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MHC class I variation associates with parasite resistance and longevity in tropical pythons

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Abstract

Using restriction fragment length polymorphism (RFLP) we identified 26 unique major histocompatibility complex (MHC) genotypes in 104 water pythons. We observed a significant independent association between reduced blood parasite load (*Hepatozoon* sp.) and python body length/age, presence of a specific RFLP fragment (C-fragment) and the overall number of fragments. The parasite has a negative impact on several python life-history traits such as growth, nutritional status and longevity. Thus, the C-fragment could be considered a 'good gene' (a fitness-enhancing genetic element). However, while the number of fragments affected parasite load, the association between level of parasitaemia and fragment number was not linear, and, hence, minimum parasite infection level was achieved at an intermediate number of fragments. Intermediate MHC fragment numbers were also observed among the largest/oldest pythons, suggesting that both a specific fragment and intermediate levels of MHC polymorphism enhanced python longevity. Thus, our results suggest python MHC is subject to both frequency-dependent and balancing selection.

Introduction

In 1996 Peter C. Doherty and Rolf M. Zinkernagel were awarded the Nobel prize for their discovery that cellular immunity to viral infections was restricted by the highly variable proteins of the major histocompatibility complex (MHC) (Doherty & Zinkernagel, 1974, 1975a; Zinkernagel & Doherty, 1974a,b). The immense importance of their discovery not only laid the foundation for novel research of the human immune system but, during recent decades, has also generated numerous studies investigating the significance of MHC diversity in other vertebrates such as fish (Langefors *et al.*, 2001; Reusch *et al.*, 2001; Lohm *et al.*, 2002; Wegner *et al.*, 2003a: Wedekind *et al.*, 2004), lizards (Olsson *et al.*, 2003), snakes (Madsen *et al.*, 1999), birds (Bonneaud *et al.*, 2004; Jarvi *et al.*, 2004; Westerdahl *et al.*, 2004, 2005) and mammals (Paterson *et al.*, 1998; Hedrick *et al.*, 2002; Kundu & Faulkes, 2003; Aquilar *et al.*, 2004; Schad *et al.*, 2005).

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Class I and II MHC molecules are responsible for the presentation of intracellular and extracellular peptides to T cells, respectively (Zinkernagel, 1979; Klein, 1986). In vertebrates the MHC contains the most variable set of coding genes, with up to 349 alleles described for a single locus (Robinson *et al.*, 2000) and heterozygosity values that generally exceed those predicted by neutrality (Edwards & Hedrick, 1998). Two, not mutually exclusive, hypotheses have been suggested to explain the high level of MHC variability: (i) MHC-based mating preferences (Potts *et al.*, 1994; Wedekind *et al.*, 1995; Wedekind & Füri, 1997; Reusch *et al.*, 2001; Olsson *et al.*, 2003) and (ii) parasite-driven selection (Paterson *et al.*, 1998; Carrington *et al.*, 1999; Langefors *et al.*, 2001; Lohm *et al.*, 2002; Wegner *et al.*, 2003a; Harf & Sommer, 2005; Schad *et al.*, 2005). Given the central role of MHC in the vertebrate immune system, the latter may be a more likely candidate for explaining the high MHC diversity observed in most vertebrates, and may serve as the underlying reason for MHC-based mate choice.

The relationship between MHC genotype and resistance to infectious agents can take several forms. Recently, it has been demonstrated that individual MHC alleles may be especially effective at presenting antigens from a particular infection, and, hence, in combating specific pathogens in fish (Langefors *et al.*, 2001; Lohm *et al.*, 2002), birds (Westerdahl *et al.*, 2005) and mammals (Paterson *et al.*, 1998; Harf & Sommer, 2005; Meyer-Lucht & Sommer, 2005; Schad *et al.*, 2005). A second, distinct, but compatible hypothesis was suggested by Doherty & Zinkernagel (1975b): as each MHC allele provides an infected individual with the ability to present a particular set of antigens, heterozygous individuals at certain MHC loci may mount a more vigorous immune response to a given infection, compared with homozygous individuals.

An association between MHC heterozygosity and infectious diseases has been observed in birds (Martin *et al.*, 1989; Westerdahl *et al.*, 2005), mice (Doherty & Zinkernagel, 1975a; McLeod *et al.*, 1989; Messaoudi *et al.*, 2002; Penn *et al.*, 2002) and in studies of hepatitis B and HIV infections in humans (Thursz *et al.*, 1997; Carrington *et al.*, 1999). Although increased level of heterozygosity enables wider recognition of pathogens (Doherty & Zinkernagel, 1975b; Penn *et al.*, 2002), it may also result in an inability to delete T cells that react with self-peptide-MHC combinations (Vidovic & Matzinger, 1988). Thus, selection against multiple gene combinations may be controlled by selection against autoimmunity (Råberg *et al.*, 1998). Within an individual, theoretical models predict that MHC alleles can be subjected to two opposing selective forces, resulting in optimal disease resistance at an intermediate number of MHC alleles (Nowak *et al.*, 1992; De Boer & Perelson, 1993). In an elegant study on sticklebacks (*Gasterosteus aculeatus*) Wegner *et al.* (2003a) empirically demonstrated that an intermediate level of MHC class II B diversity indeed resulted in optimal parasite resistance. In the present study, we explore how MHC class I diversity may affect the ability of free-ranging water pythons (*Liasis fuscus*) to counteract pathogenic effects of haematozoan (*Hepatozoon* sp.) parasite infections.

Materials and methods

Study area and species

The study was conducted in the Fogg Dam conservation reserve, in the lower reaches of the Adelaide River floodplain, 60 km south-east of Darwin in the Northern Territory of Australia.

Water pythons are large (up to 3 m), nonvenomous snakes widely distributed across tropical Australia (Cogger, 1992). The results in the present paper are based on a capture–mark–recapture study of pythons collected in the Fogg Dam conservation reserve between 2001 and 2003. The snakes were captured at night by spotlighting (on foot, or from a slow-moving vehicle), and released the following day after they had been measured, weighed, sexed, blood sampled and individually marked for later recognition.

Although the water pythons at our study site show considerable among-individual variation in annual growth rates, python body size still constitutes a robust predictor of python age, i.e. large snakes are older than small individuals (Madsen & Shine, 2000). Thus, in the present study, body length is used as a proxy for python age.

Detection of parasites

Blood parasites were examined by placing a drop of blood, obtained by cutting off approximately 2 mm of the snake's tail tip, directly onto a glass slide and smearing it with a second slide to produce a one cell thick blood layer. The blood smears were air dried, fixed in methanol and stained with Giemsa. Each slide was examined under oil immersion (×100). Two thousand red blood cells were analysed per slide and infection intensity quantified as number of infected erythrocytes. To determine whether a single blood sample provided reliable data on parasite load, 15 pythons were sampled twice within a month. The two parasite counts revealed a highly significant correlation ($r = 0.99$, $P = 0.0001$, d.f. = 14), strongly suggesting that the infection level recorded when performing only a single count yields a robust measure of *Hepatozoon* infection levels.

Screening of MHC class I variation

Genomic DNA was isolated from 100 μl of whole blood by phenol-chloroform extraction (Sambrook *et al.*, 1989). Restriction fragment length polymorphism (RFLP) of MHC class I genes was analysed in 104 pythons using a species-specific probe. The probe was a cloned and sequenced PCR fragment spanning 261 bp of the hypervariable exon 3 of a water python class I gene. Initially six pythons were tested in a Southern blot analysis using five different restriction enzymes; *Hin*dIII, *Pst*I, *Sac*I, *Taq*I and *Pvu*II. However, *Pvu*II revealed the highest degree of polymorphism and was subsequently used in the RFLP analysis. Ten milligrams of genomic DNA was digested with 24 U of restriction enzyme for 3 h and then run in a 0.8% agarose gel so that all fragments longer than 500 bp remained in the gel. Lambda DNA, digested with *Hin*dIII, was used as a size marker. The DNA in the gel was transferred to a nylon membrane (Micron Separtions Inc., Westborough, MA, USA) using a vacuum blotter model (Bio-Rad, Hercules, CA, USA) and the membranes were crosslinked in an XL-1500 UV crosslinker (Spectronics Corporation, Westbury, NY, USA). The membranes were prehybridized in a prehybridization solution [0.5 M Na_2HPO_4, 1% sodium dodecyl sulphate (SDS)] for 45 min at 62°C and then hybridized overnight at 62°C in a solution containing 0.2 M Na_2HPO_4, 1.0% SDS, 1.0%

bovine serum albumin, 6% PEG 6000 and the probe labelled with (α–^{32}P) dCTP (Amersham, Little Chalfont, UK). The membranes were washed at 62°C for 15 min. in preheated 2X SSPE, 20 min in 1X SSPE and finally for 20 min in 0.5X SSPE. The washed membranes were exposed to X-ray film (Eastman Kodak, Rochester, NY, USA) in intensifying screens for 1–5 days at −80°C.

In order to compare RFLP fragments among gels, one individual (having 15 fragments) was run in duplicate (well numbers 4 and 15) in all the gels analysed (a total of 18 samples were run on each gel).

Statistical analysis

The distribution of parasite infection level was highly skewed, and was therefore ln(x + 1) transformed before being submitted for statistical analysis. Effects of MHC genotype on parasite load were analysed using a Generalized Linear Model (Type III statistics presented) with presence/absence of each specific RFLP fragment as factors, total number of fragments (entered in the model as a linear and a quadratic term) and python size/age as covariates. The rationale for including number of fragments as a quadratic term in the model was based on our observations that the relationship between parasite load and number of RFLP fragments was most likely not linear. We used Schwarz Bayesian Information Criterion (BIC) to determine which model that best fitted our data (Quinn & Keough, 2002). Our analyses revealed a lower BIC in the model including a quadratic predictor compared with a model excluding the term (BIC = 108.84 and 113.04, respectively), and, hence a quadratic predictor was included in the full model. A Shapiro and Wilke's test of residuals revealed no violation of the model's underlying assumptions ($P > 0.05$). Backward elimination was set at $P > 0.25$ (Quinn & Keough, 2002).

In order to explore whether the relationship between python parasite load/python body length and number of RFLP fragments was best described by quadratic or linear function we compared the BIC-values obtained from the different models. In both analyses the BIC-values based on a quadratic function were lower than those obtained using a linear predictor (parasite load and number of RFLP fragments: quadratic BIC = 125.64, linear BIC = 137.24; python body length and number of RFLP fragments: quadratic BIC = 723.76, linear BIC = 734.08). The lower BIC-values obtained from the quadratic models suggest a better fit, and this predictor was therefore employed in our subsequent analyses.

Results

We identified 26 unique MHC class I genotypes among the 104 screened water pythons. The number of RFLP fragments ranged between 10 and 15 (mean ± SD: 12.4 ± 1.09, size range 1–9 kb) of which six displayed among-individual variability (henceforth referred to as fragments A, B, C, D, E and F). The frequencies of the fragments ranged from 4.8% (A) to 70% (D) (Table 1). Only presence of the C-fragment had a significant association with lower blood parasite load (Table 2). Number of RFLP fragments was also significantly associated with levels of parasitaemia (Table 2). Interpolation of the quadratic function suggests that minimum parasite infection rate was observed at 13.2 MHC fragments (Fig. 1).

Table 1 Number and frequency of the six variable restriction fragment length polymorphism fragments.

Fragment	Number	Frequency (%)
A	5	4.8
B	33	31.7
C	61	58.7
D	73	70.2
E	48	46.2
F	28	26.9

Table 2 Generalized linear model analyses of effects of major histocompatibility complex class I genotype and python body length/age on *Hepatozoon* sp. parasite load. Backward elimination was set at $P > 0.25$.

	F	d.f.	P
C-fragment	8.79	1	0.0038*
F-fragment	1.85	1	0.1766
Number of fragment (linear)	5.61	1	0.0198*
Number of fragments (quadratic)	5.33	1	0.0230*
Python body length/age	13.86	1	0.0003*
Full model	11.66	5, 98	0.0001

*Significant after sequential Bonferroni correction (Rice, 1989).

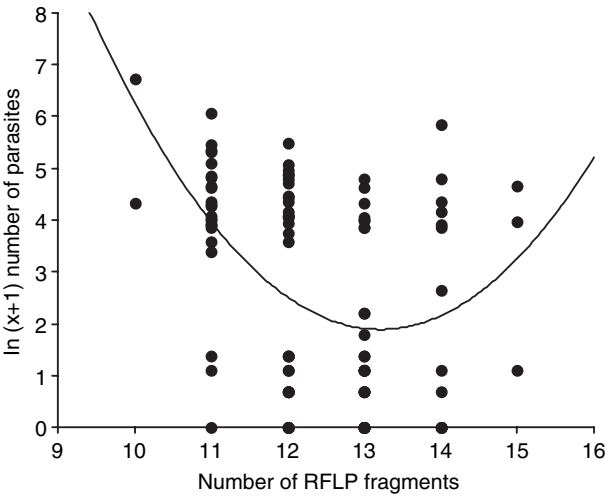

Fig. 1 Relationship between parasite load [ln (x+1) transformed] and number of major histocompatibility complex class I restriction fragment length polymorphism fragments. Quadratic function: $y = 76.296 - 11.266x + 0.426x^2$.

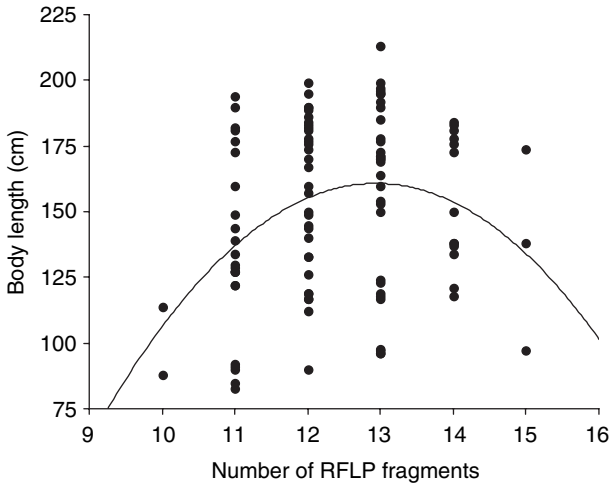

Fig. 2 Relationship between python body length (cm) and number of major histocompatibility complex class I restriction fragment length polymorphism fragments. Quadratic function: $y = -895.838 + 163.315x - 6.311x^2$.

Furthermore, the results from the model also demonstrate that python body length/age was significantly associated with parasite infection level, i.e. larger/older pythons harboured lower levels of parasitaemia compared with smaller younger snakes (Table 2).

As python body length/age was associated with parasite load (Table 2), we also explored whether number of MHC fragments was associated with python length/age. Again a nonlinear relationship was evident, and a quadratic regression revealed a significant relationship between these two parameters ($R = 0.34$, $P = 0.0024$, d.f. = 103), and intermediate fragment numbers were observed in the largest/oldest pythons (Fig. 2).

Discussion

The comparatively large number of RFLP fragments observed suggest that water pythons have several class I genes. Consequently, the individual number of RFLP fragments can be used as an index of heterozygosity. The exact number of class I genes cannot be determined on the basis of the RFLP patterns, and, furthermore, some of the fragments may be pseudogenes. At present we have no data indicating that the fragments are expressed and, hence, the significant lower level of parasite load in pythons harbouring a C-fragment may not be a direct effect of this fragment. However, a similar association between pathogens and microsatellites located within or adjacent to the MHC have been documented in Soay sheep (*Ovis aries*; Paterson *et al.*, 1998) and between pathogens and specific MHC class II alleles in Atlantic salmon (*Salmo salar*; Langefors *et al.*, 2001; Lohm *et al.*, 2002), hairy-footed gerbil (*Gerbillurus paeba*; Harf & Sommer, 2005) and Malagasy mouse lemur (*Microcebus murinus*; Schad *et al.*, 2005). Thus, we suggest that pythons having this fragment are directly or epistatically part of a genetic–phenotypic interaction that enhances the ability of pythons with the C-fragment to combat parasite infections compared with snakes lacking this fragment.

Madsen *et al.* (2005) demonstrated that hematozoan parasite load (*Hepatozoon* spp.) had a negative impact on several python life-history traits such as growth, nutritional status, juvenile survival and female reproductive output. Thus, the results of the present study suggest that the C-fragment could be considered a 'good gene' (a genetic element with fitness-enhancing effects). However, if all individuals in the population would benefit from having the C-fragment, why has not this gene (or genes) gone to fixation?

The prevalence of haematozoan parasite infections in the water python population appears to be very high as examination of 100 randomly chosen samples, all revealed *Hepatozoon*-specific PCR products (Ujvari *et al.*, 2004). Furthermore, *Hepatozoon* parasites with identical nucleotide sequences were identified in several host organisms such as goannas (*Varanus* spp.) and water pythons, suggesting that parasite host shifts have occurred between squamate taxa in the study area (Ujvari *et al.*, 2004). Thus, if such a host shift occurred recently, one potential explanation why not all pythons have this fragment may be that pathogen resistance linked to the C-fragment is evolving, but lagging behind current selection. Thus, only future work can reveal whether the C-fragment frequency is increasing in the water python population.

A second potential explanation is that multiple genes may have similar function and that the fitness loss from lacking the C-fragment is compensated for by the presence of some other gene or gene combinations. Indeed our results suggest that pythons with an intermediate number of RFLP fragments also exhibited reduced parasite loads (Fig. 1).

Major histocompatibility complex diversity has also been demonstrated to correlate with parasite infections level in hairy-footed gerbils (*Gerbillurus paeba*; Harf & Sommer, 2005) and in sticklebacks (*Gasterosteus aculeatus*; Wegner *et al.*, 2003a,b; Kurtz *et al.*, 2004). However, the latter result was only obtained when sticklebacks had been infected by three parasites simultaneously, and therefore Wegner *et al.* (2003a) suggested that a single allele was expected to counteract the pathogenic effect of a single disease whereas multiple alleles would achieve resistance to several infectious agents. At present we have no data on the number of different parasites that may infect water pythons, but apart from *Hepatozoon* sp., most snakes appear to be infected by gastrointestinal parasites such as nematodes and cestodes (T. Madsen & B. Ujvari, pers. obs). However, our results suggest that pythons having either a specific fragment (Table 2) or an intermediate number of MHC fragments exhibited

reduced *Hepatozoon* sp. parasite load (Fig. 1). The latter result therefore mirrors results obtained in a study of sticklebacks (Wegner *et al.*, 2003a, but see Harf & Sommer, 2005). Wegner *et al.* (2003a) suggested that maximal heterozygosity does not necessarily confer the highest fitness in sticklebacks, as too high MHC diversity may limit T-cell diversity and, hence, reduce pathogen recognition efficiency. Our results suggest that similar negative selection on T-cell repertoire size may also operate in pythons.

Individual pythons maintain similar among-year parasite loads and the level of parasitaemia in larger/older pythons is lower compared with younger snakes, suggesting that only snakes with lower levels of parasitaemia were able to survive to old age (Madsen *et al.*, 2005). Pythons having intermediate fragment numbers were indeed larger/older compared with snakes with fewer and greater numbers of fragments (Fig. 2). The results from the present study suggest that both a specific MHC molecule and MHC polymorphism may enhance parasite resistance and longevity in a vertebrate host. Thus, our results therefore suggest that python MHC is subject to both frequency dependence and balancing selection.

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