.5	1.15 ± 1.32	1.076	43.20	0.95 / 0.91	17	14.51
Total	159	62.0	1.62 ± 1.40	1.000	41.43	0.80 / 0.89	27	12.09

Parasite load

In 159 *A. flavicollis* individual faecal samples eight different morphotypes of gastrointestinal nematodes were detected. Five of these were strongyle nematodes, two were from the Trichuridae family (*Capillaria* sp., *Trichuris* sp.) and one belonged to the Oxyuridae family (*Syphacia* sp.). 62% of the examined individuals hosted infections of one to three nematode morphotypes. The remaining 38% did not show any infection. The number of different nematode morphotypes in the populations ranged from four to six. Both, the strongyle nematodes and those from the family Trichuridae were detected frequently (in 49.7% and 25.2% of all individuals, respectively), whereas *Syphacia* sp. occurred rarely (in 5.7%). The sex of the host had neither an effect on nematode prevalence (χ^2 = 1.279; df = 1; P = 0.26) nor on infection intensity measured as FEC (Mann-Whitney-U-Test, z = -0.323; P = 0.75). Among the populations nematode prevalence ranged from 45.5% to 80.0%, mean infection intensity varied from 1.13 ± 1.22 to 2.23 ± 1.80 (FEC, Table 1). Nematode prevalence and mean nematode infection intensity were correlated (Spearman's rho = 0.782, N = 8, P < 0.05).

Table 2: Effects of genetic diversity on nematode load in eight populations of *A. flavicollis*, calculated by generalised linear models. Given are the full models for (a) nematode prevalence and (b) nematode infection intensity. The capture year was included in each model. ß ± SE stands for the coefficient ± standard error, r^2 is the proportion of variance explained by each predictor. *P* - values were calculated by removing each predictor from the model and testing the change in deviance for significance.

(a) Nematode prevalence	ß ± SE	r^2	*P*
MODEL 1			
YEAR	-0.034 ± 0.373	0.09%	0.93
MLH	-2.278 ± 4.106	4.50%	0.53
MODEL 2			
YEAR	-0.111 ± 0.336	1.22%	0.74
D^2	0.015 ± 0.011	22.91%	0.16
MODEL 3			
YEAR	-0.145 ± 0.406	1.44%	0.72
MHC H_{OBS}	0.046 ± 1.312	0.01%	0.97
MODEL 4			
YEAR	-0.103 ± 0.342	1.03%	0.76
ALLELIC RICHNESS	-0.170 ± 0.074	**60.64%**	**0.02**

(b) Nematode infection intensity	ß ± SE	r^2	*P*
MODEL 1			
YEAR	0.116 ± 0.383	1.78%	0.77
MLH	0.304 ± 4.208	0.10%	0.95
MODEL 2			
YEAR	0.149 ± 0.283	3.59%	0.62
D^2	0.013 ± 0.008	32.35%	0.18
MODEL 3			
YEAR	0.069 ± 0.407	0.56%	0.87
MHC H_{OBS}	0.352 ± 1.310	1.39%	0.80
MODEL 4			
YEAR	0.059 ± 0.114	2.26%	0.63
ALLELIC RICHNESS	-0.128 ± 0.051	**54.28%**	**0.05**

Effects of population genetic diversity on nematode load

By means of generalized linear models we investigated the effects of population genetic diversity on nematode load (Table 2). We calculated models for the influence of each genetic predictor separately. Neutral genetic diversity did not have significant effects on the nematode load. Neither the mean MLH nor the mean d^2 significantly influenced the nematode prevalence, as removing these variables from the models did not lead to a significant increase in deviance (MLH: z = -0.63, df = 5, P = 0.53; d^2: z = 1.40, df = 5, P = 0.16; Fig. 1a & b, Table 2). Likewise there were no effects on the intensity of infection (MLH: t = 0.07, df = 5, P = 0.95; d^2: t = 1.58, df = 5, P = 0.18; Fig. 2a & b, Table 2).

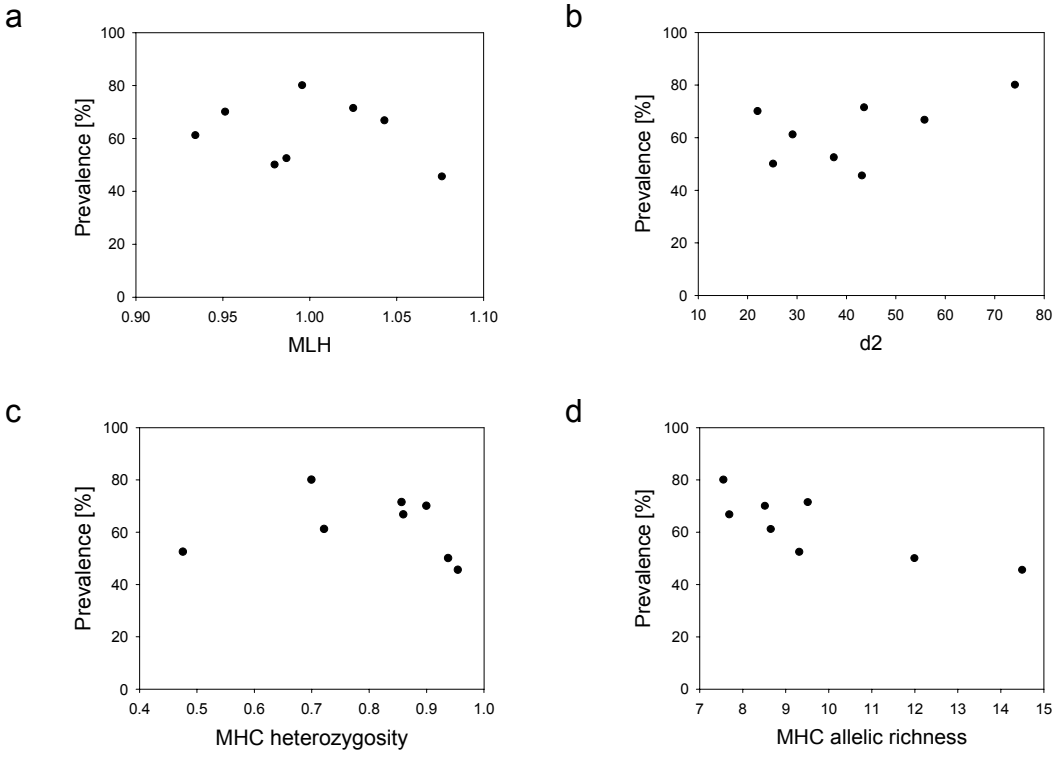

Figure 1: Nematode prevalence in relation to different measures of genetic diversity in populations of *A. flavicollis*. Microsatellite markers: (a) mean multilocus heterozygosity (MLH), (b) mean averaged differences in repeat units (d^2) per population; MHC marker: (c) MHC-DRB heterozygosity, (d) allelic richness per population.

Moreover, regarding the coding MHC marker, we did not detect an effect of observed MHC heterozygosity on nematode prevalence nor intensity of infection (prevalence: z = 0.04, df = 5, *P* = 0.97, Fig. 1c; infection intensity: t = 0.27, df = 5, *P* = 0.80, Fig. 2c, Table 2). However, the allelic richness in a population had a significant effect and explained a substantial part of the variation in nematode load. A high number of different MHC alleles in a population significantly decreased the nematode prevalence in the population (z = -2.29, df = 5, *P* = 0.02, Fig. 1d). Likewise, a high allelic richness reduced the mean nematode infection intensity in the population, although at the margin of significance (t = -2.51, df = 5, *P* = 0.05, Fig. 2d; Table 2).

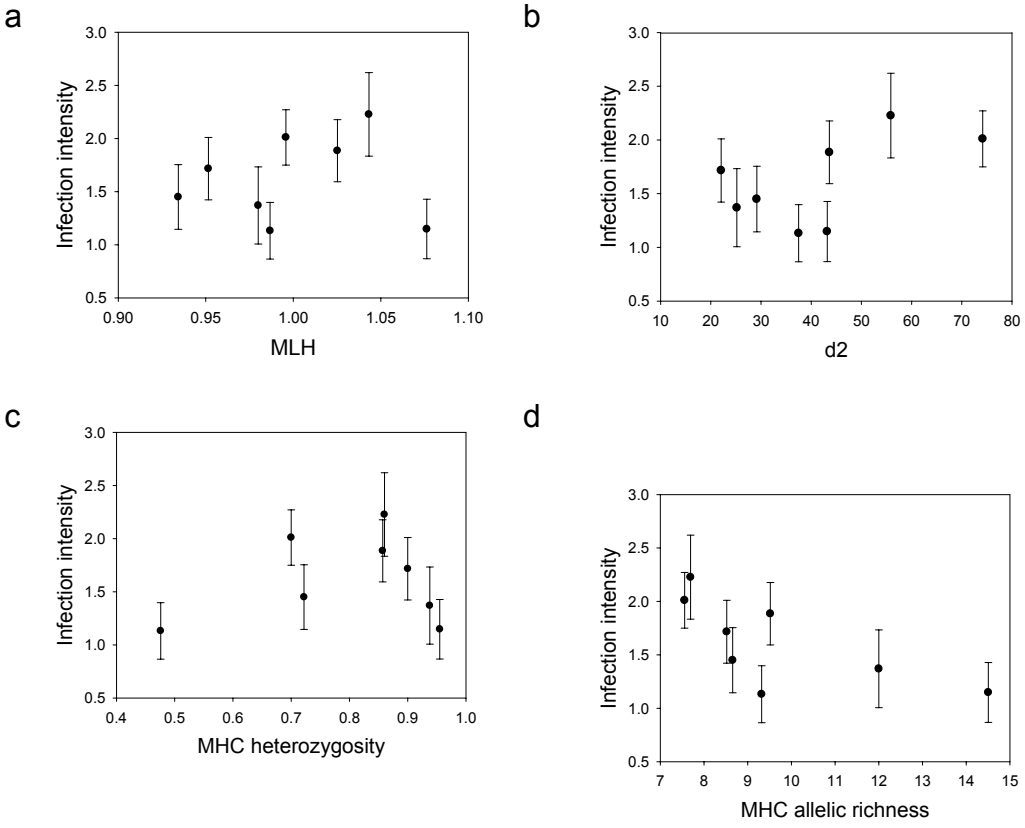

Figure 2: Nematode infection intensity in relation to different measures of genetic diversity in populations of A. flavicollis. Bars represent the standard errors of the means, a-d according to Fig.1.

DISCUSSION

The importance of host genetic diversity in pathogen defence was pointed out in a number of studies, whereby mainly single infectious agents were used. For natural populations these effects are expected to be even stronger due to the presence of a variety of infectious pathogens (Spielman et al., 2004). Here, we studied the importance of genetic diversity for nematode resistance in natural populations of yellow necked mice. We investigated neutral and adaptive genetic variation and tested the influence of interpopulation genetic variation on parasite burden. No significant effects of neutral genetic diversity or MHC heterozygosity on parasite burden could be detected. However, we discovered a significant effect of the MHC allelic richness within a population on nematode resistance. Mice populations with a large MHC allele repertoire displayed lower parasite loads.

The number of nematode morphotypes and their prevalence detected in the study populations are within the range of results from other parasitological studies in the genus *Apodemus* (3 to 7 nematode species, 24% to 92% helminth prevalence, dominated by nematodes, Abu-Madi *et al.*, 2000; Behnke *et al.*, 1999; Stefancíková *et al.*, 1994). In wild rodents, parasite prevalence and intensity are known to underlie spatial and temporal variation, beside intrinsic factors as the immune state, sex or age of the host (Abu-Madi *et al.*, 2000; Behnke *et al.*, 2001; Behnke *et al.*, 2005). However, in our data the sampling year had no significant effect on nematode prevalence and intensity.

So far, most studies have targeted MHC diversity within single populations (Meyer & Thompson, 2001), whereas MHC diversity between different populations has rarely been investigated. One of those is, for instance, the study of Prugnolle et al. (2005), who investigated the relationship between MHC diversity (HLA in humans) in different human populations worldwide and their corresponding pathogen regimes sharing a long-term coevolutionary history (evolutionary equilibrium between hosts and pathogens). They showed that, beside a strong effect of the human colonization history, MHC diversity is shaped by the local pathogen richness. Human populations exposed to a more diverse pathogen array show higher HLA diversity but not higher neutral genetic diversity than those exposed to fewer pathogens. Analogously, Wegner and co-authors (2003) detected in a study on eight stickleback populations (*Gasterosteus aculeatus*) that parasite diversity was positively correlated with MHC *IIB* variation in a population but not with genetic neutral variation. In both studies, populations from very different habitats with different levels of pathogen richness were explored but neither prevalence nor infection intensity were taken

into account. In contrast, our study populations originate from similar habitats within the same geographic region and featured very similar parasite communities and diversities. Hence, no association between parasite richness and MHC diversity was detectable (data not shown) but populations differed in prevalence and infection intensity. We assume that in our study populations the pronounced differences in MHC diversity are likely caused by isolation effects in the investigated park areas, as suggested by the association of allelic richness (but no other genetic parameters) with the distance to the city. Some host populations seemed to be in an evolutionary unbalanced situation and probably experienced a recent loss of genetic diversity.

An individual itself will not directly benefit from its population's large MHC allele pool in terms of parasite resistance. But constantly challenged by multiple pathogens in the wild, populations with a large MHC allele pool are more likely to harbour individuals that possess protective alleles for each of the different infections. Moreover, several protective alleles in a population will hinder the easy spreading of pathogens because the adaptation to diverse resistance genotypes is more complex than in a genetically uniform population. Thus, our findings are in line with our previous findings in *A. flavicollis* and a number of studies in other natural mammal populations, which pointed out the functional importance of specific MHC alleles for resistance or susceptibility to parasite infections in individual-based analyses (Axtner & Sommer, 2007; Deter *et al.*, 2008; Froeschke & Sommer, 2005; Harf & Sommer, 2005; Meyer-Lucht & Sommer, 2005; Paterson *et al.*, 1998; Schad *et al.*, 2005).

Another debated selection mechanisms is the '*heterozygote advantage hypothesis*' (Doherty & Zinkernagel, 1975). It is based on the suggestion that heterozygotes are able to recognise two suits of pathogens, one for each allele, and are therefore favoured due to their ability to resist a broader array of pathogens than homozygotes. Studies supporting the heterozygote advantage hypothesis were mostly carried out under laboratory conditions (Doherty & Zinkernagel, 1975; McClelland *et al.*, 2003; Penn *et al.*, 2002). Only few studies on wild populations disclosed an advantage of MHC heterozygote individuals in terms of parasite infections (e.g. Froeschke & Sommer, 2005; Oliver *et al.*, 2009). For our study, however, we suggest to treat the insignificant effect of MHC heterozygosity on nematode resistance with caution because the small number of observations (eight populations) infers low statistical power. On the other hand, this strengthens the positive result of a significant association between MHC allelic richness and nematode resistance as it was detectable despite the low statistical power. Our findings thereby underline recent simulations and theoretical models, which showed that heterozygote advantage on its own is insufficient to

explain the large number of MHC alleles as commonly observed (Borghans *et al.*, 2004; De Boer *et al.*, 2004).

An influence of neutral genetic diversity on nematode burden was not indicated although we are aware that six microsatellite loci only fragmentary describe genome-wide variation. Despite being widely used as a surrogate for inbreeding, it was shown that both microsatellite diversity measures MLH and d^2 are only very weakly correlated with the inbreeding coefficient, even at a high number of typed loci (Balloux *et al.*, 2004; Slate *et al.*, 2004). Therefore, we cannot draw conclusions whether inbreeding depression in the investigated populations is present or not. There was no correlation between neutral and MHC diversity in our data, which one would expect if genetic drift and inbreeding had predominantly shaped the genetic structure of the populations. It seems that nematode resistance in the studied populations is rather a matter of genetic variation at loci involved in pathogen defence than related to overall genetic diversity. Similarly, Spielman and colleagues (2004) identified that lowered pathogen resistance in *Drosophila* populations was not related to generalized inbreeding effects but to the absence of specific resistance alleles.

In conclusion, our study supports the hypothesis that populations with a high MHC allele diversity are better armed against high parasite burdens and highlights the significance of adaptive genetic diversity in the field of conservation genetics. A population with a large MHC allele reservoir is more likely to possess resistance alleles to the multitude of pathogens present in the wild.

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