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A gull species recognizes mhc-ii diversity and dissimilarity using odor cues

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27 **1. Introduction**

28 Most birds do not display any apparent olfactory behavior, such as sniffing or scent-marking. Therefore, it
29 was long thought that birds had little or no sense of smell and relied primarily on visual or acoustic cues.
30 However, there is now abundant evidence that birds have a functional olfactory system (Bang and Cobb
31 1968; Steiger et al. 2008) and use olfaction in various non-social contexts, including foraging, navigation or
32 nest sanitation (Caro and Balthazart 2010; Abankwah et al. 2020; Potier 2020; Bonadonna and Gagliardo
33 2021). In contrast, the role of olfactory cues in avian social life is still poorly known (Caro et al. 2015), albeit
34 being a rapidly growing research area (Whittaker and Hagelin 2021).

35 As in other vertebrates, recent research suggests that the major histocompatibility complex (MHC)
36 may be an important force involved in mate choice in birds (Zelano and Edwards 2002; Løvlie et al. 2013).
37 The MHC is a polymorphic group of genes that plays a critical role in immunity. In general, higher MHC
38 diversity is considered to provide resistance to a wider range of parasites and to increase fitness (Milinski
39 2006). Accordingly, species from diverse taxa have developed MHC-based mate choice, such as mate choice
40 for MHC-dissimilar partners (i.e., MHC-compatible partners), thereby avoiding the production of offspring
41 with low MHC diversity (Strandh et al. 2012; Kamiya et al. 2014; Hoover et al. 2018), or mate choice for
42 MHC-diverse partners, which may provide both genetic and non-genetic benefits (Richardson et al. 2005;
43 Griggio et al. 2011). However, high MHC diversity may also be associated with costs, such as an increased
44 risk of autoimmune disorders or a reduction in the potential for inducing an immune reaction due to negative
45 selection of T-cells (Nowak et al. 1992; Simmonds and Gough 2007; Woelfing et al. 2009; Migalska et al.
46 2019). There is therefore increasing support for an intermediate optimum of MHC-diversity (Madsen and
47 Ujvari 2006; Kalbe et al. 2009; Rekdal et al. 2019).

48
49 Whatever the optimum in MHC-diversity, MHC characteristics needs to be encoded in phenotypic
50 cues for animals to assess the MHC characteristics of potential partners during mate choice. However, while
51 several mammals, lizards or fish have been shown to assess MHC characteristics using odor cues (reviewed
52 in Schubert et al. 2021), this ability in birds has been poorly explored. To the best of our knowledge, only the
53 blue petrel (*Halobaena caerulea*), a Procellariiform seabird, and the song sparrow (*Melospiza melodia*) and
54 the house sparrow (*Passer domesticus*), two Passeriform birds, have been tested. While blue petrels and
55 song sparrows were shown to discriminate MHC-II characteristics using odor cues (Leclaire et al. 2017;
56 Grieves et al. 2019), house sparrows did not discriminate MHC-I characteristics using olfaction (Amo et al.
57 2022). To estimate the taxonomic range of olfactory assessment of MHC in birds, more avian taxa need to be
58 studied.

59 The black-legged kittiwake (*Rissa tridactyla*) is a genetically monogamous Charadriiform seabird
60 with a functional sense of smell (Leclaire et al. 2009). In this species, preen gland secretions encode
61 information about MHC dissimilarity (Leclaire et al. 2014) and offspring sex-ratio varies with the MHC
62 dissimilarity between parents (Pineaux et al. 2022), suggesting that kittiwakes might be able to recognize the

63 MHC characteristics of conspecifics. Here, we used behavioral tests to investigate whether female kittiwakes
64 can use odor cues to distinguish the MHC characteristics of males. We used a protocol inspired by Leclaire
65 et al. (2009), which showed that kittiwakes discriminate between several odors deposited in their nest. Our
66 test is based on the pecking response of incubating females to one single odor sample put in their nest.
67 Results cannot therefore be interpreted as female preference. However, we consider that the fact that
68 different females react on average differently along a continuum of MHC characteristics indicate
69 recognition. Because the MHC-based mating pattern of kittiwake is unknown, we tested recognition of both
70 MHC diversity and MHC dissimilarity, and always included both the linear and the quadratic term of MHC
71 diversity.

72

73 **2. Materials and methods**

74 *2.1 Study site*

75 The study was conducted during the 2018 incubating period (May-June) in a population of black-legged
76 kittiwakes nesting on an abandoned US Air Force radar tower on Middleton Island, Alaska (59°26'N,
77 146°20'W). Nests on artificial ledges were visible from inside the building through sliding one-way glass
78 windows (Gill and Hatch 2002).

79

80 *2.2 Behavioral tests*

81 We assessed female's response to male odor samples placed on the edge of the nest bowl. Odor samples were
82 a mixture of cloacal samples (potentially related to fecal odor) and nest materials (potentially related to fecal
83 odor deposited on the nest during defecation and fecal, skin, plumage and preen gland odor passively
84 deposited on the nest when birds are sitting). We collected odor samples from 43 incubating males. We
85 collected cloacal samples as in Leclaire et al. (2023). Briefly, after capture, we injected 1 mL of sterile
86 phosphate-buffered saline solution into male's cloaca with a sterile needleless syringe and drew the solution
87 out. At the time of capture, a piece of nest material (i.e., a blend of mosses and grasses of ca. 4 cm diameter)
88 was collected from the nest of the captured male, using clean nitrile gloves. The piece of nest material was
89 immediately mixed with the cloacal sample and stored in plastic bags at -20°C until the test. Odor samples
90 were stored on average for 17 ± 1 days (range: 8 – 24 days). The time the odor sample was kept in the
91 freezer did not affect the behavioral response of the females (Spearman's correlation test between the time
92 the sample was kept in the freezer and the time the female took to peck at the odor sample during the test: S
93 = 13678, $P = 0.077$, $r = -0.28$).

94 Fifty-two incubating females were each tested once. They were never tested with the odor of their
95 partner. All odor samples ($n = 43$) were used at least once. Just before the test, a small piece (ca. 2 cm
96 diameter) of an odor sample was defrosted for 15 minutes. The remaining odor sample was kept frozen. Nine
97 of these remaining odor samples were used in tests later on. The odor sample was placed on the edge of the
98 nest bowl, just under the beak of an incubating female. Because we had to open the sliding window of the

99 nesting site to put the odor sample on the nest, all females, except one, took off. Behavioral observations
100 started when the female landed back on her nest. Females returned to their nest on average \pm se: 14 ± 5 sec
101 after the odor sample was placed on the nest (range: 0-462 sec). For each test, we recorded whether the
102 female pecked at the odor sample and the time she took to peck at the odor sample for the first time.
103 Observation lasted until the female pecked at the odor sample or for 15 min when she did not peck at the
104 odor sample. Most females who pecked at the odor sample took the sample with their bill and moved it along
105 the nest bowl, as they often do with nest material when maintaining the nest structure. Tests were carried out
106 by 3 observers who were blind to MHC-characteristics of the males concerned.

107

108 2.3 MHC analyses

109 Details about MHC analyses are given in Pineaux et al. (2020). Briefly, DNA was extracted from blood
110 samples, and 258 bp fragments of exon 2 of MHC class-IIB were amplified using PCR and sequenced in 4
111 runs with an Illumina MiSeq platform. Amplicon sequences were analyzed with AmpliSAS (substitution
112 errors: 1%, indel errors: 0.001%, minimum frequency with respect to dominant: 33%, minimum amplicon
113 sequence frequency: 2.5%) (Sebastian et al. 2016). Considering the larger set of samples analysed in the 4
114 Illumina MiSeq runs, we detected a maximum of 6 alleles per individual, suggesting the presence of a
115 minimum of 3 MHC class-IIB loci. We focused on the amino acid sequences of the peptide binding region
116 (Leclaire et al. 2014). MHC-IIB diversity and MHC-IIB dissimilarity were each estimated using two indices.
117 First, MHC-IIB diversity was estimated using the number of MHC-IIB alleles, while MHC-IIB dissimilarity
118 between the female and the sampled male was estimated on a pure shared/non-shared allele basis. The
119 percent difference in MHC-IIB allele sharing was calculated as $100 * \text{TOT}_{\text{FM}} / (\text{N}_{\text{F}} + \text{N}_{\text{M}})$, where TOT_{FM} =
120 total number of different alleles present in the male and the female (shared allele count as 1) and N = number
121 of alleles within an individual (Strandh et al. 2012). Second, we estimated MHC-IIB diversity as the degree
122 of functional diversity (or divergence) of alleles across all loci, and MHC-IIB dissimilarity as the functional
123 distance between two individuals. We described the chemical binding properties of each amino acid in the
124 PBR with Sandberg's five physicochemical descriptors (z-descriptors; Sandberg et al. 1998). This Sandberg's
125 matrix was used to construct a UPGMA dendrogram of alleles (function `hclust` in R), which represents
126 clusters of functionally similar MHC-IIB sequences. The dendrogram was used as a reference to calculate
127 the functional MHC-IIB diversity of an individual as the minimum total length of all the branches required to
128 span its MHC-IIB alleles (function `treedive` in package *vegan*) (Petchey and Gaston 2006). MHC-IIB
129 functional dissimilarity between the male odor donor and the female was estimated using the tree distance
130 (function `treedist` in package *vegan*), which is found by combining species in a common dendrogram and
131 seeing how much of the dendrogram height is shared and how much is unique. Because five females were
132 not genotyped at the MHC-IIB, we could not calculate the MHC-IIB dissimilarity between the female and
133 the sampled male for five tests. The number of alleles was correlated with the functional diversity
134 (Spearman's rank correlation test: $S = 1110$, $P < 0.0001$, $r = 0.92$, $n = 43$ males), and the percent difference

135 in allele sharing was correlated with the functional dissimilarity (Spearman's rank correlation test: $S = 2619$,
136 $P < 0.0001$, $r = 0.85$, $n = 47$ male-female pairs).

137

138 2.4 Statistics

139 To test for difference in females' behavioral response according to the MHC-II diversity of the male odor
140 donor and to the MHC-II dissimilarity between the female and the male odor donor, first we used a
141 generalized linear mixed model (GLMM) with the probability to peck at the odor sample (0 when the female
142 did not peck at the sample or 1 when the female pecked at the sample) as the binomial response variable.
143 Fixed effects included MHC-II dissimilarity, male MHC-II diversity and the quadratic term of male MHC-II
144 diversity. Although the MHC-II diversity of the male odor donor was negatively related to the MHC-II
145 dissimilarity to the female (Spearman rank test between the number of alleles and the percent difference in
146 allele sharing: $S = 22305$, $r = -0.29$, $P = 0.048$ and between the functional diversity and the functional
147 dissimilarity: $S = 24936$, $r = -0.44$, $P = 0.002$; $n = 47$ tests for which we have both MHC-II diversity and
148 dissimilarity), we included the two variables together in the models as the variance inflation factors were $<$
149 1.5 (Zuur et al. 2010). Fixed effects also included the difference between the date of the test and the laying
150 date, as female reaction to male MHC-II characteristics might potentially change across the breeding season
151 (Manning et al. 1992; Leclaire et al. 2017). The identity of the male odor donor was included as a random
152 effect, while the observer was not included as a random effect because its variance estimate was 0. Second,
153 for females who pecked at the samples, we used a linear mixed model (LMM) with the time taken to peck at
154 the odor sample as the response variable (log-transformed to meet normality of residuals), and the same fixed
155 effects as above. Both the observer and the identity of the male odor donors were included as random effects.

156 We performed all statistical analyses using the R statistical software (R Core Team 2019). In
157 GLMMs, we used Wald's tests to assess the significance of variables. In LMMs, significance of each
158 predictor variable was calculated using the Satterthwaite approximation as implemented in the package
159 *lmerTest* (Kuznetsova et al. 2017). Normality and homogeneity of variance were checked visually (Zuur et
160 al. 2010). Data can be found at doi.org/10.17605/OSF.IO/H2KFN.

161

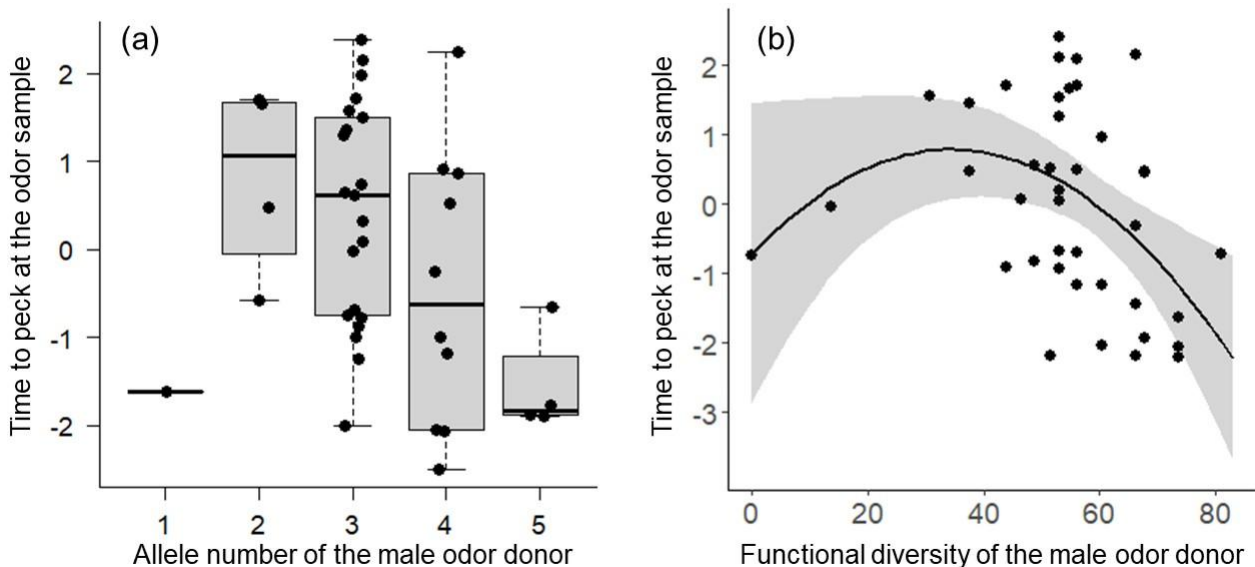
162

163 3. Results

164 When considering the tests for which we had both the MHC-II diversity of the male odor donor and the
165 MHC-II dissimilarity between the male odor donor and the female ($n = 47$ tests), seven females (15 %) did
166 not peck at the odor sample. The probability of pecking at the sample did not vary with male MHC-II
167 diversity (allele number: quadratic term: $\chi^2_1 = 0.01$, $P = 0.94$ and linear term: $\chi^2_1 = 0.17$, $P = 0.68$; functional
168 diversity: quadratic term: $\chi^2_1 = 0.01$, $P = 0.93$ and linear term: $\chi^2_1 = 0.12$, $P = 0.73$) or MHC-II dissimilarity
169 (percent difference in allele sharing: $\chi^2_1 = 0.06$, $P = 0.80$ and functional dissimilarity: $\chi^2_1 = 0.01$, $P = 0.94$).

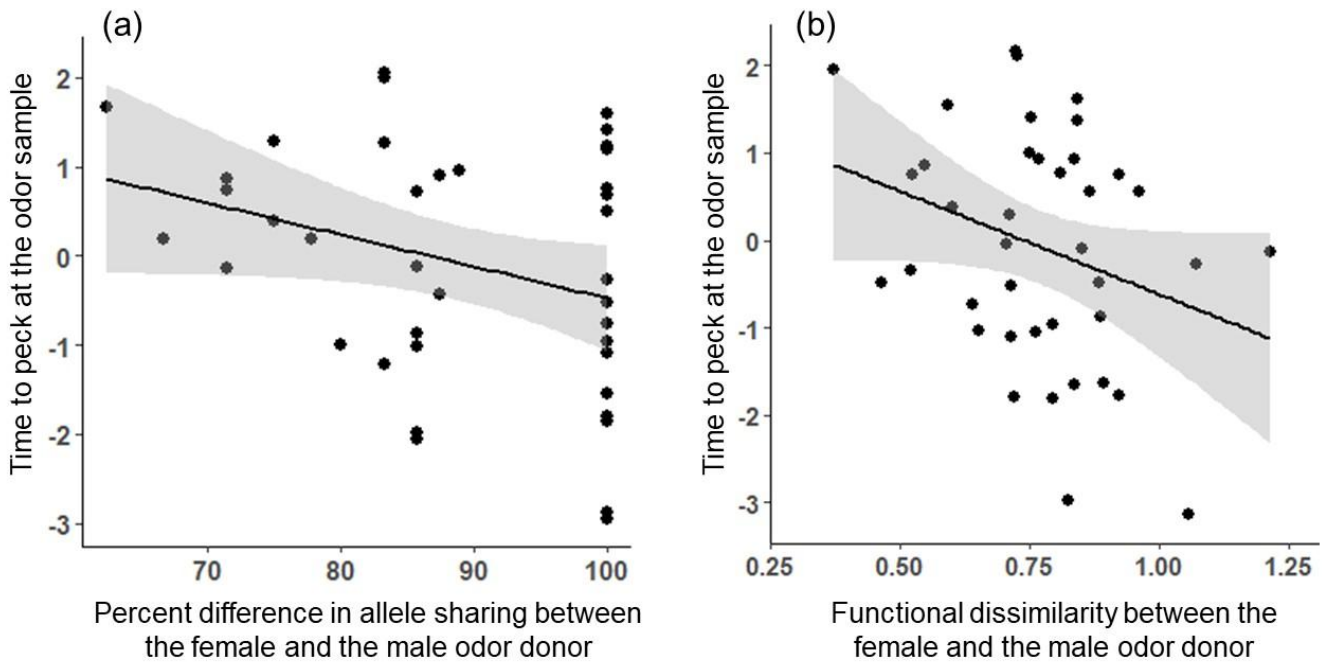
170 Among the females who pecked at the sample (n = 40 tests for which we had both the male MHC-II
 171 diversity and the MHC-II dissimilarity between male and female), females took more time to peck at the
 172 odor sample when the male had an intermediate MHC-II diversity (allele number: quadratic term: $\chi^2_1 = 5.49$,
 173 P = 0.025 and linear term: $\chi^2_1 = 6.60$, P = 0.015, Fig. 1a; functional diversity: quadratic term: $\chi^2_1 = 5.97$, P =
 174 0.020 and linear term: $\chi^2_1 = 3.91$, P = 0.056; Fig. 1b). When excluding the test where the single male odor
 175 donor had only one MHC-II allele (Fig. 1a), females took less time to peck at the odor sample when the male
 176 had higher MHC-II diversity (allele number: linear term: $\chi^2_1 = 13.73$, P < 0.001 and quadratic term: $\chi^2_1 =$
 177 0.36, P = 0.55; functional diversity: linear term: $\chi^2_1 = 6.98$, P = 0.012 and quadratic term: $\chi^2_1 = 2.43$, P =
 178 0.13; Fig. 1). This result suggests that this male carrying a single MHC-II allele drives the quadratic
 179 relationship between the time taken to peck at the sample and the MHC-II diversity. Females who pecked at
 180 the sample took also less time to peck at the odor sample when they were more MHC-II dissimilar to the
 181 male odor donor (percent difference in allele sharing: $\chi^2_1 = 6.08$, P = 0.019 and functional dissimilarity: $\chi^2_1 =$
 182 6.89, P = 0.013; Fig. 2).

183



184

185 **Fig. 1:** Time taken by females to peck at the odor sample according to (a) the number of MHC-II alleles and
 186 (b) the functional MHC-II diversity of the male odor donor. The time to peck at the sample was represented
 187 by the residuals of a linear mixed model in which the time to peck at the sample was explained by all
 188 variables except the MHC-II diversity of the male. The black line and the grey shadow in (b) represent
 189 prediction line and 95% confidence interval.



190

191 **Fig. 2:** Time taken by females to peck at the odor sample according to (a) the percent difference in MHC-II
 192 allele sharing and (b) the functional MHC-II dissimilarity to the male odor donor. The time to peck at the
 193 sample was represented by the residuals of a linear mixed model in which the time to peck at the sample was
 194 explained by all variables except the MHC-II dissimilarity. The black lines and grey shadows represent
 195 prediction line and 95% confidence interval.

196

197 **5. Discussion**

198 Using behavioral tests, we investigated whether, in black-legged kittiwakes, females recognized the MHC-II
 199 characteristics of males. First, we found that females took more time to peck at the odor sample when it came
 200 from a male with intermediate MHC-II diversity. However, the quadratic relationship between MHC-II
 201 diversity and the time taken to peck at the sample was only due to the single male who had only one MHC-II
 202 allele (Fig. 1). When excluding this male, the relationship between male MHC-II diversity and female
 203 behavior became linear. In this kittiwake population, very few individuals carry a single MHC-II allele (< 1
 204 % of the individuals; unpublished data), potentially suggesting that carrying a single MHC-II allele is
 205 counter-selected. Recently, several studies in fish, snakes and birds have tested the "golden mean hypothesis"
 206 and have shown that individuals with intermediate MHC-diversity have higher reproductive success (Kalbe
 207 et al. 2009), better immunity (Rekdal et al. 2021) and lower parasite load (Wegner et al. 2004; Madsen and
 208 Ujvari 2006). In kittiwakes, the optimum in MHC-II diversity has been tested in chicks only, and was found
 209 to be at maximum diversity in females (Pineaux et al. 2020). However, the optima in MHC diversity can
 210 vary according to individual traits that affect the trade-off between the benefits and costs associated with
 211 high MHC diversity (Roved et al. 2018; Pineaux et al. 2020). Therefore, the optimum in MHC-II diversity

212 may differ between adults and chicks, because of age-differences in exposure to parasites, acquired
213 immunity and predisposition to infection (Benskin et al. 2009). Further studies are needed to determine
214 whether, in kittiwakes, intermediate or maximum MHC-II diversity is optimum in adult males.

215 We also found that females took less time to peck at an odor sample coming from a male with higher
216 MHC-II dissimilarity. Although female pecking response cannot be interpreted as preference for certain
217 odors, our results show that female kittiwakes may use odor cues to recognize MHC-II diversity and MHC-II
218 dissimilarity. Further studies are needed to determine the contexts in which females may use this ability (e.g.,
219 mate choice, adjustment of parental investment, offspring sex-ratio distortion). Although the role of olfaction
220 in the recognition of MHC dissimilarity has been shown in several vertebrate species (reviewed in Schubert
221 et al. 2021), its role in the recognition of MHC diversity has been less studied, probably because MHC
222 diversity, by influencing health, can be reflected in any condition-dependent traits (Lie et al. 2008; Dunn et
223 al. 2013; Slade et al. 2017). However, three-spined sticklebacks (*Gasterosteus aculeatus*), ring-tailed lemurs
224 (*Lemur catta*) and song sparrows (*Melospiza melodia*) can discriminate MHC diversity using odor cues
225 (Milinski et al. 2005; Grieves et al. 2019; Grogan et al. 2019).

226 In birds, the sources of the body odors used in social communication are unknown. Here, we tested
227 whether females could discriminate male MHC-II characteristics using a mixture of odor sources (i.e.,
228 cloacal samples mixed with nest material), thereby preventing us to identify precisely the sources of MHC-
229 related odors. In kittiwakes, preen secretions encode information about MHC-II dissimilarity (Leclaire et al.
230 2014), but studies are required to determine the role of other sources such as feces or plumage microbiota
231 (Leclaire et al. 2019; Schubert et al. 2021). In addition, while our results are consistent with odor-based
232 discrimination of MHC-II characteristics, our methods do not allow us to determine whether discrimination
233 is based on broader genome-wide genetic variation or on other polymorphic genes whose variations covary
234 with those in the MHC, such as the major urinary protein (MUP) genes in natural populations of mice
235 (Sherborne et al. 2007).

236 Altogether, our results, by adding evidence for olfactory discrimination of MHC characteristics in
237 birds, suggest that this capacity may be widespread in this taxon. However, whether birds use it in mate
238 choice or more generally to maximize fitness has still to be demonstrated.

239

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244

245

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