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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 was  
29 long thought that birds had little or no sense of smell and relied primarily on visual or acoustic cues. However,  
30 there is now abundant evidence that birds have a functional olfactory system (Bang and Cobb 1968; Steiger et  
31 al. 2008) and use olfaction in various non-social contexts, including foraging, navigation or nest sanitation  
32 (e.g., Caro and Balthazart 2010; Abankwah et al. 2020; Potier 2020; Bonadonna and Gagliardo 2021). In  
33 contrast, the role of olfactory cues in avian social life is still poorly known (Caro et al. 2015), albeit being a  
34 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 in  
52 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 song  
55 sparrows were shown to discriminate MHC-II characteristics using odor cues (Leclaire et al. 2017; Grieves et  
56 al. 2019), house sparrows did not discriminate MHC-I characteristics using olfaction (Amo et al. 2022). To  
57 estimate the taxonomic range of olfactory assessment of MHC in birds, more avian taxa need to be studied.

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

63 odor cues to distinguish the MHC characteristics of males. We used a protocol inspired by Leclaire et al.  
64 (2009), which showed differential pecking response of incubating females to different odor samples (skunk,  
65 banana, fish or control odor) put in their nest. Here, we put a single odor sample of a male to the nest of an  
66 incubating female and assessed her behavioral response. Although differential response of females cannot be  
67 interpreted as female preference, we consider that the fact that different females react on average differently  
68 along a continuum of males' MHC characteristics indicate discrimination. Because the MHC-based mating  
69 pattern of kittiwake is unknown, we tested recognition of both MHC diversity and MHC dissimilarity, and  
70 always included both the 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). This experiment was conducted under the approval of the USGS Alaska  
78 Science Centre Animal Care and Use Committee, in accordance with US law and under permits from the US  
79 Fish and Wildlife Service and the State of Alaska.

80

### 81 *2.2 Behavioral tests*

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

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

99 just under the beak of an incubating female (Figure 1). Because we had to open the sliding window of the  
100 nesting site to put the odor sample on the nest, all females, except one, took off. Behaviors were recorded by  
101 an observer as soon as the female landed back on her nest. Females returned to their nest on average  $\pm$  se: 14  
102  $\pm$  5 sec after the odor sample was placed on the nest (range: 0-462 sec). For each test, we recorded the time  
103 taken by the female to peck at the odor sample for the first time. 93 % of the females who pecked at the odor  
104 sample took the sample with their bill and moved it along the nest bowl, as they often do with nest material  
105 when maintaining the nest structure. We therefore also recorded the time taken by the female to move the odor  
106 sample for the first time. Observation lasted until the female pecked at the odor sample or for 15 min when she  
107 did not peck at the odor sample. Tests were carried out by 3 observers who were blind to MHC-characteristics  
108 of the males concerned.



109

110 **Figure 1:** Picture of a kittiwake's nest in which an odor sample (encircled in red) was put on the edge of the  
111 nest. This picture was taken during a preliminary test on a non-incubating bird. However, in all tests considered  
112 in this study, the female was incubating.

113

### 114 2.3 MHC analyses

115 Details about MHC analyses are given in Pineaux et al. (2020). Briefly, DNA was extracted from blood  
116 samples, and 258 bp fragments of exon 2 of MHC class-IIB were amplified using PCR and sequenced in 4  
117 runs with an Illumina MiSeq platform. Amplicon sequences were analyzed with AmpliSAS (substitution  
118 errors: 1%, indel errors: 0.001%, minimum frequency with respect to dominant: 33%, minimum amplicon  
119 sequence frequency: 2.5%) (Sebastian et al. 2016). Considering the larger set of samples analysed in the 4  
120 Illumina MiSeq runs, we detected a maximum of 6 alleles per individual, suggesting the presence of a  
121 minimum of 3 MHC class-IIB loci. We focused on the amino acid sequences of the peptide binding region  
122 (Leclaire et al. 2014). MHC-IIB diversity and MHC-IIB dissimilarity were each estimated using two indices.  
123 First, MHC-IIB diversity was estimated using the number of MHC-IIB alleles, while MHC-IIB dissimilarity  
124 between the female and the sampled male was estimated on a pure shared/non-shared allele basis. The percent

125 difference in MHC-IIB allele sharing was calculated as  $100 * \text{TOT}_{\text{FM}} / (\text{N}_{\text{F}} + \text{N}_{\text{M}})$ , where  $\text{TOT}_{\text{FM}}$  = total number  
126 of different alleles present in the male and the female (shared allele count as 1) and  $\text{N}$  = number of alleles  
127 within an individual (Strandh et al. 2012). Second, we estimated MHC-IIB diversity as the degree of functional  
128 diversity (or divergence) of alleles across all loci, and MHC-IIB dissimilarity as the functional distance  
129 between two individuals. We described the chemical binding properties of each amino acid in the PBR with  
130 Sandberg's five physicochemical descriptors (z-descriptors; Sandberg et al. 1998). This Sandberg's matrix was  
131 used to construct a UPGMA dendrogram of alleles (function `hclust` in R), which represents clusters of  
132 functionally similar MHC-IIB sequences. The dendrogram was used as a reference to calculate the functional  
133 MHC-IIB diversity of an individual as the minimum total length of all the branches required to span its MHC-  
134 IIB alleles (function `treedive` in package *vegan*) (Petchey and Gaston 2006). MHC-IIB functional dissimilarity  
135 between the male odor donor and the female was estimated using the tree distance (function `treedist` in package  
136 *vegan*), which is found by combining species in a common dendrogram and seeing how much of the  
137 dendrogram height is shared and how much is unique. Because five females were not genotyped at the MHC-  
138 IIB, we could not calculate the MHC-IIB dissimilarity between the female and the sampled male for five tests.  
139 The number of alleles was correlated with the functional diversity (Spearman's rank correlation test:  $S = 1110$ ,  
140  $P < 0.0001$ ,  $r = 0.92$ ,  $n = 43$  males), and the percent difference in allele sharing was correlated with the  
141 functional dissimilarity (Spearman's rank correlation test:  $S = 2619$ ,  $P < 0.0001$ ,  $r = 0.85$ ,  $n = 47$  male-female  
142 pairs).

143

#### 144 2.4 Statistics

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

161 identity of the male odor donors were included as random effects. Third, for females who moved the odor  
162 sample, we used a binomial GLMM model to test whether the probability to delay the move of the odor sample  
163 after the first pecking (0 when the female immediately moved the sample after the first pecking or 1 when the  
164 female delayed the move of the sample) varied with MHC-II characteristics. Fixed and random effects were  
165 similar as above.

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

171

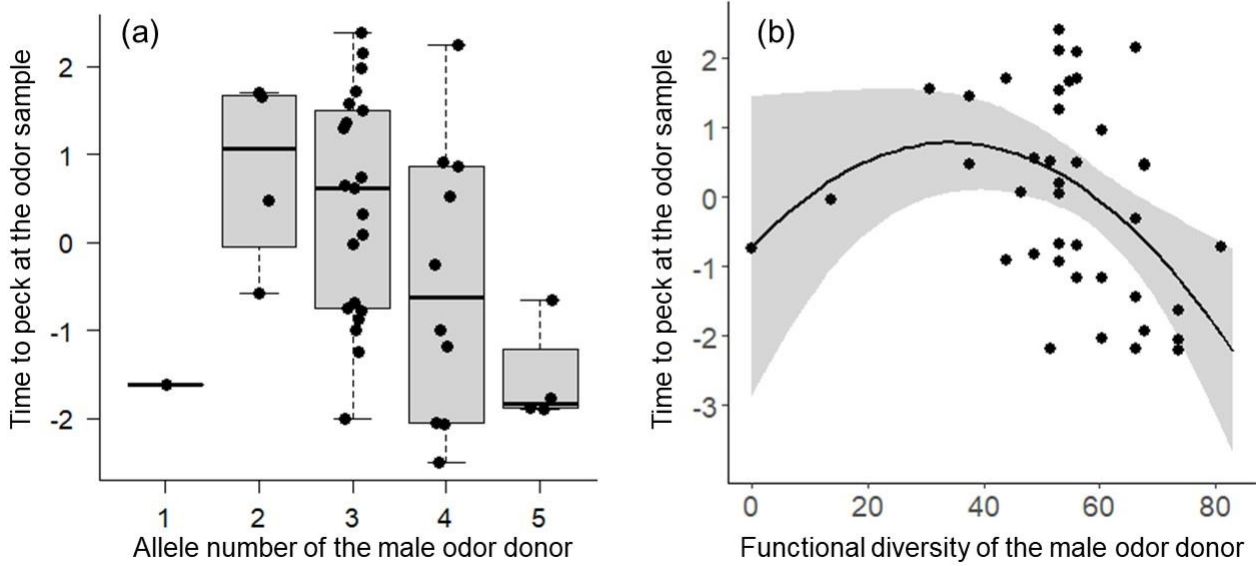
172

### 173 3. Results

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

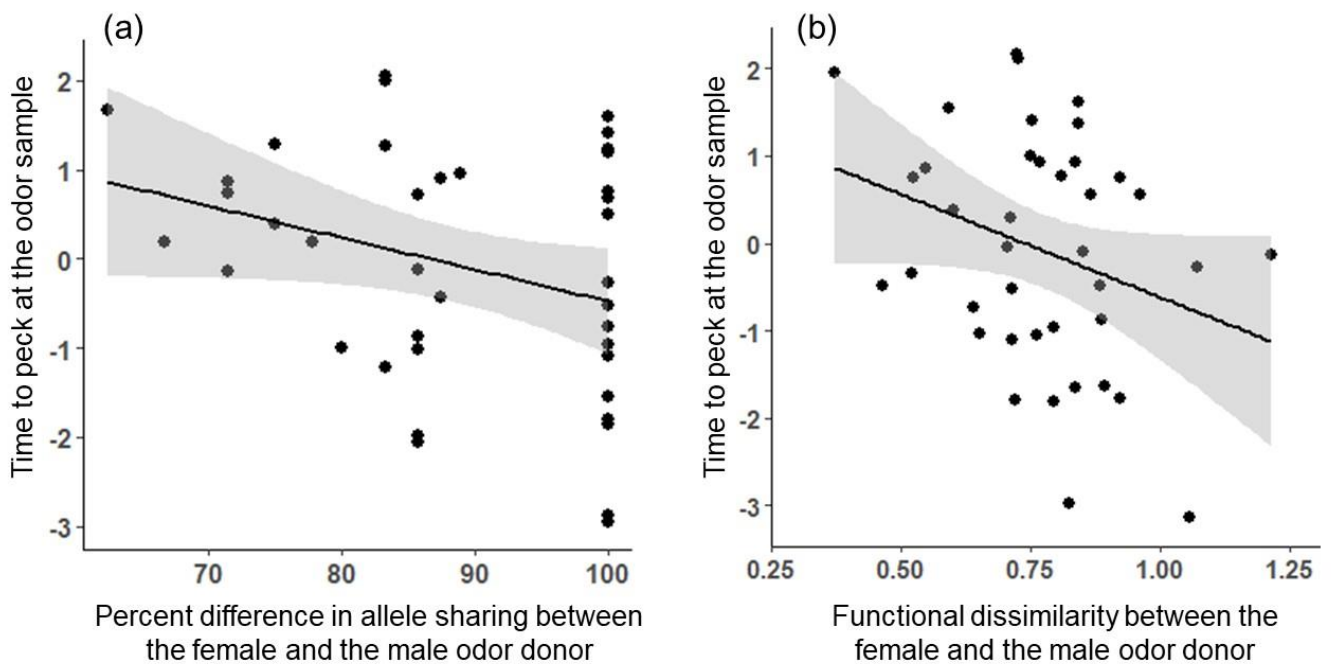
180 Among the females who pecked at the sample (n = 40 tests for which we had both the male MHC-II  
181 diversity and the MHC-II dissimilarity between male and female), females took more time to peck at the odor  
182 sample when the male had an intermediate MHC-II diversity (allele number: quadratic term:  $\chi^2_1 = 5.49$ , P =  
183 0.025 and linear term:  $\chi^2_1 = 6.60$ , P = 0.015, Figure 2a; functional diversity: quadratic term:  $\chi^2_1 = 5.97$ , P =  
184 0.020 and linear term:  $\chi^2_1 = 3.91$ , P = 0.056; Figure 2b). When excluding the test where the single male odor  
185 donor had only one MHC-II allele (Fig. 2a), females took less time to peck at the odor sample when the male  
186 had higher MHC-II diversity (allele number: linear term:  $\chi^2_1 = 13.73$ , P < 0.001 and quadratic term:  $\chi^2_1 = 0.36$ ,  
187 P = 0.55; functional diversity: linear term:  $\chi^2_1 = 6.98$ , P = 0.012 and quadratic term:  $\chi^2_1 = 2.43$ , P = 0.13; Figure  
188 2). This result suggests that this male carrying a single MHC-II allele drives the quadratic relationship between  
189 the time taken to peck at the sample and the MHC-II diversity. Females who pecked at the sample took also  
190 less time to peck at the odor sample when they were more MHC-II dissimilar to the male odor donor (percent  
191 difference in allele sharing:  $\chi^2_1 = 6.08$ , P = 0.019 and functional dissimilarity:  $\chi^2_1 = 6.89$ , P = 0.013; Figure 3).

192



193

194 **Figure 2:** Time taken by females to peck at the odor sample according to (a) the number of MHC-II alleles  
 195 and (b) the functional MHC-II diversity of the male odor donor. The time to peck at the sample was represented  
 196 by the residuals of a linear mixed model in which the time to peck at the sample was explained by all variables  
 197 except the MHC-II diversity of the male. The black line and the grey shadow in (b) represent prediction line  
 198 and 95% confidence interval.



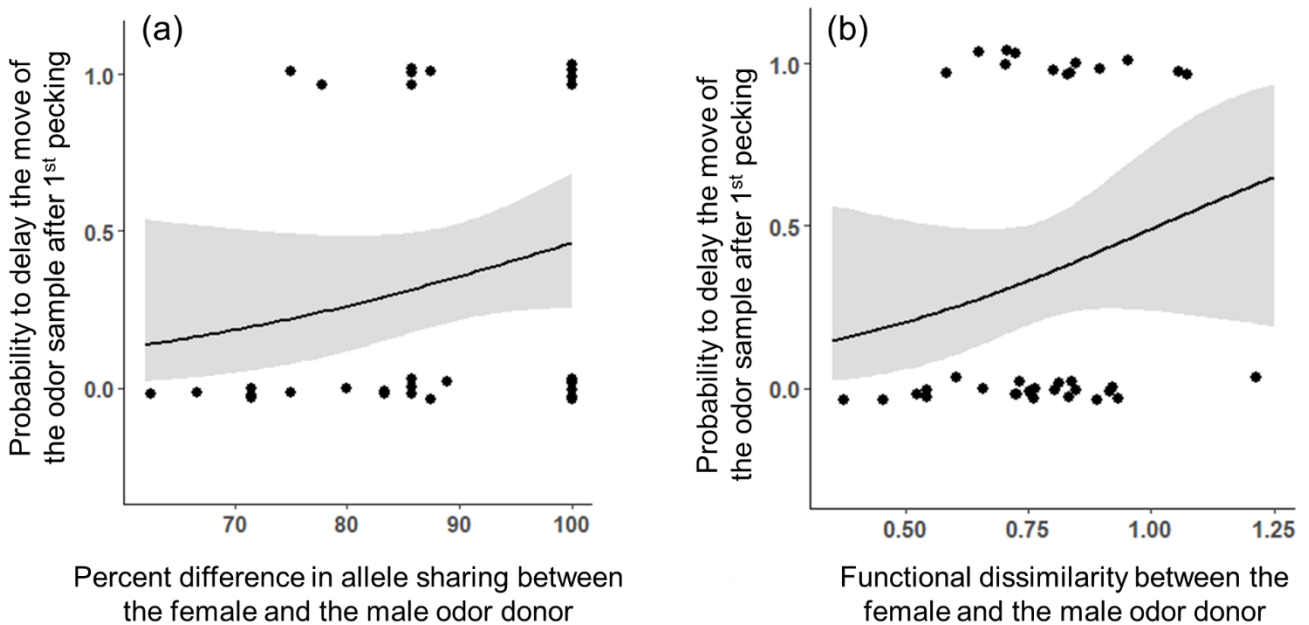
199



200 **Figure 3:** Time taken by females to peck at the odor sample according to (a) the percent difference in MHC-  
 201 II allele sharing and (b) the functional MHC-II dissimilarity to the male odor donor. The time to peck at the  
 202 sample was represented by the residuals of a linear mixed model in which the time to peck at the sample was  
 203 explained by all variables except the MHC-II dissimilarity. The black lines and grey shadows represent  
 204 prediction line and 95% confidence interval.

205

206 93 % of the females who pecked at the odor sample took the sample with their bill and moved it along  
 207 the nesting bowl (n = 37 tests for which we have both the MHC-II diversity and MHC\_II dissimilarity). 65%  
 208 of those females (n = 24 females) moved the sample just after the first pecking, while the others (n = 13  
 209 females) delayed the move of the odor sample. The probability to delay the move of the odor sample after the  
 210 first pecking increased with the dissimilarity to the male odor donor (percent difference in allele sharing:  $\chi^2_1 =$   
 211 5.39, P = 0.020 and functional dissimilarity:  $\chi^2_1 = 8.35$ , P = 0.004; Figure 4). The probability to delay the move  
 212 of the odor sample did not vary with the MHC-II diversity of the male odor donor (all P values > 0.10).



213

214 **Figure 4:** Probability for the females to delay the move of the odor sample after the first pecking (0: the female  
 215 moved the sample just after the first pecking and 1: the female delayed the move of the sample after the first  
 216 pecking) according to (a) the percent difference in MHC-II allele sharing and (b) the functional MHC-II  
 217 dissimilarity to the male odor donor. The black lines and grey shadows represent prediction line and 95%  
 218 confidence interval of a GLMM between the probability to delay the move of the sample after the first pecking  
 219 and the MHC-II dissimilarity to the male odor donor.

220

## 221 5. Discussion

222 Using behavioral tests, we investigated whether, in black-legged kittiwakes, females discriminated the MHC-  
223 II characteristics of males. First, we found that females took more time to peck at the odor sample when it  
224 came from a male with intermediate MHC-II diversity. However, the quadratic relationship between MHC-II  
225 diversity and the time taken to peck at the sample was only due to the single male who had only one MHC-II  
226 allele (Fig. 1). When excluding this male, the relationship between male MHC-II diversity and female behavior  
227 became linear. Clearly, more tests including male odor donors carrying a single MHC-II allele would have  
228 been needed to ascertain the quadratic nature of the relationship between male MHC-II diversity and the  
229 female's reaction to the odor sample. However, in this kittiwake population, very few individuals carry a single  
230 MHC-II allele (< 1 % of the individuals; unpublished data), suggesting that carrying a single MHC-II allele is  
231 counter-selected. Recently, several studies in fish, snakes and birds have tested the "golden mean hypothesis"  
232 and have shown that individuals with intermediate MHC-diversity have higher reproductive success (Kalbe et  
233 al. 2009), better immunity (Rekdal et al. 2021) and lower parasite load (Wegner et al. 2004; Madsen and Ujvari  
234 2006). In kittiwakes, the optimum in MHC-II diversity has been tested in chicks only, and was found to be at  
235 maximum diversity in female chicks (Pineaux et al. 2020). However, the optima in MHC diversity can vary  
236 according to individual traits that affect the trade-off between the benefits and costs associated with high MHC  
237 diversity (Roved et al. 2018; Pineaux et al. 2020). Therefore, the optimum in MHC-II diversity may differ  
238 between adults and chicks, because of age-differences in exposure to parasites, acquired immunity and  
239 predisposition to infection (Benskin et al. 2009). Further studies are needed to determine whether, in  
240 kittiwakes, intermediate or maximum MHC-II diversity is optimum in adult males.

241 We also found that females took less time to peck at an odor sample coming from a male with higher  
242 MHC-II dissimilarity. Almost all females who pecked at the odor sample moved it along the nesting bowl, as  
243 they commonly do with nest material during nest maintenance. However, while some females moved the odor  
244 sample just after the first pecking, other delayed the move of the sample. We found that the probability to delay  
245 the move of the odor sample was higher when the odor sample came from a male with higher MHC-II  
246 dissimilarity. Female pecking response and sample moving response cannot be interpreted as preference or  
247 aversion for certain odors. However, our results show that female kittiwakes may use odor cues to discriminate  
248 MHC-II diversity and MHC-II dissimilarity. Further studies are needed to determine the contexts in which  
249 females may use this ability (e.g., mate choice, adjustment of parental investment, offspring sex-ratio  
250 distortion). Although the role of olfaction in the recognition of MHC dissimilarity has been shown in several  
251 vertebrate species (reviewed in Schubert et al. 2021), its role in the recognition of MHC diversity has been less  
252 studied, probably because MHC diversity, by influencing health, can be reflected in any condition-dependent  
253 traits (Lie et al. 2008; Dunn et al. 2013; Slade et al. 2017). However, three-spined sticklebacks (*Gasterosteus*  
254 *aculeatus*), ring-tailed lemurs (*Lemur catta*) and song sparrows (*Melospiza melodia*) can discriminate MHC  
255 diversity using odor cues (Milinski et al. 2005; Grieves et al. 2019; Grogan et al. 2019).

256 In birds, preen gland secretions are often suggested to be the main source of body odors and have been  
257 shown to be used in social communication in a few species (Hirao et al. 2009, Zhang et al. 2010). However,  
258 in most birds, the sources of body odors are unknown (Hagelin and Jones 2007). Here, we tested whether

259 females could discriminate male MHC-II characteristics using a mixture of odor sources (i.e., cloacal samples  
260 mixed with nest material), thereby preventing us to identify precisely the sources of MHC-related odors. In  
261 kittiwakes, preen secretions encode information about MHC-II dissimilarity (Leclaire et al. 2014), but studies  
262 are required to determine the role of other sources such as feces, skin or plumage microbiota (Leclaire et al.  
263 2019; Schubert et al. 2021). For instance, in kittiwakes, the outside edge of the nest is usually full of feces  
264 suggesting that fecal odor might play a role in communication. In addition, while our results are consistent  
265 with odor-based discrimination of MHC-II characteristics, our methods do not allow us to determine whether  
266 discrimination is based on broader genome-wide genetic variation or on other polymorphic genes whose  
267 variations covary with those in the MHC, such as the major urinary protein (MUP) genes in natural populations  
268 of mice (Sherborne et al. 2007). Finally, the development of inventive odor discrimination tests based on more  
269 relevant behavioral responses is key to better appreciate the contexts in which social odor discrimination  
270 abilities can be used in kittiwakes, but also more generally in non-burrowing large wild birds.

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

274

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279

280

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