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A GULL SPECIES RECOGNIZES MHC-II DIVERSITY AND DISSIMILARITY USING ODOR CUES

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Abstract

The major histocompatibility complex (MHC) plays a crucial role in resistance to parasite, and is thus often suggested to be an important force driving social interactions, including mating preference, in vertebrates. However, the phenotypic cues used by individuals to assess the MHC characteristics of conspecifics is generally unknown. Here we used behavioral tests to investigate whether, in black-legged kittiwakes, females use odor cues to distinguish male MHC-II diversity and MHC-II dissimilarity. We found that females took less time to peck at the odor sample when it came from a male with high MHC-II dissimilarity. They also took less time to peck at an odor sample coming from a male with high or low MHC-II diversity compared to intermediate MHC-II diversity. However, this result is due to the single individual who has only one MHC-II allele. When excluding this individual, females took less time to peck at an odor sample coming from a male with high MHC-II diversity. Altogether, our study adds evidence for olfactory recognition of MHC characteristics in birds, but further studies are required to determine whether kittiwakes use this ability to optimize fitness.

Keywords: Birds; Olfaction; Sexual selection; Intraspecific communication; Major histocompatibility complex; Black-legged kittiwake; *Rissa tridactyla*

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 (Abankwah et al. 2020; Caro and Balthazart 2010; 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, an important force driving mate choice in birds is the major
36 histocompatibility complex (MHC) (Zelano and Edwards 2012; Løvlie et al. 2013), a polymorphic cluster of
37 genes that plays a critical role in immunity. In general, higher MHC diversity is considered to provide
38 resistance to a wider range of parasites and to increase fitness (Milinski 2006). Accordingly, species from
39 diverse taxa have therefore developed MHC-based mate choice, such as mate choice for MHC-dissimilar
40 partners (i.e., MHC-compatible partners), thereby avoiding the production of offspring with low MHC
41 diversity (Strandh et al. 2012; Kamiya et al. 2014; Hoover et al. 2018) or mate choice for MHC-diverse
42 partners, which may provide both genetic and non-genetic benefits (Richardson et al. 2005; Griggio et al.
43 2011). However, high MHC diversity may also be associated with costs, such as an increased risk of
44 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,
51 while several mammals, lizards or fish have been shown to assess MHC characteristics using odor cues
52 (reviewed in Schubert et al. 2021), this ability in birds has been poorly explored. To the best of our
53 knowledge, only the blue petrel (*Halobaena caerulea*), a Procellariiform seabird, and the song sparrow
54 (*Melospiza melodia*) and the house sparrow (*Passer domesticus*), two Passeriform birds, have been tested.
55 While blue petrels and song sparrows were shown to discriminate MHC-II characteristics using odor cues
56 (Leclaire et al. 2017; Grieves et al. 2019), house sparrows did not discriminate MHC-I characteristics using
57 olfaction (Amo et al. 2022). To estimate the taxonomic range of olfactory assessment of MHC in birds, more
58 avian taxa need to be studied.

59 The black-legged kittiwake (*Rissa tridactyla*) is a genetically monogamous Charadriiform seabird
60 which has 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 varied with the MHC
62 dissimilarity between parents (Pineaux et al. 2022), suggesting that kittiwakes might be able to recognize
63 the MHC characteristics of conspecifics. Here, we used behavioral tests to investigate whether female
64 kittiwakes can use odor cues to distinguish the MHC characteristics of males. We used a protocol inspired
65 by Leclaire et al. (2009), which showed that kittiwakes discriminate between several odors deposited in
66 their nest. Our test is based on the pecking behavioral response of incubating females to an odor cue put in
67 their nest. Results cannot therefore be interpreted as female preference. However, a differential response
68 to different odors will indicate recognition. Because the MHC-based mating pattern of kittiwake is
69 unknown, we tested recognition of both MHC diversity and MHC dissimilarity, and always included both the
70 linear and the quadratic term of MHC diversity.

71

72 **2. Materials and methods**

73 *2.1 Study site*

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

78

79 *2.2 Behavioral tests*

80 We assessed female's response to male odor samples placed on the edge of the nest bowl. Odor samples
81 were a mixture of cloacal samples (potentially related to fecal odor) and nest materials (potentially related
82 to fecal odor deposited on the nest during defecation and fecal, skin, plumage and preen gland odor
83 passively deposited on the nest when birds are sitting). We collected odor samples from 43 incubating
84 males. After capture, we collected cloacal samples as in Leclaire et al. (2022). Briefly, we injected 1 mL of
85 sterile phosphate-buffered saline solution into male's cloaca with a sterile needleless syringe and drew the
86 solution out. At the time of capture, a piece of nest material (i.e., a blend of mosses and grasses of ca. 4 cm
87 diameter) was also collected from the nest of the captured male, using clean nitrile gloves. The piece of
88 nest material was immediately mixed with the cloacal sample and stored in plastic bags at -20°C until the
89 test. Odor samples were stored on average for 17 ± 1 days (range: 8 – 24 days).

90 Fifty two incubating females were each tested once. They were never tested with the odor of their
91 partner. All odor samples (n = 43) were used at least once. Just before the test, a small piece (ca. 2 cm
92 diameter) of a random odor sample was defrosted for 15 minutes. The remaining odor sample was kept
93 frozen. None of these remaining odor samples were used in tests later on. The odor sample was placed on
94 the edge of the nest bowl, just under the beak of an incubating female. When placing the odor sample on

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

103

104 *2.3 MHC analyses*

105 Details about MHC analyses are given in Pineaux et al. (2020). Briefly, DNA was extracted from blood
106 samples, and 258 pb fragments of exon 2 of MHC class-II β were amplified using PCR and sequenced with an
107 Illumina MiSeq platform. Amplicon sequences were analyzed with AmpliSAS (Sebastian et al. 2016). We
108 focused on the amino acid sequences of the peptide binding region (Leclaire et al. 2014). MHC-II diversity
109 was estimated using the number of MHC-II alleles. MHC-II dissimilarity between the female and the
110 sampled male was estimated on a pure shared/non-shared allele basis. MHC-II dissimilarity was calculated
111 as $TOT_{FM} / (N_F + N_M)$, where TOT_{FM} = total number of variable alleles present in the male and the female
112 (shared allele count as 1) and N = number of alleles within an individual, which represents the percent
113 difference in allele sharing (Strandh et al. 2012). Because five females were not genotyped at the MHC-II,
114 we could not calculate the MHC-II dissimilarity between the female and the sampled male for five tests.

115

116 *2.4 Statistics*

117 To test for difference in females' behavioral response according to the MHC-II diversity of the male odor
118 donor and to the MHC-II dissimilarity between the female and the male odor donor, first we used a
119 generalized linear mixed model (GLMM) with the probability to peck at the odor sample (0 when the
120 female did not peck at the sample or 1 when the female pecked at the sample) as the binomial response
121 variable. Fixed effects included MHC-II dissimilarity, male MHC-II diversity and the quadratic term of male
122 MHC-II diversity. Although the MHC-II diversity of the male odor donor was negatively related to the MHC-II
123 dissimilarity to the female (Spearman rank test: $S = 22305$, $r = -0.29$, $P = 0.048$, $n = 47$ tests), we included
124 the two variables together in the models as the variance inflation factors were < 1.5 (Zuur et al. 2010).
125 Fixed effects also included the difference between the date of the test and the laying date, as female
126 reaction to male MHC-II characteristics might potentially change across the breeding season (Maning et al.
127 1992; Leclaire et al. 2017). The observer and the identity of the male odor donor were included as random
128 effects. Second, for females who pecked at the samples, we used a linear mixed model (LMM) with the

129 time taken to peck at the odor sample as the response variable (log-transformed to meet normality), and
130 the same fixed and random effects as above. The time the odor sample was kept in the freezer tended to
131 have a negative effect on the time taken to peck at the sample (Spearman's correlation test: $S = 13678$, $P =$
132 0.077 , $r = -0.28$, $n = 40$ tests). However, we decided not to include this variable in the final models to avoid
133 over-parameterization of the models and to increase the sample size (we did not record the date of sample
134 collection for 4 tests). Including this variable in the models did not change the results.

135 We performed all statistical analyses using the R statistical software (R Core Team 2019). In all
136 models, we assessed significance of each predictor variable by the change in deviance after removal of that
137 variable (Likelihood-Ratio Test) using a chi-square test. Normality and homogeneity of variance were
138 checked visually (Zuur et al. 2010).

139

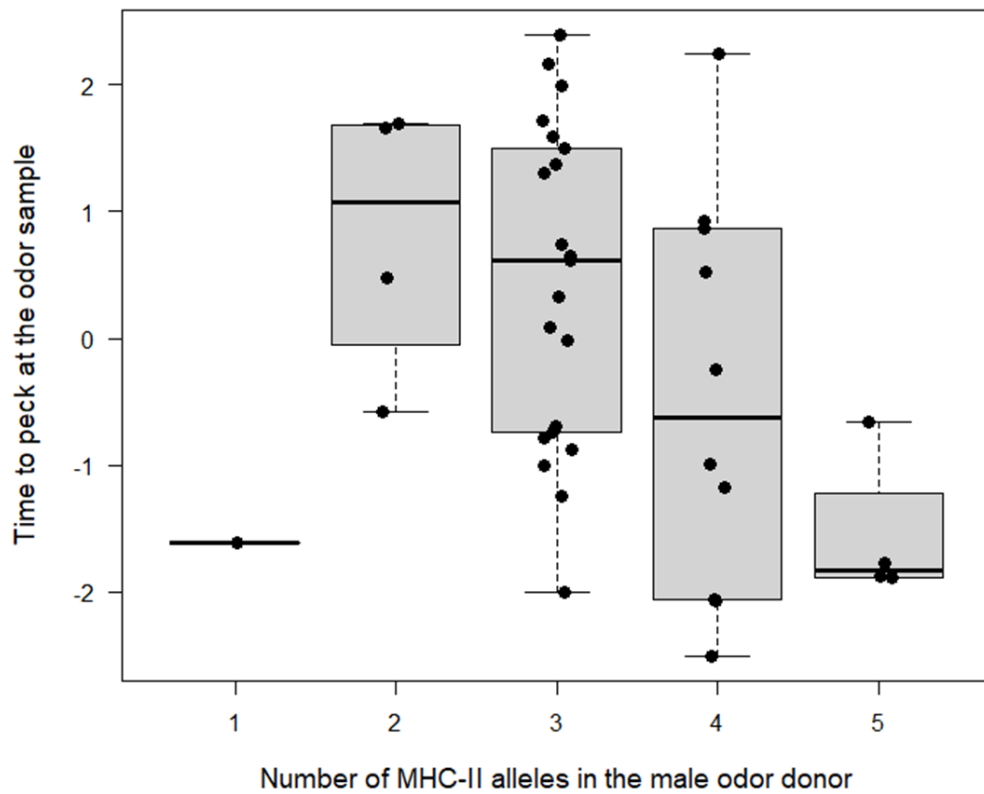
140

141 **3. Results**

142 8 females (18 %) did not peck at the odor sample. The probability of pecking at the sample did not vary with
143 male MHC-II diversity (quadratic term: $\chi^2_1 = 0.02$, $P = 0.88$ and linear term: $\chi^2_1 = 0.11$, $P = 0.73$) or MHC-II
144 dissimilarity ($\chi^2_1 = 0.001$, $P = 0.97$).

145 Among the females that pecked at the sample, females took more time to peck at the odor sample
146 when the male had an intermediate number of MHC-II alleles (quadratic term: $\chi^2_1 = 5.87$, $P = 0.015$ and
147 linear term: $\chi^2_1 = 6.88$, $P = 0.009$; Fig. 1). When excluding the test where the single male odor donor had
148 only one MHC-II allele (Fig. 1), females took less time to peck at the odor sample when the male had a
149 higher number of functional MHC-II alleles (linear term: $\chi^2_1 = 12.87$, $P < 0.001$ and quadratic term: $\chi^2_1 =$
150 0.41 , $P = 0.52$; Fig. 1). This result suggests that this male carrying a single MHC-II allele drives the quadratic
151 relationship between the time taken to peck at the sample and the MHC-II diversity. Similar results were
152 obtained when excluding the MHC-II dissimilarity between the female and the male odor donor from the
153 models, thereby increasing the sample size by 4 tests ($n = 44$ tests). Females that pecked at the sample took
154 also less time to peck at the odor sample when they were more MHC-II dissimilar to the male odor donor
155 ($\chi^2_1 = 6.40$, $P = 0.011$; Fig. 2).

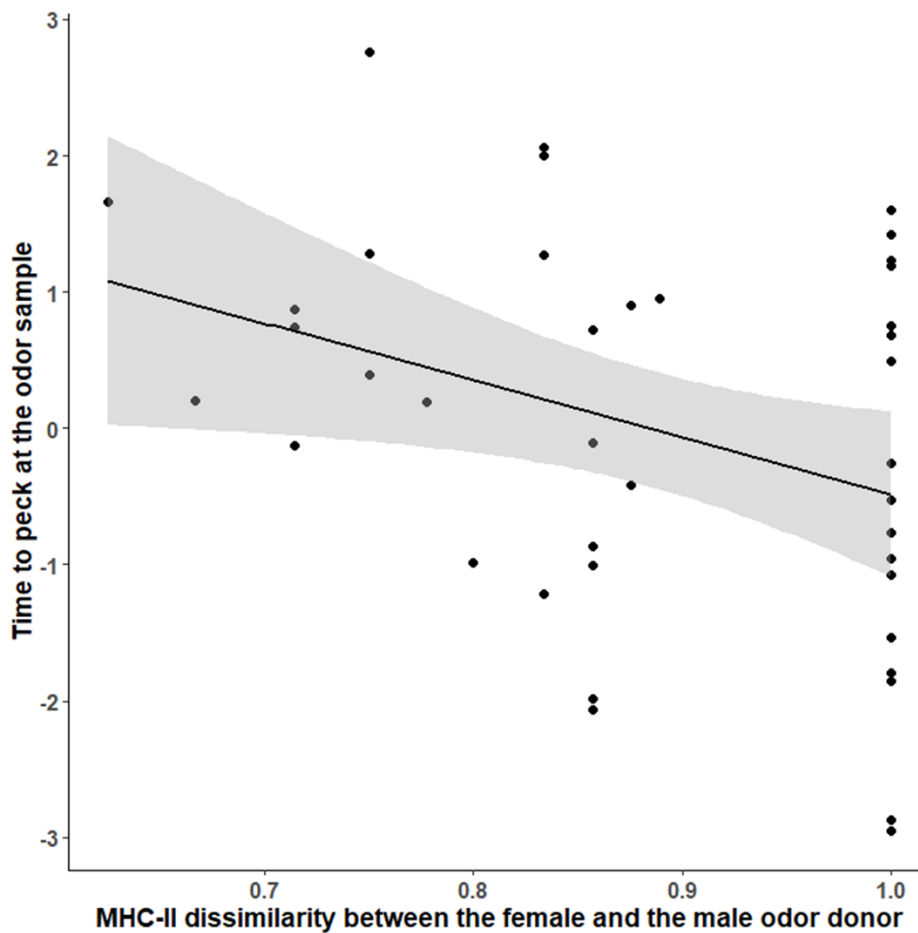
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157

158

159 **Fig. 1:** Time taken by females to peck at the odor sample according to the number of MHC-II alleles of the
 160 male odor donor (i.e., the MHC-II diversity of the male). The 8 females that did not peck at the odor sample
 161 during the 15-minutes test were excluded. The time to peck at the sample was represented by the residuals
 162 of a linear mixed model in which the time to peck at the sample was explained by all variables except the
 163 MHC-II diversity of the male.



164

165

166 **Fig. 2:** Time taken by females to peck at the odor sample according to the MHC-II dissimilarity to the male
 167 odor donor. The 8 females that did not peck at the odor sample during the 15-minutes test were excluded.
 168 The time to peck at the sample was represented by the residuals of a linear mixed model in which the time
 169 to peck at the sample was explained by all variables except the MHC-II dissimilarity. Similar results were
 170 obtained when the MHC-II dissimilarity was coded as a binomial variable (i.e., 0 for pairs that shared at
 171 least one allele and 1 for pairs that shared no allele). The black line and the grey shadow represent
 172 prediction line and 95% confidence interval.

173

174 **5. Discussion**

175 Using behavioral tests, we investigated whether, in black-legged kittiwakes, females recognized the MHC-II
 176 characteristics of males. First, we found that females took more time to peck at the odor sample when it
 177 came from a male with intermediate MHC-II diversity. However, the quadratic relationship between MHC-II
 178 diversity and the time taken to peck at the sample was only due to the single male that had only one MHC-
 179 II allele (Fig. 1). When excluding this male, the relationship between male MHC-II diversity and female
 180 behavior became linear. In this kittiwake population, very few individuals carry a single MHC-II alleles (< 1 %

181 of the individuals; unpublished data), potentially suggesting that carrying a single MHC-II allele is counter-
182 selected. More tests including male odor donors carrying a single MHC-II allele would have been needed to
183 ascertain the quadratic nature of the relationship between male MHC-II diversity and the female's reaction
184 to the odor sample. Recently, several studies in fish, snakes and birds have tested the "golden mean
185 hypothesis" and have shown that individuals with intermediate MHC-diversity have higher reproductive
186 success (Kalbe et al. 2009), better immunity (Rekdal et al. 2021) and lower parasite load (Wegner et al.
187 2004; Madsen and Ujvari 2006). In kittiwakes, the optimum in MHC-II diversity has been tested in chicks
188 only, and was found to be maximum in females (Pineaux et al. 2020). However, the optima in MHC-
189 diversity can vary according to individual traits that affect the trade-off between the benefits and costs
190 associated with high MHC-diversity (Roved et al. 2018; Pineaux et al. 2020). Therefore, the optimum in
191 MHC-II diversity may differ between adults and chicks, because of age-differences in exposure to parasites,
192 acquired immunity and predisposition to infection (Benskin et al. 2009). Studies are needed to determine
193 whether, in kittiwakes, intermediate or maximum MHC-II diversity is optimum in adult males.

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

205 In birds, the sources of the body odors used in social communication are unknown. Here, we tested
206 whether females could discriminate male MHC-II characteristics using a mixture of odor sources (*i.e.*,
207 cloacal samples mixed with nest material), thereby preventing us to identify precisely the sources of MHC-
208 related odors. In kittiwakes, preen secretion encode information about MHC-II dissimilarity (Leclaire et al.
209 2014), but studies are required to determine the role of other sources such as feces or plumage microbiota
210 (Leclaire et al. 2019; Schubert et al. 2021). In addition, while our results are consistent with odor-based
211 discrimination of MHC-II characteristics, our methods do not allow us to determine whether discrimination
212 is based on broader genome-wide genetic variation or on other polymorphic genes whose variations covary
213 with those in the MHC, such as the major urinary protein (MUP) genes in natural populations of mice
214 (Sherborne et al. 2007).

215

216 Altogether, our results, by adding evidence for olfactory discrimination of MHC characteristics in
217 birds, suggest that this capacity may be widespread in this taxa. However, whether birds use it in mate
218 choice or more generally to optimize fitness has still to be demonstrated.

219

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224

225

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