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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 parasites in vertebrates, and is thus often suggested to be an important force driving social interactions, including mating preference. However, the phenotypic cues used by individuals to assess the MHC characteristics of conspecifics are 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 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. In addition, when the odor sample came from a male with higher MHC-II dissimilarity, females took less time to peck at the sample, but once they pecked at it, delayed the use of the sample. Altogether, our results add 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*

1. Introduction

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

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

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

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

odor cues to distinguish the MHC characteristics of males. We used a protocol inspired by Leclaire et al. (2009), which showed differential pecking response of incubating females to different odor samples (skunk, banana, fish or control odor) put in their nest. Here, we put a single odor sample of a male to the nest of an incubating female and assessed her behavioral response. Although differential response of females cannot be interpreted as female preference, we consider that the fact that different females react on average differently along a continuum of males' MHC characteristics indicate discrimination. Because the MHC-based mating pattern of kittiwake is unknown, we tested recognition of both MHC diversity and MHC dissimilarity, and always included both the linear and the quadratic term of MHC diversity.

2. Materials and methods

2.1 Study site

The study was conducted during the 2018 incubating period (May-June) in a population of black-legged kittiwakes nesting on an abandoned US Air Force radar tower on Middleton Island, Alaska (59°26'N, 146°20'W). Nests on artificial ledges were visible from inside the building through sliding one-way glass windows (Gill and Hatch 2002). This experiment was conducted under the approval of the USGS Alaska Science Centre Animal Care and Use Committee, in accordance with US law and under permits from the US Fish and Wildlife Service and the State of Alaska.

2.2 Behavioral tests

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

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

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

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

2.3 MHC analyses

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

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

2.4 Statistics

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

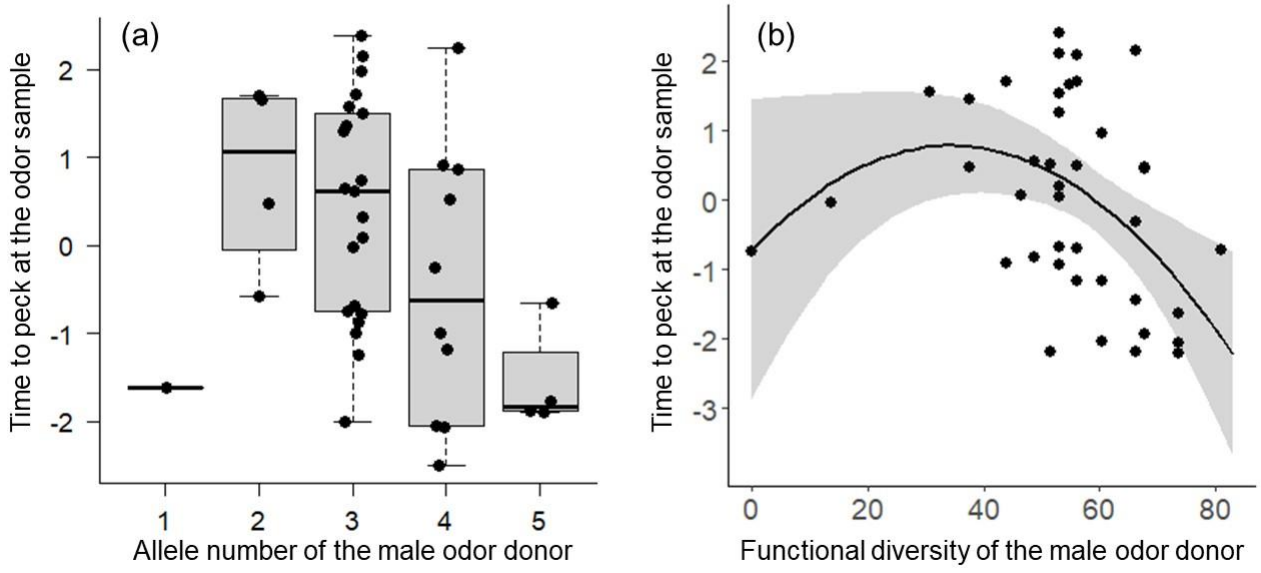
identity of the male odor donors were included as random effects. Third, for females who moved the odor sample, we used a binomial GLMM model to test whether the probability to delay the move of the odor sample after the first pecking (0 when the female immediately moved the sample after the first pecking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

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

3. Results

When considering the tests for which we had both the MHC-II diversity of the male odor donor and the MHC-II dissimilarity between the male odor donor and the female (n = 47 tests), seven females (15 %) did not peck at the odor sample. The probability of pecking at the sample did not vary with male MHC-II 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 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 (percent difference in allele sharing: $\chi^2_1 = 0.06$, P = 0.80 and functional dissimilarity: $\chi^2_1 = 0.01$, P = 0.94).

Among the females who pecked at the sample (n = 40 tests for which we had both the male MHC-II diversity and the MHC-II dissimilarity between male and female), females took more time to peck at the odor sample when the male had an intermediate MHC-II diversity (allele number: quadratic term: $\chi^2_1 = 5.49$, P = 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 = 0.020 and linear term: $\chi^2_1 = 3.91$, P = 0.056; Figure 2b). When excluding the test where the single male odor donor had only one MHC-II allele (Fig. 2a), females took less time to peck at the odor sample when the male had higher MHC-II diversity (allele number: linear term: $\chi^2_1 = 13.73$, P < 0.001 and quadratic term: $\chi^2_1 = 0.36$, 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 2). This result suggests that this male carrying a single MHC-II allele drives the quadratic relationship between the time taken to peck at the sample and the MHC-II diversity. Females who pecked at the sample took also less time to peck at the odor sample when they were more MHC-II dissimilar to the male odor donor (percent 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).



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.

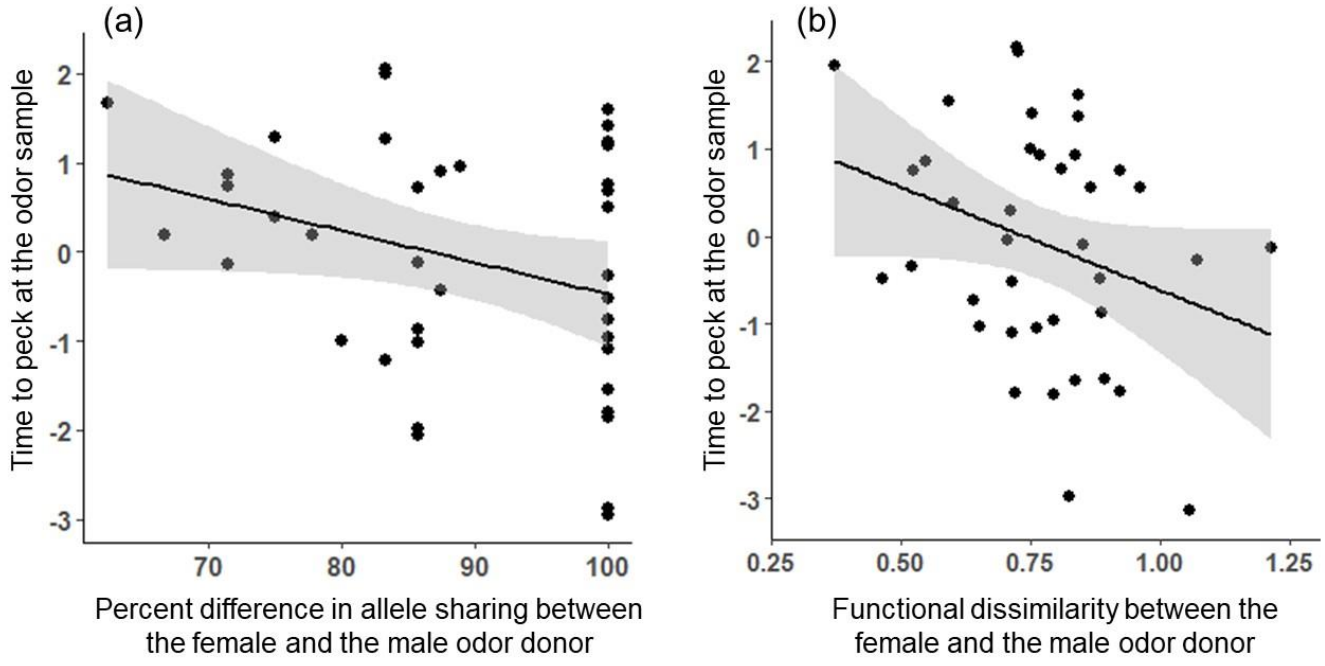


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Figure 3: Time taken by females to peck at the odor sample according to (a) the percent difference in MHC-II allele sharing and (b) the functional MHC-II dissimilarity to the male odor donor. The time to peck at the sample was represented by the residuals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

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

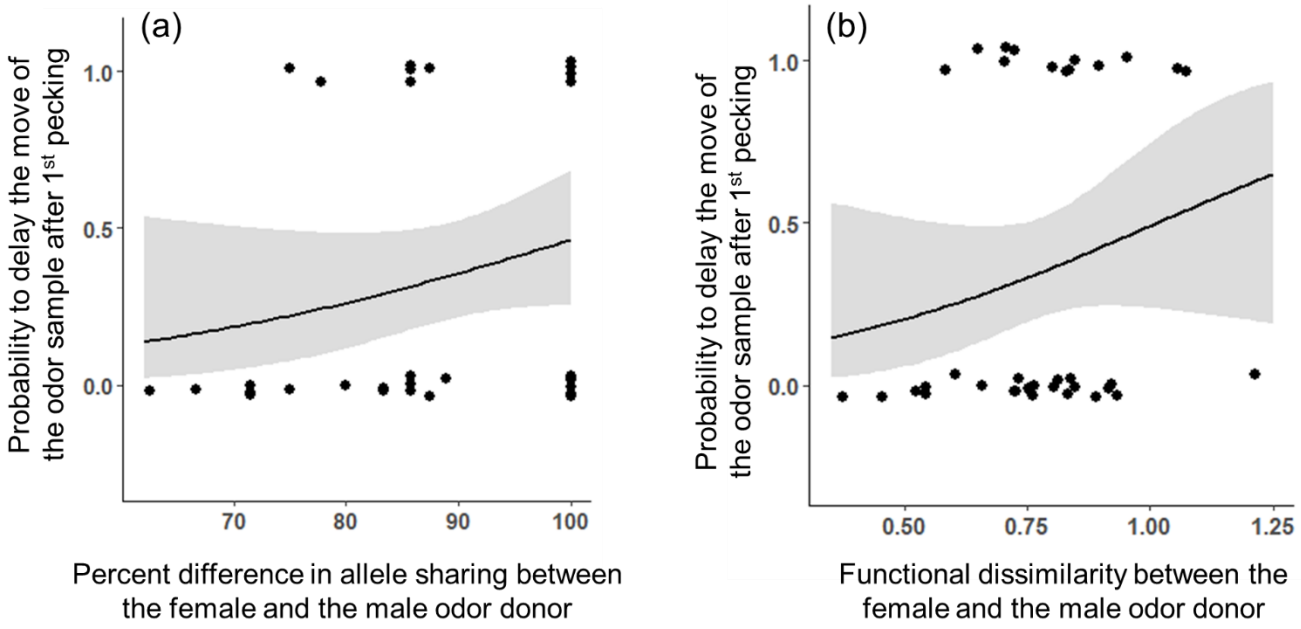

Figure 4: Probability for the females to delay the move of the odor sample after the first pecking (0: the female moved the sample just after the first pecking and 1: the female delayed the move of the sample after the first pecking) according to (a) the percent difference in MHC-II allele sharing and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

5. Discussion

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

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

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

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

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

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