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Author for correspondence:

Sarah Leclaire
e-mail: sarah.leclaire@free.fr

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Odour-based discrimination of similarity at the major histocompatibility complex in birds

Sarah Leclaire¹, Maria Strandh², Jérôme Mardon¹, Helena Westerdahl² and Francesco Bonadonna¹

¹Centre d'Ecologie Fonctionnelle et Evolutive, CNRS–CEFE, 1919 route de Mende, 34293 Montpellier, France

²Molecular Ecology and Evolution Lab, Lund University, Ecology building, 22362 Lund, Sweden

SL, 0000-0002-4579-5850

Many animals are known to preferentially mate with partners that are dissimilar at the major histocompatibility complex (MHC) in order to maximize the antigen binding repertoire (or disease resistance) in their offspring. Although several mammals, fish or lizards use odour cues to assess MHC similarity with potential partners, the ability of birds to assess MHC similarity using olfactory cues has not yet been explored. Here we used a behavioural binary choice test and high-throughput-sequencing of MHC class IIB to determine whether blue petrels can discriminate MHC similarity based on odour cues alone. Blue petrels are seabirds with particularly good sense of smell, they have a reciprocal mate choice and are known to preferentially mate with MHC-dissimilar partners. Incubating males preferentially approached the odour of the more MHC-dissimilar female, whereas incubating females showed opposite preferences. Given their mating pattern, females were, however, expected to show preference for the odour of the more MHC-dissimilar male. Further studies are needed to determine whether, as in women and female mice, the preference varies with the reproductive cycle in blue petrel females. Our results provide the first evidence that birds can use odour cues only to assess MHC dissimilarity.

1. Introduction

The major histocompatibility complex (MHC) is an extremely polymorphic cluster of genes that encode antigen-presenting molecules of central importance in vertebrate adaptive immunity. Individuals with a broad antigen-binding repertoire can recognize and eliminate a wider range of pathogens and therefore have fitness advantages over individuals with a narrow MHC repertoire [1–4]. As MHC-dissimilar parents are more likely to produce offspring with a broad MHC repertoire, mating preference for MHC-dissimilar partners is a common strategy in numerous taxa, including mammals, fish, lizards and birds [5–9].

Most studies, investigating the cues used in the assessment of MHC dissimilarity to potential mates, have showed the importance of odour cues [6,10–12]. For instance, in species as diverse as humans, bank voles (*Myodes glareolus*), sand lizards (*Lazerta agilis*) and house mice (*Mus musculus*), females prefer the scent of MHC-dissimilar males [6,10,11,13]. In birds, although European storm petrels (*Hydrobates pelagicus*), Humboldt penguins (*Spheniscus humboldti*) and zebra finches (*Taeniopygia guttata*) can discriminate kinship based on odour cues [14–16] and the scent secretions of black-legged kittiwakes (*Rissa tridactyla*) covary with MHC similarity [17], the ability of birds to assess MHC similarity using olfactory cues has not yet been explored.

The blue petrel (*Halobaena caerulea*) is a monogamous seabird with a particularly good sense of smell [18–20] that has been shown to preferentially mate with partners dissimilar at the MHC class II loci [9]. In this monomorphic species, males and females share parental duties equally [21,22], and mate choice is therefore thought to be reciprocal. Potential cues for olfactory recognition of genetic

relatedness exist in blue petrels as their odours carry a kin label [23]. This finding does not indicate, however, a relationship between odour and MHC and the capacity of blue petrels to use this label. In this study, we carried out binary choice tests to determine whether in blue petrels, males and females can discriminate the odour of opposite-sex individuals that vary in their MHC similarity.

2. Methods

(a) Study site

A colony of about 80 nest burrows of individually banded blue petrels (*H. caerulea*) was studied at île Verte in the Kerguelen archipelago (southern Indian Ocean; 49°51'S, 70°05'E). We performed this study while birds were incubating eggs between 8 and 11 December 2007 ($n = 28$ tests), between 14 and 23 December 2009 ($n = 4$ tests) and between 20 November and 22 December 2015 ($n = 5$ tests).

(b) Y-maze experiment

Using a Y-maze apparatus, incubating birds (females: $n = 26$ tests, males: $n = 11$ tests; electronic supplementary material, table S1) were presented with the odours of two individuals whose MHC genotype was unknown. Male odours were presented to females, whereas female odours were presented to males. In the field, birds were sexed based on song characteristics or because they were previously sexed using molecular methods [24]. For birds sexed based on song characteristics, sexes were later confirmed using molecular methods [24]. We had to exclude two tests where odour donors (one male and one female) were of the same sex as the recipient. Individuals were MHC genotyped years later, so that the experiment was carried out blind to MHC similarity between the odour donors and the recipient. Only one trial per individual was performed within a breeding season.

Odours were collected from 14 males and 14 females (electronic supplementary material, table S1). In 2007 and 2009, odours were collected using cotton swabs (in 2007: $n = 7$ males and 5 females; in 2009: $n = 7$ females), whereas in 2015, odours were collected using cotton bags ($n = 7$ males and 2 females). To collect odours with cotton swabs, one observer, wearing clean nitrile gloves, gently rubbed the back, rump, wings, chest and head of birds with cotton swabs. These swabs, which are used in forensic police procedures, are described by Prada *et al.* [25]. Cotton swabs were stored individually in opaque glass jars and kept in the dark at ambient temperatures (5–10°C). To collect odours with cotton bags, birds were held individually in a cotton bag (21 × 20 cm; 10 g) for 1 h before being returned to the burrow where they were found. Odour bags were stored separately in plastic storage bags and kept in the dark at ambient temperatures (5–10°C). Swabs and cotton bags were only used within the season of collection. For each test, the odour donors were determined randomly, so that combinations of odour stimuli were used only once, except three combinations that were used twice.

We used the plastic Y-maze apparatus described in Bonadonna & Mardon [19]. In the field, blue petrels were removed from burrows, transported to the Y-maze in a cotton bag, placed in the temporary holding compartment of the maze's entrance and allowed to settle for 5 min. At the end of this period, a trap door was lifted, and the bird was allowed to make a choice between one of the two ends of the maze. The end of each choice arm was equipped with a compartment for the odour source and was equipped with a central processing unit cooling fan that provides controlled airflow. The choice was easily assessable by the noise of the bird walking in the maze, and was considered when the bird

walked down one of the maze's arms to the end and stayed there for more than 30 s. No-choice birds (removed after 15 min) either never settled down, passing continuously from one arm to the other or sat calmly in the holding compartment. The maze was carefully washed after each trial with 90% methanol or ethanol to remove odour residues.

Binary choice tests make it impossible to conclude whether birds chose an arm because of preference or avoidance of the odour. For the ease of the reading, we will, however, use the term preference over avoidance in the rest of the manuscript.

(c) Molecular analysis of major histocompatibility complex

During the course of other studies, blood was collected with syringe or capillaries from the brachial vein and kept in Queen's lysis buffer at +4°C. DNA was extracted from the blood samples, using the Qiagen DNeasy® Blood and Tissue Kit and the manufacturer's protocol (Qiagen, Venlo, The Netherlands; July 2006).

We amplified 200 bp fragments (161 bp excluding primers) of the exon 2 of the blue petrel MHC class IIB genes. Amplicon sequencing was performed, using the Illumina technology. Barcoded amplicons that contained Illumina adaptors were prepared in two consecutive PCR runs. The first PCR primers were composed of two parts: the Illumina Read Miseq adaptors (forward: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and reverse: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the MHC class IIB-specific primers (P141: 5' CAACGGCACCGAGCGGGTGAG and P144: 5' CACCYCGT AGTTGKCCCGG designed in Strandh *et al.* [26]). This first PCR amplification was performed in a volume of 25 µl which contained 25 ng of genomic DNA, 0.5 µM of each primer and 1× Phusion High Fidelity PCR Master Mix (Thermo Scientific). The first PCR programme consisted of 30 s initial denaturation at 98°C, followed by 25 cycles of 10 s denaturation at 98°C and 30 s annealing/extension at 72°C. A final elongation step was run at 72°C for 10 min. Amplicons were then purified using the AMPure® XP Beads protocol (Agencourt Bioscience Corporation). Purified amplicons were used in a second PCR in which the Nextera XT index primers were added to the amplified MHC fragments. The second PCR amplification was performed in 25 µl reaction mixtures, which contained 2.5 µl of purified amplicon product, 0.5 µM of each primer and 1× Phusion High Fidelity PCR Master Mix (Thermo Scientific). The second PCR programme consisted of 30 s initial denaturation at 98°C, followed by eight cycles of 10 s denaturation at 98°C, 30 s annealing at 62°C and 30 s extension at 72°C. A final elongation step was run at 72°C for 10 min. Products of the second PCR were then purified using the AMPure XP Beads protocol. Amplicons were sequenced in two runs on an Illumina MiSeq apparatus which produced 2 × 250 bp reads.

To analyse amplicon sequences, we used ampliSAS, a three-step pipeline that consists of read demultiplexing, unique sequence clustering and erroneous sequence filtering [27]. First, the Illumina data were filtered to remove low-quality sequences (sequences with Phred scores less than 20 were removed). Sequences were then clustered using the default ampliSAS parameters for Illumina sequences (substitution errors: 1%, indel errors: 0.001%, minimum frequency with respect to dominant: 25%), and sequences that were potential chimeras or that had less than 3% frequency were discarded. Considering the larger set of samples that we analysed in the run ($n = 122$ samples, including 41 samples that were part of this study), the reproducibility of alleles between technical duplicates ($n = 11$ DNA samples that were split and processed in independent PCRs) was 100%. After processing, we had an average ($\pm$ s.e.) of 4824 ± 23 reads per individual (range: 2115–4955). We obtained 96 different MHC class II alleles. The number of alleles per

Figure 1. Y-maze tests where blue petrel (a) females and (b) males were challenged to choose the odour of individuals that had either functionally-dissimilar MHC or functionally-similar MHC. (Online version in colour.)

individual was 3.59 ± 0.06 (average $\pm$ s.e.; range: 1–4). Two individuals included in this study were only genotyped, using the method described in Strandh *et al.* [9]. The correspondence between the two methods was tested by genotyping 36 individuals of the population using the two methods. An average of $93.5 \pm 2.5\%$ similarity was detected between the two methods, and 100% correspondence was found in 83% of samples.

Amino acid and functional distances between individual MHC class IIB genotypes were used to describe MHC similarity between individuals. The two types of distances were calculated, using the approaches described in Strandh *et al.* [9]. To calculate amino acid distances, a maximum-likelihood tree was inferred for all translated MHC sequences, using the RAXML software (v. 7.0.4) under the PROTMIX model and the JTT substitution matrix, with default settings [28]. To calculate functional distances, the chemical binding properties of the amino acids in the peptide binding regions (PBRs) [26] were described by five physico-chemical descriptor variables (z-descriptors) for each amino acid [29], and the resulting matrix was used to construct an alternative maximum-likelihood tree with contml in the PHYLIP-package, v. 3.695. This tree represents clusters of functionally-similar MHC sequences rather than clusters of evolutionary-similar MHC sequences. The amino acid and functional trees were used as references from which the amino acid and functional distances between MHC-sequence repertoires were calculated, using unweighted UniFrac analyses (GUniFrac package in R) [30]. Amino acid distances were positively correlated with functional distances among the 43 individuals used in this study (Mantel test: $r = 0.59$, $p = 0.001$).

(d) Statistics

We used binomial tests to assess choice preferences. Tests were performed with the R software [31].

3. Results

Twenty out of 25 females made a choice (80%), and seven out of 10 males made a choice (70%) in the Y-maze. Considering amino acid distances between MHC class IIB genotypes, neither females nor males showed a preference for individuals that were more MHC dissimilar. Half of the females ($n = 10$) oriented to the odour of the more MHC-dissimilar male ($\chi^2_1 = 0$, $p = 1$), whereas 86% of the males ($n = 6$) oriented to the odour of the more MHC-dissimilar female ($\chi^2_1 = 3.57$, $p = 0.059$).

In contrast, considering functional distances between MHC class IIB genotypes, females and males showed significant

but opposite preferences. Seventy-five per cent of the females ($n = 15$) preferred the odour of the male that was more MHC-similar to them, whereas 25% of the females ($n = 5$) oriented to the odour of the more MHC-dissimilar male ($\chi^2_1 = 5$, $p = 0.025$; figure 1a). In contrast, all the seven males ($n = 7$) who made a choice oriented to the odour of the more MHC-dissimilar female ($\chi^2_1 = 7$, $p = 0.008$; figure 1b).

4. Discussion

Our results provide the first evidence that birds can detect MHC similarity based on odour cues alone, and suggests that, similar to humans, rodents or lizards [6,7,10], an odour-based mechanism of MHC-related mate choice may occur in petrels and potentially in birds in general.

Blue petrels preferentially mate with MHC-dissimilar partners [9], and we therefore predicted that males and females would prefer the scent of MHC-dissimilar potential partners in this study. Although incubating males preferentially approached the scent of the more MHC-dissimilar female, incubating females showed the opposite preference, preferentially approaching the scent of the more MHC-similar male. These preferences were detected when considering functional distances of MHC class IIB genes between the odour donors and the recipients, but were not detected when considering amino acid distances. Similarly, while blue petrels preferentially mate with partners who have functionally dissimilar MHC class IIB genes, there was no significant evidence for mate choice according to amino acid distances [9]. The functional binding specificity of an MHC molecule determines what antigens can be bound and hence what pathogens can be eliminated. When partners have MHC genes with large functional distances, their chicks are expected to be particularly well prepared to eliminate a wide range of pathogens. Recently, the anchor residues of these antigens have been suggested to be the target odourants in the olfactory assessment of MHC class II genotypes (reviewed in [32,33]). In contrast to functional distance, the information conveyed from amino acid distances is therefore expected to less well reflect the biologically relevant differences between individuals.

The sex-specific preference for the odour of MHC-dissimilar individuals might suggest that males are the choosy sex in blue petrels. However, mate choice in this species is suggested to be reciprocal. An alternative explanation for this sex-specific

preference stems from the period when our study was carried out: the egg-incubating period. In humans, the menstrual cycle can strongly impact women social perception and preference (reviewed in [34]). Whereas women commonly prefer the odour of MHC-dissimilar men, this preference is reversed when women use oral contraceptives which mimic the hormonal state during pregnancy [11,35]. Similarly, in female mice, inbreeding avoidance depends on oestrogen levels and varies with the reproductive cycle [36,37]. Although female mice prefer mates with dissimilar MHC odour signatures [38], pregnant mice prefer to nest with individuals who share their MHC odour [39]. In birds, incubation is associated with several hormonal changes, including decreased levels of progesterone and oestradiol in females [40,41]. As we tested blue petrels during the incubating period, one cannot exclude that a hormonal-based mechanism exists in petrels and that females would have shown the opposite preference, if tested during the mate choice period. The adaptive benefits of this reversion during the reproductive cycle is unknown. It has been suggested that attraction to kin during pregnancy may be associated with increased benefits in the form of altruism from family members. Petrels share parental care with their breeding partner exclusively, suggesting that altruism is not a selecting force acting on kin preference. Our study is based on a binary choice test in laboratory conditions, and one cannot exclude that females would have shown other preferences under more natural mate choice conditions. A similar Y-maze study carried out during the blue petrel mate choice period should help unravelling this unexpected finding.

Although MHC-dependent mate choice has been shown in several bird species including great frigatebirds (*Fregata*

minor), red junglefowls (*Gallus gallus*), ring-necked pheasants (*Phasianus colchicus*), savannah sparrows (*Passerculus sandwichensis*) and blue petrels ([9,42–45], but see [46]), the phenotypic cues used by birds to assess MHC similarity have never been determined. Our results strongly suggest that olfactory cues are involved in this process. Odour cues seem of primary importance in MHC-similarity recognition in mammals, fish or lizards [6,11,13,47], but they have long been ignored in birds [48]. Our finding suggests that chemical communication, the most ancient communication channel, is still present in birds, and may be critical for fitness optimization also in this taxon.

To conclude, our study, by revealing that birds can discriminate MHC similarity based on odour cues, provides a mechanism that links mate choice and immunity in birds and suggests that MHC odour cues might be vertebrate-wide.

Data accessibility. The dataset supporting this study is stored in the electronic supplementary material.

Authors' contributions. F.B., J.M. and S.L. designed the study and carried out the choice tests. M.S. and H.W. conceived the molecular laboratory work, and M.S. and S.L. carried out the molecular analyses. S.L. performed the statistical analyses and wrote the manuscript. F.B., M.S., H.W. and F.B. helped draft the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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