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The best smellers make the best choosers: mate choice is affected by female chemosensory receptor gene diversity in a mammal

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The products of the genes of the major histocompatibility complex (MHC) are known to be drivers of pathogen resistance and sexual selection enhancing offspring genetic diversity. The MHC further influences individual odour types and social communication. However, little is known about the receptors and their volatile ligands that are involved in this type of chemical communication. Here, we have investigated chemosensory receptor genes that ultimately enable females to assess male genes through odour cues. As a model, we used an invasive population of North American raccoons (*Procyon lotor*) in Germany. We investigated the effect of two groups of chemosensory receptor genes—trace amine-associated receptors (TAARs) and olfactory receptors (ORs)—on MHC-dependent mate choice. Females with more alleles of the TAAR or OR loci were more likely to choose a male with a diverse MHC. We additionally found that MHC class I genes have a stronger effect on mate choice than the recently reported effect for MHC class II genes, probably because of their immunological relevance for viral resistance. Our study is among the first to show a genetic link between behaviour and chemosensory receptor genes. These results contribute to understanding the link between genetics, olfaction and associated life-history decisions.

1. Introduction

Females of many vertebrate species are believed to use their sense of smell to assess the genetic quality of potential reproductive partners [1,2]. The genes of the major histocompatibility complex (MHC) are now widely accepted to play a central role in sexual selection, as they are targeted by females who aim at boosting the genetic variability of their offspring by preferring to mate with males that are particularly diverse (or dissimilar to the female) in their MHC [3–6]. Although examples of MHC-dependent mate choice have been reported for almost all major vertebrate taxa [7–10], and although females are probably able to smell the odours that reveal the MHC composition of males [4,5,11–13], the odorants involved in this type of communication and their corresponding chemosensory receptors remain mysterious.

If olfaction builds a bridge between female mate choice and MHC loci [14,15], the alleles of the polymorphic chemosensory receptor genes of females should show a signature of this relationship. A recent study in bats has corroborated this hypothesis by providing evidence for the participation of trace amine-associated receptor (TAAR) genes in MHC-dependent mating of a bat species [4]. Females with a more diverse TAAR gene repertoire were more likely to perform MHC-dependent mating, probably because of enhanced olfactory resolution. TAARs are a small family of G-protein-coupled receptors that are involved in chemical communication [16,17] and that are highly sensitive to

low concentrations of volatile amines [18]. Vertebrate olfaction is, however, also strongly influenced by 'canonical' olfactory receptor (OR) genes [19]. Whereas several hundreds of these loci are scattered over many chromosomes in most vertebrate genomes [20–22], a relatively small and conserved subset of OR genes is physically linked to the MHC region [23]. As a consequence of this tight physical linkage, the effect of linkage disequilibrium (LD) might lead to a relatively fixed correspondence between OR and MHC alleles [24]. TAAR and MHC-linked OR genes are therefore good candidates for mediating the female perception of still unidentified MHC-dependent male odours.

Apart from their role in mate choice, the molecules encoded by the MHC are primarily known for their role in the adaptive immune response of vertebrates. Essentially, classic MHC receptors can help in maintaining homeostasis or trigger inflammation by interacting both with small peptides (of self or non-self origin) and with T-lymphocytes [25]. Although MHC class I molecules present peptides of intracellular origin to the immune system, class II receptors specialize in presenting peptides that are 'captured' from the extracellular environment [25,26]. We have recently described a correlation between MHC class II genes and mate choice behaviour among invasive raccoons in Europe [5]. Since both MHC classes are generally linked [27], MHC class I genes might be the primary cause for the previously found association. This is especially plausible because raccoons have evolved under strong selection by both rabies and the canine distemper virus in their natural North American distribution, in which cyclical outbreaks of these diseases can kill up to around 80% of individuals in local populations [28,29]. Outside of North America, raccoons are also under the selective pressure of canine distemper [30–32] and are relevant reservoirs of other viral pathogens [33]. Surprisingly, no data are currently available for raccoon MHC class I loci, despite the public health relevance of the diseases mentioned above. Similarly, chemosensory receptors have not yet been investigated in this species, although the social relevance of olfaction for both male and female raccoons is undisputed [34].

We report here the first characterization of MHC class I genes for raccoons, together with seven TAAR and two OR loci. Specifically, we have aimed at answering the following questions. (i) Which MHC class I alleles are present in an invasive European raccoon population? (ii) What is the role of MHC class I loci in mate choice? (iii) How does the effect of MHC class I compare with that of the MHC class II genes that we have previously reported? (iv) How do TAAR and certain OR genes influence the dependency between MHC and mate choice? The characterization of the chemosensory receptors and the identification of loci affecting mate choice shed light on the link between an individuals' immunogenetic composition, chemical communication and associated life-history decisions.

2. Material and methods

(a) The study area, sampling and radio tracking

We took advantage of 6 years of observation, radio tracking and sampling of 146 raccoons captured between 2006 and 2011 in an area of approx. 1100 ha in the Müritz National Park in the north-eastern German state of Mecklenburg-West Pomerania (53°15′–22′ N, 13°8′–21′ E). All animals were captured with live-traps as previously described [5,35] and in accordance with current German law (*Tierschutzgesetz*). Sampling included ear-tagging, weighing, ageing [5,36,37], sexing and the collection of blood, buccal swabs and one 6-mm skin punch from each animal.

Raccoons in this area can live for about 10 years (highest age recorded for one individual = 14 years). The estimated raccoon density (summer, including offspring) was five to six animals per 100 ha [30]. We analysed 31 202 individual location data points from 69 radio-collared individuals (36 males and 33 females). Locations were determined by using radio antennas that were hand-held or mounted on automobiles or boats. Fifteen camera traps placed across the area yielded additional location data (5365 photo-trapping nights and 15 339 images of 145 individuals).

(b) Genotyping of MHC class I and chemosensory receptor genes

DNA was extracted from skin punches with the NucleoSpin Tissue-Kit (Macherey-Nagel, Düren, Germany). The names and sequences of all primers used in this work are given in the electronic supplementary material, table S1. Concerning the MHC, we amplified and sequenced a target of 226 base pairs (bp) in the exon 2 of MHC class I loci. For the chemosensory receptor genes, we targeted eight TAAR loci (TAAR1, TAAR2, TAAR3, TAAR4, TAAR5, TAAR7, TAAR8 and TAAR9) and three OR loci (OR14J1, OR2J3 and OR2W1). The choice of target genes was based on a preliminary sequencing run of 10 unrelated individuals in order to identify polymorphic loci. Each individual was processed for each locus in two independent technical replicates, from DNA amplification to allele calling. Library preparation followed a two-step PCR approach, with the first PCR (26 cycles) being dedicated to product amplification and a second PCR (10 cycles) to index (or barcode) incorporation. After initial optimization of the annealing temperatures, all PCRs for initial amplification with CS primers (Fluidigm reference) were set to a final volume of 10 µl, with 3 µl PCR buffer containing $MgCl_2$, 0.5 µl of each primer [10 µM], 1 × FastStart High Fidelity Taq DNA polymerase (Sigma-Aldrich), 0.2 mM each dNTP, 2.5 µl GC-Rich and 14.9 µl H_2O, under the following conditions: an initial 1 min denaturation at 95°C; 26 cycles of denaturation (30 s at 95°C), annealing (30 s at 55°C) and extension (1 min at 72°C), and a final elongation step (10 min at 72°C). Equimolarity for downstream steps was achieved by estimating the dsDNA concentration with a TECAN infinite F200PRO plate reader (TECAN Group AG, Männedorf, Switzerland) and adjusting volumes, accordingly, to 10 ng μl^{-1} with H_2O. The second PCR (barcode incorporation) was set at a volume of 20 µl consisting of 10 µl PCR buffer, 4 µl barcode (Access Array Barcode Library for Illumina Sequencers – 384 Single Direction, Fluidigm Corporation, South San Francisco, CA, USA), 4 µl PCR product and 2 µl H_2O under the following conditions: an initial 10 min denaturation at 95°C; 10 cycles of denaturation (30 s at 95°C), annealing (30 s at 60°C) and extension (1 min at 72°C), and a final elongation step (10 min at 72°C). Purification was performed with the NucleoMag NGS Clean-up and Size Select Kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocol. Two final 8 pM amplicon libraries (one for the MHC and one for the chemosensory receptor genes) were prepared. In order to improve sequencing quality, the chemical diversity of both libraries was increased by an 8% spike-in of standardized bacterial DNA (PhiX Control, Illumina, San Diego, CA, USA). Sequencing was performed by using a MiSeq Reagent Kit v2 Nano (Illumina) for each library, in independent runs, in an Illumina MiSeq instrument.

We employed a recently developed bioinformatic workflow [5,38] to filter, interpret and call alleles out of the raw sequences. Raw reads were initially quality-checked by removing

low-quality reads (phred quality score ≥ 30 in at least 90% of bases) and reads with any sequencing errors on primers or barcodes. The final allele calling of each sample allowed only variants that passed our conservative approach and had been identified in both replicates (i.e. originated from independent PCRs). All other steps on the bioinformatic pipeline were performed as described elsewhere [5].

(c) Female mate choice

We tested whether females chose their mating partners depending on the male MHC class I alleles. Parentage and relatedness indices of all individuals had previously been established in our earlier study, and detailed methods are described therein [5]. In general terms, all individuals were genotyped for 10 microsatellite loci that were used for parentage inference and relatedness estimation. We were thus able to identify 95 family trios and assumed each offspring to be the outcome of an actual female choice event. In 64 cases out of the 95 established family trios, at least one further sexually mature male was known to be resident in the study area during the time of conception (60–66 days before birth), and so these males were considered potential mates for female choice. Since males and females are rather mobile concerning their dens within the study area over the observation years [30], we are confident that considering all males present in the study area as potential mates does not bias the female mate choice results in any specific direction. By combining data of male/female availability in time and space with the MHC class I genotyping data generated in this work, we were able to investigate 48 MHC class I-genotyped groups, each of them consisting of one family trio and further candidate males that had not been chosen by the female. Each group had an average of 22.25 candidate males, in addition to the actual father (ranging from 15 to 26, s.d. = 4.59). Altogether, 18 different females and 16 different males were involved in the mate choices. An individual female was involved in one to four mate choice events (average = 1.71, s.d. = 0.99). The most 'attractive' male was chosen eight different times by five different females (the number of times being chosen ranged for all males from 1 to 8 times, average = 2.19, s.d. = 1.87). The final female choice dataset amounted thus, in total, to 48 real choices (mother with actual father) and 1068 potential choices (mother with candidate males).

(d) Parameters for MHC dissimilarity and diversity

MHC allele diversity and the amino acid distance among the MHC alleles of an individual can serve as a proxy for the range of pathogenic antigens that can potentially be presented to T-lymphocytes [6]. We calculated three MHC dissimilarity parameters, pairwise, among all individuals. Two MHC diversity parameters were additionally calculated for all candidate males of the sample. Whereas the dissimilarity indices implied a comparison between the alleles (as defined by their amino acid sequences) of the assessing females and the assessed males, the diversity indices were inherent to each assessed male. The five MHC parameters adopted here have been previously employed [4,5] and are defined as follows:

1. Male allele dissimilarity (MALDis): number of MHC alleles present in the assessed male individual but not shared by the assessing female individual.
2. Couple allele dissimilarity (CALDis): sum of the number of non-shared MHC alleles in each pair of individuals.
3. Mean amino acid dissimilarity (μAADis): mean amino acid distance (defined as the number of amino acid substitutions per site, using the Poisson correction model) among the non-shared MHC alleles in each pair of individuals.
4. Male allele diversity (MALDiv): number of different MHC alleles present in each male.
5. Male amino acid diversity (MAADiv): sum of amino acid distances among the MHC alleles of each male.

(e) Statistics

We followed the two-step approach described recently [4], in which we used both generalized linear mixed modelling (GLMMs) and Monte Carlo randomization tests (MCRT) in order to evaluate the possible presence and extent of an effect.

We used GLMMs in order to determine whether the outcome of female choice events could be predicted by any of the MHC dissimilarity or diversity indices. To achieve this, we calculated, for each offspring, all five MHC genetic parameters corresponding to its real parents (real female choice) and to each of its potential parents (substituting the father by each potential male). Thus, each offspring accounted, on average, to 23.25 possible mate choices, out of which only one was the actually observed female choice. The results of the MHC comparison parameters were then organized into one row per choice, and the value of a binary variable named 'CHOSEN' was set to '0' or '1', depending on the pair being only potential or real, respectively. GLMMs were used here because the same females and the same males were involved in several 'choice' events, which were not necessarily independent of each other. In order to account for the confounding effects of litter sizes and the fact that some parents had more offspring than others over their lifetimes, we considered the identity of mothers and potential fathers (as well as sampling year) as random factors in the models. While the GLMMs were built and performed using the *glmer* function of the R 'lme4' package [39], we analysed the interactions among explanatory variables with the packages 'effects' [40] and 'sjplot' (https://CRAN.R-project.org/package=sjPlot). In R syntax, our full model for mate choice (before collinearity analysis and model selection) was built with the following expression: `glmer(CHOICE~MALDis + CALDis+μAADis + MALDiv + MAADiv + (1|FATHER) + (1|MOTHER) + (1|SAMPLING_YEAR), family = binomial (link='cloglog'))`. The non-default cloglog link function aimed at accounting for overdispersion.

We employed MCRT in order to test if females chose mating partners in a way that differed significantly from randomness, concerning the genetic differences between females and males (MALDis, CALDis and μAADis) and the diversity of males (MALDiv and MAADiv). To implement it, we first calculated the mean dissimilarity or diversity considering all 48 real mating pairs observed here. This was done once for each MHC index. We then created, for each MHC index, a distribution of dissimilarity/diversity means by disassembling the real mating pairs and creating new ones 10^5 times, with replacement. The set of candidate males found to be available to each female (considering space and time) was used for the re-samplings. *P*-values were then calculated directly as the proportion of times that simulated means were further away from the mean of their distribution that was the real, observed mean, in any direction (two-tailed). If the real mating partners would have dissimilarity or diversity indices seldom achieved by randomly found indices, then this would be indicative for an effect of the respective MHC index that cannot be explained by chance.

GLMMs and MCRTs are largely different statistical ways to test related, but different things. GLMMs 'suffer' from dealing with zero-inflated data as in the case of mate choice, but, given the proper correctors (cloglog link function), they have the advantage of making it possible to infer effect sizes and confidence intervals [41]. Meanwhile, the MCRT is 'immune' to zero-inflation, but it ignores the extent of the difference between males and females, being thus blind to effect size (as in a classical null-hypothesis testing approach). In the cases when both GLMMs and MCRTs agree on the effect of an explanatory

variable, MCRTs can give us enough confidence of the existence of an effect, while the GLMMs are best in inferring the strength of that effect. Therefore, as discussed before [4,5], we believe this 'dual' statistical approach to be the ideal procedure in mate choice tests like the one described here.

Finally, we estimated two chemosensory receptor gene-related indices for every individual in the dataset: (1) chemosensory receptor gene diversity and (2) heterozygosity. Diversity was calculated directly as the number of different alleles (defined by amino acid sequences) found per individual, in each locus. Heterozygosity was inferred as the proportion of heterozygote loci in each individual. The diversity of those females that sired offspring was considered further for interaction analysis with the MHC and mate choice, while heterozygosity was used solely for the purpose of locus characterization. For the interaction analysis, each female was assigned a mean diversity value concerning TAARs, ORs and their combination (mean for all assessed chemosensory receptor genes). Whenever we found evidence that any of the five MHC indices mentioned before influenced mate choice (by being used by females as a way to choose males), we tested if an interaction effect between that MHC index (in the couples) and mean chemosensory diversity (of choosing females) was detectable. In practice, we have (post hoc) tested the existence (and extent) of an interaction effect between MAADiv and chemosensory diversity on the probability of females to choose males based on MAADiv, because the latter was found to be the only relevant MHC index. We considered TAARs alone, ORs alone and their combination as explanatory variables. In R syntax, we used the following expression for MHC classes I and II: `glmer(CHOICE~female_chem_div:MAADiv + (1|FATHER) + (1|MOTHER) + (1|SAMPLING_YEAR), family = binomial (link='cloglog'))`.

3. Results

(a) Genotyping

Genotyping was successful for all individuals submitted to the MHC class I sequencing procedure, which yielded 1 026 700 pairs of raw 251 bp-long reads (ranging from 267 to 49 137 reads per individual, with an average of 9167 and a standard deviation (s.d.) of 6253). Quality control, primer and barcode trimming, and chimaera and BLAST filters led to the final number of 917 648 merged 226 bp-long reads (ranging from 1394 to 42 096 reads/individual, average = 8478, s.d. = 5.330), which were used to define alleles. The fragment corresponded to 75 amino acid residues or approx. 84% of the full exon 2 encoding for the alpha 1 domain of the folded MHC receptor.

We found 10 alleles in the whole sample and named them Prlo-B*01 to Prlo-B*10, in decreasing order of abundance (nucleotide and amino acid alignments shown in the electronic supplementary material, figure S1). Allele sequences could be translated into eight unique amino acid sequences. Indels were found in two positions. Taking the most common Prlo-B*01 as a reference, indels corresponded to deletions of seven bases (alleles Prlo-B*05, Prlo-B*07 and Prlo-B*08) or of nine bases (Prlo-B*10). The latter was an in-frame indel, whereas the former led to a frameshift that, at least within the amplified fragment, did not include a stop codon. Between four and eight alleles were found per individual (average = 5.71, s.d. = 1.29), indicating the amplification and genotyping of at least four homologous loci. The mean repeatability index was 0.9955 (i.e. over 99% of preliminary allele callings were identical among individual replicates). Accordingly, the application of the technical replicate filter for final allele calling only excluded 0.45% of sequence variants.

Table 1. Alleles detected at each locus in the dataset ($n = 146$) and individually.

locus	total number of alleles detected in the sample	number of alleles per individual	mean heterozygosity
MHC-I	10	4–8	0.74
TAAR1	4	1 or 2	0.82
TAAR2	2	1 or 2	0.40
TAAR3	2	1 or 2	0.11
TAAR4	5	1 or 2	0.64
TAAR5	2	1 or 2	0.42
TAAR7[a]	1	1	0
TAAR8	3	1 or 2	0.61
TAAR9	3	1 or 2	0.61
OR14J1[a]	1	1	0
OR2J3	24	5–8	0.80
OR2W1	16	4–8	0.69

[a]Monomorphic loci.

The loci TAAR7 and OR14J1 were found to be monomorphic after their initial genotyping (10 unrelated individuals) and were thus not considered in any further analyses. Genotyping of the loci TAAR1, TAAR2, TAAR3, TAAR4, TAAR5, TAAR8, TAAR9, OR2J3 and OR2W1 was successful for all individuals, except for the locus TAAR3 in one female and the loci OR2J3 and OR2W1 in four females. The genotyping of all chemosensory receptors started with 1 068 196 pairs of raw 251 bp-long reads, accounting for an average of 5182 pairs or reads per individual and locus (ranging from 1258 to 98 095, with an s.d. of 10 304). Quality control, primer and barcode trimming, and chimaera and BLAST filters led to the final average of 1993 merged reads per individual and locus (ranging from 205 to 84 737 reads per individual, s.d. = 6.086), which were used to define alleles. No significant differences were found in terms of amplification or genotyping efficiency among the different loci. Accounting for the number of alleles from each locus found in each individual, we estimated to have amplified alleles from single loci in all TAAR genes and alleles from at least four homologous loci for both odorant receptors (table 1). No individual was genotyped for less than four alleles in each OR locus, suggesting a lack of copy number variation among individuals. We named the alleles according to the gene symbol and order of abundance in our sample (e.g. Prlo-TAAR1*01, Prlo-TAAR1*02, etc.). The total number of alleles and the mean heterozygosity determined for each chemosensory locus is given in table 1.

(b) Female mate choice

Because collinearity between the two explanatory variables MALDiv and MAADiv was very high (Pearson correlation = 0.98), we dropped the variable MALDiv from all analyses to avoid redundancy. All remaining parameters had Pearson correlations among each other below 0.60

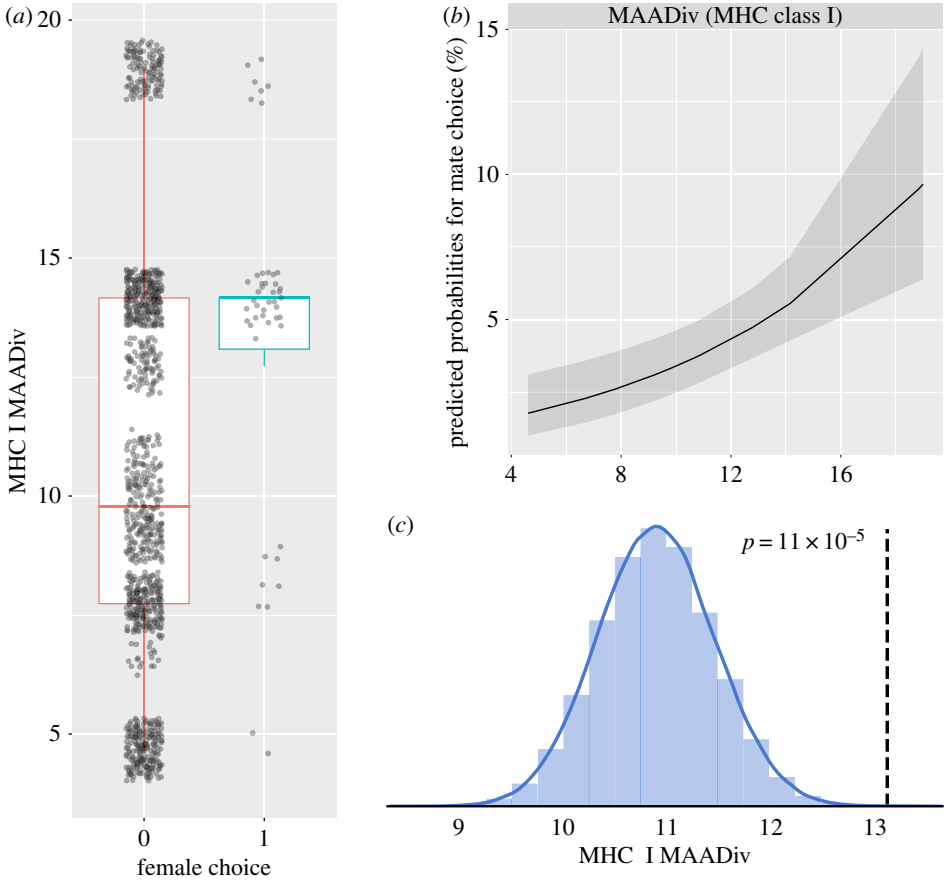

Figure 1. Effect of MHC class I amino acid diversity (MAADiv) among sexually mature males on their probability of siring offspring. The three panels refer to the same data. (*a*) Boxplot of the MAADiv (MHC class I) of males which were chosen (blue) opposed to non-chosen males (red) in 48 mate choices events, as inferred from offspring presence. (*b*) Result of the GLMM approach: the predicted probabilities to be chosen depending on MAADiv. (*c*) Result of the randomization approach: the dashed vertical line denotes the mean MAADiv of actually chosen males, while the blue histogram represents the distribution of the means of randomly chosen males (10^5 simulations).

(electronic supplementary material, figure S2) and were kept as fixed effects.

We found evidence that females chose mating partners according to the MHC class I composition of the males, measured as amino acid diversity (MAADiv), using both statistical approaches. First, according to the GLMMs, the probability of a male being chosen by a female was best predicted by the diversity index MAADiv (p-value = 6.11×10^{-5}). More amino acid diversity corresponded to a higher probability of mate choice (figure 1*a*,*b*). The effect size (odds-ratio) of this relationship was 1.13 (with the Wald confidence interval at the 95% level = 1.07–1.20). Although only 18 different females and 16 different males were involved in the mate choices, the amount of variance assigned to the random effects (identity of males or females) was, for MAADiv, smaller than 0.01. For all other explanatory variables (the dissimilarity indices CALDis, MALDis and μAADis), we were not able to detect any correlation with female choice unexplainable by chance alone (all p-values greater than 0.12 during model selection). Then, according to the MCRTs, the MHC class I amino acid diversity (MAADiv) of males chosen by females of this sample was far higher than expected by chance (p-value = 1.1×10^{-4}, figure 1*c*). Concerning the other MHC dissimilarity indices, mere chance could not be ruled out as explanatory of the observed results (p-values of 0.1937, 0.3025 and 0.2040 for MALDis, CALDis and μAADis; respectively, electronic supplementary material, figure S3).

By combining this dataset with results from the same raccoon sample that had previously been generated for the MHC class II [5], we obtained strong evidence for an interaction between the variables MAADiv of MHC class I and MAADiv of MHC class II of candidate males on their probability of being chosen by any female as a mating partner (p-value = 5.1×10^{-5}). For both MHC-I and MHC-II, more amino acid diversity among alleles possessed by males led to increased chances of fathering offspring, but these chances were enhanced when diversity was simultaneously high in the loci of both MHC classes (figure 2).

(c) Interaction of chemosensory loci with the MHC on the outcome of mate choice

We found evidence for an interactive effect of male diversity (MAADiv, taken from MHC-I or MHC-II) and the chemosensory loci diversity of females on the probability of mate choice (p-values = 7.4×10^{-4} and 9.7×10^{-3} for MHC-I and MHC-II, respectively). The evidence of interaction held true when we split chemosensory diversity in TAAR diversity (p-values = 6.1×10^{-4} and 4.7×10^{-3} for MHC-I and MHC-II, respectively) and in OR diversity (p-value = 4.8×10^{-3} for MHC-I), being borderline in the case of OR diversity and MHC class II (p-value = 0.056). As a general rule, females with more alleles of TAAR or OR loci were more likely to choose a male that was more diverse in his MHC. The interaction between female chemosensory diversity and male

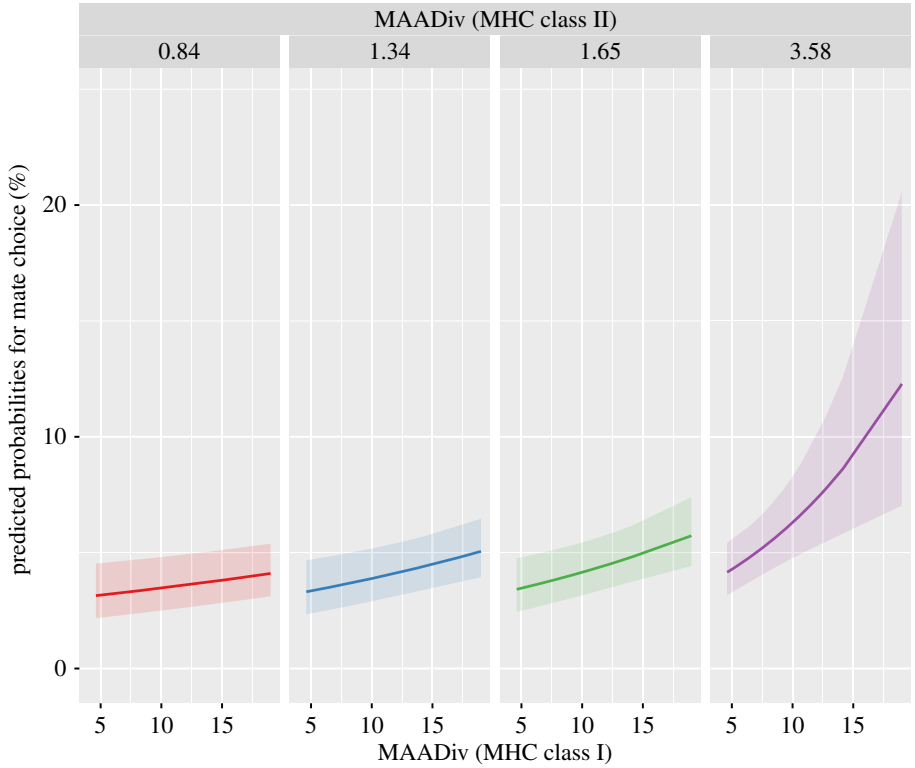

Figure 2. Interaction of MHC class I MAADiv and MHC class II MAADiv of male raccoons on their probability of siring offspring. The four subpanels denote the four quartiles of MHC class II MAADiv. The relationship between MHC class I MAADiv and the probability of a male siring offspring are given in each subplot.

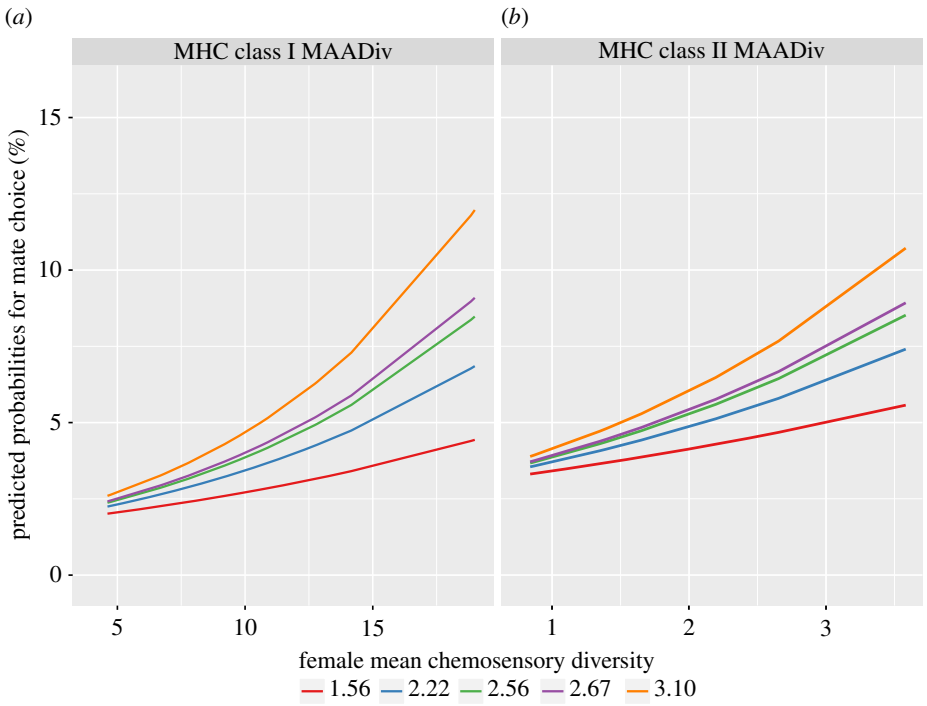

Figure 3. Interaction effect of MAADiv of males and chemosensory diversity of females on the probability of males siring offspring. The five colours denote four quartiles and the mode of female mean chemosensory allele diversity.

MHC diversity on mate choice probability is depicted in figure 3.

4. Discussion

If polymorphic chemosensory receptor genes influence the ability to detect smells/scents, then they should correlate with the ability of females to perform MHC-dependent mate choice. Here, we have found evidence confirming this hypothesis, as female variability at chemosensory receptor genes and male MHC diversity affect female choice together. The most probable scenario seems to be the one in which females with more chemosensory alleles have enhanced olfactory resolution and are thus more able to perform mate choice reacting to a male's smell (i.e. indirectly, his MHC composition). After bats [4], this is the second report pointing to the important role of chemosensory receptor genes in mate choice among mammals

and now revealing two further olfactory receptor loci. In an earlier study with the same raccoon sample, we were able to rule out the participation of genome-wide diversity or dissimilarity on mate choice, using microsatellites [5].

Although MHC-dependent mate choice has been the subject of intense research for nearly 40 years [14,15,42], the molecules that make it possible for females to 'read' the genes of males are still unknown. Both OR loci alone and TAAR loci alone contribute to this interaction in the case of MHC class I (p-values = 4.8×10^{-3} and 6.4×10^{-4} for ORs and TAARs, respectively), but less certainty has been observed in the case of MHC class II (p-values = 0.057 and 4.9×10^{-3} for ORs and TAARs, respectively). As a consequence of the statistical limitations associated with overparametrization, we did not analyse the effect of each TAAR and OR locus on mate choice separately. It appears likely that TAAR1 is not involved in odour recognition, as it is the only TAAR not expressed in the main olfactory epithelium [16,17,43]. However, because of the confounding effects of LD between MHC classes I and II, we considered any combination of the eight remaining polymorphic chemosensory receptor loci assessed here (TAAR2-9 and ORs) as valid candidates for building a link between male odour and female olfaction leading to mate choice. For example, TAAR5 is a good candidate among mice, because of its known affinity to trimethylamine and participation in social communication [44]. The biosynthesis of trimethylamine needs commensal microflora, whose composition, in turn, probably reflects the MHC composition of the host animal [45]. Moreover, a recent study [46] in mice was able to show experimentally that a urinary volatile amine named isobutylamine (a ligand of TAAR3) can elicit sexual behaviour. We believe, therefore, that the future search for ligands of polymorphic TAAR or specific OR loci will lead to the identification of the odorants involved in MHC-dependent mate choice. In other words, the deorphanization of the set of chemosensory receptors mentioned here is expected to reveal the chemical nature of the links between female choice, MHC and male odour in mammals.

We have additionally provided evidence that MHC class I alleles have a stronger effect on mate choice than MHC class II [5], although in the same 'direction': increased MHC diversity among males correlates with increased probability of fathering offspring. The receptors encoded by the MHC class I genes are known to present peptides of intracellular origin to T-lymphocytes [25], and in turn, viral infections are expected to elicit an immune response (and shape selection) primarily of MHC class I receptors. Viral infections play a dominant role in the population dynamics of raccoons and perhaps also in its recent evolution and thus might explain the prominence of MHC class I over class II. Under the strong selective pressure from rabies [28] and canine distemper [29,32] viruses, the following predictions can be made and tested in upcoming investigations: (i) the dramatic cyclic outbreaks of rabies and canine distemper among raccoons, some of which kill more than 80% of local populations [28,47], is expected to have left a comparably strong signature on MHC class I allele frequencies; (ii) strains of rabies and canine distemper viruses probably overlap geographically with certain MHC class I alleles or allele families; and (iii) selection pressure on MHC class I alleles in North American populations has probably shaped allele frequencies more strongly than in invasive populations of Europe and Asia, which have not been in contact with rabies for many generations. In addition, the possibility that, because of LD, the MHC class I alleles are the primary factor explaining the correlation previously found between MHC class II alleles and rabies [47] seems intriguing and worth investigating.

The finding that the alleles of both classes are linked to MHC-dependent mate choice can be, to some extent, a consequence of strong LD across the region containing the loci of both classes. Genetic linkage, as a prerequisite for LD, is the case in the few carnivore species that have had their MHC regions fully sequenced and well annotated, such as cats and dogs [48,49]. On the other hand, the interaction between amino acid diversity in MHC-I and in MHC-II suggests a scenario in which both MHC classes I and II receptors contribute to the individual-specific odour type of raccoons. A future thorough sequencing and assembly of the whole MHC region will be crucial in confirming or rejecting the LD hypothesis. Bioassays assessing the reaction of females to a panel of males should shed light on the matter of smell and reveal how the repertoire of classes I and II receptors 'translates' into individual odours.

Ethics. All animals were handled in accordance with current German law (*Tierschutzgesetz*).

Data accessibility. All sequences characterized here are available in the GenBank database under the accession numbers MH330235–MH330315.

Authors' contributions. S.S., P.S.C.S. and F.-U.M. designed the study. F.-U.M. was responsible for fieldwork and contributed all samples. The wet laboratory analyses were carried out by M.M. and M.K. under the supervision of P.S.C.S. P.S.C.S. applied the bioinformatic pipeline to the raw data and its statistical interpretation. The manuscript was written by P.S.C.S. and edited by S.S. All authors contributed significantly to the final version of the manuscript.

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