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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  $17 \pm 1$  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:  $14 \pm 5$  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  $\text{TOT}_{\text{FM}}$  =  
120 total number of different alleles present in the male and the female (shared allele count as 1) and  $\text{N}$  = 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](https://doi.org/10.17605/OSF.IO/H2KFN).

161

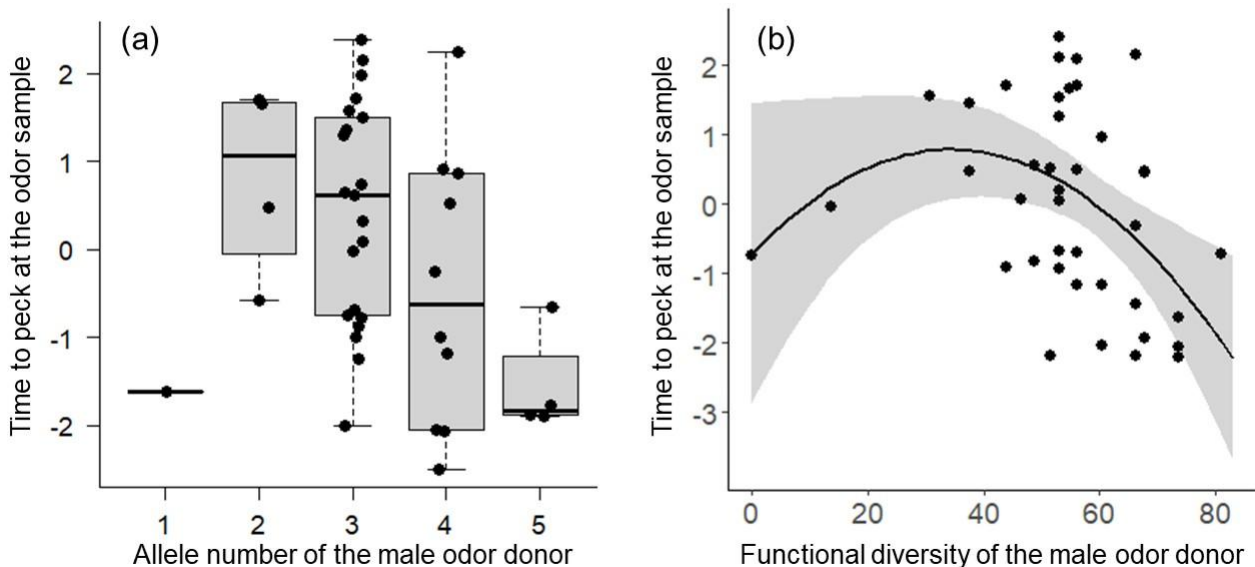
162

## 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:  $\chi^2_1 = 0.01$ ,  $P = 0.94$ ).

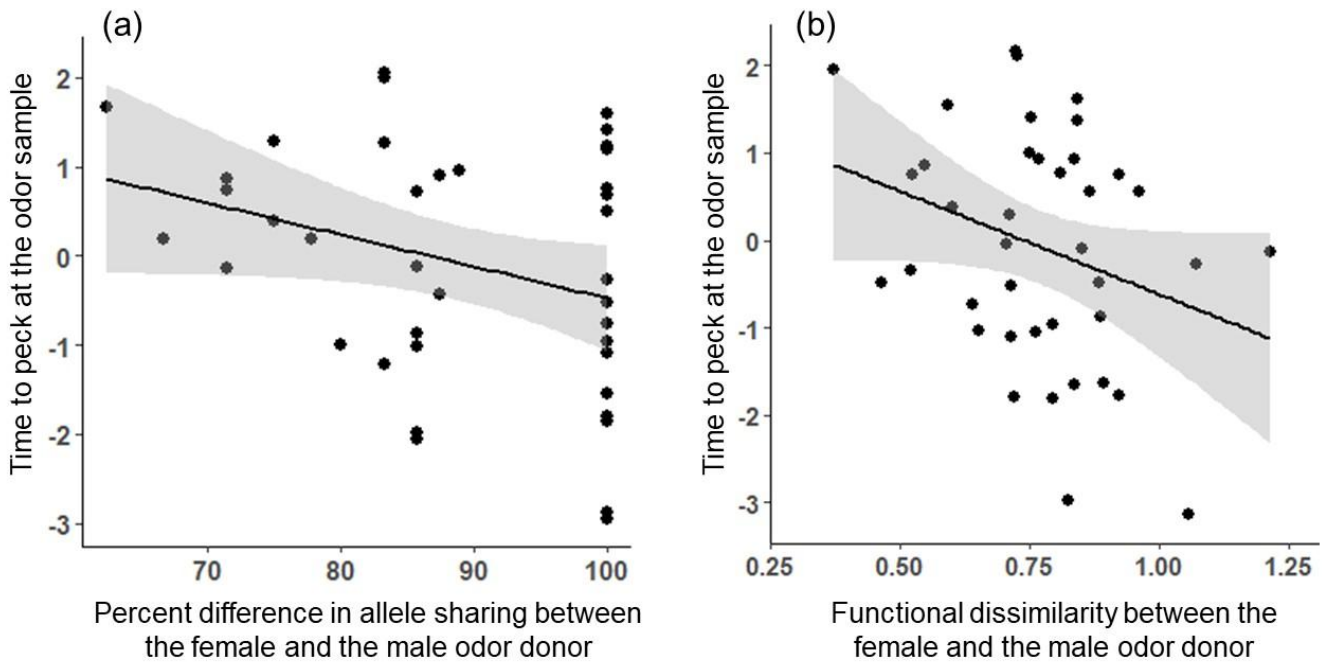
170 Among the females who pecked at the sample (n = 40 tests for which we had both the male MHC-II  
 171 diversity and the MHC-II dissimilarity between male and female), females took more time to peck at the  
 172 odor sample when the male had an intermediate MHC-II diversity (allele number: quadratic 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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