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1 Title

2 Do changes in gene expression contribute to sexual isolation and
3 reinforcement in the house mouse?

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22

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24 vomeronasal receptors

25

26 Abstract

27 Expression divergence, rather than sequence divergence, has been shown to be important in
28 speciation, particularly in the early stages of divergence of traits involved in reproductive
29 isolation. In the two European subspecies of house mice, *Mus musculus musculus* and *M. m.*
30 *domesticus*, earlier studies have demonstrated olfactory-based assortative mate preference
31 in populations close to their hybrid zone. It has been suggested that this behaviour evolved
32 following the recent secondary contact between the two taxa (~3,000 years ago) in response
33 to selection against hybridisation. To test for a role of changes in gene expression in the
34 observed behavioural shift, we conducted a RNA-sequencing experiment on mouse
35 vomeronasal organs. Key candidate genes for pheromone-based subspecies recognition, the
36 vomeronasal receptors, are expressed in these organs. Overall patterns of gene expression
37 varied significantly between samples from the two subspecies, with a large number of
38 differentially expressed genes between the two taxa. In contrast, only ~200 genes were
39 found repeatedly differentially expressed between populations within *M. m. musculus* that
40 did, or did not display assortative mate preferences (close to, or more distant from the
41 hybrid zone, respectively), with an overrepresentation of genes belonging to vomeronasal
42 receptor family 2. These receptors are known to play a key role in recognition of chemical
43 cues that handle information about genetic identity. Interestingly, four out of five of these
44 differentially expressed receptors belong to the same phylogenetic cluster, suggesting
45 specialisation of a group of closely-related receptors in the recognition of odorant signals
46 that may allow subspecies recognition and assortative mating.

47

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Introduction

Understanding the genetic basis of speciation remains a key challenge in evolutionary biology (Seehausen *et al.* 2014), in particular determining the genetic architecture of differentiation and reproductive isolation (Nosil & Feder 2012), the types of genes involved (Nosil & Schluter, 2011), and the types of genetic changes responsible for the evolution of reproductive isolation between diverging taxa (Hoekstra & Coyne 2007). Although evidence is rapidly accumulating for the role of protein coding and structural changes in adaptation and reproductive isolation (Stapley *et al.* 2010), it has also long been argued that gene expression differences may be of prime importance in species differentiation (King & Wilson 1975; Carroll *et al.* 2005), allowing rapid divergence (Wolf *et al.* 2010). Moreover, measures of gene expression provide an important bridge between genotype and phenotype (Huestis & Marshall 2009). In recent years, the study of gene expression has been greatly facilitated by high-throughput sequencing-based methods such as RNA-seq (Mortazavi *et al.* 2008; Wang *et al.* 2009), contributing to understand the importance of expression variation in both local adaptation and speciation.

Gene expression changes and the evolution of reproductive isolation

There are many examples of differential gene expression causing adaptive phenotypic changes (e.g., Chan *et al.* 2010a; McBride *et al.* 2014), and large-scale transcriptomics studies have shown heritable expression differences between diverging populations (e.g., Wolf *et al.* 2010; Uebbing *et al.* 2016; Davidson & Balakrishnan, 2016). How these components of gene expression can be associated with reproductive isolation is still a largely unexplored question. The major advances on this point come from studies investigating the role of gene regulation in the evolution of postzygotic isolation and, in particular, how misexpression in hybrids can reflect Dobzhansky-Muller incompatibilities (e.g. Haerty & Singh, 2006; Ortiz-Barrientos, Counterman, & Noor, 2007; Dion-Cote, Renaut, Normandeau,

& Bernatchez, 2014; Davidson & Balakrishnan, 2016; Mack *et al.* 2016). A recent study also identified significant changes in expression associated with a postmating-prezygotic reproductive barrier between two *Solanum* plant species, that involve effector molecules possibly responsible for pollen tube arrest (Pease *et al.* 2016). As far as premating isolation is concerned, evidence is mostly found in phytophagous insect species that are reproductively isolated due to host plant preference and specialisation. For example, recent studies have shown expression differences between populations specialised on different plants, involving genes related to host selection (odorant receptors, salivary proteins, detoxification genes) (e.g. cactus-specialised populations of *Drosophila mettleri*: Hoang, Matzkin, & Bono, 2015; *Rhagoletis* host races: Ragland *et al.* 2015; pea aphid host races: Eyres *et al.* 2016). These differences in expression have therefore been proposed to play a key role for adaptive genetic divergence in traits causing reproductive isolation during ecological speciation (Pavey, Collin, Nosil, & Rogers, 2010; Etges, 2014). Despite growing evidence for the role of gene regulation in mate choice and sexual selection (Wilkinson *et al.* 2015), support for expression changes contributing to sexual isolation is relatively scarce, and again concerns insect species. Examples include sex-specific gene expression changes underlying shifts in sex-pheromone preferences between populations of the mosquito *Anopheles gambiae* (Cassone *et al.* 2008), the silkworm *Bombyx mori* (Fujii *et al.* 2011) and *D. melanogaster* (Bailey *et al.* 2011), and a change in expression of a fatty acid synthase responsible for cuticular hydrocarbon production inducing sexual isolation between *D. serrata* and *D. birchii* (Chung *et al.* 2014).

Gene expression and the evolution of assortative mate preference in a house mouse hybrid zone

In this study, we investigate the role of expression divergence in the evolution of olfactory-based assortative mate preference between two subspecies of the house mouse (*Mus*

101 *musculus*) in order to gain insights into the genetic basis of sexual isolation in this
102 mammalian system. The two European subspecies of the house mouse, *M. m. musculus* and
103 *M. m. domesticus* have diverged in allopatry for 0.5 million years in the Indian sub-continent
104 (Boursot *et al.* 1996), before meeting secondarily in Europe around 5,000 years ago (and
105 around 3,000 years ago in Denmark) (Cucchi *et al.* 2012). They now form a hybrid zone
106 stretching from Norway to the Black Sea. Assortative mate preference between the two
107 subspecies has been demonstrated in populations at the edges of the hybrid zone in
108 Denmark in both subspecies, and in both males and females, but appears particularly strong
109 in *M. m. musculus* populations (Smadja & Ganem 2002, 2005; Smadja *et al.* 2004a; Ganem *et*
110 *al.* 2008). These results suggest a degree of sexual isolation between the two taxa. In
111 contrast, populations of both subspecies occurring in distant allopatry or slightly further
112 away from the Danish hybrid zone do not display any directional mate preference (Smadja &
113 Ganem 2005; Smadja *et al.* 2015). This pattern of reproductive character displacement has
114 been confirmed in the Czech part of the hybrid zone (Bimova *et al.* 2011). Since selection
115 against hybrids is acting in the mouse hybrid zone (reduced hybrid fertility: Britton-Davidian
116 *et al.* 2005; Albrechtova *et al.* 2012; Turner *et al.* 2012; Bhattacharyya *et al.* 2013; Turner &
117 Harr 2014; sexual selection against hybrids: Latour *et al.* 2014; hybrid microbiome
118 dysfunction: Wang *et al.* 2015) a reinforcement process has been hypothesised in this
119 system, by which premating barriers could have evolved as a response to selection against
120 hybrids (Bimova *et al.* 2011; Smadja & Ganem, 2005; Smadja *et al.* 2015).

121
122 A current challenge is to determine the genetic basis of assortative mate preferences
123 between the two subspecies and of the behavioural shift within each subspecies between
124 populations distant from or close to the hybrid zone. Previous studies have shown that
125 chemical signals present in urine are sufficient to elicit discrimination and assortative mate
126 preferences between the two subspecies (Smadja & Ganem 2002, 2005; Smadja *et al.*

2004a; Ganem *et al.* 2008; Bimova *et al.* 2011), suggesting a role of the olfactory system in this behavioural shift. In mammals, two organs are responsible for odorant signal recognition: the main olfactory epithelium (MOE), primarily involved in recognition of airborne chemicals present in the environment (odorants and pheromones), and the accessory olfactory organ or vomeronasal organ (VNO), known to play a key role in both volatile and non-volatile kairomone and pheromone recognition (Isogai *et al.* 2011; Chamero, Leinders-Zufall, & Zufall, 2012; Liberles, 2014; Bear *et al.* 2016). Recognition in these organs involves chemoreceptors (olfactory receptors (ORs) in the MOE and vomeronasal receptors (VRs) in the VNO) that are organised in very large multigene families in the mouse genome (more than 1,200 ORs and 530 VRs, Ibarra-Soria, Levitin, & Logan, 2013; Ibarra-Soria, Levitin, Saraiva, & Logan, 2014; Zhang & Firestein, 2002). Although duplications, mutations, and adaptive evolution certainly play an important role in the evolution of chemoreceptors and their associated behaviours (Horth, 2007; Karn *et al.* 2010; Smadja *et al.* 2015), variation in expression at these genes can also strongly influence mouse behaviour (Keller *et al.* 2009; Zhang *et al.* 2010; Bear *et al.* 2016). A recent study has demonstrated that changes in expression patterns of vomeronasal receptors can induce changes in patterns of mate preference between laboratory strains of the house mouse (Broad & Keverne 2012), confirming the key role of the vomeronasal organ and its receptors in the recognition and transduction of olfactory signals used in mate choice, as well as the possible link between variation in VR expression and variation in mate preference in mice.

Predictions and experimental design

We hypothesized that the observed behavioural divergence between populations close to the hybrid zone and displaying strong assortative mate preferences (hereafter ‘Choosy’ populations) and populations further away from the hybrid zone and not displaying any directional mate preferences (hereafter ‘Non-Choosy’ populations) could originate from

divergence in expression of some vomeronasal receptors. To test this hypothesis, we conducted deep RNA-sequencing of whole vomeronasal organs, an approach that has already been validated for detection of expression variation at VRs (Ibarra-Soria *et al.* 2014). We compared adult female and male mice originating from different ‘Non-Choosy’ and ‘Choosy’ populations of *M. m. musculus* and *M. m. domesticus*. Estimates of differential expression allowed us to identify specific sets of vomeronasal receptors and quantify differences in receptor expression potentially related to the shift toward assortative mate preference in the hybrid zone.

MATERIAL AND METHODS

Samples

Mice used in the study

Patterns of gene expression were studied in adult mice that were born in the wild, trapped in Jutland, Denmark in October 2010 in several sites (indoor farms and other human dwellings) and then maintained in the laboratory under controlled conditions before being behaviourally tested and euthanised for dissection. As it is known that postnatal odour exposure can influence patterns of mate choice and VNO gene expression of adult mice (Broad & Keverne 2012; Chamero *et al.* 2012; Cadiou *et al.* 2014), this experimental strategy allowed us to study behavioural and expression patterns characterising wild animals, while controlling for some factors (odorant environment, age range, diet) just before dissection that could inflate variance in expression among samples.

Mice were trapped in three distinct sampling areas, characterised by populations with distinct mate preference behaviours (Figure S1): (1) the border of the hybrid zone on the *M. m. musculus* side (50 km North to the genetic centre of hybrid zone (defined in Raufaste *et al.* 2005)) where strong assortative mating has been documented ('*musculus* Choosy' samples) (Smadja *et al.* 2004b, 2015; Smadja & Ganem 2005; Ganem *et al.* 2008), (2) another area in Denmark but further away from the hybrid zone, where mate preference was not assortative ('*musculus* Non-Choosy' samples) (Smadja *et al.* 2015); and (3) the border of the hybrid zone on the *M. m. domesticus* side (50 km South to the genetic centre of the hybrid zone), where weak assortative mating was documented ('*domesticus*' Choosy samples) (Smadja & Ganem 2005; Ganem *et al.* 2008; Latour *et al.* 2014). The *M. m. domesticus* samples were used as a control for the mapping of *M. m. musculus* reads against the house mouse reference genome, which is primarily of *M. m. domesticus* origin, and for

interspecific comparisons. We decided to focus on the *M. m. musculus* side for comparisons between 'Choosy' and 'Non-Choosy' populations, because the strongest behavioural contrasts were observed there (Smadja *et al.* 2004b; Smadja & Ganem 2005; Ganem *et al.* 2008). Two populations per geographical area were included as biological replicates, each composed of several trapping sites (six populations in total).

Mice were brought back to the laboratory in Montpellier (France) and maintained in quarantine under controlled and homogenized conditions for two months (same facility room, odorant environment, 12/12 photoperiod, and diet - food ad libitum). Only mice trapped as young adults were selected for this study, so that the ages of individuals participating to the behavioural and expression analyses were likely to have been within the age range at which VR expression in the VNO is optimal (between 90 and 120 days old, Zhang *et al.* 2010). During the period in the laboratory, mice were tested for sex odour preferences to confirm assortative preferences in the '*musculus* Choosy' group and an absence of directional preference in the '*musculus* Non Choosy' group (protocol and results reported in Smadja *et al.* 2015). We also confirmed weak assortative mate preferences in the '*domesticus* Choosy' group (Latour *et al.* 2014). We then selected eight males and eight females per population, resulting in a total of 96 adult male and female mice taking part to the RNA-seq experiment. The average mate preference of RNA-seq samples did not deviate from the average mate preference assessed in larger population samples. Geographical, genetic and behavioural characteristics of the mice used in this study are summarised in Table S1 (the method used to estimate hybridisation index of each mouse is described in detail in Latour *et al.* 2014 and Smadja *et al.* 2015 and summarised in Table S1).

Vomeroneasal organ dissection and RNA extraction

For each of the 96 female and male mice, the VNO was dissected rapidly after death by cervical dislocation. The VNO was immediately immersed in RNAlater and stored at -80C. Total RNA was extracted using Macherey-Nagel XS kits or the phenol-chloroform based TRIzol procedure. RNA extracts were subsequently quantified and checked for quality with Nanodrop to assess sample concentration and purity and with Agilent Bioanalyzer to assess sample integrity. To constitute samples representative of each of the six populations and sexes, and to obtain enough material for library preparation, equimolar mixes from the eight males or eight females per population were pooled and again checked for quantity and quality using the Agilent Bioanalyzer and Nanodrop, to obtain 3 to 5 µg per pool (200ng/µL).

Trapping and transport of mice were performed under permission from the French Ministry of Agriculture (authorization n°C34-265). Housing took place in the facilities of the University of Montpellier and followed the ethical and animal welfare guidelines of the University of Montpellier and the regional ethical committee.

Transcriptome sequencing and raw data analysis

Library preparation and sequencing

RNA-seq libraries were prepared using the TruSeq RNA sample preparation kit (Illumina) and quantified using Agilent DNA 1000 Labchip kits (Agilent Technologies, Palo Alto, CA). The libraries were then sequenced on an Illumina HiSeq 2000 platform using a paired-end 100bp read-length protocol (3 libraries per lane, 4 lanes in total). Library preparation and sequencing were carried out at the MGX -Montpellier Genomix platform (Institut de Génomique Fonctionnelle- Institut de Génétique Humaine, Montpellier, France).

Filtering and mapping

Raw reads were filtered in order to discard reads with low quality sequence. The remaining

reads were trimmed to subtract primer sequences and low quality 3' ends using *cutadapt* (Martin 2011). The package *RSEM* (Li & Dewey 2011), with default mapping parameters, was used to map the reads against the house mouse reference genome and its known transcript annotation database (<http://www.ensembl.org/>, version GRCm38.p2) and to estimate gene expression abundance. Since the reference genome used for mapping corresponds to an inbred strain of the laboratory mouse (C57BL/6J) (primarily of *M. m. domesticus* origin), we compared the distribution of expression estimations between wild *M. m. domesticus* and *M. m. musculus* samples to detect any potential mapping biases among samples.

Identification of Vomeronasal Receptor and Olfactory Receptor transcripts

Coding sequences belonging to family 1 (V1R) and family 2 (V2R) of vomeronasal receptors (Yang & Zhang 2007; Zhang *et al.* 2007) as well as coding sequences of ORs were extracted with the BioMart tool from Ensembl (<http://www.ensembl.org/>). Information on chromosome location, genomic coordinates and strand for each gene was according to the NCBI-Build-37 mouse assembly (mm9).

Expression analyses

To compare the amount of gene expression among different classes of genes (e.g. VR genes versus non-VR genes), we normalised coverage values using the Bioconductor package *CQN* (conditional quantile normalization) (version 1.2.0) (Hansen *et al.* 2012), which accounts for possible bias due to library size, gene length and GC content. For comparisons of gene expression patterns among samples and differential expression analyses, read counts were normalised using the Bioconductor package *edgeR* (version 2.13) (McCarthy *et al.* 2012) implemented for the software *R* v.3.0.1 (<http://www.r-project.org/>) and the method of trimmed mean of M values (TMM) (Robinson *et al.* 2010), which normalises for the library size and for the total number of expressed transcripts in each sample. A gene was

265 considered as expressed in one sample if the number of reads was higher than 1 count per
 266 million (CPM).
 267
 268 Multidimensional scaling (MDS) plots were generated from the 500 genes maximizing the
 269 distances between points, to visualise similarity of global gene expression between samples.
 270 Coverage at the gene level was obtained by summing up transcript coverage values
 271 associated with a given gene. To assess whether global patterns of gene expression vary
 272 between subspecies, mate preference profile groups ('*musculus* Choosy' versus '*musculus*
 273 Non-Choosy') and sexes, we first verified equal dispersions among samples and then
 274 performed permutation-based MANOVA analyses (Anderson 2001) using the PERMDISP and
 275 PERMANOVA programs of the Vegan package *Adonis* (Dixon 2003) with 1000 permutations.
 276 Two models were used: the effects of the explanatory variables "Subspecies" and "Sex"
 277 were tested on the whole dataset (counts ~ Subspecies + Sex + Subspecies*Sex) and the
 278 effects of the explanatory variables "Choosiness" (two levels: Choosy and Non-Choosy) and
 279 "Sex" were tested on *M. m. musculus* samples only (counts ~ Choosiness + Sex +
 280 Choosiness*Sex). All explanatory variables were defined as fixed factors in our models.
 281
 282 Differential expression analyses were conducted using *edgeR* (version 2.13) (McCarthy *et al.*
 283 2012). The analyses compared the two subspecies ('*domesticus* Choosy' versus '*musculus*
 284 Choosy' samples) or the two groups of *M. m. musculus* mice showing distinct behavioural
 285 profiles ('*musculus* Choosy' versus '*musculus* Non-Choosy'). Since males and females display
 286 similar patterns of mate preference within each population (e.g., Smadja *et al.* 2004b, 2015;
 287 Smadja & Ganem 2005), we considered four biological replicates per categorical group (two
 288 sexes x two populations). Only genes expressed in all samples but one of the same
 289 categorical group were included, leading to a different number of genes considered for each
 290 comparison. Because simulation analyses have shown the tendency of *edgeR* to identify a

high number of false differentially expressed genes (Soneson & Delorenzi 2013), the significance level was reduced to 0.01. Additionally, a false discovery rate (FDR) method was used to correct the threshold of *P*-values for multiple testing (Benjamini & Yekutieli 2001). The outcome of this analysis was a list of up- and down-regulated differentially expressed (DE) genes.

Enrichment analyses

Functional annotation of the significantly differentially expressed genes was performed using the functional classification program *DAVID* (Version 6.8) (Huang *et al.* 2009). Enrichment analyses were performed for DE genes between the two subspecies and for DE genes between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*. Functional categories were considered significantly enriched when $FDR < 0.05$ and we analysed functional annotation clusters, which group similar functional categories together, to identify enriched biological functions. In the case of VR genes, we ran an independent hypergeometric test, as some of the genes are not annotated as VR genes in the database. In all cases, the reference background genome included only expressed genes for each comparison, instead of using the whole reference mouse genome.

Phylogenetic and clustering analyses

To map DE genes onto the phylogenies of vomeronasal receptor gene family 1 (V1R) and 2 (V2R), we first reconstructed these gene phylogenies using available coding sequences of V1R and V2R (191 and 110 sequences respectively). Sequences were aligned using *MACSE* (Multiple Alignment of Coding Sequences; Ranwez *et al.* 2011), with manual adjustments. *jModelTest* v0.1.1 (Posada 2008) identified GTR+G+I (General Time Reversible + Gamma + Invariable sites) as the model of sequence evolution that best described the observed pattern of sequence variation for both V1R and V2R, and this model was used to perform

maximum likelihood phylogenetic analyses using *RAxML* version 8 (Stamatakis 2014). We then reported positions of DE genes identified previously between the two subspecies and between ‘Choosy’ and ‘Non-Choosy’ samples within *M. m. musculus* on the phylogenetic trees of the V1R and V2R families.

Since the phylogenetic analyses indicated a close relationship among five V2R genes that were found to be differentially expressed between the ‘Choosy’ and ‘Non-Choosy’ samples within *M. m. musculus*, we assessed the probability of phylogenetic clustering using a randomisation procedure. We randomly sampled five receptor genes in the phylogenetic tree of vomeronasal receptor family 2 and computed the total genetic distance among them using the *ape* R package (Paradis *et al.* 2004). We performed this random sampling 500,000 times to obtain an expected distribution of genetic distance. The observed value of genetic distance among the five DE V2R genes was then compared to this distribution.

Since all VR genes belong to only two different gene families, they share a significant amount of genetic identity. Since mapping was based on sequence identity, we were concerned that reads mapping to one truly differentially expressed gene may also map to other VR paralogs with close sequence identity, leading to false positive results in the clustering analysis.

Therefore, for each VR gene found to be differentially expressed in the ‘*musculus* Choosy’ versus ‘*musculus* Non-Choosy’ comparison, we retrieved the 10 closest paralogs with the program *cd-hit-est* (<http://weizhong-lab.ucsd.edu/cd-hit/>). Sequences were aligned using the program *MACSE* (Ranwez *et al.* 2011) and positions that were specific to the differentially expressed genes were recorded. Then, using mapping information, we retrieved the coverage at each position for each of the DE genes in the relevant samples, and checked that positions specific to individual genes were covered at the same level as shared positions.

RESULTS

Variation in gene expression among samples

All samples were sequenced successfully, except one from the '*Musculus* Non-Choosy' group ('*Musculus* Non-Choosy population2 Females', Table S1) due to failure in library construction. For the eleven sequenced samples, the percentage of mapped reads per sample was between 79% and 85%, and the total number of transcripts per sample varied from 33457 to 39770 (Table S2). Table S3 reports the raw and normalised counts for all genes and all samples.

The MDS plots, representing variation in overall gene coverage among samples, indicated a clear separation along dimension 1 between samples of the two subspecies (Figure 1A) and some degree of separation between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* (Figure 1B). Multivariate analysis of variance showed that the overall gene expression levels differed significantly between *M. m. musculus* and *M. m. domesticus* samples but not between sexes (perMANOVA Adonis test: $F_{\text{subspecies}} = 6.423$; $P = 0.004$; $F_{\text{sex}} = 0.804$; $P = 0.547$; $F_{\text{subspecies} \times \text{sex}} = 0.322$; $P = 0.916$). The trend for a separation between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* was not statistically significant (perMANOVA Adonis test: $F_{\text{choosiness}} = 2.424$; $P = 0.089$; $F_{\text{sex}} = 0.782$; $P = 0.537$; $F_{\text{choosiness} \times \text{sex}} = 0.506$; $P = 0.835$).

The number of expressed VR genes varied slightly among samples (Table S4a) but remained comparable (range between 197 to 252), with no significant difference between samples from the most divergent groups, i.e. the two subspecies (Wilcoxon rank sum test, $P = 0.886$). We also detected expression of Olfactory Receptors (OR) in the vomeronasal organ samples

(Tables S3 and S4). The mean levels of expression of VR and OR genes were lower than the mean coverage for the rest of the genes (mean CPM_{VR} = 3.414; mean CPM_{OR} = 0.804; mean CPM_{other_genes} = 27.198), with ORs particularly lowly expressed (Table S4b and Figure S2). In contrast, the mean of median CPM values across all samples was higher for VRs than for the other genes (mean median_{VR} = 0.57; mean median_{other_genes} = 0.29) (Figure S2).

Finally, we report expression of some mouse lipocalins in our VNO samples (Table S3). Seven Odorant Binding Proteins (OBP) were expressed in all samples (including *Obp3-p* annotated as a pseudogene in the mouse reference genome Stopková *et al.* 2016). Among the 12 Major Urinary Proteins (MUP) annotated as functional genes and pseudogenes in the reference genome and that belong to Group A (i.e. the ancestral group of MUPs for which mapping information is reliable, Logan *et al.* 2008), we detected expression of three MUPs in all samples (*Mup4*, *Mup5* and to a lesser extent *Mup6*), and four other MUPs were lowly expressed, mostly in *M. m. musculus* samples (*Mup3*, *Mup20* (also known as Darcin), *Mup-ps19* and *Mup-ps20*).

Differential expression among samples

Differential expression analyses were performed on the comparison between the two subspecies and the comparison between ‘Choosy’ and ‘Non-Choosy’ samples within *M. m. musculus*. DE genes showed log fold changes from 1 to 10 and average coverage between 1 and 12 logCPM (counts per million) (Figure 2). We found a large number of genes (2624) differentially expressed between *M. m. domesticus* and *M. m. musculus* samples (Figure 2A, Table S5a). In contrast, we only found 236 genes differentially expressed between the ‘Choosy’ and ‘Non-Choosy’ *M. m. musculus* samples (Figure 2B, Table S5b). For this comparison, there were more differentially expressed genes up-regulated in the ‘Choosy’ samples (156 genes) than the ‘Non-Choosy’ samples (90 genes) (Table S5b), and in general

the values of log fold change were also higher in the ‘Choosy’ samples (Figure 2B).

We identified 90 VR genes and one OR gene (*Olfr460*) differentially expressed between the two subspecies (Table S5a). We also identified three lipocalins that were differentially expressed between the two taxa (Table S5a): two MUP genes from Group A (*Mup6* and *Mup20*) expressed mostly in *M. m. musculus* samples, and one OBP gene (*Obp6*) only expressed in *M. m. domesticus* samples. More generally, functional analyses revealed an enrichment in 35 functional categories (GO terms) in the set of genes differentially expressed between the two subspecies, with the most enriched clusters of annotation terms corresponding to signal-related functions, vomeronasal receptors of type 2 (V2Rs) and other G-protein-coupled receptors (Table S6a). For the comparison between ‘Choosy’ and ‘Non-Choosy’ samples within *M. m. musculus*, we identified 34 enriched functional categories, with the most enriched clusters of annotation terms corresponding to immune-related functions, signal-related functions and sensory perception of smell (Table S6b). Among the genes found to be differentially expressed between ‘Choosy’ and ‘Non-Choosy’ samples, we found five V2R genes (*V2r17*, *V2r69*, *V2r70*, *V2r73* and *V2r75*), which corresponds to a significant enrichment of this category of genes (hypergeometric test: $P < 0.01$ with 5 out of 140 in a sample of 236 DE genes). Among the five DE V2Rs, four were up-regulated in the ‘Choosy’ samples and one in the ‘Non-Choosy’ samples (Figure 3). Differentially expressed genes between ‘Choosy’ and ‘Non-Choosy’ samples within *M. m. musculus* also included two OR genes (*Olfr15* and *Olfr449*) but no members from family 1 of the VR genes (V1R) (Table S5b).

Phylogenetic relationships among differentially expressed vomeronasal receptor genes

When plotting the differentially expressed genes on the vomeronasal receptor phylogenies, we observed that genes differentially expressed between the two subspecies mapped

almost everywhere on the V1R and V2R phylogenies (Figure 4A and 4B). In contrast, genes differentially expressed between 'Choosy' and 'Non-Choosy' samples only mapped to family 2 of vomeronasal receptors (Figure 4B). One DE VR gene, *V2r17*, belongs to clade 6 of the V2R family. Interestingly, the other four DE VR genes belong to the same phylogenetic clade, clade 3 (genes *V2r69*, *V2r70*, *V2r73* and *V2r75*). The phylogenetic distance between the five DE VR genes was found to be significantly smaller than expected by chance (random sampling test, observed phylogenetic distance = 3.69; $p < 0.004$ and Figure S3).

The fact that closely related VR genes were found to be differentially expressed between the 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* could reflect a bias in mapping due to sequence similarity among closely-related paralogs. Indeed, these four VR belong to the same genomic cluster on chromosome 7, and are probably tandem-repeat paralogs with relatively high sequence similarity. However, we found no evidence for such a bias. Although the coverage along the gene was variable, there was no difference between the coverage of conserved and variable regions in any gene, and there were numerous variable sites with high coverage (Figure S4). This was true even when DE genes were compared to their most closely-related paralogs (same phylogenetic cluster). For example, analysis of coverage information along *V2r70* (differentially expressed) and its closest relative *V2r74* (ENSMUSG00000090774) (not differentially expressed) showed prominent differences in coverage between these two genes for both variable and conserved regions, which indicates that the mapping was able to differentiate reads from different but closely related VR genes.

DISCUSSION

Changes in expression are expected to play an important role in the evolution and divergence of traits involved in reproductive isolation. Here we have used RNA-seq on whole vomeronasal organs collected from wild mice sampled in Denmark to test for the role of changes in expression of vomeronasal receptors in the behavioural shift toward olfactory-based assortative mating observed in the hybrid zone between the two European subspecies of the house mouse. Overall expression levels did not vary significantly between males and females in our dataset, a result in agreement with previous comparisons of male and female mouse VNO transcriptomes (Ibarra-Soria *et al.* 2014). Since assortative mate preferences are displayed by both males and females in the studied mouse populations (Smadja *et al.* 2004b; Smadja & Ganem 2005), we did not analyse differential expression between males and females further. Overall expression levels differed between the two subspecies, and we found a very large number of genes differentially expressed between the two taxa (more than 2,000 in total, and 90 VRs), consistent with the degree of divergence between the two taxa (Phifer-Rixey *et al.* 2014). In contrast, only 236 genes were found repeatedly differentially expressed between the *M. m. musculus* populations showing contrasting patterns of mate preference. Among these DE genes between 'Choosy' and 'Non-Choosy' samples, we found an overrepresentation of genes belonging to family 2 of vomeronasal receptors, known to play a key role in pheromone recognition in mice (Chamero *et al.* 2012). Interestingly, four out of five of these differentially expressed VRs belong to the same phylogenetic cluster. In the following paragraphs, we focus on discussing these findings related to expression divergence between populations of *M. m. musculus* showing contrasting mate preference behaviours.

How confident can we be that differences in expression are associated with differences in behaviour?

Comparative transcriptomics has previously been used to reveal candidate genes for behavioural differences or to reconstruct preference-gene networks (reviewed in Wilkinson *et al.* 2015). In our study, how confident can we be that these differences in gene expression are, at least in part, associated with the observed behavioural shift toward assortative mating in the populations nearer to the hybrid zone? First, the use of several biological replicates for each of the two types of natural populations known to differ in their mate preference patterns minimizes the risk of observing population-specific differences that could reflect different regimes of selection or demographic events among populations. Second, we sampled *M. m. musculus* populations with contrasting mate preference behaviours but are geographically close in Denmark. The different populations thus share a very recent common ancestor, have similar low rates of introgression from *M. m. domesticus* and live in similar environments. Our aim was to minimize any other possible natural sources of divergence between these populations and therefore maximize the chance that genetic/expression differences reflect, at least in part, the known behavioural difference between the groups. Since VR expression at the adult stage is known to be influenced by postnatal (from birth to 19 days old) exposure to odours (environment, parental imprinting) at least at the kin recognition level (Broad & Keverne 2012), we decided to study wild-caught mice to ensure assessment of patterns of gene expression as close as possible to natural populations, i.e. potentially influenced by a combination of innate and imprinted mechanisms. However, to homogenise replicates further and avoid any possible variation in gene expression due to differing physiological conditions, we maintained wild-caught mice in the laboratory before dissection to control for the main factors known to influence expression in general and VR expression in particular (diet, temperature, photoperiod, the olfactory environment, age range). Although we could not strictly control

for the age of these wild-caught individuals, we selected only mice trapped as young adults expected to fall in the age range corresponding to the peaked plateau of VR expression in adult mice (Zhang *et al.* 2010). This ensured that age-related variation in VR expression would not strongly influence our results.

Considering that the experiment was performed using wild animals from natural populations, we cannot completely exclude other sources of variation in the observed profiles of gene expression. However, we consider that this series of experimental settings, combined with a targeted approach to candidate genes likely to be involved in the behaviour of interest, offers optimal conditions to detect association between assortative mate preferences and differential gene expression.

Identity and functions of differentially expressed genes

Our main objective was to assess patterns of expression of vomeronasal receptors. However, we identified several other classes of genes showing repeated patterns of differential expression between 'Choosy' and 'Non-Choosy' samples. Several of these genes have physiological functions related to signal transportation, perception and transduction. Enrichment analyses showed an overrepresentation of genes involved in regulation of odorant selectivity in the extracellular space and olfactory lumen. This includes genes coding for androgen-binding proteins (ABP), previously suggested to act as subspecific signals in mice (Laukaitis *et al.* 1997; Bimova *et al.* 2011), and some organic anion transporters (OAT) expressed in the mouse olfactory mucosa. The latter are potentially involved in odour signalling by binding and transporting organic anions present in urine (Kaler *et al.* 2006). Other enriched categories include genes involved in regulation of signal transduction (cyclic-nucleotide-gated channel (CNG) gene family; Francia, Pifferi, Menini, & Tirindelli, 2014). We also identified two olfactory receptors differentially expressed between the two behavioural groups. Although ORs are primarily expressed in the MOE, our results are congruent with a

recent study reporting expression of ORs in mouse VNOs (Ibarra-Soria *et al.* 2014). We did not detect differential expression of MUPs (from Group A) or OBPs between ‘Choosy’ and ‘Non-Choosy’ samples, but could confirm previous findings evidencing their expression in VNO tissues (Utsumi *et al.* 1999; Ibarra-Soria *et al.* 2014; Stopková *et al.* 2016) and expression divergence at some MUP and OBP genes between the two subspecies (although not affecting the same genes as in Stopková *et al.* 2016). These lipocalins expressed in the VNO may play a role in sequestering volatile pheromones and possibly transporting them to vomeronasal receptors (Sharrow *et al.* 2002; Stopková *et al.* 2016). Interestingly, the diversity of genes and functions identified as differentially expressed between ‘Choosy’ and ‘Non-Choosy’ samples suggests that the evolution of assortative mating involves a complex network of VNO genes that will need further investigation. Additionally, genes expressed in other olfactory tissues (MOE) or involved in the integration of the signal in the brain, which we did not study here, may also play an important role in the expression of assortative mate preference.

As far as VR are concerned, we identified five receptor genes (*V2r17*, *V2r69*, *V2r70*, *V2r73* and *V2r75*) that showed repeated differential expression between the two behavioural groups. Since in most cases only one functional receptor is expressed per neuron in the vomeronasal organ (Dalton & Lomvardas 2015; Ishii & Mombaerts 2011), the observed expression divergence at individual VRs could result from differences in transcript level per neuron (Young *et al.* 2003), but also from differences in the numbers of neurons expressing a given receptor, a parameter that shapes the olfactory map in the accessory olfactory bulb and provides to the brain the signal for a behavioural response (Mombaerts 2004; Bear *et al.* 2016). Since we used RNA-seq on whole vomeronasal organs, our experiment does not allow us to distinguish these two levels of expression, but is informative about the overall pattern of variation and divergence at individual VRs that likely influence odour perception.

546 Four of the differentially expressed VR were found to be up-regulated in the 'Choosy'
 547 samples and one was up-regulated in the 'Non-Choosy' samples. A majority of VR genes
 548 upregulated in the 'Choosy' samples is consistent with the idea that some receptors are
 549 recruited in the zone of contact with the other subspecies to allow the expression of
 550 assortative mate preferences.

551 In contrast to differentially expressed VRs between the two subspecies belonging to both
 552 families of VRs (family 1 and family 2), expression differentiation within *M. m. musculus*
 553 between 'Choosy' and 'Non-Choosy' samples only affects members of family 2 (V2R). This
 554 corresponds to a significant enrichment of this category of genes among all DE genes.

555 Traditionally, V2Rs were thought to be involved primarily in pheromone recognition (as
 556 opposed to V1Rs that were thought to be involved in the detection of the physiological
 557 status of other animals). Recent findings have shown that individual V2Rs are specifically
 558 tuned to detect the nature of the signal emitter (e.g. conspecifics, heterospecifics, individual
 559 recognition), whereas individual V1Rs are activated by cues from multiple species (Isogai *et al.*
 560 2011). Additionally, V2R-positive Vomeronasal Sensory Neurons detect members of
 561 several large peptide or protein families (in particular MHC class I peptides and some Major
 562 Urinary Proteins (MUPs) secreted in urine) that are thought to inform the receiver about the
 563 genetic identity of a signaler (Hurst *et al.* 2001; Leinders-Zufall *et al.* 2004; Papes *et al.* 2010;
 564 Chamero *et al.* 2012). In agreement with this, urines have been shown to diverge in their
 565 composition of MUPs between the two subspecies (Hurst *et al.* 2017; Stopková *et al.* 2007)
 566 and between 'Choosy' versus 'Non-Choosy' samples within *M. m. musculus* (Hurst *et al.*
 567 2017), which establishes MUPs as potential candidates acting as subspecific signals and
 568 potentially recognised by V2Rs. Since our study also confirms previous findings that indicate
 569 that some MUPs are also expressed in the VNO, a future challenge will be to understand
 570 better the respective role of VNO-expressed MUPs and MUPs expressed in the liver and
 571 excreted in urine in mouse communication.

Phylogenetic clustering and functional specialisation of differentially expressed vomeronasal receptors

We found that four out of five of the differentially expressed V2Rs belong to the same phylogenetic cluster (V2r69, V2r70, V2r73 and V2r75 in clade 3 of the V2R family). Although these four DE genes are in the same genomic cluster on chromosome 7 and share a relatively high degree of sequence similarity, we have evidence to exclude the possibility of a methodological artefact. We did not find any evidence for a bias when aligning these DE genes with other closely-related paralogs and comparing coverage information in specific versus shared positions along these genes. Moreover, some of the closely-related paralogs belonging to the same genomic cluster as the DE genes did not show significant differential expression. If this pattern of phylogenetic clustering of DE VRs is true, it suggests that the evolution of assortative mate preference in *M. m. musculus* could involve the specialisation of a group of closely-related receptors in the clade 3 of V2Rs. This hypothesis is consistent with the study by Isogai *et al.* (2011) which showed that distinct mouse VR receptor subfamilies have evolved towards the specific recognition of certain animal groups (heterospecific signals from predators or sympatric closely-related species/subspecies, male or female conspecific signals) or chemical structures (volatile steroids, non-volatile proteins). Interestingly, the VNOs analysed in their study were from *M. m. domesticus* (laboratory mice), and when tested using *M. m. musculus* scents, several V2Rs were activated including V2r17 (DE gene in our study) and its close relatives and V2r66 (belonging to the same clade 3 as the four VRs differentially expressed in our study). Other receptors identified as differentially expressed in our study could not be individually functionally characterised. Based on these results, these authors and others (Chamero *et al.* 2012; Wynn *et al.* 2012) hypothesized a role of these VRs in reproductive isolation. An interesting follow-up from our work would be to apply the methods developed by Isogai *et al.* to test whether the same set

of closely-related V2Rs, identified by these authors as activated by *M. m. musculus* scents when expressed in *M. m. domesticus* VNOs, would be activated by *M. m. domesticus* stimuli when expressed by *M. m. musculus* VNOs, as our RNA-seq results suggest.

Evolution of expression changes and the role of selection

Regulatory changes in genes involved in reproduction are thought to be prime targets for divergence during speciation. They are expected to evolve under natural and/or sexual selection. Previous studies had shown that expression divergence in testis-associated genes seems to establish during a later phase of the speciation process when reproductive isolation is complete (Voolstra, Tautz, Farbrother, Eichinger, & Harr, 2007; Bryk, Somel, Lorenc, & Teschke, 2013). In our study, we provide evidence for expression divergence in VNO-related genes between populations within the *M. m. musculus* subspecies, in a context of secondary contact with the subspecies *M. m. domesticus*. This result may suggest that expression divergence at these genes has evolved rapidly in the ‘Choosy’ populations from the border of the hybrid zone. Whatever the source of expression divergence (new mutations, standing variation, hybridisation), changes in expression related to the evolution of assortative mate preferences are expected, in the context of reinforcement, to evolve rapidly under diverging selection (natural and sexual selection against hybridisation in the house mouse system, Britton-Davidian *et al.* 2005; Latour *et al.* 2014). Although accumulating evidence suggests a predominantly neutral model of gene expression evolution (e.g. in house mice, Staubach, Teschke, Voolstra, Wolf, & Tautz, 2010), positive selection has also been shown to shape changes in expression for some genes and lineages (e.g. Wittkopp, Haerum, & Clark, 2008; Jeukens & Bernatchez, 2012; Chapman *et al.* 2013). In a recent study, we identified several genomic regions surrounding VR genes in the house mouse with signatures of positive selection specifically in populations from the border of the hybrid zone that display assortative mate preference (same samples as the ‘Choosy’ *M. m.*

musculus samples analysed in the present study). Interestingly, one of these genomic regions corresponds to the downstream and putative regulatory region of the cluster containing the candidate VRs identified in the present study, in the vicinity of *V2r75* (Smadja *et al.* 2015). Another study on wild-derived strains of the two subspecies has suggested that V2Rs identified by Isogai *et al.* (2011) as detecting subspecific cues are prone to evolve under positive selection, in contrast to V2Rs detecting other classes of signals (Wynn *et al.* 2012). Analysing genome-wide patterns of nucleotide polymorphism and divergence in the same biological samples as the ones used in the present study should allow us to confirm whether some differentially expressed vomeronasal receptors or their *cis*-regulatory regions evolve under divergent selection in the populations displaying assortative mate preferences.

To conclude, this study suggests that changes in expression at key receptors for pheromone recognition may play an important role in the evolution of assortative mating and reinforced sexual isolation in the house mouse. Evidence for differential expression at some closely-related vomeronasal receptors between populations within *M. m. musculus* that do or do not display assortative mate preferences (close to or more distant from the hybrid zone, respectively) implicates them in this behavioural shift. This is consistent with previous findings suggesting the specialisation of this group of receptors in the recognition of odorant signals that may allow subspecies recognition and assortative mating.

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894 DATA ACCESSIBILITY

895 Raw reads have been archived in the NCBI SRA public database under the bioproject
896 identifier PRJNA356041 and through the biosample identifiers SAMN06212561 to
897 SAMN06212563 and SAMN06212565 to SAMN06212572. Expression values (raw and
898 normalised counts) for each gene in each sample are provided in Table S3.

899

900 Author contributions

901 C.S., P.B. and G.G. conceived the experiment. C.S., G.G. and P.C. conducted fieldwork and
902 dissections. P.C. and D.S. performed RNA sample and library preparation and sequencing.
903 E.L. and S.T. analysed the data. C.S. wrote the manuscript and E.L., S.T. and G.G. provided
904 comments and revisions on it.

905

MAIN FIGURES

Figure 1: Multi-Dimensional Scaling (MDS) plots indicating variation in gene expression among (A) all *M. m. musculus* and *M. m. domesticus* samples and (B) *M. m. musculus* samples only.

932

933 **Figure 2:** Smear plots indicating significantly differentially expressed genes (red dots) and
 934 vomeronasal receptors (blue dots) between (A) *M. m. musculus* 'Choosy' and *M. m.*
 935 *domesticus* 'Choosy' samples; (B) 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*.
 936 Non-differentially expressed genes are shown as black dots. The blue vertical lines indicate 1
 937 log fold change. The x-axis indicates genes that are up regulated in each sample (plot A: dots
 938 on the right and on the left corresponding to genes up regulated in *M. m. musculus* and in
 939 *M. m. domesticus* respectively; plot B: dots on the right and on the left corresponding to
 940 genes up regulated in 'Choosy' *M. m. musculus* and in 'Non-Choosy' *M. m. musculus*
 941 respectively). The y-axis indicates the level of average expression of the gene (highly
 942 expressed with higher values). CPM: counts per million; FC: fold change.

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947 **Figure 3:** Heatmap plot of differentially expressed vomeronasal receptor (VR) genes in the
948 comparison between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples. The values are
949 presented in log fold change (FC) per gene, in which the sample with the lowest expression
950 value corresponds to 0.

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956 **Figure 4:** Phylogenetic trees of the two families of vomeronasal receptors (VR) (A: family 1;

957 B: family 2). Branches coloured in blue indicate differentially expressed VR between *M. m.*

958 *musculus* and *M. m. domesticus*. The number of differentially expressed VR genes between

959 the two subspecies and the total number of VR genes in each subfamily (clade) are

960 presented between brackets (e.g. 8/29). Branches coloured in orange indicate differentially

961 expressed VR between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*. Branches

962 coloured in red indicate VR genes with significant differential expression in both

963 comparisons. In family 2, clade 3 is shown in detail with receptor IDs (MGI nomenclature,

964 see Methods).

965

Supplemental Information for:

Do changes in gene expression contribute to sexual isolation and reinforcement in the house mouse?

Etienne Loire, Sergio Tusso, Pierre Caminade, Dany Severac, Pierre Boursot, Guila Ganem and
Carole M. Smadja

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Table S2: General mapping and gene expression statistics (separated Excel file)

Table S3: Raw and normalised (CPM) counts for all genes and all samples (separated Excel file, sheet 1: ALL_GENES_raw_counts; sheet 2: ALL_GENES_normalised_counts, sheet 3: OR_normalised_counts; sheet 4: VR_normalised_counts; sheet 5: MUP_normalised_counts; sheet 6: OBP_normalised_counts)

Table S4: Variation in mean count and in expressed gene number among samples (separated Excel file, sheet 1: Table S4a: number and percentage of expressed genes per sample; sheet 2: Table S4b: average counts per sample)

Table S5: List of differentially expressed genes (separated Excel file, sheet 1: Table S5a: Differentially expressed genes between "Choosy" *Mus musculus musculus* and "Choosy" *M. m. domesticus*; sheet 2: Table S5b: Differentially expressed genes between "Choosy" and "Non-Choosy" samples within *Mus musculus musculus*)

Table S6: Functional enrichment results (separated Excel file, sheet 1: Table S6a: enriched functional categories in differentially expressed genes between 'musculus Choosy' and 'domesticus Choosy' samples; sheet 2: Table S6b: enriched functional categories in differentially expressed genes between 'musculus Choosy' and 'musculus Non-Choosy' samples)

Figure S1: Map indicating the geographical origins of the mice used in this study.

Figure S2: Violin plots of log-transformed normalised gene coverage.

Figure S3: Distribution of phylogenetic distances among random sampling of five vomeronasal receptor genes.

Figure S4: Coverage per position (bp) for the five differentially expressed VR genes in the comparison between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples.

Supplementary information

Discussion:

Robustness of differential expression inferences

RNA-seq on pooled samples is a cost-effective strategy widely used to assess variation in gene expression among experimental conditions or biological groups while producing transcriptome-wide data (reviewed in Todd *et al.* 2016). This pooling design offers an advantage by increasing sample size into the analysis, and when working with samples that may exhibit a large amount of biological variation, analysing several sample pools, rather than a few single samples per condition, also lessens the impact of single aberrant samples (Todd *et al.* 2016). Although sample pooling can theoretically result in lower precision in gene expression estimation (for lowly expressed transcripts) and increased false positive rates in differential expression (DE) analyses as compared to individual sequencing (Rajkumar *et al.* 2015), this limitation is overcome when the number of individuals per pool is sufficiently large ($n > 5$) and the number of biological replicates per group is maximized (Todd *et al.* 2016). By using eight individuals per pool and from three to four biological replicates per group for DE analyses, we placed ourselves in favourable conditions to reliably identify changes in gene expression between groups with a pooling design. To minimize further the risk of false positives in DE analyses, we also applied a stringent false discovery correction and selected the method for DE detection (*edgeR*) with the highest sensitivity and specificity compared to other methods (e.g. *DESeq2*, *Cuffdiff2*) (Rajkumar *et al.* 2015). Altogether, the stringent DE analysis and the consistency of differential expression among biological replicates strongly support the idea of divergence in gene expression between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples.

Figure S1: Map indicating the geographical origins of the mice used in this study (adapted from Latour et al. 2014). Trapping sites in Jutland (Denmark) used in Latour et al. 2014 (the bold black line represents the genetic centre of the hybrid zone as defined in Raufaste et al 2005). The colored circles indicate the trapping sites defining the populations analysed in this study (red: “Non-Choosy *M. m. musculus* populations”; orange : “Choosy *M. m. musculus* populations”; blue: “Choosy *M. m. domesticus* populations”)

Figure S2: Violin plots of log-transformed normalised gene coverage (CPM) per population for vomeronasal receptor (VR) (red), olfactory receptor (OR) (green) and all other categories of genes (blue).

Figure S3: Distribution of phylogenetic distances among random sampling of five vomeronasal receptor genes. The arrow represents the observed distance between the five VRs found differentially expressed between 'Choosy' and 'Non-Choosy' samples, and its probability.

Figure S4: Coverage per position (bp) for the five differentially expressed VR genes in the comparison between ‘Choosy’ and ‘Non-Choosy’ *M. m. musculus* samples. Vertical lines represent variable sites of the gene.

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