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Research paper

MHC class II DRB diversity predicts antigen recognition and is associated with disease severity in California sea lions naturally infected with *Leptospira interrogans*

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ABSTRACT

We examined the associations between California sea lion MHC class II DRB (*Zaca-DRB*) configuration and diversity, and leptospirosis. As *Zaca-DRB* gene sequences are involved with antigen presentation of bacteria and other extracellular pathogens, we predicted that they would play a role in determining responses to these pathogenic spirochaetes. Specifically, we investigated whether *Zaca-DRB* diversity (number of genes) and configuration (presence of specific genes) explained differences in disease severity, and whether higher levels of *Zaca-DRB* diversity predicted the number of specific *Leptospira interrogans* serovars that a sea lion's serum would react against. We found that serum from diseased sea lions with more *Zaca-DRB* loci reacted against a wider array of serovars. Specific *Zaca-DRB* loci were linked to reactions with particular serovars. Interestingly, sea lions with clinical manifestation of leptospirosis that had higher numbers of *Zaca-DRB* loci were less likely to recover from disease than those with lower diversity, and those that harboured *Zaca-DRB*.C or –G were 4.5 to 5.3 times more likely to die from leptospirosis, regardless of the infective serovars. We propose that for leptospirosis, a disadvantage of having a wider range of antigen presentation might be increased disease severity due to immunopathology. Ours is the first study to examine the importance of *Zaca-DRB* diversity for antigen detection and disease severity following natural exposure to infective leptospires.

1. Introduction

An individual's genetic architecture can influence susceptibility, resistance and tolerance to pathogens (Segal and Hill, 2003), as well as limit disease severity (e.g. Cansanção et al., 2016; Toubiana et al., 2016). Empirical evidence of associations between host genetics and infectious disease in natural populations is growing. For instance, low levels of heterozygosity as measured by presumably neutral non-coding markers have been related to higher pathogen loads (e.g. Luikart et al., 2008), increased susceptibility to infections (e.g. Kaunisto et al., 2013; Ferrer et al., 2014), altered immunocompetence (e.g. Hawley et al., 2005; Voegeli et al., 2013; Brock et al., 2015), and more severe disease (e.g. Hawley et al., 2005; Acevedo-Whitehouse et al., 2006; Rijks et al., 2008) in a wide range of species.

The role of functional genetic variation in shaping pathogen resistance is also well known for natural populations. In particular, various studies have shown that genes belonging to the major histocompatibility complex (MHC) are related to risk of infection in natural populations (e.g. Kloch et al., 2010; Froeschke and Sommer, 2012; Schad et al., 2012; Osborne et al., 2015a), and that polymorphism at this genetic region is maintained via heterozygote advantage, frequency-dependence (reviewed in Sommer, 2005; Piertney and Oliver, 2006; Spurgin and Richardson, 2010) or under the optimality hypothesis (Nowak et al., 1992). In addition to the MHC, 'non-MHC' genes, such as those that encode cytokines and other innate effectors, also play a role in shaping immune responses (e.g. Coltman et al., 2001; Ezenwa et al., 2010; González-Quevedo et al., 2015; Osborne et al., 2015b). Thus, various host genes contribute towards a successful immune response, and their involvement or importance may vary according to a given pathogenic challenge, depending upon the pathophysiology of the disease as well as the immune responses that the pathogen typically elicits (Acevedo-Whitehouse and Cunningham, 2006; Friberg et al., 2010).

Given the number of infectious diseases that have emerged in the

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recent past (Jones et al., 2008; McCloskey et al., 2014) and the impact that they can have on the viability of natural populations (May and Anderson, 1979; Smith et al., 2009), it is of interest to investigate potential associations of relevant host genes with diseases that are relevant to a given population, be it by causing high levels of mortality, decreasing body condition or severely reducing fertility and reproductive success. One such disease is leptospirosis, a bacterial zoonosis caused by pathogenic species of the genus *Leptospira* (Faine, 1994). Leptospirosis can affect humans and a broad range of domestic and wild animals (McBride et al., 2005), including the California sea lion, *Zalophus californianus*, which periodically undergoes outbreaks of leptospirosis (Vedros et al., 1971; Dierauf et al., 1985; Lloyd-Smith et al., 2007) with high mortality due to renal failure (Gulland et al., 1996). In addition to its global significance, a further reason for studying the genetic regulation of susceptibility to this disease is that antigenic diversity of the genus *Leptospira* is extremely high. It has over 230 distinct agglutinating epitopes on the surface lipopolysaccharide (known as serovars), which in turn are grouped into 24 serogroups based on antigenic similarities (Cerqueira and Picardeau, 2009). This makes *Leptospira* an interesting pathogen model for testing the hypothesis that MHC diversity bestows a selective advantage by enhancing resistance to infection and development of disease.

Following infection, pathogenic leptospires may disseminate throughout the host's organs, exerting damage to the endothelial lining of capillaries and leading to localised ischemia which can result in renal tubular necrosis, hepatocellular damage, placentitis and meningitis, depending upon the host species (Guerra, 2009). In the California sea lion, leptospires affect the kidneys, causing marked swelling, loss of cortical differentiation, and occasional subcapsular and corticomedular haemorrhages (Gulland et al., 1996). Immune responses to infective leptospires have not been studied in depth for the California sea lion, however, their response is likely to similar to what has been reported for other mammals. Namely, host responses initiate with macrophage ingestion of leptospires, followed by antibody production (Palaniappan et al., 2007). As macrophages are antigen-presenting cells, it is likely that leptospiral antigens are processed and presented for immune recognition on class II MHC molecules (see Radaelli et al., 2009), essential for the presentation of extracellular peptides and subsequent production of antibodies. Recent studies offer some evidence of the MHC's involvement in leptospirosis, and have highlighted associations between MHC genotypes and susceptibility to leptospirosis in humans (Lingappa et al., 2004; Guo et al., 2010; Esteves et al., 2014), Furthermore, histological observations of degraded leptospires within macrophages offer further evidence that leptospiral antigens can be processed and presented for immune recognition on class II MHC molecules (Lingappa et al., 2004).

The mammalian MHC is typically divided into gene classes (I–IV, of which 1 and II are 'classical MHC genes') that differ in their structure and function (Gruen and Weissman, 2001; Goldberg and Rizzo, 2015). The class II MHC genes are grouped as DP, DQ and DR genes, all of which encode for polymorphic cell surface receptor proteins (Engelhard, 1994). The California sea lion's class II MHC-DQA and -DQB genes exhibit extremely low polymorphism (Bowen et al., 2002), but DRB loci maintain high diversity by possessing multiple DRB loci and varying individual gene configurations (Bowen et al., 2004). Furthermore, California sea lion MHC-DRB (hereafter *Zaca-DRB*) sequences are compatible with antigen-binding and antigen-presentation functions (Bowen et al., 2004). Taking into account the immunobiology of leptospirosis, which involves immune interactions with leptospiral surface proteins (Cullen et al., 2004), and that the diversity of MHC class II molecules of an individual determines the range of antigens to which an individual is capable of responding (Kaufmann and Schaible, 2005), we hypothesize that in vitro detection of antigenic determinants of pathogenic leptospires is enhanced in sea lions with higher levels of *Zaca-DRB* diversity. Furthermore, as having higher diversity at the MHC-II is posited to be advantageous for pathogen recognition and subsequent immune responses (Sommer, 2005; Piertney and Oliver, 2006), we expect leptospirosis to be less severe in sea lions that have more *Zaca-DRB* loci. We tested these hypotheses by examining individual configurations of *Zaca-DRB* in leptospirosis-affected California sea lions that had stranded along the northern California coast.

2. Materials and methods

2.1. Study animals

We examined medical records of California sea lions stranded along the California and Oregon coasts between 1995 and 2005, and whose phylogeographic origin had been assigned to the Northern Channel Islands in California, USA (assignment based on 18 unlinked microsatellites and a 394 bp-long fragment of the mitochondrial control region I; data not shown). Based on the medical records of these individuals, two groups were defined as follows: Group 1 "Leptospirosis afflicted" (n = 77; adult, subadult and juvenile sea lions with clinical diagnosis of leptospirosis) and Group 2 "controls" (n = 28; group composed of adult sea lions that showed no clinical manifestations of leptospirosis or of any other ongoing infectious disease, and that had stranded due to trauma). None of the control sea lions showed any evidence of infectious disease upon thorough medical examination, particularly of signs associated with leptospirosis, and thus were considered to be adequate representatives of individuals that were not infected by leptospiral serovars or any other known disease. Criteria for assignment of animals into Group 1 were serum chemistry results indicative of clinical leptospirosis (blood urea nitrogen > 100 mg/dl, creatinine > 2 mg/dl, sodium > 155 meq/l and phosphorus:calcium > 1; Colagross-Schouten et al., 2002), or gross (swollen kidneys with loss of renule differentiation and pale cortices) and microscopic lesions (interstitial nephritis) detected during post mortem examination consistent with the disease (Greig et al., 2005).

Blood (live sea lions) or skin (dead sea lions) samples were collected at the Marine Mammal Center and preserved for genetic analyses (see below). Sampling was conducted by approval of the IACUC at the Marine Mammal Center and was authorised by the Bioethics Committee of the Autonomous University of Querétaro. All procedures conducted adhered to guidelines stipulated by US laws of animal research, where animal handling and sampling was undertaken, and to those outlined by SAB in their ethics statement on the use of animals for research and teaching. No animals suffered any observable consequences as a result of being used in this study. Samples were collected under MMPA permit no 932-1905/MA-009526.

2.2. Serology

As part of ongoing research at The Marine Mammal Center, serum agglutination reactions against six *L. interrogans* serovars had been quantified by microscopic agglutination test (MAT) for all of the 77 sea lions diagnosed with leptospirosis. This test, considered the standard reference test for diagnosis of leptospirosis (Faine, 1994), is used for the detection of leptospira-specific antibodies at the serovar levels (i.e. antibodies produced against different leptospiral epitopes; Borg-Petersen and Fagraeus, 1949; Levett, 2003). MAT relies on exposing suspensions of live cultured *L. interrogans* serovars to serial dilutions of an individual's serum in microtiter plates and quantifying antibody-driven agglutination of the leptospires. To ensure consistency with previous studies, the endpoint titer of each assay was recorded as the serum dilution at which 50% of the leptospires were agglutinated (Colagross-Schouten et al., 2002; Lloyd-Smith et al., 2007). All of the assays were conducted at the California Animal Health and Food Safety Laboratory (Davis, CA).

Based on the MAT results, for each sea lion, we recorded their MAT repertoire (i.e. panel of serovars that yielded positive agglutination). The diversity of responses to antigenic determinants was calculated for

leptospirosis-affected sea lions as the number of positive reactions (0–6) with an endpoint titer ≥ 1:800 (Lloyd-Smith et al., 2007). The rationale for using this measure was that although cross-reactions between serovars can occur when an individual's initial immune response is directed towards a heterologous (i.e. antigenically-similar) serovar (Levett, 2003; Andre-Fontaine, 2006), the serum samples used here were tested against six *L. interrogans* serovars each belonging to a different serogroup (Levett, 2003). In that sense, the agglutination reactions with a high endpoint titer (Lloyd-Smith et al., 2007) are likely to reflect reactions against distinct epitopes. The serovars tested against were: *L. interrogans* serovar Pomona (serogroup 18: Pomona), *L. interrogans* serovar Bratislava (serogroup 1: Australis), *L. kirschneri* serovar Grippotyphosa (serogroup 9: Grippotyphosa), *L. interrogans* serovar Copenhageni (serogroup 11: Icterohaemorrhagiae), *L. interrogans* serovar Canicola (serogroup 5: Canicola), and *L. interrogans* serovar Hardjo type Hardjoprajitno (serogroup 22: Sejroe), all considered of diagnostic interest for the California sea lion in the US (Lloyd-Smith et al., 2007).

As a measure of disease severity, we classified each of the Leptospirosis-affected sea lions in two outcome groups: *i*) survived (i.e. sea lions with clinical signs of leptospirosis that survived during rehabilitation up to their release) and, *ii*) did not survive (i.e. sea lions with signs of leptospirosis that were dead upon arrival at The Marine Mammal Center or that died during treatment). As proxies for 'strength of immunity' for each sea lion we $\log_{10}$ transformed the maximum endpoint titer and multiplied the resulting value by -1. In this way, the value was an indicator of the amount of specific anti-leptospira antibodies produced by an individual. Additionally, based on the maximum endpoint titer, we grouped leptospirosis-affected individuals as low-responders (maximum endpoint titer < 1:800) and high-responders (maximum endpoint titer ≥ 1:800) (Lloyd-Smith et al., 2007).

2.3. MHC genotyping

We extracted DNA from skin or blood samples collected from each individual at The Marine Mammal Center. Extraction was accomplished by Chelex-Proteinase K digestion as done previously (Acevedo-Whitehouse et al., 2006). In all cases, the DNA was diluted to 10 ng·μl^{-1}. Individual *Zaca-DRB* configuration (repertoire of MHC class II DRB loci present) and diversity (number of MHC class II DRB loci present) was determined by running eight sequence-specific primer (SSP) PCRs, as previously reported (Bowen et al., 2004). Each 30 μl reaction contained < 1 μg DNA, 30 pmol of one of eight primer pairs (in which the forward primer was fluorescently labelled with one of the following dyes: FAM, ROX [Applied Biosystems, UK], ATTO560 or ATTO565 ROX [ATTO-TECH, GmbH, Germany]), 1 × PCR buffer, 200 pmol dNTPs and 0.4 U/C HotStar Plus Taq DNA Polymerase (Qiagen, UK). Amplification was performed in triplicate, on separate days, in a 3700 Applied Biosystems thermal cycler under the following conditions: 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C, and a final extension step of 72 °C for 10 min. The presence or absence of each SSP-derived sequence type was resolved by running the amplified products on an ABI 3100 automatic Sequencer (Applied Biosystems, UK). Reactions were inspected and scored manually after visualization in GeneMapper v3.7 (Applied Biosystems, UK). The protocol developed by Bowen et al. (2004) is highly specific for each *Zaca-DRB* locus identified to date, and each unique primer pair used to amplify a give gene does not amplify any other gene. For each *Zaca-DRB* locus, we randomly sequenced four positive reactions as continued confirmation that the correct gene was being amplified.

2.4. Neutral genetic variation

In order to account for differences in neutral genetic variation, we estimated homozygosity by locus (HL) for each individual. This measure weights the contribution of each locus to the homozygosity index according to their allelic variability (Aparicio et al., 2006) and varies between 0 (all loci are heterozygous) and 1 (all loci are homozygous). To calculate HL, we genotyped each sample at 14 pinniped microsatellites (see Table S1 in Supplementary Material for details on markers used). Multiple reactions were performed using four to five pairs of fluorescently-labelled primers, grouped according to optimized annealing temperatures and expected product sizes. We followed the PCR protocols described in Acevedo-Whitehouse et al. (2006). Amplified products were run on an ABI 3100 automatic Sequencer (Applied Biosystems, UK). Alleles were inspected manually and scored using GeneMapper v3.7 (Applied Biosystems, UK). In order to detect discrete size variants, microsatellite allele binning was accomplished using Flexibin (Amos et al., 2007). Homozygous individuals were genotyped twice to reduce errors due to allele dropout. Before calculating HL for each individual we tested all loci for departures from Hardy–Weinberg expectations, linkage disequilibrium and null alleles using Genepop version 3.4 (Raymond and Rousset, 1995). HL was calculated using the Excel Macro I*RMacroN4* developed by W. Amos.

2.5. Statistical methods

The main aim of this study was to examine the relationship between a sea lion's MHC class II DRB configuration and its response to leptospirosis, while taking into account levels of neutral genetic variability and controlling for potential confounding factors, when possible. We initially explored our dataset graphically to establish the spread and distribution of the data. Continuous response variables that deviated from the normal distribution were examined with Cullen and Frey graphs to determine their distribution. Prior to analysing our data, we checked for collinearity between *Zaca-DRB* diversity and HL and found no relationship between these explanatory variables (Pearson's correlation; r = 0.45, t = 0.46227, df = 102, p = 0.64).

Different approaches were used to address each of our hypotheses. Effects of 'host genetics' on disease status (yes/no category; binomial error distribution) were examined by building a generalized linear mixed effect model (GLMM). These models are an extension to the generalized linear models (Crawley, 2002) in which the linear predictor contains random effects as well as fixed effects. Fixed explanatory variables were *Zaca-DRB* diversity (i.e. the number of *Zaca-DRB* loci present in an individual) and background levels of genetic variation (HL). To account for stochastic variation among sampling years (e.g. environmental conditions) that could influence susceptibility to infection, we defined 'Year' (1995–2005) as a factor and included it as a random effect in the model. The GLMM was fitted by maximum likelihood (Laplace approximation).

A series of generalized linear models (GLMs; Crawley, 2002) were built to address whether in vitro detection of antigenic determinants of pathogenic leptospires is enhanced in individuals with higher levels of *Zaca-DRB* diversity, and to determine if individuals with higher MHC polymorphism are at an advantage for pathogen recognition and subsequent immune responses. Response variables were, in turn: *i*) number of low-titer positive agglutination reactions (0–6, Poisson error distribution), *ii*) number of high-titer positive agglutination reactions (0–6, Poisson error distribution), *iii*) strength of immunity (continuous variable with a beta distribution – modelled using a quasibinomial error distribution and logit link), and leptospirosis outcome (survived/died, binomial error distribution). Explanatory variables fitted were *Zaca-DRB* diversity (i.e. the number of *Zaca-DRB* loci present in an individual) and background levels of genetic variation (HL). Homogeneity of variance was inspected using plots of the standardised residuals against the fitted values and normality of error was checked with probability plots. In each case, the initial model included two-way interactions. Using standard deletion-testing, the interaction was dropped from each model unless doing so significantly reduced the amount of deviance explained and increased the Akaike information criterion (AIC). We checked for overdispersion by comparing the residual deviance to the residual degrees of freedom of the models

(Crawley, 2002).

Contingency tables were built and examined by Fisher exact tests in order to investigate associations of specific *Zaca-DRB* configuration or genes with *i*) leptospirosis (yes/no), *ii*) reactions against specific serovars, and *iii*) survival. This allowed us to calculate an exact probability value for each observed relationship. We adjusted for multiple comparisons by applying Hochberg procedures (Roback and Askins, 2005). Once the probability was established, we calculated the odds ratio (OR) as a measure of association between the identified *Zaca-DRB* configuration or gene and the response variable. All analyses were performed in R version 3.3.1 (R Development Core Team, 2016). GLMMs were built with R package *lme4*, and contingency tables, Fisher exact *p*-values and odds ratios were calculated with R package *Epitools*.

3. Results

3.1. Associations between Zaca-DRB loci and infection

Twenty-three distinct *Zaca-DRB* configurations were identified in the 105 California sea lions included in this study (see Table S2 in Supplementary Material). Of these, only one configuration, '*ABCDEFGH*', differed in frequency between leptospirosis-affected and control individuals, being more common in sea lions with leptospirosis (40.26%) than in sea lions with no clinical manifestation of disease (10.71%) (Pearson's $Chi^2 = 8.19$; Fisher's exact test, $p = 0.002$). Sea lions with this configuration were 5.6 times (OR; 95% CI = 1.56–20.22) more likely to be affected than sea lions with any other configuration. Odds ratios for all *Zaca-DRB* configurations are shown in Table S2. In terms of individual *Zaca-DRB* loci (A to H; see Table S3 in Supplementary Material), after accounting for multiple comparisons, the frequency of *Zaca-DRB*.B was higher in sea lions with leptospirosis (Pearson's $Chi^2 = 14.32$; Fisher's exact test, $p = 0.0005$), with individuals that had this gene being 8 (95% CI = 2.43–26.33) times more likely to be affected. Odds ratios for all *Zaca-DRB* loci are shown in Table S3 (Supplementary Material).

All of the leptospirosis-affected CSL had positive reactions to serovar Pomona. Agglutination reactions against the other serovars varied among individuals, and a number of *Zaca-DRB* loci were found to be significantly associated with responses to specific serovars (Table 1). In particular, *Zaca-DRB*.B was linked to *L. interrogans* serovars Icterohaemorrhagiae, Grippotyphosa and Canicola, while *Zaca-DRB.D* was linked to serovar Icterohaemorrhagiae and *Zaca-DRB*.A was linked to Grippotyphosa. In all cases the relationship was positive, with serum from sea lions that had the abovenamed *Zaca-DRB* locus reacting to the serovar.

Table 1
Association of each *Zaca-DRB* gene locus (*Zaca-DRB.A* to *Zaca-DRB.A.H*) with specific *Leptospira interrogans* serovars assessed by in vivo microscopic agglutination in serum from stranded California sea lions with a clinical diagnosis of leptospirosis (n = 77). Only those relationships that were significant at $\alpha = 0.05$ are included. Values in bold remained significant after accounting for multiple comparisons.

Zaca-DRB	Serovar	OR	Wald 95% CI	Fisher exact p-Value
B	Icterohaemorrhagiae	34.5	4.09–290.35	**0.003**
B	Gryppotyhosa	20.1	2.70–149.52	**0.007**
B	Bratislava	23.33	2.39–227.05	0.019
B	Canicola	12	1.73–83.03	**0.018**
D	Icterohaemorrhagiae	16.75	2.53–111.07	**0.008**
D	Bratislava	13.6	1.57–117.95	0.037
D	Gryppotyhosa	9.75	1.69–56.20	0.020
D	Hardjo	5.85	1.16–29.35	0.033
A	Gryppotyhosa	10.5	2.08–53.01	**0.007**
A	Icterohaemorrhagiae	9.14	1.54–54.24	0.025
C	Hardjo	4.15	1.29–13.33	0.011

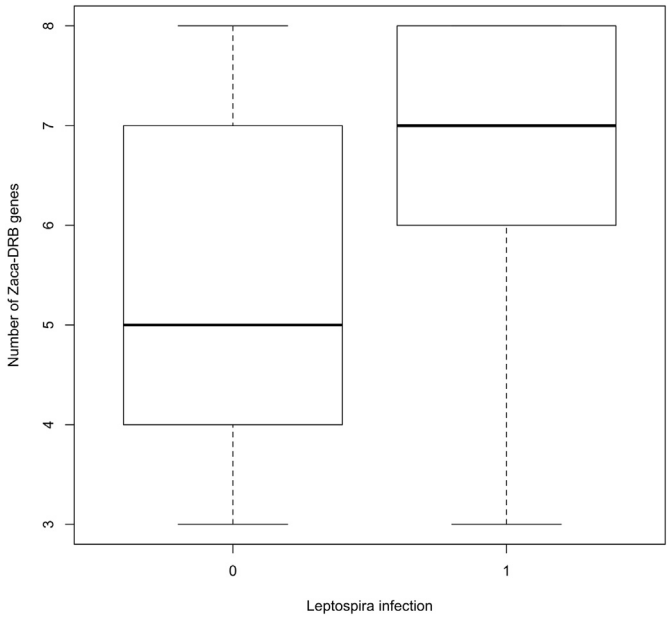

Fig. 1. *Zaca-DRB* diversity in leptospirosis-affected (1) and control (0) California sea lions.

3.2. Zaca-DRB diversity of leptospirosis-affected and control sea lions

Disease status was significantly influenced by *Zaca-DRB* diversity ($z = 2.81$, $p = 0.0049$), regardless of differences in neutral genetic variation, with affected sea lions tending to have more *Zaca-DRB* loci than those in the control group (Fig. 1).

3.3. Strength of response against infective leptospires

Sea lions with leptospirosis had up to 10 distinct MAT repertoires, of which the most common (42%) was reactions against all of the six serovars tested. The number of agglutination reactions ($\geq$ 1:800 titer) was predicted by *Zaca-DRB* diversity (GLM; Deviance = 5.32, $p = 0.02$), with serum from sea lions with more *Zaca-DRB* loci tending to react to more serovars (Fig. 2). Maximum endpoint titers were inversely predicted by *Zaca-DRB* diversity (df = 72, RDev = 16.62, $p = 0.029$) and by neutral genetic variation (df = 71, RDev = 15.12, $p = 0.022$).

3.4. Disease severity

Zaca-DRB diversity was inversely related to survival of leptospirosis-affected sea lions, with those that survived having fewer *Zaca-DRB* loci (GLM; $z = -1.493$, $p = 0.015$; Fig. 3). Neutral genetic variation did not help explain differences in survival (Full model is shown in Table S4 in Supplementary Material). Leptospirosis-affected sea lions that harboured either *Zaca-DRB.C* or *Zaca-DRB.G* genes were less likely to survive than sea lions that lacked these genes (*Zaca-DRB.C*: OR = 6.3, 95% CI = 1.24–32.05; *Zaca-DRB.G*: OR = 4.154, 95% CI = 1.05–16.50). Odds ratios for all *Zaca-DRB* loci are shown in Table S5 in Supplementary Material.

4. Discussion

Host immune responses to leptospirosis remain mostly unexplored, and even less is known about which factors inherent to the host immune system are responsible for the varying degrees of disease severity. Although the role of cell-mediated immunity to control pathogenic leptospires has been recently explored and found to be important (Schröder et al., 2008), immunity against leptospiral infection has

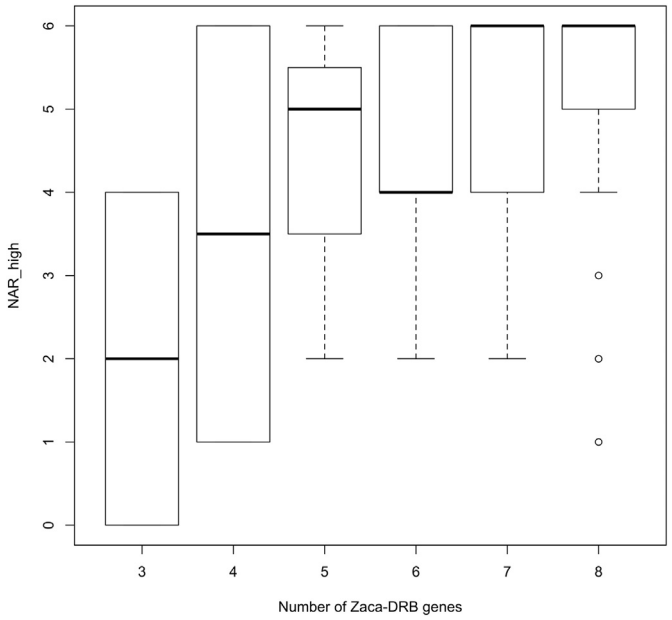

Fig. 2. Relationship between a sea lions number of *Zaca-DRB* loci and the number of positive agglutination reactions ≥ 1:800 (NAR_high) against *Leptospira interrogans* serovars. The bold lines show the median responses, the boxes encompass the quartiles, and the whiskers indicate the endpoint data.

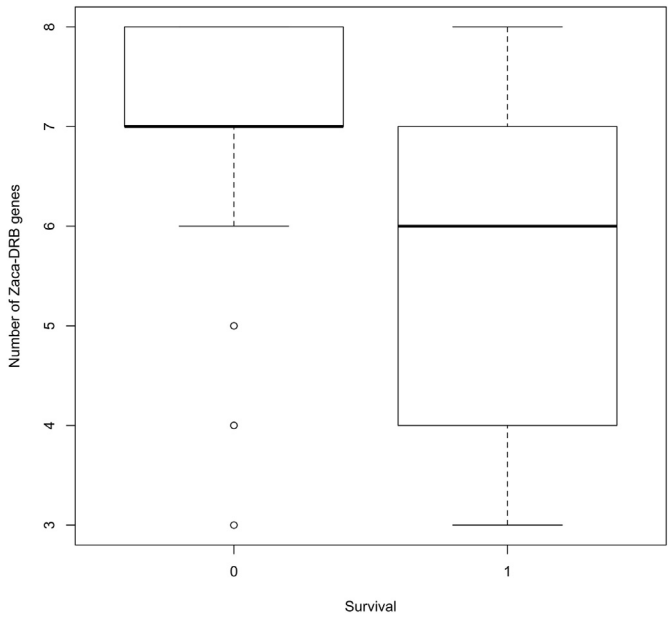

Fig. 3. *Zaca-DRB* diversity varied between leptospirosis outcome groups, with leptospirosis-affected sea lions that died (0) having significantly more *Zaca-DRB* loci than those that survived (1). The bold lines show the median responses, the boxes encompass the quartiles, and the whiskers indicate the endpoint data.

traditionally been considered humoral (Palaniappan et al., 2007). Such responses involve development and maturation of B-lymphocytes as well as the synthesis of immunoglobulins that target leptospiral lipopolysaccharides (Faine, 1994). There is also some evidence that specific MHC alleles confer protection against infective leptospires in humans (Guo et al., 2010; Fialho et al., 2009). In our study, we found evidence that *L. interrogans* infection status and disease severity in the California sea lion are associated with MHC class II DRB (*Zaca-DRB*) diversity (multiplicity) and configuration. However, the relationships observed were unexpected and warrant careful consideration.

In accordance with our prediction that sea lion MHC class II genes would be involved in the recognition of leptospiral antigens, we found that the number of *Zaca-DRB* loci of an individual directly predicted the number of distinct MAT reactions. To date, all *L. interrogans* isolates obtained from wild California sea lions from the California coast that have been characterized molecularly have been identified as serovar Pomona (Zuerner and Alt, 2009). However, comparably high MAT titers against other serovars were recorded for the sea lions in this study, and have been reported previously for sea lions from the same region (Prager et al., 2013). It is possible that although serovar Pomona is endemic to the California sea lion US stock (Lloyd-Smith et al., 2007; Prager et al., 2013), sea lions can become exposed to other serovars that are common in other areas of the species' distribution. For instance, in the Gulf of California, the most common antibodies detected by MAT (1 ≥ 800 endpoint titer) were serovars Autumnalis, Grippotyphosa, Tarassovi, Bataviae, Pyrogenes, and Canicola (Avalos-Téllez et al., 2016), and antibody responses to serovar Pomona are much less common in animals from this geographic region (Acevedo-Whitehouse et al., 2003). Movement of sea lions between colonies from the Gulf of California and the northeastern Pacific could lead to exposure to other infecting serovars while at hauling sites. Even if such hypothetical infectious contacts were sporadic and spaced, high antibody titers could be maintained for at least a year following exposure (Lloyd-Smith et al., 2007). Thus, the result herein observed is taken as indication that the number of antigenic determinants a sea lion can respond to serologically is influenced by its diversity at *Zaca-DRB*.

Previous studies have shown evidence that individuals that are heterozygote at the MHC class II peptide binding region have an advantage when facing one (Kurtz et al., 2004) or various co-infecting pathogens (Penn et al., 2002; McClelland et al., 2003; Oliver et al., 2009). While we did not examine MHC-II allele heterozygosity in the present study, the unique mechanism for generating polymorphism in the California sea lion (Bowen et al., 2004) allowed us to show that having more *Zaca-DRB* loci allows an individual to recognize more antigenic variants of a given pathogen. However, we also found that diseased sea lions had higher *Zaca-DRB* diversity than those in the control group. It is difficult to explain this unexpected finding beyond speculation given how little we know of the individuals in the control group. This is because although we were careful to select only those individuals with no clinical manifestation of leptospirosis (or any other disease) and whose known cause of stranding was trauma, it is possible that this group harboured individuals that were, in fact, experiencing subclinical leptospirosis, or were infected by an unidentified pathogen with no obvious clinical manifestation. However, if this were the case, under the hypothesis that having more *Zaca-DRB* loci allows an individual to recognize more antigens, we would have expected to see no difference in the number of *Zaca-DRB* loci between groups, rather than a significantly lower number of loci in the control group.

If the ‘control’ sea lions were actually subclinical cases of leptospirosis, it is plausible that the apparent ‘advantage’ of having less *Zaca-DRB* loci were due to the avoidance of antibody-mediated pathogenesis rather than to having a lower individual repertoire for antigen-recognition. In other words, while sea lions with more *Zaca-DRB* loci are better at recognizing different leptospiral antigens and induce antibody production, the clinical manifestation of leptospirosis could actually be caused by those immune responses against the infective leptospires. Normally, antibody production leads to clearance of an infection. However, studies conducted on humans have shown that the interaction of leptospires with antigen-recognition receptors is thought to be key to initiate immune responses against the spirochaetes but is also a cause of immunopathology (Fraga et al., 2011). For instance, intense antibody production in response to infection with virulent *L. interrogans* serovars can lead to high levels of inflammatory cytokines known as a ‘cytokine storm’ (Haake and Levett, 2015), that results in severe tissue lesions in kidneys, liver and lungs, leading to the symptoms and signs of clinical leptospirosis (Abdulkader et al., 2002). Leptospirosis-driven immunopathology has not yet been studied in depth for the California

sea lion; however, interstitial infiltration of neutrophils, lymphocytes and antibody-producing cells (plasmatic cells) in the kidneys of infected sea lions has been reported previously (Gulland et al., 1996; Colagross-Schouten et al., 2002). If leptospirosis-associated immunopathology were to occur in California sea lions, having more *Zaca-DRB* loci would increase chances of leptospiral antigen-recognition, leading to antibody production and thus ultimately raising the risk of immunopathology. Having confirmed that the number of reactions with high titers (> 1:800) was higher in sea lions with more *Zaca-DRB* loci while the risk of dying was also significantly higher in leptospirosis-affected sea lions with more *Zaca-DRB* loci seems to add support to this explanation.

Interestingly, when exploring the strength of the immune response following antigen-recognition, we found that the maximum endpoint titer was inversely related to genetic diversity, both in terms of *Zaca-DRB* loci and neutral genetic variation. As very few of the sea lions (n = 3) in our study reacted exclusively to a single serovar, it is possible that this parameter is somewhat inaccurate as a 'measure of strength of the immune response' because it ignores the rest of the responses against other serovars. However, in that case, we would have expected no pattern to be discernable.

Another possibility is that rather than highest *Zaca-DRB* diversity being advantageous for strong immune responses, those sea lions with intermediate numbers of *Zaca-DRB* loci could be the ones that tend to respond more intensely, as would be expected under the optimality hypothesis (Nowak et al., 1992), which poses that selection may disfavour individuals with high MHC diversity and instead favour an 'immunogenetic optimum' (Wegner et al., 2003). This would happen if having an increased capacity for antigen recognition due to high MHC diversity were offset by a decline in T-cell repertoire in order to avoid autoimmune reactions (Relle and Schwarting, 2012). Empirical support for this hypothesis has grown in the past decades (e.g. Wegner et al., 2003; Wegner et al., 2006; Kalbe et al., 2009; Hablützel et al., 2014), and there is evidence that in natural populations, those individuals that have an intermediate number of different MHC class II alleles tend to be the most frequent genotype (Reusch et al., 2001). Interestingly, all of the leptospirosis-affected sea lions that had five *Zaca-DRB* loci (n = 4) had high maximum endpoint titers, whereas some of the sea lions with higher (6 to 8) numbers of *Zaca-DRB* loci had low maximum endpoint titers. However, in addition to the limitation of using the maximum endpoint titer as a measure of strength of the immune response, we have limited statistical power to attempt to explore this possibility in more depth. Further studies should aim to increase sample size to have a proper representation of sea lions with low, intermediate and high numbers of *Zaca-DRB* loci, as being a species that generates diversity by polygeny, the California sea lion is an ideal model in which to test the optimality hypothesis.

Various studies conducted in a wide range of mammals have statistically linked specific MHC class II DRB alleles to parasites, reporting both 'protective' and 'harmful' roles (e.g. Untalan et al., 2007; Schad et al., 2012). Here, we found *Zaca-DRB.B* to be a high-risk gene for leptospirosis. Furthermore, this gene was linked to in vitro serum responses against three of the serovars tested, namely Icterohaemorrhagiae, Grippotyphosa and Canicola. *Zaca-DRB.A* and *Zaca-DRB.D* genes were also linked to responses against serovar Grippotyphosa and Icterohaemorrhagiae, respectively. Although each MHC class II molecule has a high peptide binding specificity (Aluvia and Margalit, 2004), resistance against a given parasite can be conferred by multiple different MHC alleles (Schad et al., 2012; Goüy de Bellocq et al., 2008). Individual serovars might have various antigenic determinants that are detected by more than one MHC peptide. However, without further studies on the MHC-driven immune responses to infective leptospira and associated immunopathologies, it is difficult to speculate about whether the identified *Zaca-DRB* loci are harmful or protective.

To date, most of the studies on MHC diversity and parasites have focused on natural populations and have sampled apparently healthy individuals. We are well aware that diseased sea lions are certainly not representative of the normal population; however, our study demanded such a group in order to test our hypotheses regarding susceptibility to leptospirosis. In this sense, we propose that this is an ideal model system in which to study the importance of MHC genes for pathogen and disease susceptibility, and have provided evidence that host-pathogen relationships are complex and that pathogen-driven selection on immune genes is likely to reflect such complexity. Furthermore, our findings highlight the need for more studies on the immune responses to specific pathogens in order to understand the role that host genetics have on shaping susceptibility to infectious disease in natural populations.

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Data accessibility

The data used for this study has been stored in Dryad. http://dx.doi.org/10.5061/dryad.7mc93.

Competing interests

The authors declare no competing interests.

Author contributions

KAW and FG conceived and designed the study; KAW performed all microsatellite and MHC genotyping and drafted the manuscript; LB assisted with statistical analyses and interpretation of the data; FG collected and curated the samples. All authors read and discussed the manuscript and gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2017.11.023.

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