

Ecology can inform genetics: Disassortative mating contributes to MHC polymorphism in Leach's storm-petrels (*Oceanodroma leucorhoa*)

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Abstract

Studies of MHC-based mate choice in wild populations often test hypotheses on species exhibiting female choice and male–male competition, which reflects the general prevalence of females as the choosy sex in natural systems. Here, we examined mutual mate-choice patterns in a small burrow-nesting seabird, the Leach's storm-petrel (*Oceanodroma leucorhoa*), using the major histocompatibility complex (MHC). The life history and ecology of this species are extreme: both partners work together to fledge a single chick during the breeding season, a task that requires regularly travelling hundreds of kilometres to and from foraging grounds over a 6- to 8-week provisioning period. Using a 5-year data set unprecedented for this species ($n = 1078$ adults and 925 chicks), we found a positive relationship between variation in the likelihood of female reproductive success and heterozygosity at Ocle-DAB2, a MHC class IIB locus. Contrary to previous reports rejecting disassortative mating as a mechanism for maintaining genetic polymorphism in this species, here we show that males make significant disassortative mate-choice decisions. Variability in female reproductive success suggests that the most common homozygous females (Ocle-DAB2*01/Ocle-DAB2*01) may be physiologically disadvantaged and, therefore, less preferred as lifelong partners for choosy males. The results from this study support the role of mate choice in maintaining high levels of MHC variability in a wild seabird species and highlight the need to incorporate a broader ecological framework and sufficient sample sizes into studies of MHC-based mating patterns in wild populations in general.

KEY WORDS

heterozygosity, major histocompatibility complex, mate choice, seabirds, sexual selection

1 | INTRODUCTION

Genes of the major histocompatibility complex (MHC) provide a compelling molecular foundation for why individuals prefer some mates over others. The MHC is highly polymorphic, with thousands of alleles at multiple loci reported in some species (Parham & Ohta, 1996; Robinson, Malik, Parham, Bodmer, & Marsh, 2000; Vogel et al., 1999).

In birds, the evidence of extreme MHC polymorphism in natural populations has recently been documented in both passerines and non-passerines, illustrating the ecological applicability of avian study systems for MHC studies (Alcaide, Edwards, Negro, Serrano, & Tella, 2008; Alcaide, Munoz, Martínez-de la Puente, Soriguer, & Figuerola, 2014; Biedrzycka et al., 2017). At the population level, variability in the MHC is associated with an adaptive immune response to

pathogens (Westerdahl, Stjernman, Råberg, Lannefors, & Nilsson, 2013) suggesting that pathogen-mediated selection contributes to maintaining this polymorphism (Doherty & Zinkernagel, 1975; reviewed further in Hedrick, 2002 and Spurgin & Richardson, 2010). Sexual selection may also influence MHC polymorphism, because individuals may be adapted to select mates with MHC genotypes that will infer genetic advantages to offspring, including increased heterozygosity or the inheritance of good genes (Edwards & Hedrick, 1998; Eizaguirre, Yeates, Lenz, Kalbe, & Milinski, 2009; Milinski, 2006).

MHC-based mate-choice studies were initially pioneered in laboratory or semi-natural settings in part to control for variability that might obscure potential effects (Jordan & Bruford, 1998; Potts, Manning, & Wakeland, 1991; Reusch, Häberli, Aeschlimann, & Milinski, 2001; Wedekind, Seebeck, Bettens, & Paepke, 1995; Yamazaki et al., 1976). As genetic methods have become more accessible, investigations have been extended to wild populations to attempt to explain how MHC diversity is maintained in nature under various selective pressures. Results so far from natural populations have produced no clear patterns in that both disassortative and assortative MHC-based mating patterns have been reported, and many studies report no significant mating patterns at all (reviewed by Hoover & Nevitt, 2016). It is possible that the range of conclusions generated by these studies does not reflect true differences in MHC-based mate-choice patterns across species, due, in part, to sample size constraints inherent to field data collection (Kamiya, O'Dwyer, Westerdahl, Senior, & Nakagawa, 2014). Reporting on results of a recent meta-analysis, Kamiya et al. point out that, to produce accurate results, studies should be designed such that sample sizes are sufficient to capture the genetic variation of MHC genes in the overall population. The logic behind this argument is that an insufficient sample size limits statistical power, which, in turn, increases the likelihood of error in interpreting mate-choice patterns. Therefore, results should be interpreted in this context before drawing broad conclusions about how MHC diversity is maintained in wild populations. The high levels of polymorphism of MHC haplotypes found in outbred populations can also make detecting associations with phenotypic traits or mating preferences challenging (Jin, Speed, & Thomson, 1995).

Furthermore, with few exceptions, studies of MHC-based mate choice in wild populations often test hypotheses on species exhibiting female choice and male–male competition, which reflects the general prevalence of females as the choosy sex in natural systems. However, there are conditions where males are also predicted to be choosy. For example, male choice could be expected if males contribute equally or more than females to rearing offspring, which is common in many burrow-nesting seabird species (Gladbach, Braun, Nordt, Peter, & Quillfeldt, 2009; Gray & Hamer, 2001; Peck & Congdon, 2006). Male choice also could be predicted in systems where female quality varies substantially, even if males do not care for young (Gwynne, 1991). These circumstances have most commonly been reported in insect systems (for review see Bonduriansky, 2001; see also Gwynne, 1981; Gwynne & Simmons, 1990; Simmons, 1992), but are not typically considered in MHC-based mate-choice studies in vertebrates. If the aim is to determine whether mate-

choice patterns contribute to maintaining MHC genetic variability, then investigations of species where male investment is high or female quality is variable should incorporate the potential for male as well as female choice.

In this context, tube-nosed seabirds (Order Procellariiformes) are useful models for investigating MHC-based mate choice as a mechanism for maintaining MHC polymorphism in a wild population (Dearborn et al., 2016; Strandh et al., 2012). Procellariiformes are highly pelagic seabirds that forage over vast expanses of open ocean (Shaffer et al., 2006; Weimerskirch, Salamolard, Sarrazin, & Jouventin, 1993). They tend to associate with nesting colonies for only a few months each year or every other year to rear a single, slow-growing chick. Procellariiformes are generally long-lived and genetically monogamous birds (Warham, 1990), and the selection of a high-quality mate is a critical choice to their lifelong reproductive success. They also have a highly developed sense of smell and some burrow-nesting species have been shown to discriminate personal odours, including the scent of their partner (Bonadonna & Nevitt, 2004; Bonadonna & Sanz-Aguilar, 2012; De León, Minguez, & Belluire, 2003; Nevitt, 2008). The molecular mechanisms underlying this ability are unknown, but MHC genes could contribute to this ability by encoding individual-specific information (Singh 2001; Penn, 2002; Milinski et al., 2005; O'Dwyer & Nevitt, 2009; Overath, Sturm, & Rammensee, 2017; Leclaire, Strandh, Mardon, Westerdahl, & Bonadonna, 2017; but see Hurst & Benyon 2004 and Hurst, Thom, Neivison, Humphries, & Benyon, 2005).

Among Procellariiformes, the Leach's storm-petrel (*Oceanodroma leucorhoa*) is especially tractable for this type of study. Leach's storm-petrels are small, sexually monomorphic, burrow-nesting birds, usually nesting in colonies on offshore islands off the Pacific and Atlantic coasts of North America where they are accessible for long-term study in semi-remote habitat. Because Leach's storm-petrels enter and exit the breeding colonies at night, investigators can easily monitor marked burrows for occupancy during daylight hours with minimal disturbance, over multiple years. They represent an extreme ecological and life history outlier among birds in that the incubation and provisioning periods last 90–100 days (Personal observation at Bon Portage Island; see also Huntington, Butler, & Mauck, 1996 and Wilbur, 1969), and chicks hatch and develop underground. Once individuals form mated pairs, they are philopatric to the nesting burrow; this is the real estate to which they return yearly to raise a chick. In many petrel species, males arrive earlier than the females at the breeding colony, to modify their burrow, as well as defend it from other prospective breeders (Warham, 1990). Following copulation, the female returns to sea to acquire the energy necessary to produce a single egg that is 22% of her body weight (Montevecchi, Kirkham, Roby, & Brink, 1983; Warham, 1990). During this prelaying exodus, the male remains at or nearby the colony to continue defending the burrow (Warham, 1990). Thus, the egg, the burrow and the parents' ability to successfully coordinate provisioning shifts constitute a significant shared investment by both parents.

The convenience of this system allowed us to reinvestigate MHC-based mating preferences using a large sample size of

established breeding pairs informed by an ecological context necessary to study a wild population. Here, we evaluate a 5-year data set comprised of a total of 1078 genotyped adults and 925 genotyped chicks, including 338 established breeding pairs, to test the hypothesis that Leach's storm-petrels mate nonrandomly, based on MHC dissimilarity. Upon demonstrating a significant variation in female reproductive success, we investigated this hypothesis using both nonbreeding and breeding adults.

2 | MATERIALS AND METHODS

2.1 | Study site and field methods

We monitored Leach's storm-petrel colonies on Bon Portage Island, Nova Scotia, Canada ($43^{\circ}26'N$, $65^{\circ}45'W$), from 2010 to 2015. Bon Portage is an easily accessible, near-shore island that houses an estimated 50,000 breeding pairs of storm-petrels (Oxley, 1999). During the July–September incubation and chick-provisioning periods, we surveyed approximately 550 burrows distributed across three distinct colony sites (Figure 1). We identified and banded breeding pairs of adults and recorded morphometrics including mass, flattened wing chord and tarsus length. To characterize MHC genotypes and confirm paternity, we collected $\sim 75 \mu\text{l}$ of blood from all adults and chicks. In our study population, the majority of chick mortality

events occur in the first week after hatching; therefore, we waited until chicks were at least 2 weeks old before collecting a blood sample. Blood was collected via brachial venipuncture and stored in 0.5 ml of Queen's lysis buffer at room temperature while in the field. All sampling was conducted in adherence to guidelines defined by the University of California, Davis Institutional Animal Care and Use Committee Protocol #19288.

2.2 | Genetic characterization

For this investigation, we focused on the antigen-binding domain of the MHC class II molecule, which is also referred to as the MHC class IIB subunit. This hypervariable β subunit contains the vast majority of sites under selection in response to pathogen selective pressures. Studies of other avian models suggest concerted evolution and selection at this region (Edwards, Wakeland, & Potts, 1995; Hess & Edwards, 2002; Sommer, 2005; Wittzell, Bernot, Auffray, & Zoorob, 1999). Scent-based mate preferences have also been historically linked to variation in this region (Aeschlimann, Häberli, Reusch, Boehm, & Milinski, 2003; Eggert, Müller-ruchholtz, & Ferstl, 1998; Milinski et al., 2005; Reusch et al., 2001; Yamazaki, Singer, Curran, & Beauchamp, 1999). We sequenced genomic fragments extending from exon 1 to two-thirds of exon 2 in two paralogous MHC class IIB genes. We refer to these fragments as Ocle-DAB1 and

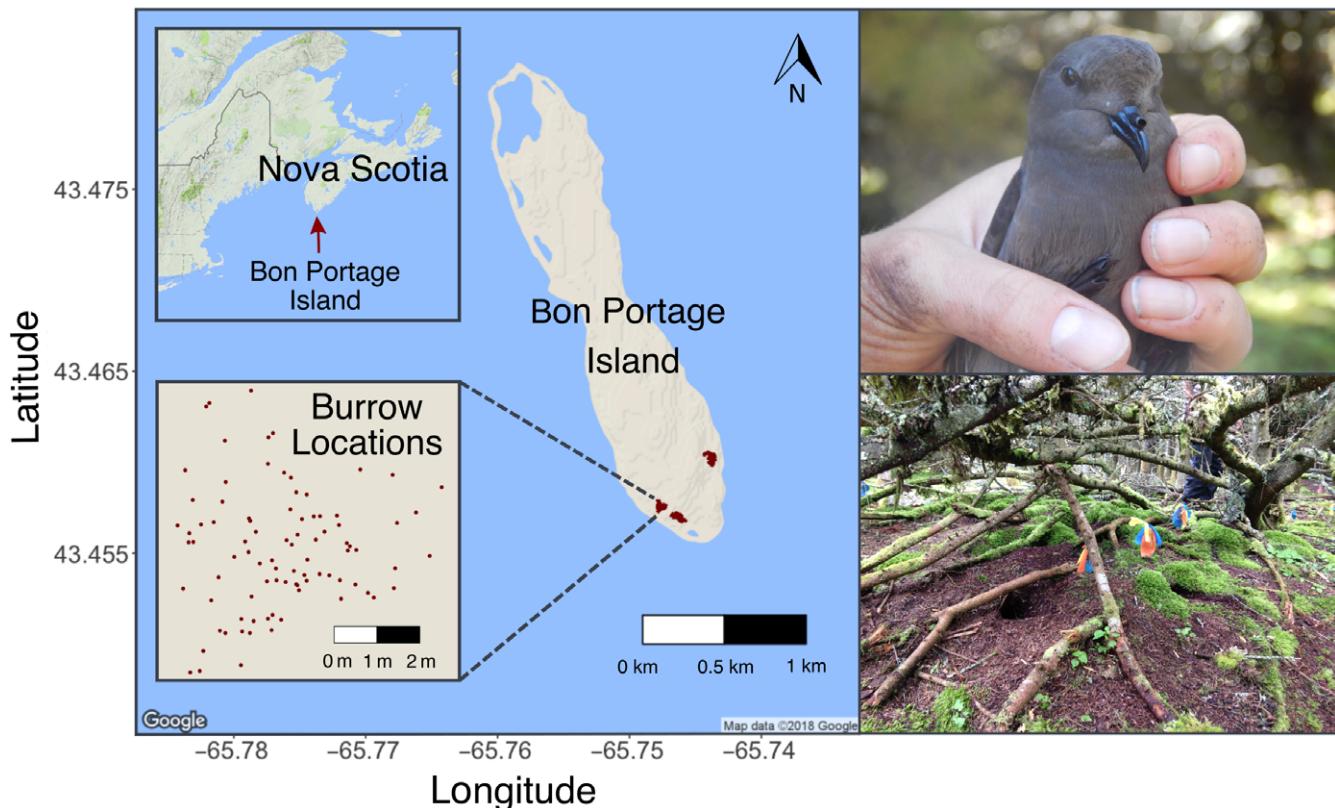


FIGURE 1 Leach's storm-petrel (*Oceanodroma leucomelas*) colonies on Bon Portage Island, Nova Scotia, Canada. Burrows at Bon Portage Island are characterized by heavy tree cover, primarily Balsam Fir (*Amygdalus balsamea*), pine (*Pinus spp.*) and alder trees (*Alnus spp.*). Approximately 550 occupied storm-petrel burrows were monitored at three colony sites between 2010 and 2015. Individual burrows at Site 1 are depicted in the inset

Ocle-DAB2 (see Supporting Information for complete characterization details).

2.3 | Sequencing and genotyping

MHC genotyping and molecular sexing were conducted at Harvard University and the University of California, Davis. To determine the sex of individual birds, we used PCR-based protocols (Griffiths, Double, Orr, & Dawson, 1998; primer pair P2 and P8; Fridolfsson & Ellegren, 1999; primer pair 2550F and 2718R) to amplify fragments of the chromo-helicase-DNA (CHD) gene in avian sex chromosomes. All PCR products were checked using 2% agarose gels (TAE: 400 mM Tris, 0.01M EDTA, pH 8.3) and stained with ethidium bromide or SYBR-Safe (Invitrogen) DNA gel stain.

MHC class IIB genes in the Leach's storm-petrel have previously been characterized by Dearborn et al. (2015), who sequenced exon 2 at two reported loci. We confirmed these two MHC class IIB loci, using locus-specific primers developed from our characterization of the two MHC class IIB genomic fragments, conducted prior to and independent of their published study (Dearborn et al., 2015; see Supporting Information for description of characterization and PCR protocol). We amplified 300-bp gene fragments at two loci (Ocle-DAB1 and Ocle-DAB2) and resolved the sequence of each fragment using BigDye 3.1 sequencing kit and ABI3130xl/ABI3730 automated sequencers (Harvard University; University of California Davis Gene Sequencing Center). Sequence chromatograms were aligned using BioEdit sequence alignment editor (Hall, 1999).

2.4 | Genetic analysis

Statistical analyses and selection tests were conducted on a subset of Leach's storm-petrel chicks ($n = 150$) sampled between 2009 and 2010. Heterozygous sites within direct sequencing chromatograms were determined using BioEdit. Ambiguous diploid genotypes were resolved into individual alleles using the PHASE module implemented in the software DNAsp version 5.0 (Librado & Rozas, 2009). Computational inferences of the gametic phase were carried out according to 1,000 iterations, 10 thinning intervals and 1,000 burn-in iterations. The software DNAsp was also used to calculate basic polymorphism statistics at each locus, including the overall number of segregating (variable) sites, nucleotide diversity (π), Watterson's estimate of the population mutation rate (θ_W) and the average number of nucleotide differences among alleles (k). The frequency distribution spectrum of variable sites was analysed through a Tajima's D test (Tajima, 1989) based on a window size of 25 nucleotides and a step size of 5 nucleotides, also run in DNAsp. Conformity to Hardy-Weinberg expectations at each locus was tested in GENALEX version 6.1 (Peakall & Smouse, 2006). Tests of linkage disequilibrium among loci were performed in the software GENETIX version 4.05 (Belkhir, Borsig, Chikhi, Raufaste, & Bonhomme, 1996).

Under conditions of positive selection, MHC genes typically exhibit high levels of genetic polymorphism and an excess of nonsynonymous to synonymous nucleotide substitutions, ($\omega: d_n/d_s > 1$). We

used the HYPHY package (Pond & Muse, 2005) implemented in the web server www.datamonkey.org to test for evidence of positive selection across our alignment of exon 2 sequences retrieved from each MHC class IIB gene duplicate. We used the random effects likelihood (REL) method to investigate site-by-site evidence of positive selection (see Kosakovsky Pond, Posada, Gravenor, Woelk, & Frost, 2006). This method, considered a generalization of the PAML method described by Yang (2007), is believed to be the most suitable approach for detection of positive selection from a low number of sequences. However, the REL method is also one of the most prone to report false positives. Because recombination can overestimate positive selection (Anisimova, Nielsen, & Yang, 2003), we ran the GARD module package (Kosakovsky Pond et al., 2006)—also implemented in the HYPHY package—to test for significant evidence of recombination breakpoints. Exon 2 coding sequences were subdivided into different partitions accordingly. Positive selection inferences were carried out with the HKY85 nucleotide substitution model, suitable for dealing with alignments comprised of a low number of sequences. In addition, we compared nonsynonymous substitution rates with synonymous substitution rates for those codons presumably comprising and not comprising the PBR. Calculations were carried out in the software MEGA 4.0 (Kumar, Nei, Dudley, & Tamura, 2008) using the modified Nei-Gojobori method with the Jukes-Cantor correction.

2.5 | Analytical methods

We defined an established pair as a breeding pair that we observed together during more than one year or as a pair that successfully hatched a chick. We matched the MHC genotypes of breeding adults with their chicks according to Mendelian inheritance pattern to ensure accurate assignment of mate partners and account for any effects of extra-pair parentage (See Supporting Information for further detail). Using these criteria, we identified and genotyped 1078 adults and 925 chicks. Of these totals, we identified and validated 338 unique established pairs. The remaining 402 adults did not meet our breeding criteria.

We next examined fitness trends with respect to MHC class IIB genes. The most direct measure of reproductive success is the ability to successfully hatch a chick. We assessed separately the probability of male and female reproductive success as a function of the individual amino acid substitutions between alleles at both the Ocle-DAB1 and Ocle-DAB2 loci, respectively. Amino acid substitutions and hatching success of all completely genotyped adult males and females in the population ($n = 1078$) were fit to generalized linear models with a binomial error distribution and a logit link function. Each model evaluated the probability of a male or female successfully hatching a chick as a function of their amino acid substitutions at either Ocle-DAB1 or Ocle-DAB2.

We used four established metrics to test for both disassortative and assortative MHC-based mating preferences: (a) heterozygosity differences, (b) MHC band-sharing coefficients (Wetton, Carter, Parakin, & Walters, 1987), (c) pairwise mean amino acid substitutions and

(d) pairwise maximum amino acid substitutions. Heterozygosity differences were calculated as the difference in overall heterozygosity between a male and a female within a pair. This metric assesses disassortative mating only, with an observed mean located outside the confidence intervals suggesting that either males or females are significantly more heterozygous than the other sex. MHC band-sharing (or “allele sharing”) coefficients were calculated as per Wetton et al. (1987) for both Ocle-DAB1 and Ocle-DAB2 loci. The two amino acid metrics were calculated from the number of amino acid substitutions between the partners’ alleles, assessing the mean difference in both loci in the first metric and the maximum value found at either locus in the second metric. In either case, the prediction for disassortative mating is that an individual should select a mate with alleles that maximize amino acid differences in offspring (Landry, Garant, Duchesne, & Bernatchez, 2001). The calculations used in assessing these metrics are further described in the supplemental information.

We then investigated what might be driving the disassortative mating effect observed in the heterozygosity difference metric, defined above as the difference in heterozygosity within a pair. We used our most common genotype (Ocle-DAB2*01/Ocle-DAB2*01), which we knew was associated with a decrease in reproductive success in females. To ensure that individuals were not selecting mates on the basis of inbreeding avoidance, we used ddRAD sequences to investigate whether individuals with this common genotype were more closely related to each other than to individuals with nonhomozygous Ocle-DAB2*01 genotypes (for sequencing methodology, see: Sin, Hoover, Nevitt, & Edwards, in preparation). We then tested whether Ocle-DAB2*01/Ocle-DAB2*01 males associate with Ocle-DAB2*01/Ocle-DAB2*01 females more or less frequently than would be expected through random mate choice, given the number of Ocle-DAB2*01/Ocle-DAB2*01 females present in the entire genotyped population ($n = 199$). At last, we investigated whether any observed effects were maintained under female choice conditions, by testing whether Ocle-DAB2*01/Ocle-DAB2*01 females associate with Ocle-DAB2*01/Ocle-DAB2*01 males more or less frequently than would be expected under random mate choice.

In addition to the Ocle-DAB2*01/Ocle-DAB2*01 categorization described above, we classified the remaining individuals in the population as retaining one Ocle-DAB2*01 allele (Ocle-DAB2*01/Ocle-DAB2*X), homozygous with no Ocle-DAB2*01 alleles (Ocle-DAB2*X/Ocle-DAB2*X Homozygotes) or heterozygous with no Ocle-DAB2*01 alleles (Ocle-DAB2*X/Ocle-DAB2*X Heterozygotes). We then used these categories to test for genotypic effects on potential proxies of fitness, including hatch date, tarsus length, wing chord and body condition index (BCI, calculated using residuals of mass regressed on tarsus; O’Dwyer, Ackerman, & Nevitt, 2008). To examine the influence of genetic quality on potential proxies of reproductive fitness, we applied linear models (type II ANOVAs) using the *lm* function in the base R package. For proxies of reproductive fitness, we used hatch date and morphometric measurements as normally distributed dependent variables and included the categorical factors of genotype and year as fixed effects. In our population, female Leach’s storm-petrels have significantly longer wing chords

than males, similar to female-biased sexual dimorphism patterns reported in Ainley (1980) and Crossin (1974). Therefore, we examined separately the effects of genotype and year on each sex.

During the course of the study, reproductive success varied widely across years, likely due to annual differences in offshore plankton productivity and sea surface temperature. To examine whether such good vs. bad years influenced the reproductive success of parents with differing genotypes, we used a two-way chi-square contingency table to test the relationship between the genotypes of successfully breeding females and year.

2.6 | Mate-choice analyses

To determine whether observed mate choice was significantly different from random, we examined the results of Monte Carlo randomization tests (Hall, 1999) using each of the four metrics of genetic dissimilarity and assessed both male and female choice models. To account for any concerns regarding multiple comparisons across these tests, we applied Bonferroni corrections ($n = 12$; p -threshold = 0.004). We also assessed the likelihood of error in the Monte Carlo randomization tests by constructing simulation curves to illustrate the effect of sample size on the consistency of obtaining a given result (for further explanation, see Hoover & Nevitt, 2016). We modelled this output with a logistic regression to show the relationship between sample size and the likelihood of finding a significant result.

We investigated male choice models by pairing known breeding males with randomly assigned females from the genotyped candidate female pool ($n = 338$ identified breeding males and females; candidate replacement males = 561; candidate replacement females = 517). As storm-petrel pairs appear to be largely genetically monogamous (Sin, Hoover, Nevitt, & Edwards in preparation), we resampled candidate males from the population without replacement. We performed 10,000 iterations of this randomization and compared the distribution of our four similarity metrics with the observed values for actual breeding pairs. Statistical significance was determined by comparing the observed mean of breeding pairs with the 2.5% and 97.5% tails of the distribution of the means of the 10,000 simulated pairings. All statistical analyses were performed in R 3.4.3 (R Core Development Team 2017).

3 | RESULTS

3.1 | Genetic characterization

Polymorphism statistics at each MHC class IIB gene are summarized in Table 1. Both the Ocle-DAB1 and Ocle-DAB2 loci showed a skewed distribution of allele frequencies that did not differ between adult males and females (Figure 2). At each of the two loci, there was one dominant allele (Ocle-DAB1*02 and Ocle-DAB2*01, respectively). While the Ocle-DAB1 gene conformed to Hardy–Weinberg expectations ($H_0 = 0.75$, $H_e = 0.74$), there were consistent heterozygosity deficits at the Ocle-DAB2 gene ($H_0 = 0.53$, $H_e = 0.66$; $p < 0.001$). We also found significant support for linkage

TABLE 1 Polymorphism statistics at two MHC class IIB genes in the Leach's storm-petrel. This table indicates the overall number of haplotypes found at the nucleotide level (h) and how many of these haplotypes translate into different amino acid sequences (aa seqs). We also show the overall number of segregating alleles (variable sites) (S), the total number of mutations (Eta), the nucleotide diversity estimator (π), the Watterson's estimator of the population mutation rate (Θ_W) and the average number of nucleotide differences among alleles (k)

Locus	h (aa seqs)	S (Eta)	π	Θ_W	k
Ocle-DAB1	10 (9)	34 (39)	0.05	0.04	14.56
Ocle-DAB2	15 (15)	38 (43)	0.06	0.04	16.87

disequilibrium between the two loci ($p < 0.001$), consistent with the two genes being closely linked.

Bayesian analysis of site-by-site positive selection revealed 11 positively selected and two negatively selected codons at the Ocle-DAB1 locus and 12 positively selected and four negatively selected codons at the Ocle-DAB2 locus (all Bayes factors >100 , Figure 3). We also found a significant excess of nonsynonymous substitutions ($d_n = 0.19 \pm 0.07$ for the Ocle-DAB1 locus; $d_n = 0.20 \pm 0.06$ for the Ocle-DAB2 locus) over synonymous substitutions ($d_s = 0.03 \pm 0.01$ for the Ocle-DAB1 locus; $d_s = 0.09 \pm 0.05$ for the DAB2 locus) for those codons presumably comprising the PBR (Z-test, $p = 0.01$). Differences were not statistically significant when comparing those codons believed not to comprise the PBR ($d_n = 0.03 \pm 0.01$ and $d_s = 0.02 \pm 0.01$ for the Ocle-DAB1 locus; $d_n = 0.04 \pm 0.01$ and $d_s = 0.04 \pm 0.02$ for the Ocle-DAB2 locus; Z-test, $p = 0.65$). At last, Tajima's D analyses revealed several stretches of exon 2 with a significant excess of high-frequency segregating sites (see Supporting Information). These results

support the action of balancing selection upon the two MHC class IIB genes investigated.

3.2 | Sex differences in quality and disassortative mating

Because nonrandom mating is a factor that can perturb Hardy-Weinberg equilibrium, we next examined mating patterns with respect to MHC class IIB. We first examined the relationship between MHC class IIB variability, as defined by the number of within-individual amino acid substitutions and the probability of reproductive success in each sex. In our study, the most direct measure of reproductive success is the ability to produce a chick. We observed a significant positive relationship between female amino acid variability and the probability of hatching a chick when we analysed the Ocle-DAB2 locus alone (Figure 4a; GLM with binomial error distribution and logit link function: slope coefficient = 0.05; $p = 0.04$). We found this relationship only in females, suggesting that increasing amino acid variability at Ocle-DAB2 correlates with female, but not male, reproductive success (Figure 4b; slope coefficient = -0.004 ; $p = 0.72$). Moreover, when limiting homozygotes to the most common Ocle-DAB2 genotype (Ocle-DAB2*01/Ocle-DAB2*01), we documented a slightly stronger correlation with female reproductive success (Supporting information Figures S3 and S4). However, we found no such correlation when we considered Ocle-DAB1 alone, or when both loci were pooled. Together, these results suggest to us that female quality, that is her ability to hatch a chick, correlates with increased heterozygosity at Ocle-DAB2. As the choicer sex tends to discriminate based on variation in mate quality (Jennions & Petrie, 1997; Parker, 1983; Real, 1991), this result led us to speculate that males may indeed be the choicer sex. We

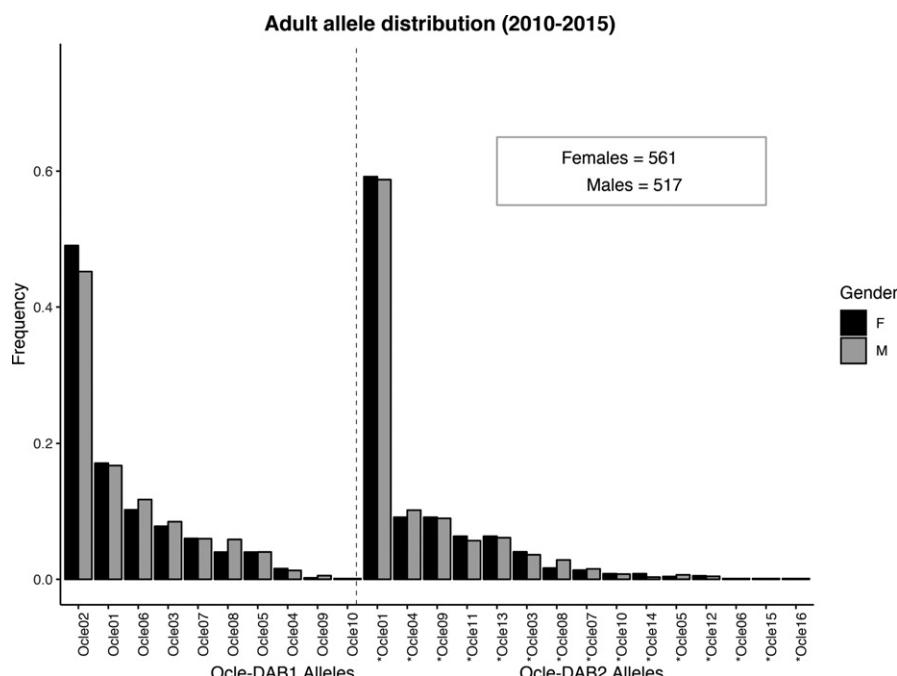


FIGURE 2 Distribution of allele frequencies at two MHC class IIB loci (Ocle-DAB1 and Ocle-DAB2; exon 2 alleles) in a Leach's storm-petrel colony on Bon Portage Island, Canada. Allele distributions were calculated from approximately 1,078 adult petrels sampled between 2010 and 2015, including breeding and nonbreeding birds. Alleles from each locus are ranked by frequency of occurrence across genotyped females

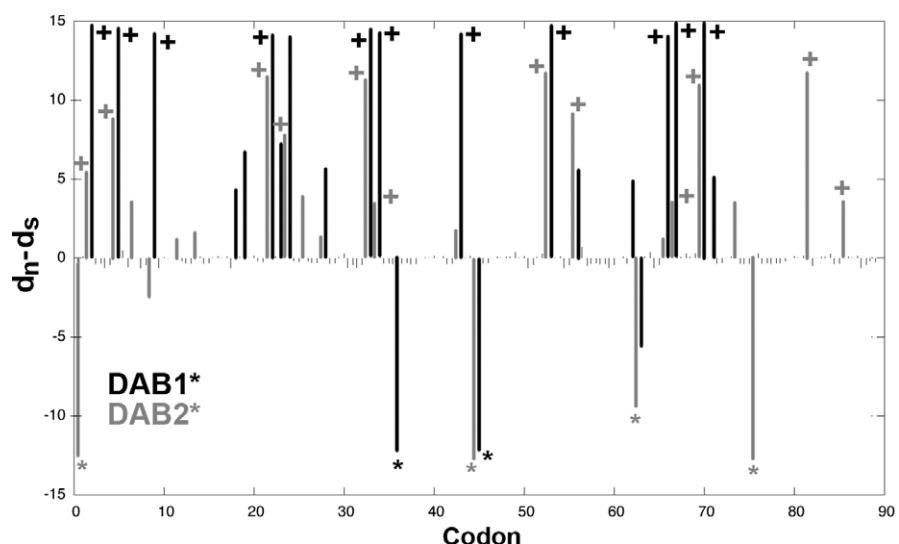


FIGURE 3 Bayesian analysis of site-by-site positive selection across an alignment of exon 2 sequences from two MHC class IIB gene duplicates in the Leach's storm-petrels. Those codons displaying statistically significant evidence of positive selection (Bayes factors > 100) are indicated by crosses. Sites showing decisive evidence of negative selection are indicated by asterisks

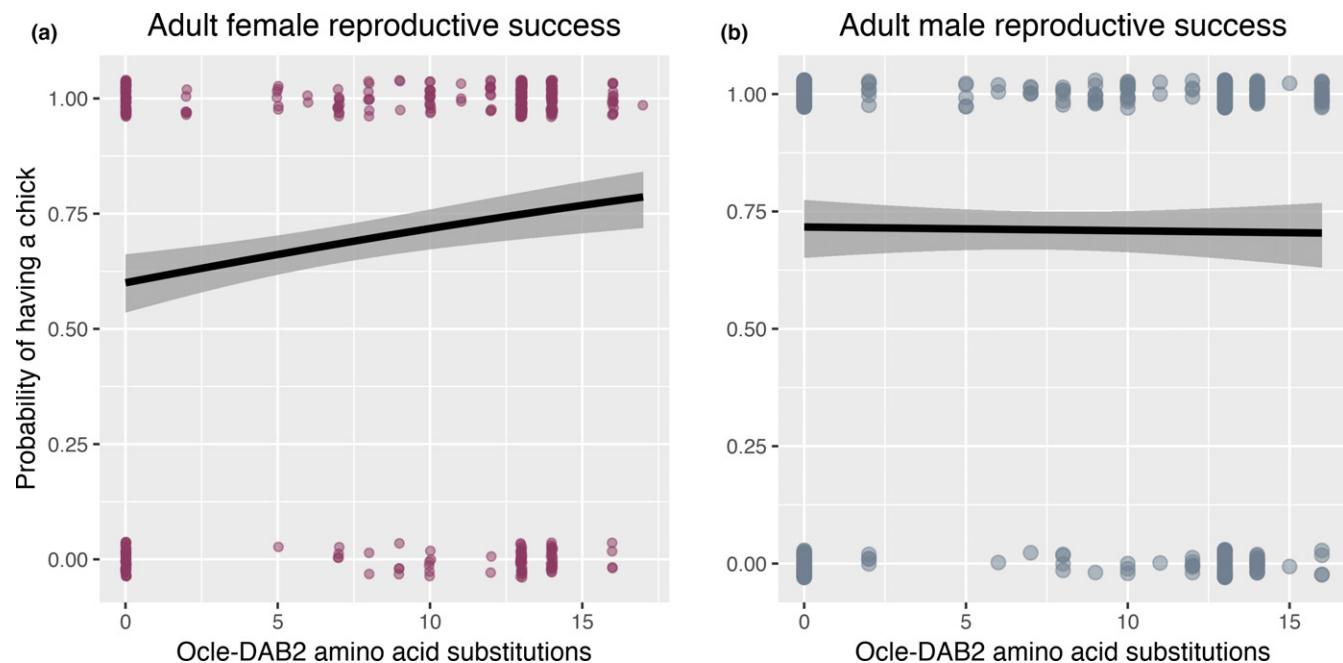


FIGURE 4 (a & b) The probability of male and female reproductive success as a function of individual amino acid differences between alleles at the DAB2 locus. Amino acid heterozygosity and hatching success of genotyped and verified adult males and females in the population ($n = 1078$) were respectively fit to a generalized linear model with a binomial error distribution and a logit link function. The models evaluate the probability of a male or female successfully hatching a chick as a function of their amino acid differences at Ocle-DAB2. For example, homozygous individuals at Ocle-DAB2 exhibit 0 amino acid differences, while the greatest difference between alleles is 16 substitutions found between Ocle-DAB2*06 and Ocle-DAB2*13. Females show a significant effect of amino acid differences on the probability of reproductive success (slope coefficient = 0.05; $p < 0.01$), whereas males exhibited no change in reproductive success relative to amino acid difference (slope coefficient = -0.004; $p = 0.72$)

therefore tested the hypotheses of nonrandom mating from the perspective of both male choice and female choice.

We tested for nonrandom (disassortative or assortative) mating patterns in our study population, using Monte Carlo simulations to represent the results of random mating. We used four established metrics of pairwise genetic similarity (see methods for calculations): (a) total heterozygosity; (b) MHC band-sharing coefficients

(Wetton et al., 1987); (c) pairwise mean amino acid substitutions; and (d) pairwise maximum amino acid substitutions (e.g., Forsberg, Dannewitz, Petersson, & Grahn, 2007). Each of these tests assumes that one sex has the potential to mate with any adult of the opposite sex in the population. When testing under female choice conditions, the results showed that females chose mates randomly with respect to MHC class IIB, irrespective of the metric

used, and these results were supported by corresponding error likelihood simulations (see Supporting information Figures S9–S13). When testing under male choice conditions, our results show that males chose females with greater MHC heterozygosity than would be expected under conditions of random mate choice ($p < 0.004$; Figure 5a). Our corresponding error likelihood simulation estimates a 92% likelihood of obtaining this result at this sample size (Figure 5b).

We next investigated whether this result might be driven by an aversion to a particular homozygous genotype in our population. We focused our investigation on 121 males with the most common homozygous genotype at DAB2, Ocle-DAB2*01/Ocle-DAB2*01, and examined their mating preferences using Monte Carlo simulations. We found that homozygous Ocle-DAB2*01/Ocle-DAB2*01 males were significantly less likely to pair with females of the same genotype ($p = 0.02$; Figure 6a). Our corresponding error simulations estimated a 76% likelihood of achieving these results using our sample size. By contrast, Ocle-DAB2*01/Ocle-DAB2*01 females did not

avoid Ocle-DAB2*01/Ocle-DAB2*01 males; their mating preferences did not deviate from random expectations ($p = 0.31$; Figure 6c). These defined Ocle-DAB2 categories did not differ significantly in relatedness, confirming these documented effects were not associated with inbreeding avoidance among closely related kin (Supporting information Figure S8). These results again suggest that the overall disassortative effect is linked to variability in female quality.

Wing chord length in adult female Leach's storm-petrels differed significantly based on Ocle-DAB2 genotype ($F_{3,566} = 4.07$, $p = 0.01$), with post hoc analysis indicating that the wing chords of Ocle-DAB2*01/Ocle-DAB2*01 averaged 1.3 mm shorter than the wing chords of Ocle-DAB2*01/Ocle-DAB2*X females (see Supporting information). No other morphometric variables, for females or males, were found to differ with genotype. However, there was a significant effect of year on the wing chord measurements of both males and females (Females: $F_{5,566} = 30.67$, $p < 0.001$; Males: $F_{5,497} = 23.21$, $p < 0.001$) with individuals recorded during the first 2 years of the study (2010–11) exhibiting

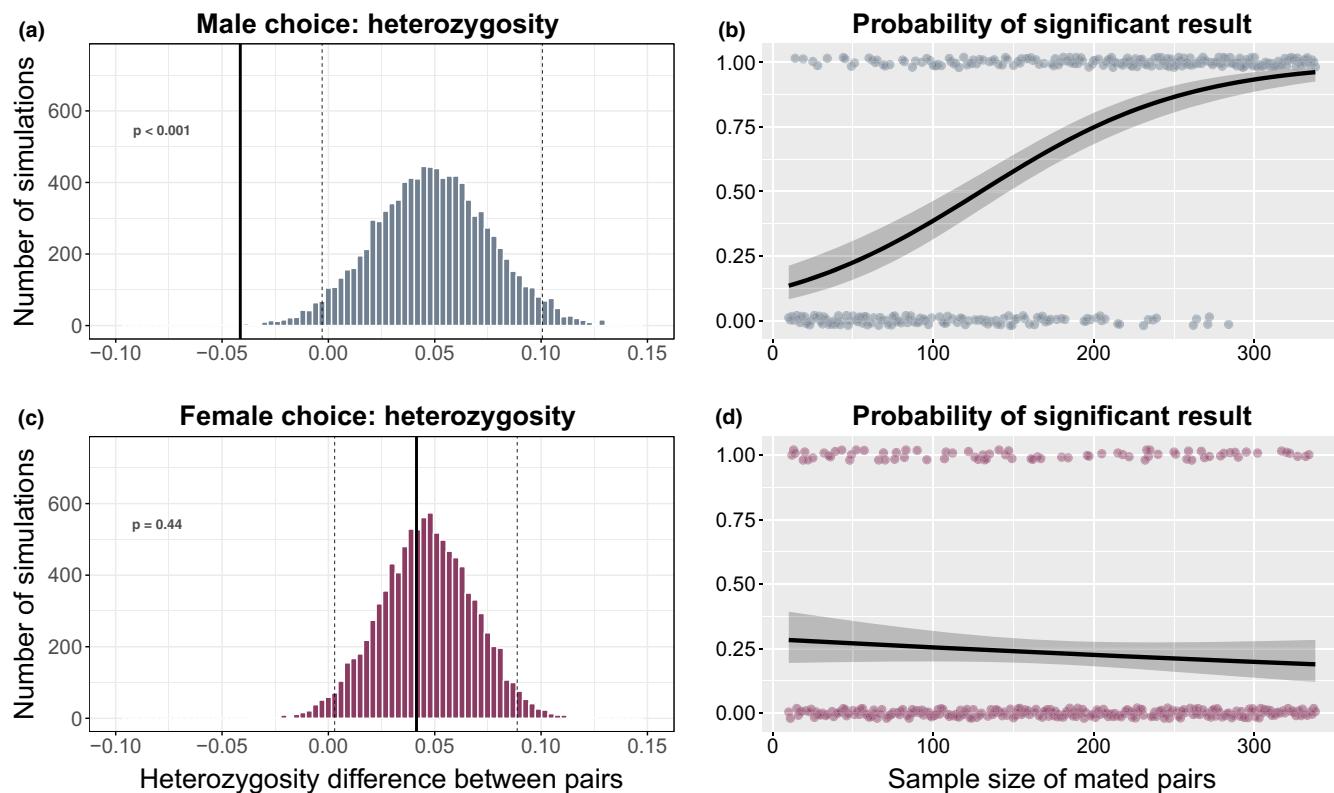


FIGURE 5 (a–d) Monte Carlo randomization tests for the difference between male and female global heterozygosity and corresponding likelihood of a type II error. If the observed mean falls outside the left-hand confidence interval for our model, this result would suggest that individuals are selecting mates that are more heterozygous than themselves. Alternatively, if the observed mean were to fall outside the right-hand confidence interval, this result would suggest that individuals are selecting mates that are less heterozygous than themselves. For males, the observed mean of pairwise differences in heterozygosity ($x = -0.041$) falls to the left of the 95% confidence intervals, suggesting that male Leach's storm-petrels select females based on increased heterozygosity at MHC class IIB if their own heterozygosity is low. The corresponding likelihood of error for the results is plotted on the right, and indicates a 92% likelihood of obtaining this result, given our sample size of 338 pairs. For females, the observed mean of pairwise differences in heterozygosity ($x = 0.041$) falls within the 95% confidence intervals, suggesting that female Leach's storm-petrels do not select males based on heterozygosity at MHC class IIB. The corresponding likelihood of error for the results is plotted on the right and indicates an 82% likelihood of obtaining this negative result given our sample size of 338 pairs.

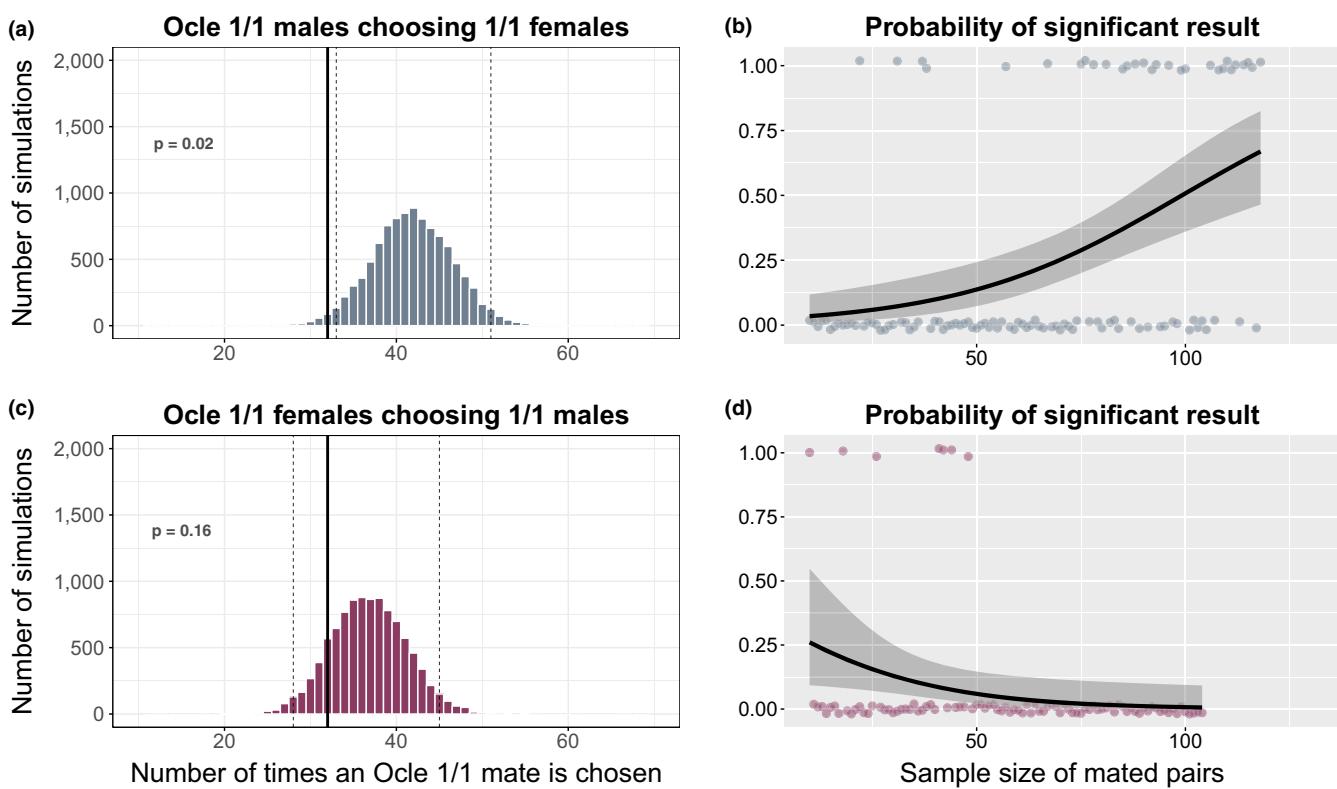


FIGURE 6 (a–d) Monte Carlo randomization test results for homozygous pairings for the most common allele at DAB2, Ocle-DAB2*01/Ocle-DAB2*01. If the observed mean falls outside the left-hand confidence interval for our simulated results, this would suggest that males are selecting significantly fewer females of this genotype than would be predicted to occur randomly. Alternatively, if the observed mean were to fall outside the right-hand confidence interval, this result would suggest that males are selecting significantly more females of this genotype than would be predicted to occur at random. In our study population, homozygous Ocle-DAB2*01 males were observed to pair with homozygous Ocle-DAB2*01 females on 32 occasions, which is significantly lower than the simulated mean of 43.2. The corresponding likelihood of error for the results is plotted on the right and indicates a 66% likelihood of obtaining this result given our sample size of 118 pairs. On the contrary, when homozygous Ocle-DAB2*01 females paired with homozygous Ocle-DAB2*01 males on 32 occasions, this was within the 95% confidence intervals of the simulated means. The corresponding likelihood of error for the results is plotted on the right and indicates a 95% likelihood of obtaining this negative result given our sample size of 118 Ocle-DAB2*01/Ocle-DAB2*01 pairs

significantly smaller wing chords compared to individuals recorded during the later years of the study (2012–2015). This result may be partially attributed to measuring error between these years, however, and is unlikely to reflect biological significance. We found no effects of Ocle-DAB2 genotype on hatch date, but do report a significant effect of year on hatch date, with the 2012 mean hatch date significantly delayed compared to the other years of the study (see SI).

We found a nonsignificant trend between the Ocle-DAB2 genotypes of successful female parents and year; χ^2 (12, $n = 338$) = 19.1, $p = 0.07$. This effect was largely driven by Ocle-DAB2*01/Ocle-DAB2*X females producing more chicks than Ocle-DAB2*01/Ocle-DAB2*01 females during years of poor overall reproductive success.

4 | DISCUSSION

Many experimental studies have investigated the role of mate choice in maintaining MHC polymorphisms in vertebrate

populations by reducing or otherwise controlling for other sources of variability (reviewed in Ziegler, Kentenich, & Uchanska-Ziegler, 2005; Piertney & Oliver, 2006; and Yamazaki & Beauchamp, 2007). However, by embracing the noisy conditions typically found in natural systems, we have exploited this variability to identify natural patterns of reproductive success in our study population. In a specific manner, we designed a multiyear project with a sufficiently large sample size to first examine patterns of breeding success in each sex and then test hypotheses informed by the natural history and ecology of the species. Within this ecological context, we identified two MHC class IIB loci (Ocle-DAB1 and Ocle-DAB2) and found both loci to exhibit evidence of positive selection in our population. We demonstrated that homozygous females have a lower probability of hatching a chick. We then showed that males pair with more heterozygous females than would be expected by random choice. Finally, we showed that this pattern can be explained by males breeding less frequently than expected with the most common genotype at Ocle-DAB2*01/Ocle-DAB2*01. Our results are thus consistent with the hypothesis that sexual

selection contributes to maintaining MHC polymorphism in this species.

The two polymorphic MHC class IIB genes identified in our study population agree with a previous characterization in this species from Kent Island, New Brunswick, Canada (Dearborn et al., 2015), which sampled the same Atlantic meta-population as our study (Bicknell et al., 2012). Dearborn et al. (2015) used locus-specific Illumina high-throughput sequencing to amplify exon 2 for each MHC class IIB locus. In comparison, we applied a single-locus typing protocol which allowed the complete characterization of the whole exon 2 region in two independent MHC class IIB gene copies. We found 27 exon 2 allele sequences compared to 24 identified by Dearborn et al. (2015, 2016). This discrepancy in allele numbers may reflect the larger sample size used in our study ($n = 1078$ vs. $n = 188$).

The low number of MHC class IIB genes reported in Leach's storm-petrels is similar to the MHC diversity of other nonpasserine birds (Alcaide, Edwards, & Negro, 2007; Burri, Hirzel, Salamin, Roulin, & Fumagalli, 2008; Hughes, Miles, & Walbroehl, 2008). The characterization of a second MHC class IIB gene, as described in our study (SI) and Dearborn et al. (2015), agrees with orthogonal MHC class IIB genes identified in other seabirds (Cloutier, 2012; Vásquez-Carillo, Friesen, Hall, & Peery, 2014; Walsh & Friesen, 2003), and supports a model of adaptive divergence of ancient avian MHC class IIB duplicates first described in owls by Burri, Salamin, Studer, Roulin, and Fumagalli (2010). While duplication of MHC genes may encompass both classical and nonclassical genes that are differentially expressed (Drews, Strandh, Råberg, & Westerdahl, 2017), there is evidence of gene expression at both MHC class IIB loci in Leach's storm-petrels, though the relative extent and functionality of this expression remain unknown (Dearborn et al., 2015). This agrees with our evidence of positive selection at those codons presumably comprising the PBR in both loci (Brown et al., 1993), as well as evidence of balancing selection maintaining high frequencies of polymorphic sites within the repertoire of exon 2 sequences. Intriguingly, departure from Hardy-Weinberg expectations at the Ocle-DAB2 gene indicates that natural selection is probably acting upon this locus, and our results here suggest that Ocle-DAB2-associated female fitness effects and corresponding male avoidance may be a selection mechanism.

In a conceptual manner, understanding the ecology and natural history of this species provides insights into why there is so much variation in female reproductive success related to MHC class IIB genotype. Leach's storm-petrels are not only a long-lived monogamous seabird species, but they also have an extreme reproductive lifestyle, particularly for the females. Although a female produces only one egg each season, it is nearly a quarter of her mass (Montevecchi et al., 1983). Unlike most nesting birds, Leach's storm-petrels travel hundreds of kilometres on a single foraging trip (Pollet et al., 2014) and must coordinate trips with their partners to provision the chick for as long as 50 days. Under these conditions, we hypothesize that adults who are MHC-homozygous, and therefore possibly immunologically deficient, might be operating under a

severe handicap that is likely to be more pronounced in females because they are physiologically depleted by producing a large egg. Thus, considering our results in a broader ecological framework of our study system, females who are MHC-compromised may be less preferred by males for lifelong partnership. At the same time, both partners rely on their nesting burrow for their lifelong reproductive success, which is also a basis for investment by the male (Warham, 1990).

In contrast to our results, a recent study reported no evidence for disassortative mating in Leach's storm-petrels and concluded that MHC polymorphism is not maintained by mate choice (Dearborn et al., 2016). This study was performed on a nearby island and sampled the same Atlantic meta-population as our study (Bicknell et al., 2012). However, this study sampled fewer pairs and did not incorporate variability in female and male reproductive quality into the experimental design. Randomization tests only assessed mating patterns among paired birds without considering the wider variability in reproductive success in the study population, including unpaired adults. In contrast to these results, our study found evidence of disassortative mating when additionally incorporating nonbreeding individuals using Monte Carlo analyses. Neither study found evidence that the observed mate-choice patterns could be explained by relatedness, although different methodologies were used to assess this (ddRAD-Seq vs. microsatellites). We found no genetic structure among the genotyped population (Supporting information Figure S8) and found no evidence that specific individuals represented genetic outliers to our population (Sin, Hoover, Nevitt, & Edwards, in preparation). Comparing our approaches illustrates the effects that study design and sample size can have on conclusions, even in the same study species.

4.1 | MHC mate choice: A mechanism for selecting “good genes”

In the broader field of MHC mate-choice studies, several hypotheses have been proposed to explain how sexual selection promotes MHC diversity (see Milinski, 2006). These hypotheses typically propose mate-choice preferences for “good genes” that maximize allele diversity (Brown, 1997) or preferences for genetic dissimilarity (i.e., compatibility; reviewed in Penn & Potts, 1999; Tregenza & Wedell, 2000). These pairings should benefit offspring by enhancing pathogen resistance, due to either increased MHC heterozygosity (heterozygote advantage: see Doherty & Zinkernagel, 1975; also Potts & Wakeland, 1990) or the inclusion of uncommon alleles that pathogens are not yet adapted to (Penn & Potts, 1999; Potts & Slev, 1995). Previous studies have also found correlations between heterozygosity in MHC and increased reproductive success (house mice: Thoss, Ilmonen, Musolf, & Penn, 2011; magellanic penguins: Knafler, Clark, Boersma, & Bouzat, 2012; gray wolf: Galaverni et al., 2015). In our study, we found significantly fewer homozygous females producing chicks than would be expected given their frequency in the overall population. These results suggest that MHC polymorphism in this system could be driven by a male preference for females showing “good genes” as

heterozygosity,” or by a rejection of females that have a particular homozygous Ocle-DAB2*01/Ocle-DAB2*01 genotype. We have not yet determined how males identify or discriminate female heterozygosity. We might predict, for example, that Ocle-DAB2*01/Ocle-DAB2*01 females are physically smaller or are in poorer physiological condition than heterozygous females. This prediction is partially born out in that Ocle-DAB2*01/Ocle-DAB2*01 females appeared to have a significantly smaller wing cord as compared to other groups, but we observed no differences in overall body condition (see Supporting information).

4.2 | Potential links to personal odours

It has also been proposed that MHC genotype influences personal odours that impact mate-choice preferences (Boehm & Zufall, 2006; Penn, 2002; Penn & Potts, 1998). In support of this idea, linkages between the MHC locus and olfactory receptor genes have been demonstrated across several vertebrate groups (e.g., Younger et al., 2001), including bird species (Galliformes: Miller et al., 2013). These and other authors have speculated that MHC-linked olfactory receptor genes could be involved in MHC-influenced mate choice, providing a possible mechanism for the observed mating bias. In addition, the chemical composition of preen gland secretions covaries with MHC similarity in both black-legged kittiwakes (*Rissa tridactyla*, Leclaire et al., 2014) and song sparrows (*Melospiza melodia*, Slade et al., 2016). However, whether these species are able to discriminate MHC similarity using olfactory cues has not been explored. Leach’s storm-petrels have an excellent sense of smell (Nevitt & Haberman, 2003; O’Dwyer & Nevitt, 2009; O’Dwyer et al., 2008), and other burrow-nesting procellariform species have been shown to detect and discriminate individual-specific odours (Bonadonna & Nevitt, 2004; Bonadonna & Sanz-Aguilar, 2012). This leads us to speculate that individuals might be able to smell differences in MHC heterozygosity in potential mates. Males might, for example, be able to detect and avoid females who have the same MHC genotype as themselves, using mechanisms that have been proposed for rodents in the context of kin recognition, such as self-referential phenotype matching (Hauber & Sherman, 2001; Mateo, 2010). According to this theory, an individual compares its own personal scent against the scent of other individuals. Our sample size was sufficiently large to examine mating preferences of a subset of homozygous males (Ocle-DAB2*01/Ocle-DAB2*01), and, consistent with this hypothesis, we found that they avoided females of the same genotype (Figure 6a). However, our observation that Ocle-DAB2*01/Ocle-DAB2*01 females have a reduced wing cord also presents a link to personal scent that may be contributing to MHC class IIB genotype recognition. Pearce, Hoover, Jennings, Nevitt, and Docherty (2017) have recently demonstrated that wing cord length in adult Leach’s storm-petrels predicts a significant amount of variation in female but not male feather bacterial community structure. This result suggests that females with shorter wing chords have more variable feather microbiota which could impact personal scent (Pearce et al.,

2017). This study lacked sufficient sample size to test for a relationship between specific MHC genotypes and bacterial community structure. However, the overall findings suggest an alternative hypothesis that low-quality females (i.e., females with reduced hatching success) that are disproportionately represented in the homozygous Ocle-DAB2*01/Ocle-DAB2*01 genotype may express predictable features in their personal odour profile that males learn to recognize and avoid. As male and female feather bacterial community structures were also found to vary significantly (Pearce et al., 2017), this hypothesis should also be investigated. While these are compelling ideas, the mechanism for discrimination is purely hypothetical and needs to be further explored.

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DATA ACCESSIBILITY

MHC DNA allele sequences are deposited in GenBank Acc. Nos. JN613229: JN613253, and genotyping data and mate-choice data are available via Dryad (<https://doi.org/10.5061/dryad.5g76b10>).

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

G.A.N. and S.V.E. designed the research project and initially set up the study colony for this investigation; M.A. performed the characterization and supervised or performed initial genotyping; B.H., S.J., G.A.N., S.V.E. and helpers collected field data, B.H. performed or supervised genotyping and sexing; B.H. spearheaded the analysis with assistance from M.A. and S.Y.; B.H., M.A. and G.A.N. wrote the manuscript; all authors contributed to editing the manuscript.

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