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Influence of major histocompatibility complex genotype on mating success in a free-ranging reptile population

Hilary C. Miller*, Jennifer A. Moore, Nicola J. Nelson and Charles H. Daugherty

Allan Wilson Centre for Molecular Ecology and Evolution, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington 6140, New Zealand

Major histocompatibility complex (MHC) genes are highly polymorphic components of the vertebrate immune system, which play a key role in pathogen resistance. MHC genes may also function as odour-related cues for mate choice, thus ensuring optimal MHC diversity in offspring. MHC-associated mate choice has been demonstrated in some fish, bird and mammal species but it is not known whether this is a general vertebrate phenomenon. We investigated whether MHC-associated mate choice occurs in a wild population of tuatara (*Sphenodon punctatus*), a territorial and sexually dimorphic reptile. We found weak evidence for MHC-disassortative mating, based on amino acid genotypic distance between pairs, when mated pairs were directly compared with potential pairs in close spatial proximity. No significant association was found between male mating success, number of MHC sequences, microsatellite heterozygosity or MHC lineage. The major determinant of mating success in tuatara was male body size, which was not related to MHC lineage or microsatellite heterozygosity. Our results suggest that male competitive ability is the primary driver of mating success in tuatara. However, MHC-associated preferences also appear to play a role, possibly as a kin avoidance mechanism during territory formation.

Keywords: tuatara; major histocompatibility complex class I; mate choice; reptile; *Sphenodon punctatus*

1. INTRODUCTION

The genes of the major histocompatibility complex (MHC) have frequently been invoked as a possible mechanism by which individuals choose mates to maximize the viability of their offspring (reviewed in Penn 2002; Milinski 2006). MHC genes are a central component of the vertebrate immune system as they present short pathogen-derived peptides on the cell surface for recognition by circulating T cells. The high polymorphism usually observed in MHC genes is linked to their role in disease resistance, where selective pressures from the diversity of pathogens in the population maintain high levels of polymorphism (Piertney & Oliver 2006). MHC genes may also play a role in generation and recognition of individual odours, as recent research on mice and fishes has shown that MHC-derived peptides also function as individual-specific olfactory signals that influence mate choice decisions (Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005; Spehr *et al.* 2006). The link between the olfactory and immune systems provided by MHC genes makes them good candidates for the basis by which individuals choose mates for indirect genetic benefits.

Indirect genetic benefits may be obtained through mate choice in two ways: choice for 'good genes' or genetic compatibility (reviewed in Neff & Pitcher 2005). Under the hypothesis of choice for good genes, individuals (regardless of their own genotype) will choose mates that display increased vigour; for instance, higher body condition or stronger expression of costly secondary sexual characteristics. These individuals may be more disease resistant (Hamilton & Zuk 1982), and thus pass 'good' MHC alleles that confer resistance to pathogens to their offspring. In choice for genetic compatibility, individuals will prefer mates with a complementary genotype to their own (Tregenza & Wedell 2000). In the context of MHC, where increased genetic diversity is thought to increase disease resistance, this may result in individuals choosing mates with a different genotype to their own (MHC-disassortative mating) to maximize MHC diversity in their offspring, or choosing mates with an intermediate level of MHC dissimilarity to optimize the MHC diversity and avoid problems associated with the hypothesized increased loss of T cells when a large number of different MHC molecules are present (e.g. Reusch *et al.* 2001; Milinski 2006).

The role of indirect genetic benefits in mate choice is contentious (Kotiaho & Puurtinen 2007; Uller & Olsson 2008) and few studies have actually demonstrated a link between disassortative mating and offspring fitness (but see Consuegra & de Leaniz 2008). It is thus unclear whether MHC-associated mate choice is a general vertebrate phenomenon. The best evidence comes from mammals (e.g. Yamazaki *et al.* 1976; Potts *et al.* 1991; Ober *et al.* 1997, but see Hedrick & Black 1997; Paterson & Pemberton 1997) and fishes (Landry *et al.* 2001; Reusch *et al.* 2001; Consuegra & de Leaniz 2008). In birds, there is little evidence for MHC-disassortative

* Author for correspondence (hilary.miller@vuw.ac.nz).

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mating (Ekblom *et al.* 2004; Westerdahl 2004; Bonneaud *et al.* 2005; Richardson *et al.* 2005), but some studies have found evidence for preference for specific MHC allelic lineages (e.g. Ekblom *et al.* 2004) or high individual heterozygosity (Richardson *et al.* 2005, but see Westerdahl 2004). Mate choice based on MHC may be more likely in species where there is a lack of social context for mate recognition, a high probability of inbreeding (Jordan & Bruford 1998) and few direct benefits (such as parental care) to mate choice (Zelano & Edwards 2002). Reptiles may be good candidates for MHC-associated mate choice, as they exhibit little parental care and few species exhibit pair bonding (Bull 2000), so there are likely to be few direct benefits of mate choice (Uller & Olsson 2008). Olfactory cues also appear to play a role in mate choice in some species (Lopez *et al.* 2003; Olsson *et al.* 2004). However, in general, precopulatory choice appears to be rare in reptiles (Tokarz 1995; Uller & Olsson 2008).

Only a single study has investigated whether MHC genes are involved in mate choice in reptiles (Olsson *et al.* 2003) and found that females preferentially associated with males that were less similar at MHC. However, this study only used an indirect method of measuring MHC polymorphism (Southern blot restriction fragment length polymorphism) rather than basing analyses on peptide-binding region (PBR) sequences, and did not specifically compare MHC markers with neutral genome-wide markers (e.g. microsatellites). Thus, it is unclear whether the apparent disassortative mating observed was linked directly to MHC genotypes, or represented a more general genome-wide effect of kin avoidance.

In this study, we investigate whether MHC genotypes correlate with mating success in tuatara (*Sphenodon punctatus*), a long-lived, medium-sized reptile, which is the sole extant representative of the order Rhynchocephalia. Tuatara have a seasonally monogamous mating system with low levels of polyandry and polygyny, but do not exhibit long-term pair bonding or parental care (Moore 2008). Tuatara are sexually dimorphic, and mating involves a conspicuous courtship ritual followed by copulation (Gillingham *et al.* 1995; Moore 2008). Both mate choice and male–male competition may be important factors in the mating system of tuatara. Fights between males are common, and body size appears to be the primary indicator of male mating success. Only 25–30 per cent of males successfully mate, and large males predominate (Moore 2008). However, females often reject courtship attempts by males (J. A. Moore 2006, personal observation), and it is unclear whether this is due to female choice or lack of receptivity, as female tuatara are only receptive every 2–4 years whereas males mate every year (Cree *et al.* 1992).

We test whether (i) pairs of tuatara observed mating in the wild are less similar at MHC than would be expected under random mating, taking into account both number of shared alleles and functional differences between alleles (genetic compatibility) and (ii) particular MHC lineages or individual heterozygosity are associated with increased mating success (good genes hypothesis). To distinguish MHC-specific effects from genome-wide effects, we compare MHC diversity with that of neutral microsatellite markers.

2. MATERIAL AND METHODS

(a) *Study population*

We studied tuatara on Stephens Island, a 150 ha island in Cook Strait, New Zealand, which harbours the largest population (30 000–50 000 individuals). Mating activity was monitored during the peak of three mating seasons (5–30 March 2005, 28 February–28 March 2006 and 27 February–27 March 2007), as described in Moore (2008). Pairs of tuatara observed mating were captured by hand after mating concluded, and then measured, weighed and blood sampled. Blood (0.1–1.0 ml) was sampled from the caudal vein or artery and stored in 95 per cent ethanol at 4°C. Twenty-six of the mated pairs were found within one of three study plots on the island. These plots are circular areas (314–615 m^2) located in a section of remnant forest on Stephens Island, in which all tuatara have been captured, measured, weighed and blood sampled.

(b) *Genetic analysis*

Genomic DNA was extracted from 5 to 10 µl whole blood using either a Qiagen DNeasy kit or standard phenol/chloroform methods (Sambrook *et al.* 1989). Previous studies on tuatara MHC, which included analysis of inheritance of alleles, suggest that at least three MHC class I genes are present in tuatara, one of which has low polymorphism and may be a pseudogene (Miller *et al.* 2006, 2007). Exons 2 and 3 form the PBR in MHC class I genes, and hence both exons are polymorphic (Bjorkman *et al.* 1987*b*). In this study, we use two primer pairs designed to amplify MHC class I exon 2 sequences from the polymorphic loci, as described in Miller *et al.* (2007), to provide an estimate of MHC class I variation. Sequences amplified with these primers are 218 or 224 bp in length, once primer sequences are removed, and span the majority of exon 2. The first primer pair (MHC1ex2F1 and MHC1ex2UBR) amplifies a single allelic lineage (comprising two similar sequences, U*18 and U*19), which is present in approximately 50 per cent of animals from Stephens Island. PCR products from this primer pair were purified with ExoSAP-IT and sequenced directly using the BigDye Terminator Cycle sequencing kit (v. 3.1) and an ABI3730 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, USA). The second primer pair (MHC1ex2F1 and MHC1ex2-UAR) amplifies between one and four sequences per individual, so denaturing gradient gel electrophoresis (DGGE) was used to separate these sequences. A modified version of the MHC1ex2F1 primer, containing a 38 bp GC clamp at the 5′ end, was used to produce PCR products for DGGE. PCR products were run on a 9 per cent polyacrylamide gel (37.5 : 1 acrylamide : bisacrylamide) with a 55–80 per cent denaturing gradient of urea and formamide. Electrophoresis was performed at 60°C in 1× TAE at 100 V for 14 hours using the BioRad D-Code system. Gels were silver stained using the method described in Bassam *et al.* (1991). Bands were excised from the gel and eluted in TE for 4 hours at 37°C, then reamplified using MHC1ex2F1 and MHC1ex2UAR. Products were purified by digestion with ExoSAP-IT and sequenced as described above. All sequences included in the analysis were verified by amplification in an independent PCR to rule out PCR artefacts, and are available in the GenBank database (accession numbers DQ145777–DQ145784; EF546395–EF546400; FJ457091–FJ457094).

All individuals were also genotyped at seven polymorphic microsatellite loci (C2F, C11P, E11N, H5H, A12N, C12F and H4H; Aitken *et al.* 2001; Hay & Lambert 2008), using the amplification conditions described in Hay & Lambert

(2008) and Moore *et al.* (2008). Amplification products were run on an ABI 3730 Genetic Analyzer (Applied Biosystems, Inc.), and alleles were visualized using GENEMAPPER (Applied Biosystems, Inc.). Allele sizes were manually scored by the same observer. See table S1 in the electronic supplementary material for summary statistics for microsatellite data.

(c) *Data analysis*

Sequences were edited using SEQUENCHER v. 4.7 (Gene Codes Corporation). Measures of nucleotide and amino acid diversity were calculated in MEGA4 (Kumar *et al.* 2004).

All statistical analyses were performed in R v. 2.5.0 (R Core Development Team 2006). To assess whether mated pairs are less similar at MHC than would be expected under random mating, we compared per cent difference (PD) and average amino acid difference (AAdist) for the 72 mated pairs with 10 000 simulations of 72 random pairings chosen from all the mated individuals. PD measures the percentage of sequences that differ between paired individuals (Yuhki & O'Brien 1990) and is thus a basic measure of allele sharing that does not take into account how alleles are different from each other. AAdist accounts for the functional similarity between genotypes by measuring amino acid difference between all pairs of alleles carried by the mates (Landry *et al.* 2001). We have used average amino acid distance rather than total distance (as in Landry *et al.* 2001; Forsberg *et al.* 2007) because each individual has between one and five different sequences, and thus the number of pairwise comparisons differs between mated pairs. AAdist was calculated for both full exon 2 sequences and peptide-binding sites (identified in Bjorkman *et al.* 1987*a*; Reche & Reinherz 2003) only. For both these genetic dissimilarity measures, we compared both means and variances of mated pairs with random pairs. The average relatedness of mated pairs (based on microsatellite genotypes) was also compared with 10 000 simulations of randomly chosen pairs. Pairwise relatedness was calculated using the formula of Queller & Goodnight (1989) in GENALEX v. 6 (Peakall & Smouse 2005). For all randomizations, bootstrap confidence intervals were used to assess whether values for mated pairs were significantly different from random pairs. One-sided confidence intervals were used as our hypotheses are directional.

We also performed the same analyses using only the pairs that were observed mating within our study plots, to provide a more direct assessment of precopulatory female choice. For these within-plot analyses, each mated female was allowed to choose randomly between available males 10 000 times, and the distribution of values for PD, AAdist and relatedness was compared with the observed value. Randomly chosen available males were males within the same plot as the female with a snout–vent length (SVL) measurement greater than 230 mm, that had either never been observed mating, or were observed mating with other females, but not the female in question. By restricting comparisons to males within the same plot with an SVL >230 mm, we aimed to only include males that were (i) geographically close enough to the female that she is likely to have encountered them and (ii) large enough to be able to compete with other males for access to females. SVL of 230 mm was chosen as the cut-off as 93.4 per cent of mated males have an SVL of 230 mm or greater, compared with only 55 per cent of unmated males (Moore 2008).

Binary logistic regression was used to determine whether male mating success was associated with body size (SVL), microsatellite heterozygosity, MHC genotype or the overall number of MHC sequences. A previous study, which investigated phenotypic correlates of mating success in tuatara, found that only SVL was significantly associated with male mating success, and body condition, tail length, ectoparasite load and territory size were not significant predictors (Moore 2008). Therefore, only SVL was included in our analysis. The response variable was mating success ($n=61$ successful and $n=45$ unsuccessful), where unsuccessful males were those within the study plots that were monitored for more than two mating seasons and were never observed mating. Predictor variables were SVL, microsatellite heterozygosity by locus (HL: Aparicio *et al.* 2006), d^2 (Coulson *et al.* 1998), number of MHC sequences (no_sequences), overall MHC genotype and individual MHC lineages (A, B, C, D, E, G, H, L and M). MHC sequences were grouped into lineages based on their amino acid sequence, such that sequences within a lineage differ by no more than four amino acids (and no more than one PBR amino acid). Lineages that occurred at frequencies of less than 0.1 (F, I, J and K) were not included owing to their low statistical power. MHC genotypes were defined according to the major lineages present in each individual. Genotypes that were present in only a single individual were excluded from this analysis. Measures of individual microsatellite heterozygosity (HL and d^2) were calculated using an EXCEL macro (available at http://www.zoo.cam.ac.uk/zoostaff/amos/#ComputerPrograms). We also calculated standardized heterozygosity and internal relatedness, but both these were highly correlated with HL and so were removed from the analysis. Models incorporating either SVL, microsatellite heterozygosity (d^2 and HL), number of MHC sequences or MHC genotype, and SVL plus a genetic component, were compared based on their AIC value, with the lowest AIC value considered to be the top model. Akaike weights (w_i) were calculated to give the approximate probability of each model being the best model in the set (Anderson & Burnham 2002). Each predictor variable for the best model was then tested for significance within the model using analyses of variance (ANOVA). To test for associations between body size and microsatellite heterozygosity, MHC lineage or overall number of MHC sequences, we used linear regression for continuous variables (HL, d^2 and no_sequences), and ANOVA for categorical data (MHC lineages), with SVL as the response variable.

3. RESULTS

MHC class I genotypes were determined for 72 mated pairs (comprising 67 females and 61 males), and 45 unmated males. Among the 173 individuals, 81 different genotypes were identified. Twenty different MHC sequences were isolated in total (see figures S1 and S2 in the electronic supplementary material), four of which (U*20–U*23) are new to this study. One to five sequences were amplified per individual (mean 2.96). Mean pairwise nucleotide diversity among all sequences was 0.172 ± 0.013, and there was an average of 20.7 amino acid differences out of 72 sites between sequences (range 0–38).

(a) *MHC-disassortative mating*

To determine whether mated pairs are more different at MHC than would be expected under random mating, we compared average PD and average AAdist between mated pairs ($n=72$), with 10 000 simulations of randomly chosen pairs (figure 1). There was no difference between

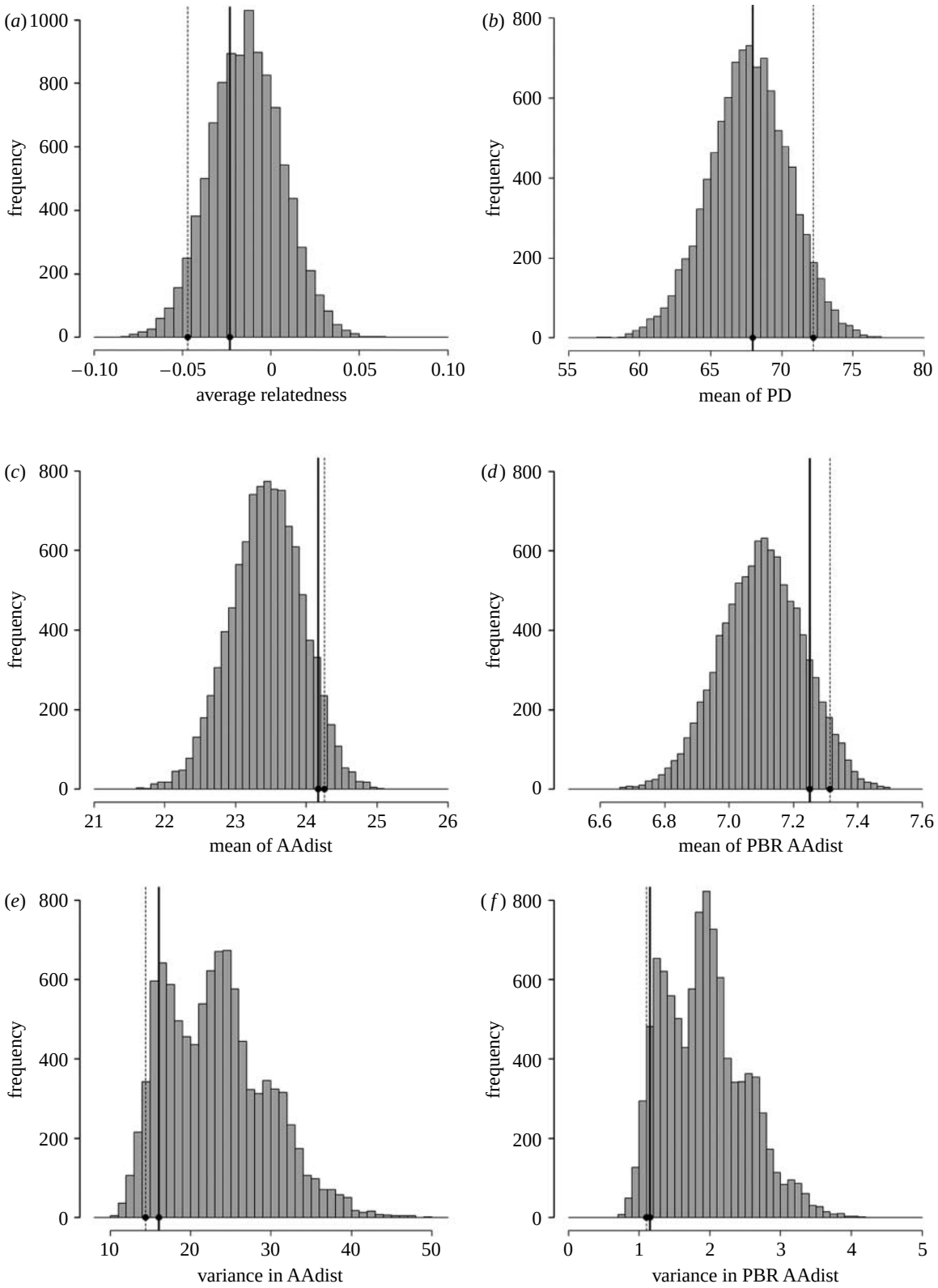

Figure 1. Frequency distributions of mean values of genetic parameters for 10 000 simulations of 72 randomly assorted pairs (grey bars), compared with the mean value for 72 mated pairs (black line) of tuatara. Genetic parameters measured are the following: (*a*) mean relatedness between pairs based on microsatellite genotypes, (*b*) average percentage of major histocompatibility complex sequences that differ between pairs (mean per cent difference), (*c*) average amino acid difference (AAdist) between pairs, based on entire exon 2 sequences, (*d*) average amino acid difference (peptide-binding region AAdist) between pairs based only on putative peptide-binding residues, (*e*) variance in AAdist for entire exon 2, and (*f*) variance in AAdist for peptide-binding sites only. One-tailed 95% confidence intervals are shown by the dotted lines.

the mated and random pairs in mean microsatellite relatedness ($\text{relatedness}_{\text{mated}} = -0.023$ versus $\text{relatedness}_{\text{random}} -0.014$, $p = 0.327$; figure 1*a*) or mean PD (mean $\text{PD}_{\text{mated}} = 67.98$ versus mean $\text{PD}_{\text{random}} = 67.69$, $p = 0.46$; figure 1*b*). For AAdist, mated pairs had higher values than random pairs ($\text{AAdist}_{\text{mated}} = 24.17$ versus $\text{AAdist}_{\text{random}} = 23.44$, $p = 0.076$; figure 1*c*). The same trend was seen when only peptide-binding residues

were considered (PBR $AAdist_{mated}=7.25$ versus $AAdist_{random}=7.1$, $p=0.129$; figure 1*d*), but again this was not significant.

The variance in these measures was assessed to determine whether individuals choose mates with a specific (i.e. maximal or intermediate) level of dissimilarity. Under this hypothesis, variance in dissimilarity measures for mated pairs should be lower than for random pairs. For PD and AAdist, the variance was not significantly lower for mated pairs compared with random pairs (variance in $PD_{mated}=688.25$ versus $PD_{random}=706.10$, $p=0.438$, variance in $AAdist_{mated}=16.03$ versus $AAdist_{random}=23.22$, $p=0.132$; figure 1*e*). However, a trend towards lower variance in AAdist for mated pairs was observed. This trend was particularly apparent when only peptide-binding residues were considered (variance in PBR $AAdist_{mated}=1.153$, $AAdist_{random}=1.918$, $p=0.072$; figure 1*f*).

For the analyses within plots, where mated pairs ($n=26$) are compared with random pairs consisting of the females and randomly chosen males within the same plot with body size (SVL) greater than 230 mm, we found no difference in mean PD between the mated and random pairs (mean $PD_{mated}=71.355$ versus mean $PD_{random}=70.025$, $p=0.4$; figure 2*b*). However, microsatellite relatedness was lower for mated pairs than random pairs ($relatedness_{mated}=-0.0498$ versus $relatedness_{random}=0.0017$, $p=0.067$; figure 2*a*), and mated pairs had higher AAdist than random pairs ($AAdist_{mated}=24.56$ versus $AAdist_{random}=23.129$, $p=0.0497$; figure 2*c*). When only PBR residues were considered, the value for AAdist for mated pairs was higher than for random (PBR $AAdist_{mated}=7.336$, $AAdist_{random}=7.094$, $p=0.177$; figure 2*d*), but the difference was not statistically significant. A trend for lower variance in AAdist in mated pairs was also observed, but not statistically significant.

(b) *MHC genotype and mating success*

It was not possible to directly measure MHC heterozygosity in this study, as we could not assign alleles to loci, so the number of MHC sequences was used as an approximation of heterozygosity. No significant difference in the number of sequences between mated ($n=61$) and non-mated ($n=45$) males was found (Fisher's exact probability test, $p=0.482$). We compared logistic regression models where the relationship between mating success and body size (measured as SVL), microsatellite heterozygosity, number of MHC sequences and MHC genotype was examined separately, and also where SVL and a genetic component were incorporated into the same model (table 1). The best model predicted by AIC incorporated only SVL. SVL was positively correlated with mating success (GLM logit: slope (β) $=0.0952\pm0.018$, $z=5.068$, $p<0.0001$). Successfully mated males had a mean SVL of 253 mm, while the mean SVL for unsuccessful males was 230 mm (figure S3*a*). The model that included microsatellite heterozygosity by locus (HL) and SVL was almost equivalent to the top model ($\Delta AIC=0.209$). However, the effect of HL was small and not significant ($\beta=2.39\pm1.753$, $z=1.363$, $p=0.173$; figures S3*b*). Including measures of MHC diversity (number of MHC sequences or MHC genotype) into the model did not improve the fit of the model to the data. We also tested associations between mating success and individual MHC lineages, both with and without SVL included in the model (see tables S2 and S3 and figure S4 in the electronic supplementary material). A model incorporating both SVL and MHC lineage D was competitive with the model with only SVL (SVL+D, $AIC_c=99.316$; SVL only, $AIC_c=99.416$). However, within this model only SVL is significant (SVL (β) $=0.098\pm0.019$, $z=5.033$, $p<0.0001$; lineage D (β) $=-0.859\pm0.583$, $z=-1.473$, $p=0.141$). This suggests that the lineage D only improves the model slightly and does not have a significant effect on mating success.

As male body size could be influenced by heterozygosity or MHC genotype, we also tested for associations between SVL and microsatellite heterozygosity, number of MHC sequences and individual MHC lineages (see table S4 in the electronic supplementary material). No significant associations were found between SVL and any of the genetic variables we measured ($p>0.3$ for all comparisons).

4. DISCUSSION

In this study, body size was the main predictor of mating success in male tuatara, but there was also some evidence for MHC-disassortative mating, particularly when mated pairs within our study plots were directly compared with potential pairs within the same plot. We also observed a trend towards inbreeding avoidance within plots, as microsatellite relatedness for mated pairs was lower than for random pairs. However, male mating success was not significantly associated with elevated microsatellite heterozygosity or particular MHC lineages.

Our primers provide an estimate of MHC class I variation, as amplification of alleles from a single MHC locus is not possible in tuatara. However, the sequences amplified in this study are likely to represent functional MHC loci, as the PCR primers used were designed from full-length, expressed MHC class I transcripts, isolated from Stephens Island tuatara (Miller *et al.* 2006), and the sequences show evidence for balancing selection on putative PBR sites (Miller *et al.* 2007). Although previous studies suggested that at least two polymorphic loci are present in tuatara, five sequences were found in 4 individuals in this study, and 18 individuals appear to only have a single MHC class I sequence. It is possible that the number of class I genes varies among individuals in tuatara, as has been found in some mammalian and fish species (Malaga-Trillo *et al.* 1998; Roos & Walter 2005). However, we cannot rule out the possibility that we have not amplified all alleles in some individuals. It is interesting to note that in all cases where a single sequence is present, that sequence was U*02. Previous studies that tracked inheritance of alleles at these loci in a family group indicated that this sequence may be duplicated and present at both loci (Miller *et al.* 2007). Also note that we have amplified only exon 2, which codes for only part of the PBR in MHC class I genes, so sequences that share the same exon 2 sequence may differ in their exon 3 sequence and still retain a functionally different PBR.

(a) *MHC-disassortative mating*

We found no evidence for MHC-disassortative mating in tuatara based on allele sharing between mates (PD), but did find a trend towards disassortative mating when the

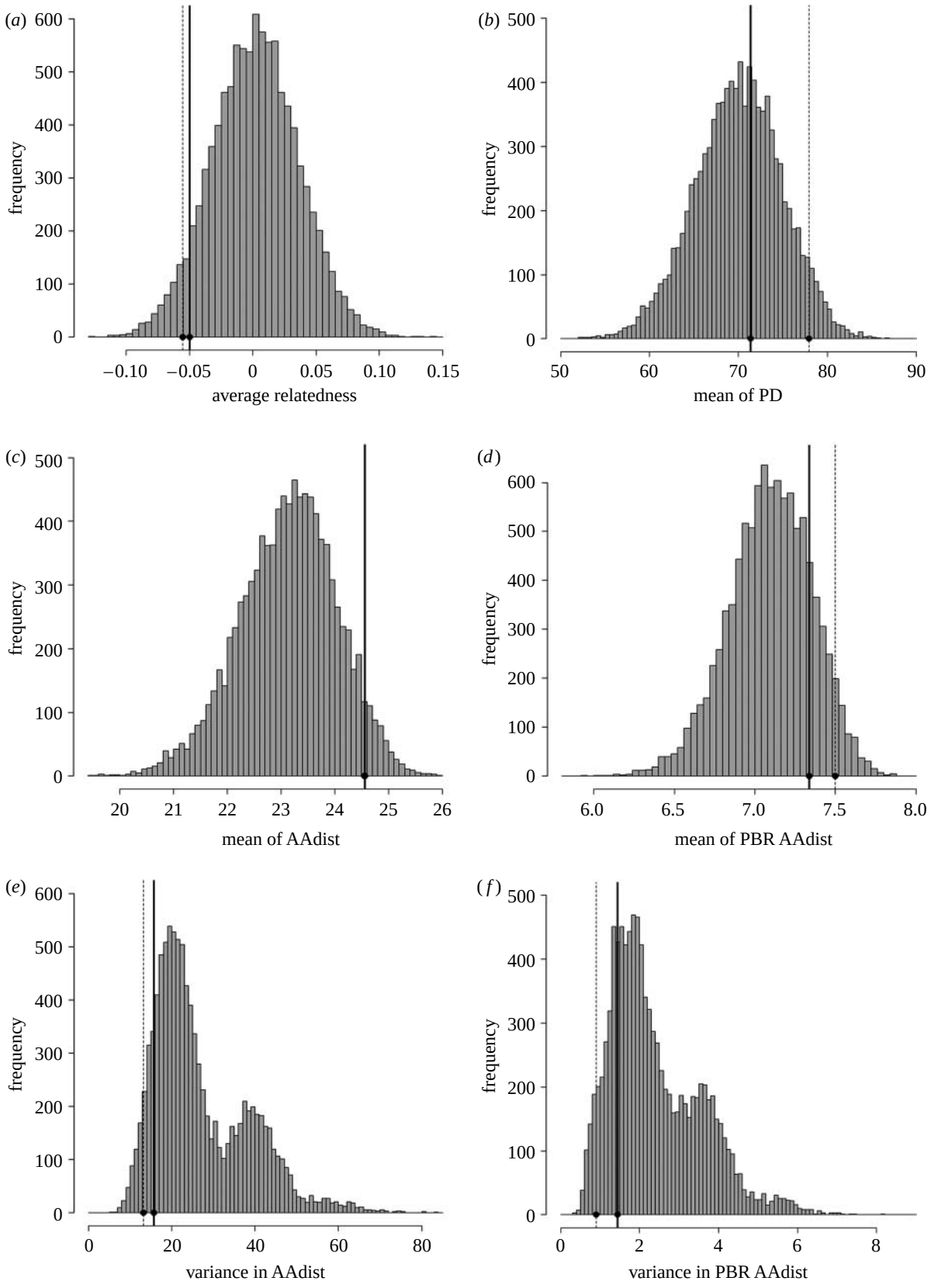

Figure 2. Frequency distributions of mean values of genetic parameters for 10 000 simulations of potential pairs (grey bars), compared with the mean value for 26 mated pairs (black line) of tuatara found within study plots. In this analysis, potential pairs comprise the female and randomly chosen males found in the same plot, which have a snout–vent length > 230 mm. Genetic parameters measured are the following: (*a*) mean relatedness between pairs based on microsatellite genotypes, (*b*) average percentage of MHC sequences that differ between pairs (mean PD), (*c*) average amino acid difference (AAdist) between pairs, based on entire exon 2 sequences, (*d*) average amino acid difference (PBR AAdist) between pairs based only on putative peptide-binding residues, (*e*) variance in AAdist for entire exon 2, and (*f*) variance in AAdist for peptide-binding sites only. One-tailed 95% confidence intervals are shown by the dotted lines (note that in (*c*), this line is obscured by the mean value for mated pairs, as $p = 0.0497$).

Table 1. Comparison of logistic regression models of the relationship between male mating success and body size (SVL), microsatellite heterozygosity (d^2 and HL), number of MHC sequences and MHC genotype ($n=106$). (The best model is shown in italics; K, number of parameters; NLL, negative log-likelihood; AIC_c, corrected AIC; ΔAIC; rescaled AIC; w_i, AIC weight.)

predictor variables	K	NLL	AIC_c	ΔAIC	w_i
SVL	*2*	*95.299*	*99.416*	*0*	*0.376*
SVL+HL	3	93.389	99.624	0.209	0.339
SVL+MHC no. seq	3	95.104	101.339	1.923	0.144
SVL+d^2	3	95.256	101.492	2.076	0.133
SVL+MHC genotype	22	44.158	106.899	7.483	0.009
MHC genotype	21	59.638	118.438	19.022	0.000
HL	2	142.978	147.095	47.679	0.000
MHC no. sequences	2	143.972	148.088	48.673	0.000
d^2	2	144.207	148.323	48.907	0.000
unconditional	1	144.523	146.561	47.145	0.000
global model	14	87.463	120.079	20.663	0.000

amino acid composition of alleles was taken into account (AAdist). Alleles with similar amino acid composition may be functionally equivalent, binding a similar spectrum of peptides and producing a similar olfactory signal (Reche & Reinherz 2004). Individuals with two divergent alleles may have better pathogen resistance than individuals with two similar alleles that essentially bind the same set of peptides. Thus, if mate choice operates to optimize pathogen resistance in offspring, the degree of difference between alleles may be an important factor. Disassortative mate choice may not be evident when using simple measures such as PD, which are based on only the presence/absence of shared alleles without taking into account the degree of difference. Similar results have been found in Atlantic salmon (*Salmo salar*) where Landry *et al.* (2001) found evidence for disassortative mating only when differences in PBR amino acids were measured, and not when the number of shared alleles was measured. We did not find a stronger tendency to disassortative mating when only PBR residues were measured instead of the whole of exon 2. The PBR sites used here were predicted from the structure of the human MHC molecule and may not match exactly the PBR sites in the tuatara molecule. However, the mismatches are probably restricted because sites showing evidence of positive selection correspond to the predicted PBR sites. Alternatively, the amino acid distance within exon 2 may reflect the distance between other linked regions (for instance, containing additional MHC genes) that are also important for mate choice.

The trend towards disassortative mating based on amino acid distance was not statistically significant ($p=0.076$) when all mated pairs were compared with randomized pairs, but was just significant at $p<0.05$ when comparisons were confined to animals within the same study plot. Our finding of stronger disassortative mating in the within-plot analyses, despite the smaller sample size, may be due to the fact that only males that could have potentially mated with each female were included and body size was controlled for (as males likely to be too small to compete for matings were excluded). This therefore provides a more direct test of precopulatory mate choice, and may have reduced the noise in the data.

We also found a trend towards lower variance in amino acid distance for mated pairs, although this was not statistically significant. If individuals are choosing mates with a specific level of MHC dissimilarity (whether maximal or intermediate), the variance in dissimilarity for mated pairs should be lower than under random choice. If choice for intermediate levels of diversity occurs (as suggested by Milinski 2006), the mean dissimilarity between mated pairs may not be significantly different from random pairs, but the variance will be significantly smaller. We found a trend towards both higher mean and lower variance in amino acid distance, which supports the idea that weak disassortative mating for maximal diversity, rather than intermediate diversity, is present in tuatara. Choice for intermediate diversity to avoid the hypothesized negative effects of increased T cell selection (Nowak 1992; but see Borghans *et al.* 2003) may be less relevant for species with few duplicate copies of each class of MHC gene.

(b) *Male body size and mating success*

Body size was the strongest determinant of mating success in male tuatara, with a positive association between SVL and mating success. Although models incorporating a genetic factor with SVL (particularly MHC lineage D and microsatellite heterozygosity by locus (HL)) were competitive with the SVL only model, the effect size of the genetic factor was small in comparison with the effect of SVL. Large body size in males may reflect vigour due to better pathogen resistance, and thus be associated with specific MHC alleles that confer resistance to common pathogens, or MHC heterozygosity. This indirect effect of MHC genotype on mate choice, mediated by a sexually selected phenotypic trait, has been shown in pheasants (*Phasianus colchicus*), where females preferred males with long tarsus spurs, a trait associated with MHC genotype (von Schantz *et al.* 1997). Similarly in deer (*Odocoileus virginianus*), signals of male quality such as antler development and body size were associated with MHC class II genotype (Ditchkoff *et al.* 2001). In tuatara, SVL is not significantly associated with either particular MHC lineages or microsatellite heterozygosity, suggesting that choice for good MHC genes or heterozygosity is not a factor in mating success. Large body size in tuatara may be more influenced by resource availability than genetic factors, and large males may be more successful because they out-compete the smaller males for food and access to females. This result also suggests that large body size in tuatara is not associated with pathogen resistance, but this needs further investigation. A recent study of tuatara

ectoparasites suggested that ectoparasites reduce the body condition of tuatara (S. Godfrey 2008, personal communication), and thus continued high levels of infestation may lead to decreased growth rates and lower SVL in the long term. Resistance to ectoparasites may be influenced more by MHC class II genes than class I (Piertney & Oliver 2006), so SVL may be associated with genetic factors not measured in this study. Analysis of a broader range of pathogens and additional MHC markers is required before the effect of pathogens on body size and subsequent mate choice can be elucidated.

(c) *Mating systems and MHC-associated mate choice*

Our results suggest that mating success in tuatara is mainly determined by male body size, but that MHC-disassortative preferences may also play a role. The effect of large body size is likely to reflect male–male competition, where large males dominate mating, rather than female choice for large males. Male–male competition is a characteristic of many reptile mating systems (e.g. Shine *et al.* 2000; Morrison *et al.* 2002). The importance of male–male competition in tuatara is supported by the finding that smaller males are more successful in open areas of Stephens Island, where population densities are 10 times lower than in forested areas, presumably because male–male competition is reduced (Moore 2008). In areas of high population density where our study was conducted, mate guarding and fights between males are common, and courtship rituals are frequently interrupted by rival males (J. A. Moore 2006, personal observation).

Determining the relative importance of male–male competition versus female choice is difficult, as our study relied solely on behavioural observations of free-ranging animals, which represent the combined effect of male competition and precopulatory mate choice. Some studies of MHC-associated mate choice have used staged mate choice experiments based on odour samples (e.g. Reusch *et al.* 2001; Olsson *et al.* 2003), to control for confounding influences that may be present in natural populations and enable choice to be measured in isolation. However, such experiments would be unlikely to produce biologically relevant results in the tuatara system because mating succeeds only when the prolonged courtship ritual (which may take several hours) is allowed to proceed. Although we found evidence for MHC-disassortative mating in tuatara, the fact that it was relatively weak may be due to the overriding effect of male–male competition. The stronger tendency towards disassortative mating observed when body size was controlled for in the within-plot analyses supports this hypothesis. There may, in fact, be little opportunity for female choice at the time of mating, as is the case for many other reptile species (Tokarz 1995; Uller & Olsson 2008). Female tuatara may encounter only a few males during the mating season, as males generally only mate with females within their territories, and mate guarding by the territorial male limits the female's access to other males (Moore 2008). However, choice may play a role in territory formation and it is here that MHC-associated preferences may operate. Male spatial structure appears to be static for long periods in tuatara, but females returning from nesting areas do not always return to the same home burrow (J. A. Moore 2007, unpublished data), and therefore have the opportunity to assess many different males and choose where they establish new territories. Similarly, when young adults begin to establish territories there may be the opportunity for choice. Thus, the MHC-disassortative mating patterns we observed within the study plots may reflect a mechanism for kin avoidance during territory formation rather than choice at the time of mating. This hypothesis is supported by the fact that within the study plots, relatedness between mated pairs is lower than for random pairs, suggesting that animals that live adjacent to one another (and hence are more likely to mate) are less related than those living further apart.

MHC-associated mate choice may also operate at the post-copulatory level through either sperm competition or cryptic female choice. MHC molecules have been implicated in selection of sperm by oocytes (Ziegler *et al.* 2002; Skarstein *et al.* 2005), and MHC-associated post-copulatory choice has been measured in a wild population of lemurs (*Microcebus murinus*; Schwensow *et al.* 2008). Many studies of MHC mate choice infer mate choice from parental analysis, rather than from behavioural observations of mating, and are thus measuring the combination of pre- and post-copulatory choice (e.g. Landry *et al.* 2001; Forsberg *et al.* 2007). Our study focuses on precopulatory mechanisms, but there is limited paternity data available for the pairs that we observed mating (Moore *et al.* submitted), which may indicate whether post-copulatory choice is a factor. Of the 12 clutches for which paternity data are available, 9 were sired by the male observed mating with the female, one showed multiple paternity (equally split between the male observed mating and an unknown male) and two were sired by an unsampled male. One female that mated with two different males in one season had a single paternity clutch. These results show that our behavioural observations of mating success mostly reflect fertilization success, but suggest that some post-copulatory mate choice or sperm competition may operate. However, a larger sample size would be required to assess the role of MHC in post-copulatory phenomena in tuatara.

In promiscuous mating systems, in particular, non-MHC-associated factors, such as male–male competition or territory quality, may predominate in determining mating success (e.g. Paterson & Pemberton 1997; Westerdahl 2004). In addition, mate choice for indirect genetic benefits usually represents a relatively weak selective force compared with choice for direct benefits (Kotiaho & Puurtinen 2007). Thus, in systems where MHC-associated effects do occur, large sample sizes of hundreds of mated pairs may be required to detect them, but such sample sizes are often difficult to obtain in natural systems. High levels of genetic variation at MHC may also weaken the statistical power of tests for MHC-associated effects, as in a large natural population such as Stephens Island, two individuals chosen at random from the population are likely to have a different genotype with few shared alleles (Jordan & Bruford 1998). It may be informative to compare the results of our study on Stephens Island with results from a tuatara population with lower diversity. However, obtaining adequate sample sizes from tuatara populations with lower diversity would be difficult owing to the much lower population densities on those islands.

In conclusion, our results suggest the mating system of tuatara is largely driven by male–male competition, but that the MHC also plays a role in determining mating

preferences. However, the apparent lack of opportunities for female choice at the time of mating suggests that the MHC-disassortative preferences we measured operate at the time of territory formation.

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