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Title

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

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**Running title:** candidate genes for sexual isolation in mice

**Keywords:** assortative mate preference, speciation, hybrid zone, RNA-seq, olfaction, vomeronasal receptors

Abstract

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

49 **Introduction**

50 Understanding the genetic basis of speciation remains a key challenge in evolutionary
51 biology (Seehausen *et al.* 2014), in particular determining the genetic architecture of
52 differentiation and reproductive isolation (Nosil & Feder 2012), the types of genes involved
53 (Nosil & Schluter, 2011), and the types of genetic changes responsible for the evolution of
54 reproductive isolation between diverging taxa (Hoekstra & Coyne 2007). Although evidence
55 is rapidly accumulating for the role of protein coding and structural changes in adaptation
56 and reproductive isolation (Stapley *et al.* 2010), it has also long been argued that gene
57 expression differences may be of prime importance in species differentiation (King & Wilson
58 1975; Carroll *et al.* 2005), allowing rapid divergence (Wolf *et al.* 2010). Moreover, measures
59 of gene expression provide an important bridge between genotype and phenotype (Huestis
60 & Marshall 2009). In recent years, the study of gene expression has been greatly facilitated
61 by high-throughput sequencing-based methods such as RNA-seq (Mortazavi *et al.* 2008;
62 Wang *et al.* 2009), contributing to understand the importance of expression variation in both
63 local adaptation and speciation.

64

65 *Gene expression changes and the evolution of reproductive isolation*

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

127 2004a; Ganem *et al.* 2008; Bimova *et al.* 2011), suggesting a role of the olfactory system in
128 this behavioural shift. In mammals, two organs are responsible for odorant signal
129 recognition: the main olfactory epithelium (MOE), primarily involved in recognition of
130 airborne chemicals present in the environment (odorants and pheromones), and the
131 accessory olfactory organ or vomeronasal organ (VNO), known to play a key role in both
132 volatile and non-volatile kairomone and pheromone recognition (Isogai *et al*. 2011;
133 Chamero, Leinders-Zufall, & Zufall, 2012; Liberles, 2014; Bear *et al.* 2016). Recognition in
134 these organs involves chemoreceptors (olfactory receptors (ORs) in the MOE and
135 vomeronasal receptors (VRs) in the VNO) that are organised in very large multigene families
136 in the mouse genome (more than 1,200 ORs and 530 VRs, Ibarra-Soria, Levitin, & Logan,
137 2013; Ibarra-Soria, Levitin, Saraiva, & Logan, 2014; Zhang & Firestein, 2002). Although
138 duplications, mutations, and adaptive evolution certainly play an important role in the
139 evolution of chemoreceptors and their associated behaviours (Horth, 2007; Karn *et al.* 2010;
140 Smadja *et al*. 2015), variation in expression at these genes can also strongly influence mouse
141 behaviour (Keller *et al.* 2009; Zhang *et al.* 2010; Bear *et al.* 2016). A recent study has
142 demonstrated that changes in expression patterns of vomeronasal receptors can induce
143 changes in patterns of mate preference between laboratory strains of the house mouse
144 (Broad & Keverne 2012), confirming the key role of the vomeronasal organ and its receptors
145 in the recognition and transduction of olfactory signals used in mate choice, as well as the
146 possible link between variation in VR expression and variation in mate preference in mice.
147
148 *Predictions and experimental design*
149 We hypothesized that the observed behavioural divergence between populations close to
150 the hybrid zone and displaying strong assortative mate preferences (hereafter 'Choosy'
151 populations) and populations further away from the hybrid zone and not displaying any
152 directional mate preferences (hereafter 'Non-Choosy' populations) could originate from

153 divergence in expression of some vomeronasal receptors. To test this hypothesis, we
154 conducted deep RNA-sequencing of whole vomeronasal organs, an approach that has
155 already been validated for detection of expression variation at VRs (Ibarra-Soria *et al.* 2014).
156 We compared adult female and male mice originating from different 'Non-Choosy' and
157 'Choosy' populations of *M. m. musculus* and *M. m. domesticus*. Estimates of differential
158 expression allowed us to identify specific sets of vomeronasal receptors and quantify
159 differences in receptor expression potentially related to the shift toward assortative mate
160 preference in the hybrid zone.
161

162 **MATERIAL AND METHODS**

163

164 **Samples**

165 *Mice used in the study*

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

175

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

188 interspecific comparisons. We decided to focus on the *M. m. musculus* side for comparisons
189 between 'Choosy' and 'Non-Choosy' populations, because the strongest behavioural
190 contrasts were observed there (Smadja *et al.* 2004b; Smadja & Ganem 2005; Ganem *et al.*
191 2008). Two populations per geographical area were included as biological replicates, each
192 composed of several trapping sites (six populations in total).
193
194 Mice were brought back to the laboratory in Montpellier (France) and maintained in
195 quarantine under controlled and homogenized conditions for two months (same facility
196 room, odorant environment, 12/12 photoperiod, and diet - food ad libitum). Only mice
197 trapped as young adults were selected for this study, so that the ages of individuals
198 participating to the behavioural and expression analyses were likely to have been within the
199 age range at which VR expression in the VNO is optimal (between 90 and 120 days old,
200 Zhang *et al.* 2010). During the period in the laboratory, mice were tested for sex odour
201 preferences to confirm assortative preferences in the '*musculus* Choosy' group and an
202 absence of directional preference in the '*musculus* Non Choosy' group (protocol and results
203 reported in Smadja *et al*. 2015). We also confirmed weak assortative mate preferences in
204 the '*domesticus* Choosy' group (Latour *et al.* 2014). We then selected eight males and eight
205 females per population, resulting in a total of 96 adult male and female mice taking part to
206 the RNA-seq experiment. The average mate preference of RNA-seq samples did not deviate
207 from the average mate preference assessed in larger population samples. Geographical,
208 genetic and behavioural characteristics of the mice used in this study are summarised in
209 Table S1 (the method used to estimate hybridisation index of each mouse is described in
210 detail in Latour *et al*. 2014 and Smadja *et al*. 2015 and summarised in Table S1).
211
212 *Vomeronasal organ dissection and RNA extraction*

213 For each of the 96 female and male mice, the VNO was dissected rapidly after death by
214 cervical dislocation. The VNO was immediately immersed in RNAlater and stored at -80C.
215 Total RNA was extracted using Macherey-Nagel XS kits or the phenol-chloroform based
216 TRIzol procedure. RNA extracts were subsequently quantified and checked for quality with
217 Nanodrop to assess sample concentration and purity and with Agilent Bioanalyzer to assess
218 sample integrity. To constitute samples representative of each of the six populations and
219 sexes, and to obtain enough material for library preparation, equimolar mixes from the eight
220 males or eight females per population were pooled and again checked for quantity and
221 quality using the Agilent Bioanalyzer and Nanodrop, to obtain 3 to 5 µg per pool (200ng/µL).
222
223 Trapping and transport of mice were performed under permission from the French Ministry
224 of Agriculture (authorization n°C34-265). Housing took place in the facilities of the University
225 of Montpellier and followed the ethical and animal welfare guidelines of the University of
226 Montpellier and the regional ethical commitee.
227
228 **Transcriptome sequencing and raw data analysis**
229 *Library preparation and sequencing*
230 RNA-seq libraries were prepared using the TruSeq RNA sample preparation kit (Illumina) and
231 quantified using Agilent DNA 1000 Labchip kits (Agilent Technologies, Palo Alto, CA). The
232 libraries were then sequenced on an Illumina HiSeq 2000 platform using a paired-end 100bp
233 read-length protocol (3 libraries per lane, 4 lanes in total). Library preparation and
234 sequencing were carried out at the MGX -Montpellier Genomix platform (Institut de
235 Génomique Fonctionnelle- Institut de Génétique Humaine, Montpellier, France).
236
237 *Filtering and mapping*
238 Raw reads were filtered in order to discard reads with low quality sequence. The remaining

239 reads were trimmed to subtract primer sequences and low quality 3' ends using *cutadapt*
240 (Martin 2011). The package *RSEM* (Li & Dewey 2011), with default mapping parameters, was
241 used to map the reads against the house mouse reference genome and its known transcript
242 annotation database (http://www.ensembl.org/, version GRCm38.p2) and to estimate gene
243 expression abundance. Since the reference genome used for mapping corresponds to an
244 inbred strain of the laboratory mouse (C57BL/6J) (primarily of *M. m. domesticus* origin), we
245 compared the distribution of expression estimations between wild *M. m. domesticus* and *M.*
246 *m. musculus* samples to detect any potential mapping biases among samples.
247
248 *Identification of Vomeronasal Receptor and Olfactory Receptor transcripts*
249 Coding sequences belonging to family 1 (V1R) and family 2 (V2R) of vomeronasal receptors
250 (Yang & Zhang 2007; Zhang *et al.* 2007) as well as coding sequences of ORs were extracted
251 with the BioMart tool from Ensembl (http://www.ensembl.org/). Information on
252 chromosome location, genomic coordinates and strand for each gene was according to the
253 NCBI-Build-37 mouse assembly (mm9).
254
255 **Expression analyses**
256 To compare the amount of gene expression among different classes of genes (e.g. VR genes
257 versus non-VR genes), we normalised coverage values using the Bioconductor package *CQN*
258 (conditional quantile normalization) (version 1.2.0) (Hansen *et al.* 2012), which accounts for
259 possible bias due to library size, gene length and GC content. For comparisons of gene
260 expression patterns among samples and differential expression analyses, read counts were
261 normalised using the Bioconductor package *edgeR* (version 2.13) (McCarthy et al. 2012)
262 implemented for the software *R* v.3.0.1 (http://www.r-project.org/) and the method of
263 trimmed mean of M values (TMM) (Robinson *et al.* 2010), which normalises for the library
264 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 ('d*omesticus* 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 categorial 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

291 high number of false differentially expressed genes (Soneson & Delorenzi 2013), the
292 significance level was reduced to 0.01. Additionally, a false discovery rate (FDR) method was
293 used to correct the threshold of *P*-values for multiple testing (Benjamini & Yekutieli 2001).
294 The outcome of this analysis was a list of up- and down-regulated differentially expressed
295 (DE) genes.
296
297 **Enrichment analyses**
298 Functional annotation of the significantly differentially expressed genes was performed
299 using the functional classification program *DAVID* (Version 6.8) (Huang *et al.* 2009).
300 Enrichment analyses were performed for DE genes between the two subspecies and for DE
301 genes between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*. Functional
302 categories were considered significantly enriched when FDR **<** 0.05 and we analysed
303 functional annotation clusters, which group similar functional categories together, to
304 identify enriched biological functions. In the case of VR genes, we ran an independent hyper-
305 geometric test, as some of the genes are not annotated as VR genes in the database. In all
306 cases, the reference background genome included only expressed genes for each
307 comparison, instead of using the whole reference mouse genome.
308
309 **Phylogenetic and clustering analyses**
310 To map DE genes onto the phylogenies of vomeronasal receptor gene family 1 (V1R) and 2
311 (V2R), we first reconstructed these gene phylogenies using available coding sequences of
312 V1R and V2R (191 and 110 sequences respectively). Sequences were aligned using *MACSE*
313 (Multiple Alignment of Coding Sequences; Ranwez *et al.* 2011), with manual adjustments.
314 *jModelTest* v0.1.1 (Posada 2008) identified GTR+G+I (General Time Reversible + Gamma +
315 Invariable sites) as the model of sequence evolution that best described the observed
316 pattern of sequence variation for both V1R and V2R, and this model was used to perform

317 maximum likelihood phylogenetic analyses using *RAxML* version 8 (Stamatakis 2014). We
318 then reported positions of DE genes identified previously between the two subspecies and
319 between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* on the phylogenetic
320 trees of the V1R and V2R families.
321
322 Since the phylogenetic analyses indicated a close relationship among five V2R genes that
323 were found to be differentially expressed between the 'Choosy' and 'Non-Choosy' samples
324 within *M. m. musculus*, we assessed the probability of phylogenetic clustering using a
325 randomisation procedure. We randomly sampled five receptor genes in the phylogenetic
326 tree of vomeronasal receptor family 2 and computed the total genetic distance among them
327 using the *ape* R package (Paradis *et al.* 2004). We performed this random sampling 500,000
328 times to obtain an expected distribution of genetic distance. The observed value of genetic
329 distance among the five DE V2R genes was then compared to this distribution.
330
331 Since all VR genes belong to only two different gene families, they share a significant amount
332 of genetic identity. Since mapping was based on sequence identity, we were concerned that
333 reads mapping to one truly differentially expressed gene may also map to other VR paralogs
334 with close sequence identity, leading to false positive results in the clustering analysis.
335 Therefore, for each VR gene found to be differentially expressed in the '*musculus* Choosy'
336 versus '*musculus* Non-Choosy' comparison, we retrieved the 10 closest paralogs with the
337 program *cd-hit-est* (http://weizhong-lab.ucsd.edu/cd-hit/). Sequences were aligned using
338 the program *MACSE* (Ranwez *et al.* 2011) and positions that were specific to the
339 differentially expressed genes were recorded. Then, using mapping information, we
340 retrieved the coverage at each position for each of the DE genes in the relevant samples,
341 and checked that positions specific to individual genes were covered at the same level as
342 shared positions.

343

344 **RESULTS**

345

346 **Variation in gene expression among samples**

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

353

354 The MDS plots, representing variation in overall gene coverage among samples, indicated a
355 clear separation along dimension 1 between samples of the two subspecies (Figure 1A) and
356 some degree of separation between 'Choosy' and 'Non-Choosy' samples within *M. m.*
357 *musculus* (Figure 1B). Multivariate analysis of variance showed that the overall gene
358 expression levels differed significantly between *M. m. musculus* and *M. m. domesticus*
359 samples but not between sexes (perMANOVA Adonis test: $F_{subspecies} = 6.423$; $P = 0.004$; $F_{sex} =$
360 $0.804$; $P = 0.547$; $F_{subspecies \times sex} = 0.322$; $P = 0.916$). The trend for a separation between
361 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* was not statistically significant
362 (perMANOVA Adonis test: $F_{choosiness} = 2.424$; $P = 0.089$; $F_{sex} = 0.782$; $P = 0.537$; $F_{choosiness \times sex} =$
363 $0.506$; $P = 0.835$).

364

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

369 (Tables S3 and S4). The mean levels of expression of VR and OR genes were lower than the
370 mean coverage for the rest of the genes (mean CPM $_{VR}$ = 3.414; mean CPM $_{OR}$ = 0.804; mean
371 CPM $_{other_genes}$ = 27.198), with ORs particularly lowly expressed (Table S4b and Figure S2). In
372 contrast, the mean of median CPM values across all samples was higher for VRs than for the
373 other genes (mean median $_{VR}$ = 0.57; mean median $_{other_genes}$ = 0.29) (Figure S2).
374
375 Finally, we report expression of some mouse lipocalins in our VNO samples (Table S3). Seven
376 Odorant Binding Proteins (OBP) were expressed in all samples (including *Obp3-p* annotated
377 as a pseudogene in the mouse reference genome Stopková *et al.* 2016). Among the 12 Major
378 Urinary Proteins (MUP) annotated as functional genes and pseudogenes in the reference
379 genome and that belong to Group A (i.e. the ancestral group of MUPs for which mapping
380 information is reliable, Logan *et al.* 2008), we detected expression of three MUPs in all
381 samples (*Mup4*, *Mup5* and to a lesser extent *Mup6*), and four other MUPs were lowly
382 expressed, mostly in *M. m. musculus* samples (*Mup3*, *Mup20* (also known as Darcin), *Mup-*
383 *ps19* and *Mup-ps20*).
384
385 **Differential expression among samples**
386 Differential expression analyses were performed on the comparison between the two
387 subspecies and the comparison between 'Choosy' and 'Non-Choosy' samples within *M. m.*
388 *musculus*. DE genes showed log fold changes from 1 to 10 and average coverage between 1
389 and 12 logCPM (counts per million) (Figure 2). We found a large number of genes (2624)
390 differentially expressed between *M. m. domesticus* and *M. m. musculus* samples (Figure 2A,
391 Table S5a). In contrast, we only found 236 genes differentially expressed between the
392 'Choosy' and 'Non-Choosy' *M. m. musculus* samples (Figure 2B, Table S5b). For this
393 comparison, there were more differentially expressed genes up-regulated in the 'Choosy'
394 samples (156 genes) than the 'Non-Choosy' samples (90 genes) (Table S5b), and in general

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

396

397 We identified 90 VR genes and one OR gene (*Olfr460*) differentially expressed between the
398 two subspecies (Table S5a). We also identified three lipocalins that were differentially
399 expressed between the two taxa (Table S5a): two MUP genes from Group A (*Mup6* and
400 *Mup20*) expressed mostly in *M. m. musculus* samples, and one OBP gene (*Obp6*) only
401 expressed in *M. m. domesticus* samples. More generally, functional analyses revealed an
402 enrichment in 35 functional categories (GO terms) in the set of genes differentially
403 expressed between the two subspecies, with the most enriched clusters of annotation terms
404 corresponding to signal-related functions, vomeronasal receptors of type 2 (V2Rs) and other
405 G-protein-coupled receptors (Table S6a). For the comparison between 'Choosy' and 'Non-
406 Choosy' samples within *M. m. musculus,* we identified 34 enriched functional categories,
407 with the most enriched clusters of annotation terms corresponding to immune-related
408 functions, signal-related functions and sensory perception of smell (Table S6b). Among the
409 genes found to be differentially expressed between 'Choosy' and 'Non-Choosy' samples, we
410 found five V2R genes (*V2r17*, *V2r69*, *V2r70*, *V2r73* and *V2r75*), which corresponds to a
411 significant enrichment of this category of genes (hypergeometric test: $P < 0.01$ with 5 out of
412 140 in a sample of 236 DE genes). Among the five DE V2Rs, four were up-regulated in the
413 'Choosy' samples and one in the 'Non-Choosy' samples (Figure 3). Differentially expressed
414 genes between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* also included two
415 OR genes (*Olfr15* and *Olfr449*) but no members from family 1 of the VR genes (V1R) (Table
416 S5b).

417

418 **Phylogenetic relationships among differentially expressed vomeronasal receptor genes**

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

421 almost everywhere on the V1R and V2R phylogenies (Figure 4A and 4B). In contrast, genes
422 differentially expressed between 'Choosy' and 'Non-Choosy' samples only mapped to family
423 2 of vomeronasal receptors (Figure 4B). One DE VR gene, *V2r17*, belongs to clade 6 of the
424 V2R family. Interestingly, the other four DE VR genes belong to the same phylogenetic clade,
425 clade 3 (genes *V2r69*, *V2r70*, *V2r73* and *V2r75*). The phylogenetic distance between the five
426 DE VR genes was found to be significantly smaller than expected by chance (random
427 sampling test, observed phylogenetic distance = 3.69; $p < 0.004$ and Figure S3).
428
429 The fact that closely related VR genes were found to be differentially expressed between the
430 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* could reflect a bias in mapping
431 due to sequence similarity among closely-related paralogs. Indeed, these four VR belong to
432 the same genomic cluster on chromosome 7, and are probably tandem-repeat paralogs with
433 relatively high sequence similarity. However, we found no evidence for such a bias. Although
434 the coverage along the gene was variable, there was no difference between the coverage of
435 conserved and variable regions in any gene, and there were numerous variable sites with
436 high coverage (Figure S4). This was true even when DE genes were compared to their most
437 closely-related paralogs (same phylogenetic cluster). For example, analysis of coverage
438 information along *V2r70* (differentially expressed) and its closest relative *V2r74*
439 (ENSMUSG00000090774) (not differentially expressed) showed prominent differences in
440 coverage between these two genes for both variable and conserved regions, which indicates
441 that the mapping was able to differentiate reads from different but closely related VR genes.
442

443 **DISCUSSION**

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

466

467

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

494 for the age of these wild-caught individuals, we selected only mice trapped as young adults
495 expected to fall in the age range corresponding to the peaked plateau of VR expression in
496 adult mice (Zhang *et al.* 2010). This ensured that age-related variation in VR expression
497 would not strongly influence our results.
498 Considering that the experiment was performed using wild animals from natural
499 populations, we cannot completely exclude other sources of variation in the observed
500 profiles of gene expression. However, we consider that this series of experimental settings,
501 combined with a targeted approach to candidate genes likely to be involved in the behaviour
502 of interest, offers optimal conditions to detect association between assortative mate
503 preferences and differential gene expression.
504
505 *Identity and functions of differentially expressed genes*
506 Our main objective was to assess patterns of expression of vomeronasal receptors.
507 However, we identified several other classes of genes showing repeated patterns of
508 differential expression between 'Choosy' and 'Non-Choosy' samples. Several of these genes
509 have physiological functions related to signal transportation, perception and transduction.
510 Enrichment analyses showed an overrepresentation of genes involved in regulation of
511 odorant selectivity in the extracellular space and olfactory lumen. This includes genes coding
512 for androgen-binding proteins (ABP), previously suggested to act as subspecific signals in
513 mice (Laukaitis *et al.* 1997; Bimova *et al.* 2011), and some organic anion transporters (OAT)
514 expressed in the mouse olfactory mucosa. The latter are potentially involved in odour
515 signalling by binding and transporting organic anions present in urine (Kaler *et al.* 2006).
516 Other enriched categories include genes involved in regulation of signal transduction (cyclic-
517 nucleotide-gated channel (CNG) gene family; Francia, Pifferi, Menini, & Tirindelli, 2014). We
518 also identified two olfactory receptors differentially expressed between the two behavioural
519 groups. Although ORs are primarily expressed in the MOE, our results are congruent with a

520 recent study reporting expression of ORs in mouse VNOs (Ibarra-Soria *et al.* 2014). We did
521 not detect differential expression of MUPs (from Group A) or OBPs between 'Choosy' and
522 'Non-Choosy' samples, but could confirm previous findings evidencing their expression in
523 VNO tissues (Utsumi *et al.* 1999; Ibarra-Soria *et al.* 2014; Stopková *et al.* 2016) and
524 expression divergence at some MUP and OBP genes between the two subspecies (although
525 not affecting the same genes as in Stopková *et al.* 2016). These lipocalins expressed in the
526 VNO may play a role in sequestering volatile pheromones and possibly transporting them to
527 vomeronasal receptors (Sharrow *et al.* 2002; Stopková *et al.* 2016). Interestingly, the
528 diversity of genes and functions identified as differentially expressed between 'Choosy' and
529 'Non-Choosy' samples suggests that the evolution of assortative mating involves a complex
530 network of VNO genes that will need further investigation. Additionnally, genes expressed in
531 other olfactory tissues (MOE) or involved in the integration of the signal in the brain, which
532 we did not study here, may also play an important role in the expression of assortative mate
533 preference.
534
535 As far as VR are concerned, we identified five receptor genes (*V2r17*, *V2r69*, *V2r70*, *V2r73*
536 and *V2r75*) that showed repeated differential expression between the two behavioural
537 groups. Since in most cases only one functional receptor is expressed per neuron in the
538 vomeronasal organ (Dalton & Lomvardas 2015; Ishii & Mombaerts 2011), the observed
539 expression divergence at individual VRs could result from differences in transcript level per
540 neuron (Young *et al.* 2003), but also from differences in the numbers of neurons expressing
541 a given receptor, a parameter that shapes the olfactory map in the accessory olfactory bulb
542 and provides to the brain the signal for a behavioural response (Mombaerts 2004; Bear *et al.*
543 2016). Since we used RNA-seq on whole vomeronasal organs, our experiment does not allow
544 us to distinguish these two levels of expression, but is informative about the overall pattern
545 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*
560 *al.* 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.

572

573 *Phylogenetic clustering and functional specialisation of differentially expressed vomeronasal*

574 *receptors*

575 We found that four out of five of the differentially expressed V2Rs belong to the same

576 phylogenetic cluster (*V2r69*, *V2r70*, *V2r73* and *V2r75* in clade 3 of the V2R family). Although

577 these four DE genes are in the same genomic cluster on chromosome 7 and share a

578 relatively high degree of sequence similarity, we have evidence to exclude the possibility of a

579 methodological artefact. We did not find any evidence for a bias when aligning these DE

580 genes with other closely-related paralogs and comparing coverage information in specific

581 versus shared positions along these genes. Moreover, some of the closely-related paralogs

582 belonging to the same genomic cluster as the DE genes did not show significant differential

583 expression. If this pattern of phylogenetic clustering of DE VRs is true, it suggests that the

584 evolution of assortative mate preference in *M. m. musculus* could involve the specialisation

585 of a group of closely-related receptors in the clade 3 of V2Rs. This hypothesis is consistent

586 with the study by Isogai *et al*. (2011) which showed that distinct mouse VR receptor

587 subfamilies have evolved towards the specific recognition of certain animal groups

588 (heterospecific signals from predators or sympatric closely-related species/subspecies, male

589 or female conspecific signals) or chemical structures (volatile steroids, non-volatile proteins).

590 Interestingly, the VNOs analysed in their study were from *M. m. domesticus* (laboratory

591 mice), and when tested using *M. m. musculus* scents, several V2Rs were activated including

592 *V2r17* (DE gene in our study) and its close relatives and *V2r66* (belonging to the same clade 3

593 as the four VRs differentially expressed in our study). Other receptors identified as

594 differentially expressed in our study could not be individually functionally characterised.

595 Based on these results, these authors and others (Chamero *et al.* 2012; Wynn *et al.* 2012)

596 hypothesized a role of these VRs in reproductive isolation. An interesting follow-up from our

597 work would be to apply the methods developed by Isogai et al. to test whether the same set

598 of closely-related V2Rs, identified by these authors as activated by *M. m. musculus* scents
599 when expressed in *M. m. domesticus* VNOs, would be activated by *M. m. domesticus* stimuli
600 when expressed by *M. m. musculus* VNOs, as our RNA-seq results suggest.
601
602 *Evolution of expression changes and the role of selection*
603 Regulatory changes in genes involved in reproduction are thought to be prime targets for
604 divergence during speciation. They are expected to evolve under natural and/or sexual
605 selection. Previous studies had shown that expression divergence in testis-associated genes
606 seems to establish during a later phase of the speciation process when reproductive
607 isolation is complete (Voolstra, Tautz, Farbrother, Eichinger, & Harr, 2007; Bryk, Somel,
608 Lorenc, & Teschke, 2013). In our study, we provide evidence for expression divergence in
609 VNO-related genes between populations within the *M. m. musculus* subspecies, in a context
610 of secondary contact with the subspecies *M. m. domesticus*. This result may suggest that
611 expression divergence at these genes has evolved rapidly in the 'Choosy' populations from
612 the border of the hybrid zone. Whatever the source of expression divergence (new
613 mutations, standing variation, hybridisation), changes in expression related to the evolution
614 of assortative mate preferences are expected, in the context of reinforcement, to evolve
615 rapidly under diverging selection (natural and sexual selection against hybridisation in the
616 house mouse system, Britton-Davidian *et al.* 2005; Latour *et al.* 2014). Although
617 accumulating evidence suggests a predominantly neutral model of gene expression
618 evolution (e.g. in house mice, Staubach, Teschke, Voolstra, Wolf, & Tautz, 2010), positive
619 selection has also been shown to shape changes in expression for some genes and lineages
620 (e.g. Wittkopp, Haerum, & Clark, 2008; Jeukens & Bernatchez, 2012; Chapman *et al*. 2013).
621 In a recent study, we identified several genomic regions surrounding VR genes in the house
622 mouse with signatures of positive selection specifically in populations from the border of the
623 hybrid zone that display assortative mate preference (same samples as the 'Choosy' *M. m.*

624 *musculus* samples analysed in the present study). Interestingly, one of these genomic
625 regions corresponds to the downstream and putative regulatory region of the cluster
626 containing the candidate VRs identified in the present study, in the vicinity of *V2r75* (Smadja
627 *et al.* 2015). Another study on wild-derived strains of the two subspecies has suggested that
628 V2Rs identified by Isogai *et al*. (2011) as detecting subspecific cues are prone to evolve
629 under positive selection, in contrast to V2Rs detecting other classes of signals (Wynn *et al.*
630 2012). Analysing genome-wide patterns of nucleotide polymorphism and divergence in the
631 same biological samples as the ones used in the present study should allow us to confirm
632 whether some differentially expressed vomeronasal receptors or their *cis*-regulatory regions
633 evolve under divergent selection in the populations displaying assortative mate preferences.
634
635 To conclude, this study suggests that changes in expression at key receptors for pheromone
636 recognition may play an important role in the evolution of assortative mating and reinforced
637 sexual isolation in the house mouse. Evidence for differential expression at some closely-
638 related vomeronasal receptors between populations within *M. m. musculus* that do or do
639 not display assortative mate preferences (close to or more distant from the hybrid zone,
640 respectively) implicates them in this behavioural shift. This is consistent with previous
641 findings suggesting the specialisation of this group of receptors in the recognition of odorant
642 signals that may allow subspecies recognition and assortative mating.
643

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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

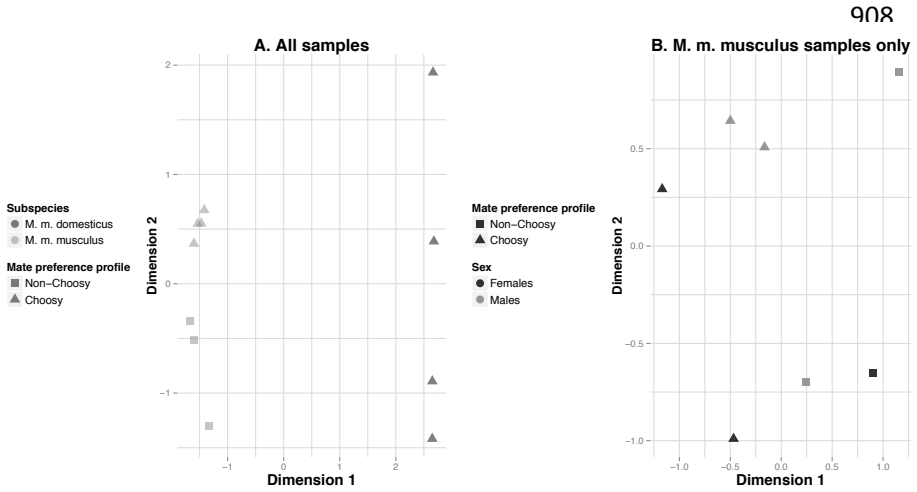
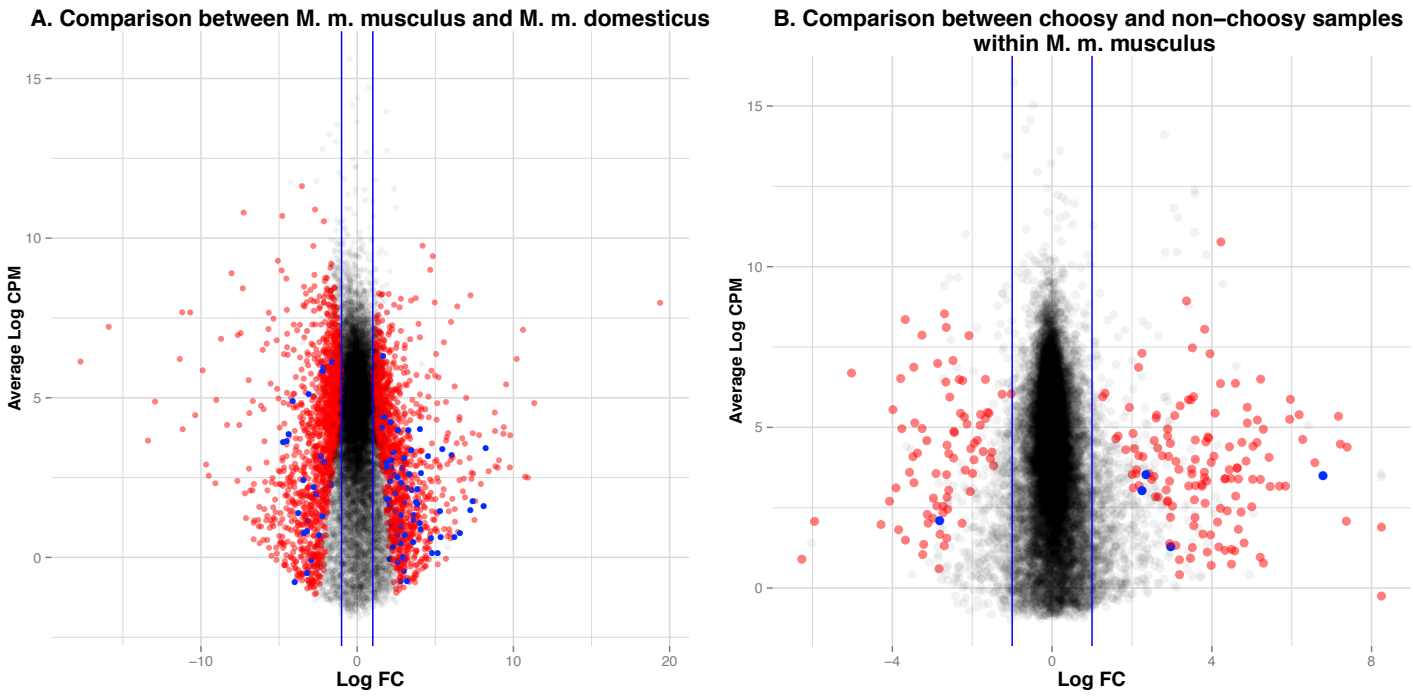



**Figure 1**: Multi-Dimensional Scaling (MDS) plots indicating variation in gene expression among (A) all M. *m. musculus* and *M. m. domesticus* samples and (B) *M. m. musculus* samples only.

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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.

943

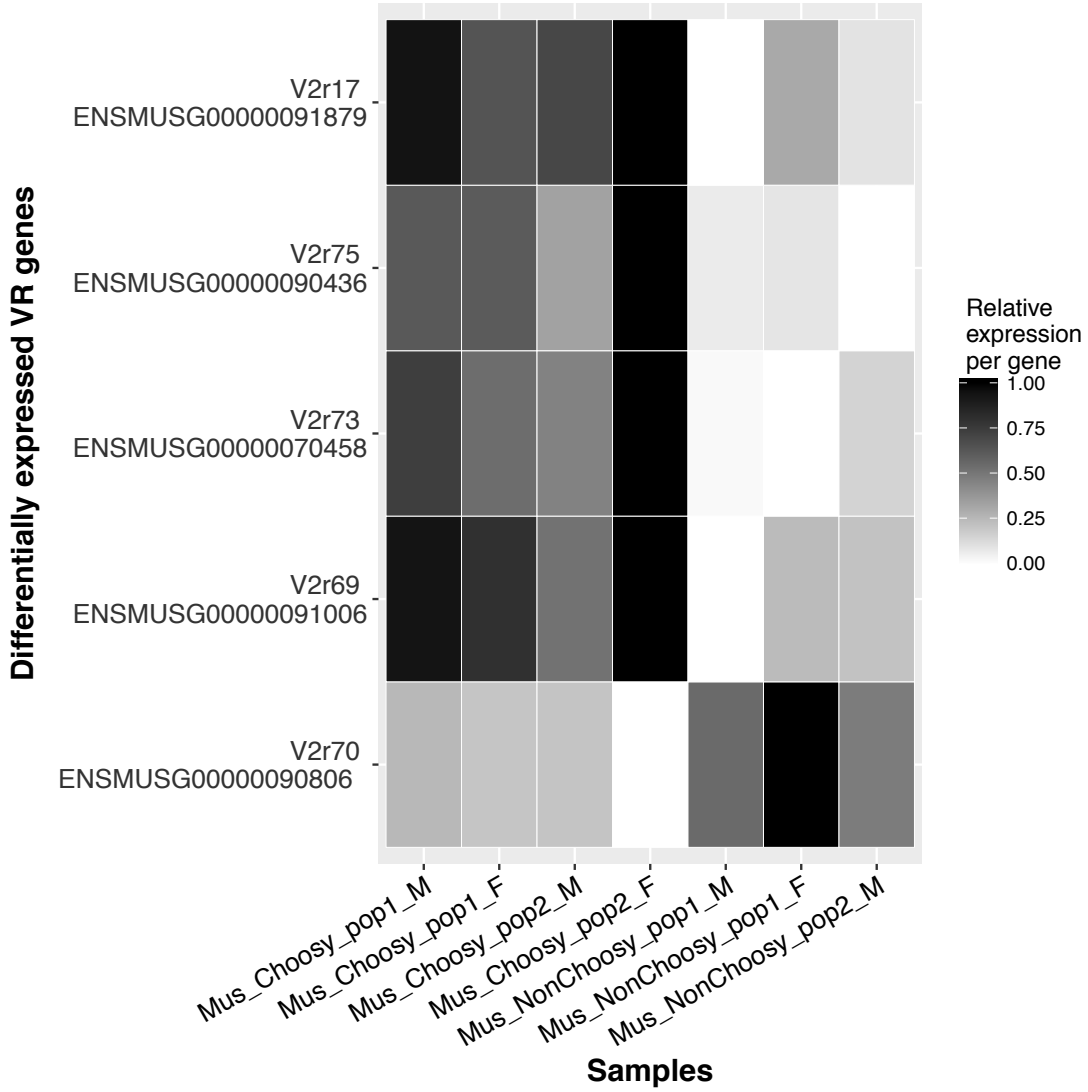
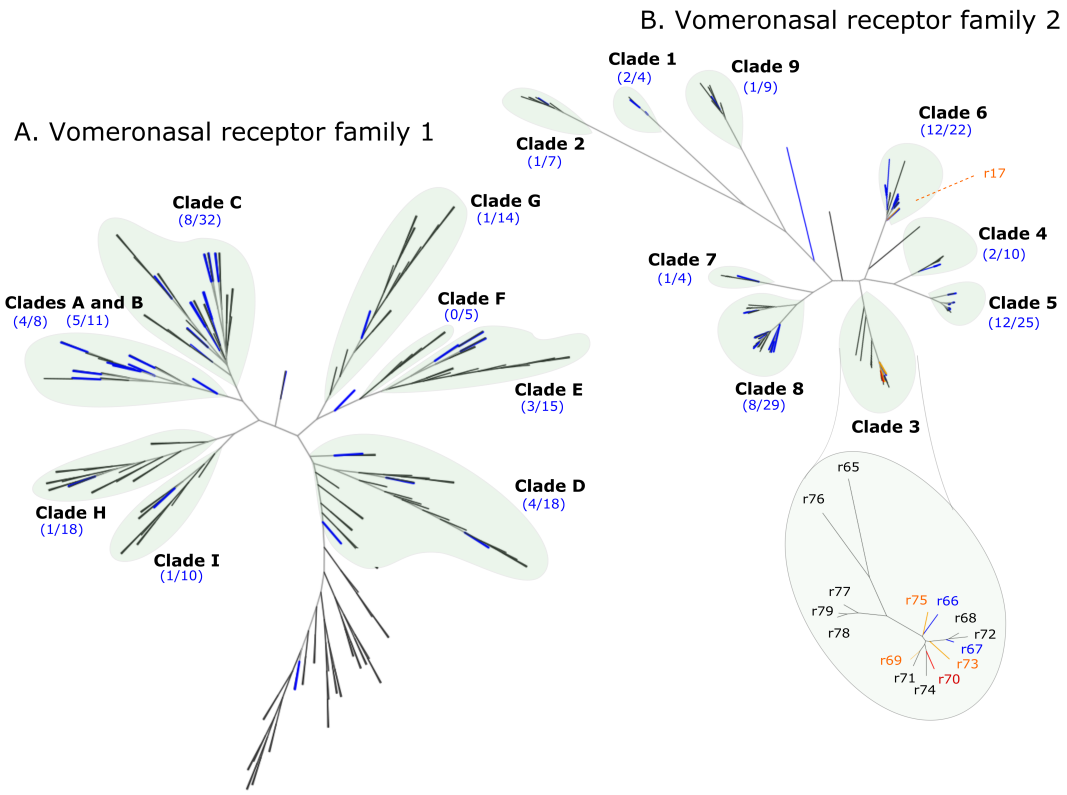



**Figure 3**: Heatmap plot of differentially expressed vomeronasal receptor (VR) genes in the comparison between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples. The values are presented in log fold change (FC) per gene, in which the sample with the lowest expression value corresponds to 0.

953

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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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| **Figure S4** | Pages 7-11 |

979 LIST OF SUPPLEMENTARY FIGURES AND TABLES:

980

981 **Table S1**: Sample description (separated Excel file)

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

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

987 **Table S4:** Variation in mean count and in expressed gene number among samples (separated
988 Excel file, sheet 1: Table S4a: number and percentage of expressed genes per sample; sheet
989 2: Table S4b: average counts per sample)

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

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

999

1000 **Figure S1:** Map indicating the geographical origins of the mice used in this study.

1001 **Figure S2**: Violin plots of log-transformed normalised gene coverage.

1002 **Figure S3:** Distribution of phylogenetic distances among random sampling of five
1003 vomeronasal receptor genes.

1004 **Figure S4**: Coverage per position (bp) for the five differentially expressed VR genes in the
1005 comparison between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples.

1006

1007

1008 **Supplementary information**

1009 Discussion:

1010 *Robustness of differential expression inferences*

1011 RNA-seq on pooled samples is a cost-effective strategy widely used to assess variation in
1012 gene expression among experimental conditions or biological groups while producing
1013 transcriptome-wide data (reviewed in Todd *et al.* 2016). This pooling design offers an
1014 advantage by increasing sample size into the analysis, and when working with samples that
1015 may exhibit a large amount of biological variation, analysing several sample pools, rather
1016 than a few single samples per condition, also lessens the impact of single aberrant samples
1017 (Todd *et al.* 2016). Although sample pooling can theoretically result in lower precision in
1018 gene expression estimation (for lowly expressed transcripts) and increased false positive
1019 rates in differential expression (DE) analyses as compared to individual sequencing
1020 (Rajkumar *et al.* 2015), this limitation is overcome when the number of individuals per pool
1021 is sufficiently large (n > 5) and the number of biological replicates per group is maximized
1022 (Todd *et al.* 2016). By using eight individuals per pool and from three to four biological
1023 replicates per group for DE analyses, we placed ourselves in favourable conditions to reliably
1024 identify changes in gene expression between groups with a pooling design. To minimize
1025 further the risk of false positives in DE analyses, we also applied a stringent false discovery
1026 correction and selected the method for DE detection (*edgeR*) with the highest sensitivity and
1027 specificity compared to other methods (e.g. *DEseq2*, *Cuffdiff2*) (Rajkumar *et al.* 2015).
1028 Altogether, the stringent DE analysis and the consistency of differential expression among
1029 biological replicates strongly support the idea of divergence in gene expression between
1030 'Choosy' and 'Non-Choosy' *M. m. musculus* samples.

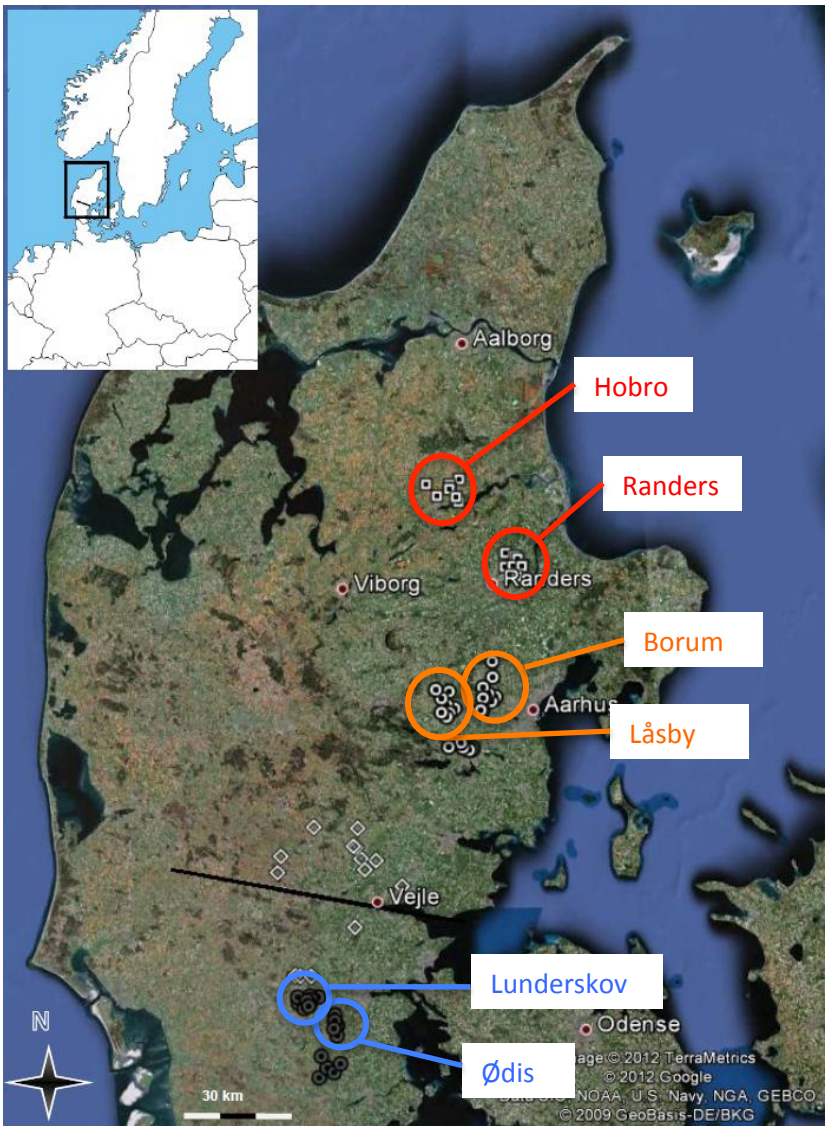



**Figure S1:** Map indicating the geographical origins of the mice used in this study (adapted from Latour et al. 2014).
Trapping sites in Jutland (Denmark) used in Latour et al. 2014 (the bold black line represents the genetic centre of the hybrid zone as defined in Raufaste et al 2005).
The colored 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

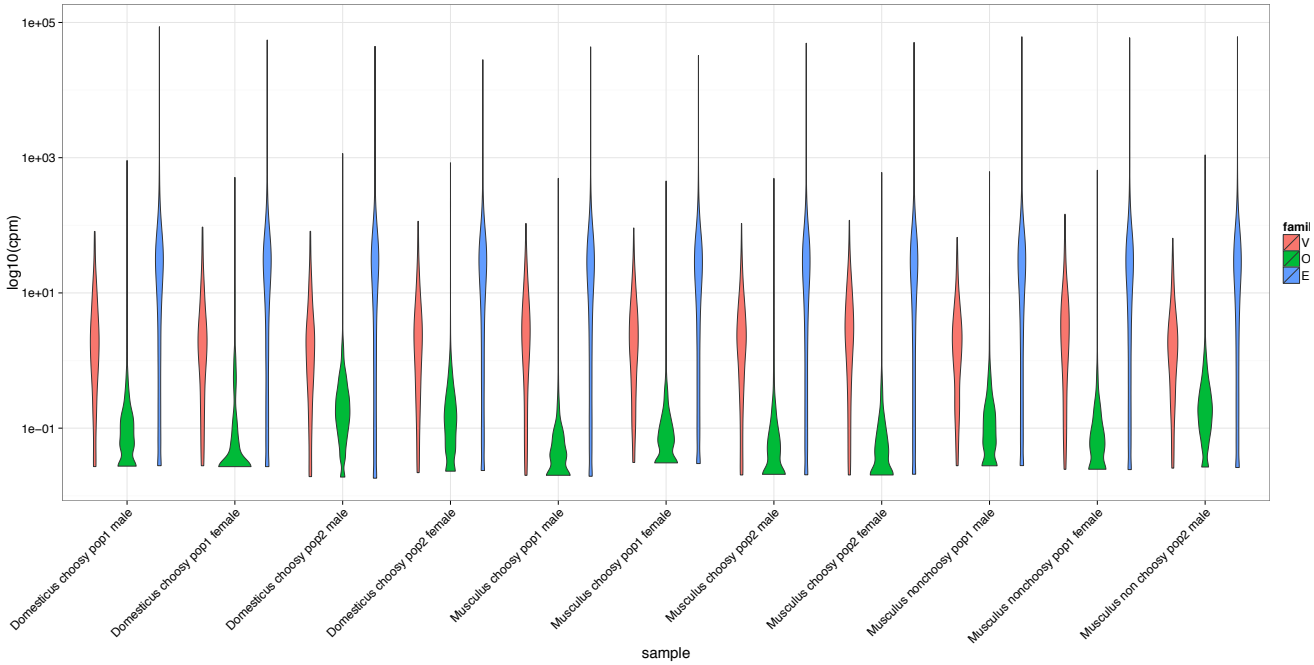



**Figure S2**: Violin plots of log-transformed normalised gene coverage (CPM) per population for vomeronasal receptor (VR) (red), olfactory receptor (OR) (green) and all other categories of genes (blue).

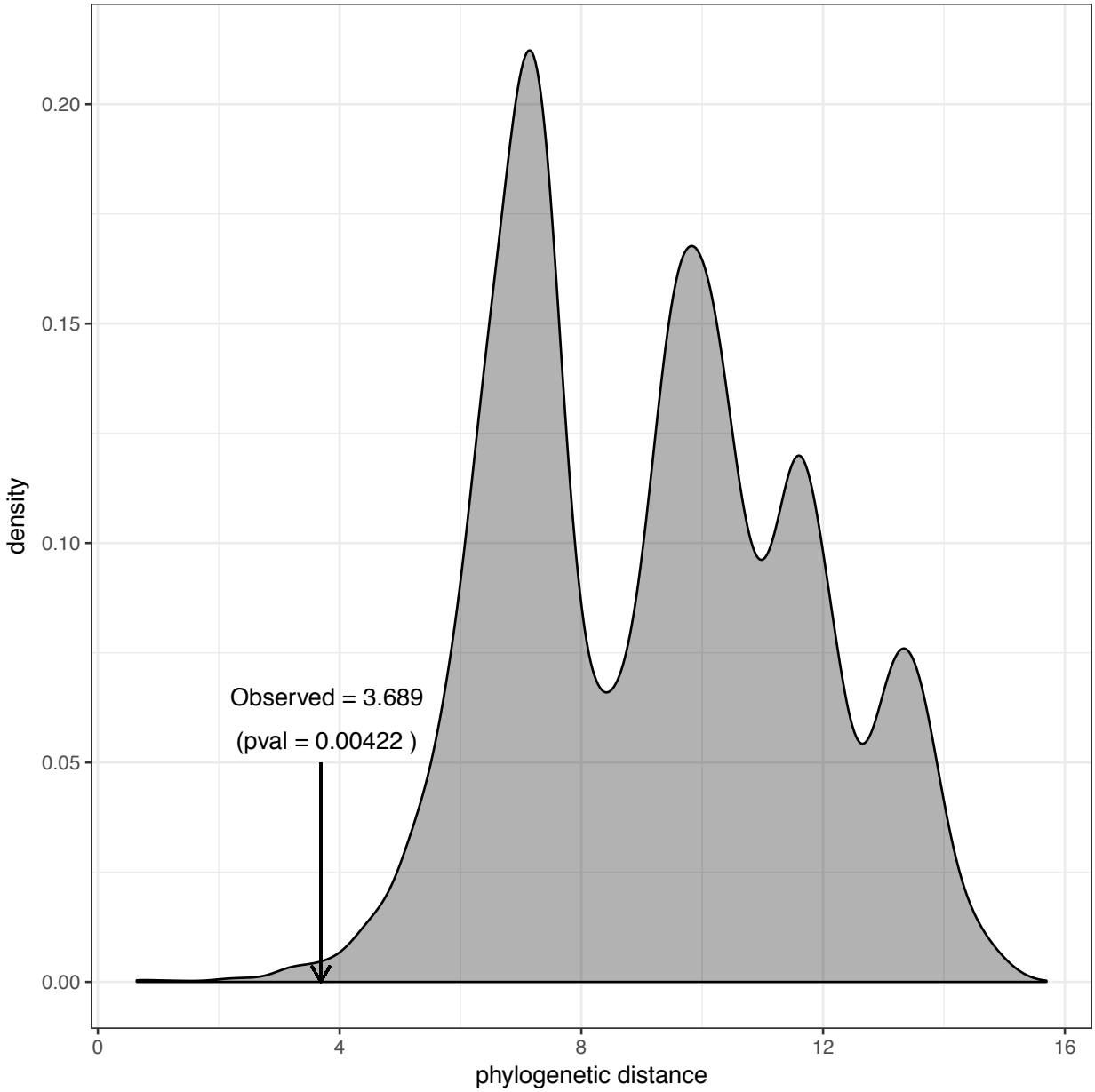
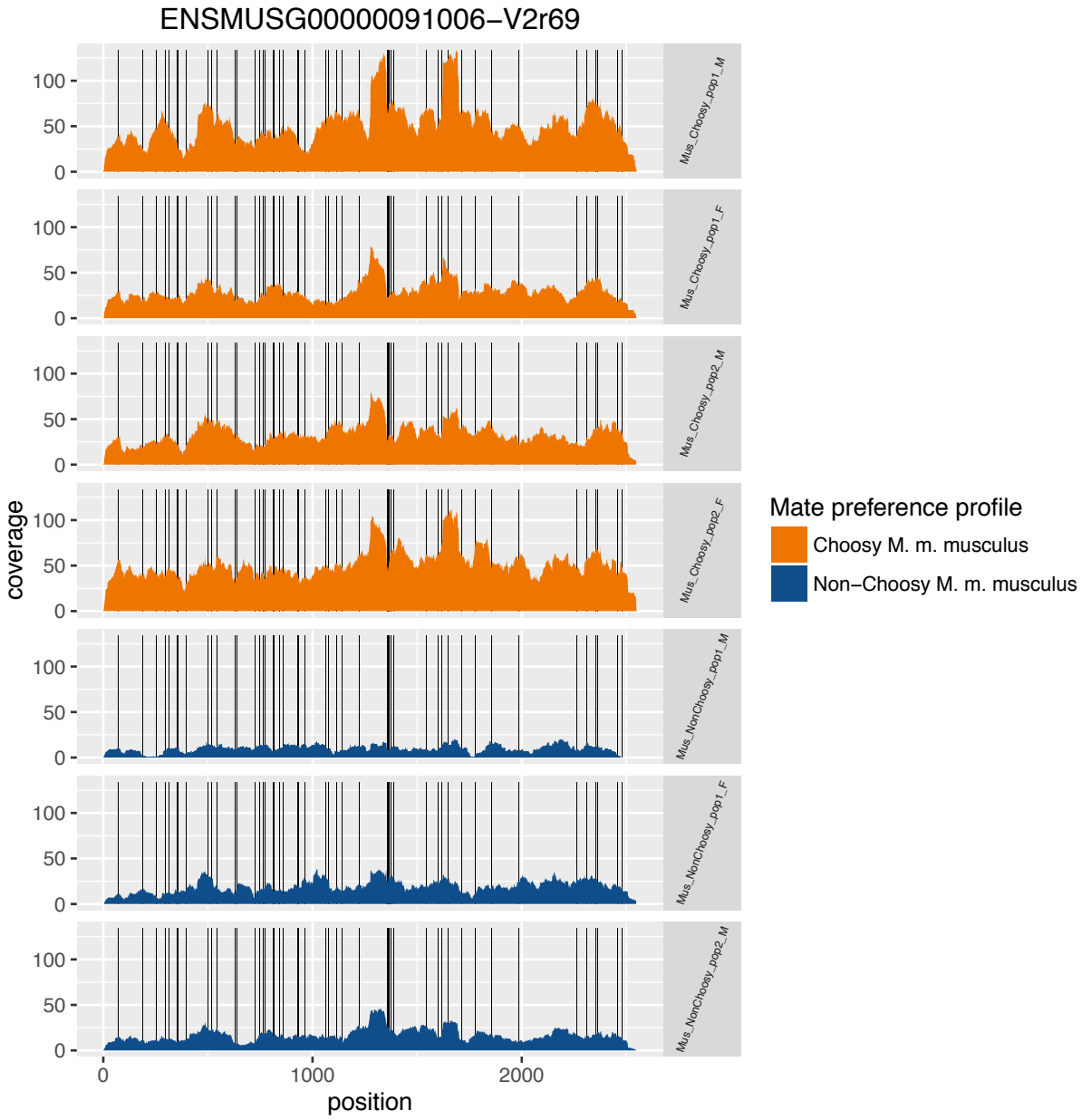



**Figure S3:** Distribution of phylogenetic distances among random sampling of five vomeronasal receptor genes. The arrow represents the observed distance between the five VRs found differentially expressed between 'Choosy' and 'Non-Choosy' samples, and its probability.

**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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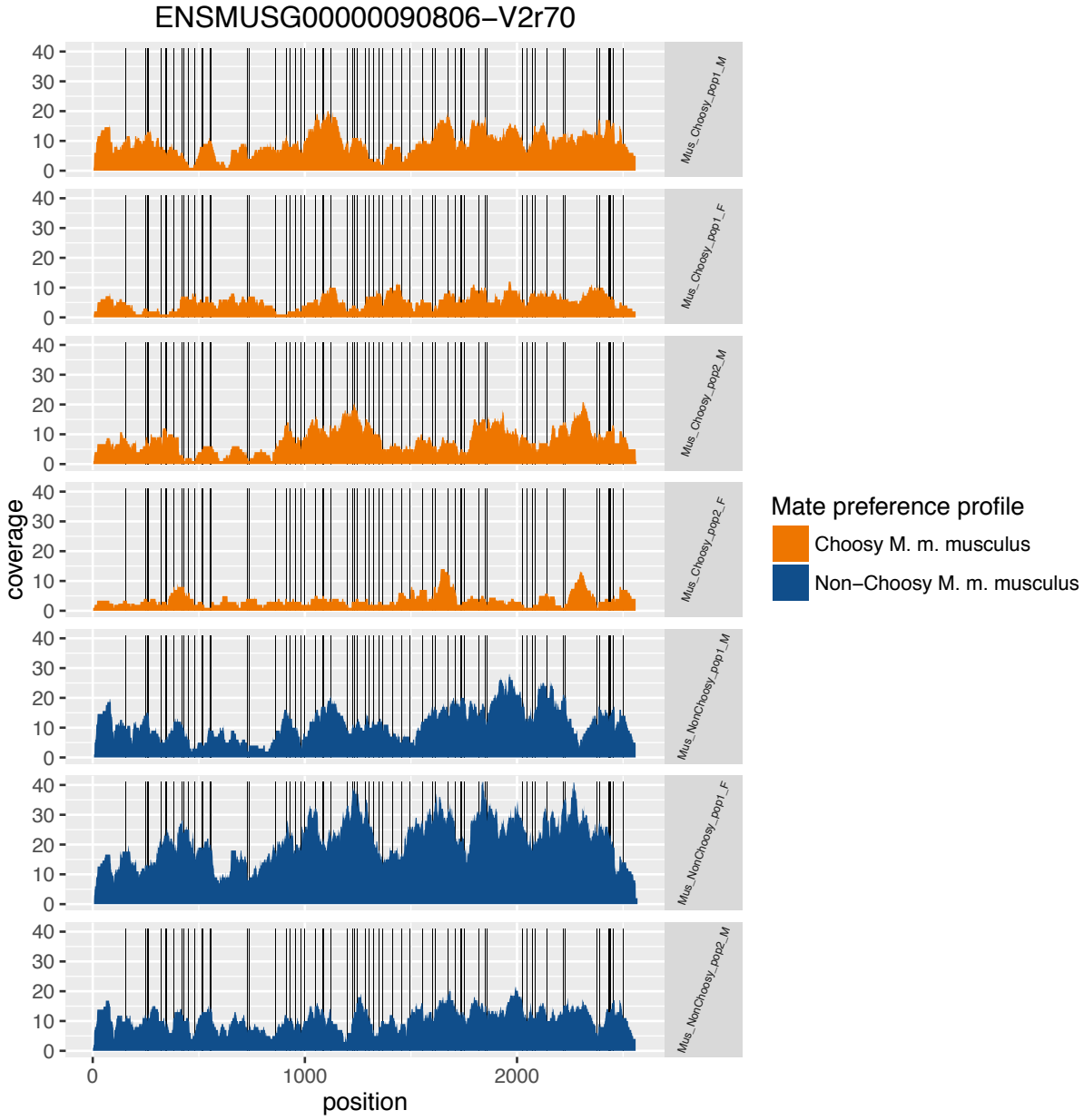


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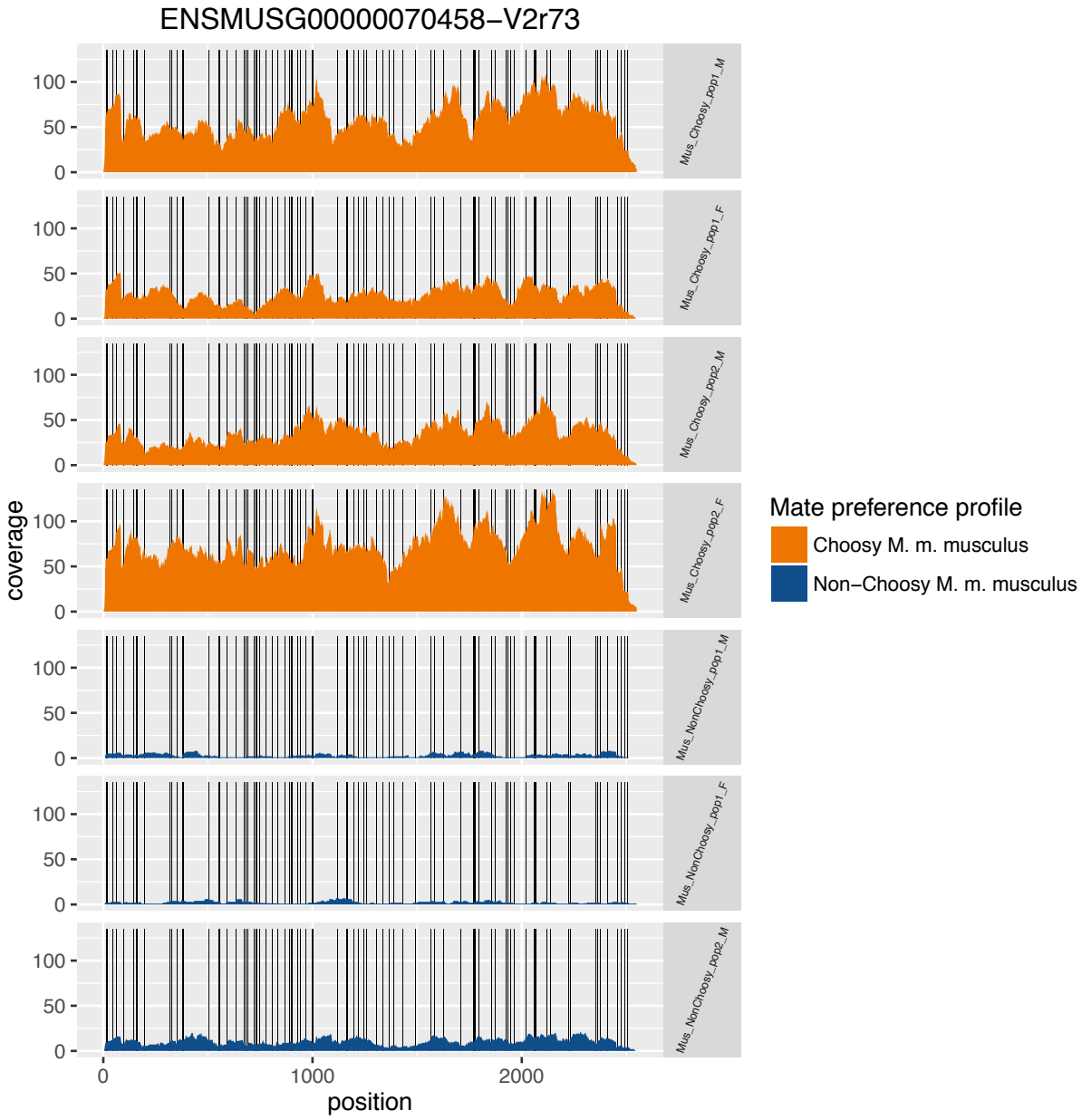
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Mus_NonChoosy_pop1_M
Mus_NonChoosy_pop1_F
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2000
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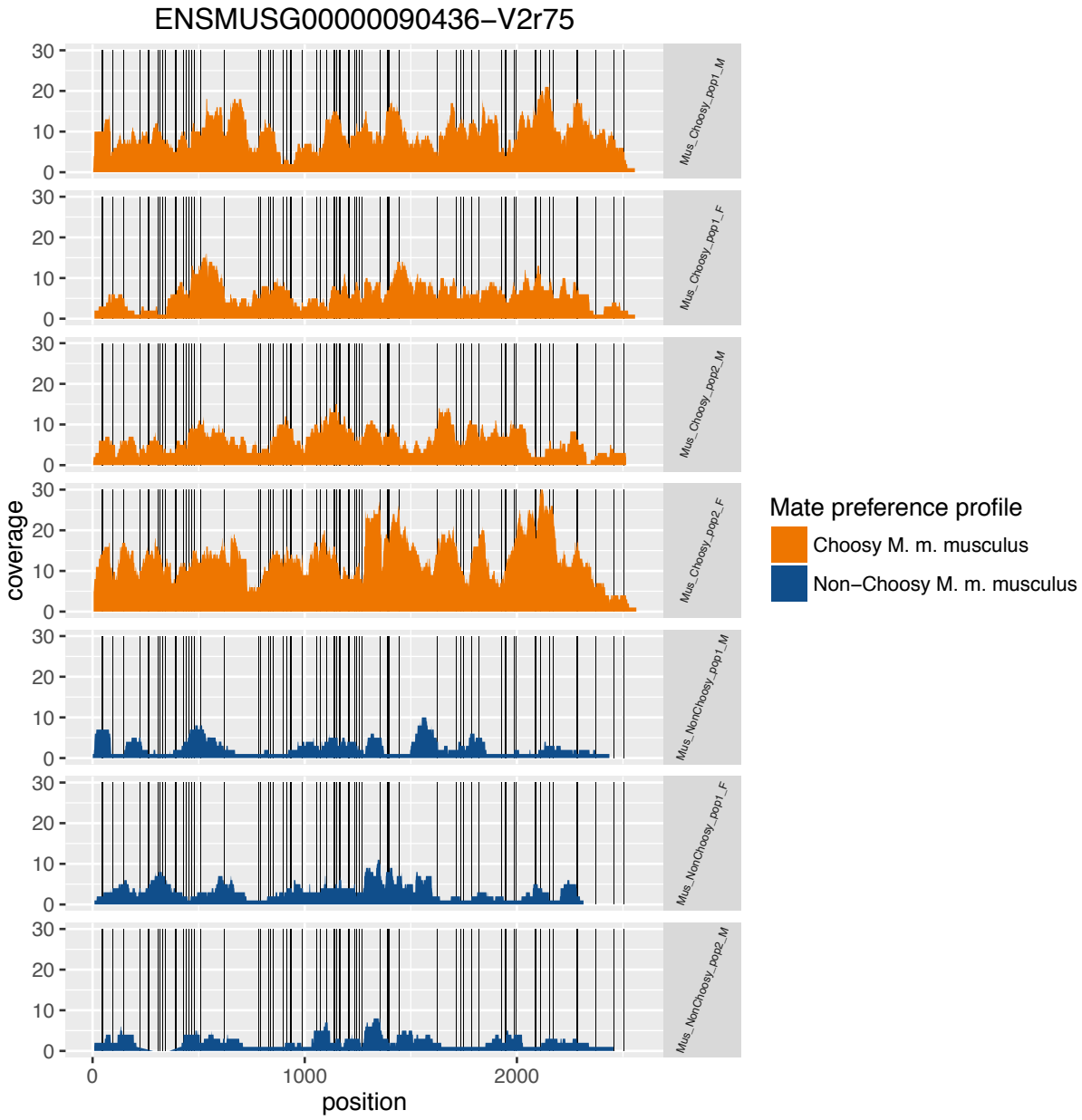
Mate preference profile
Choosy M. m. musculus
Non–Choosy M. m. musculus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Mus_Choosy_pop2_M
Mus_Choosy_pop2_F
Mus_NonChoosy_pop1_M
Mus_NonChoosy_pop1_F
Mus_NonChoosy_pop2_M
Mate preference profile
Choosy M. m. musculus
Non–Choosy M. m. musculus

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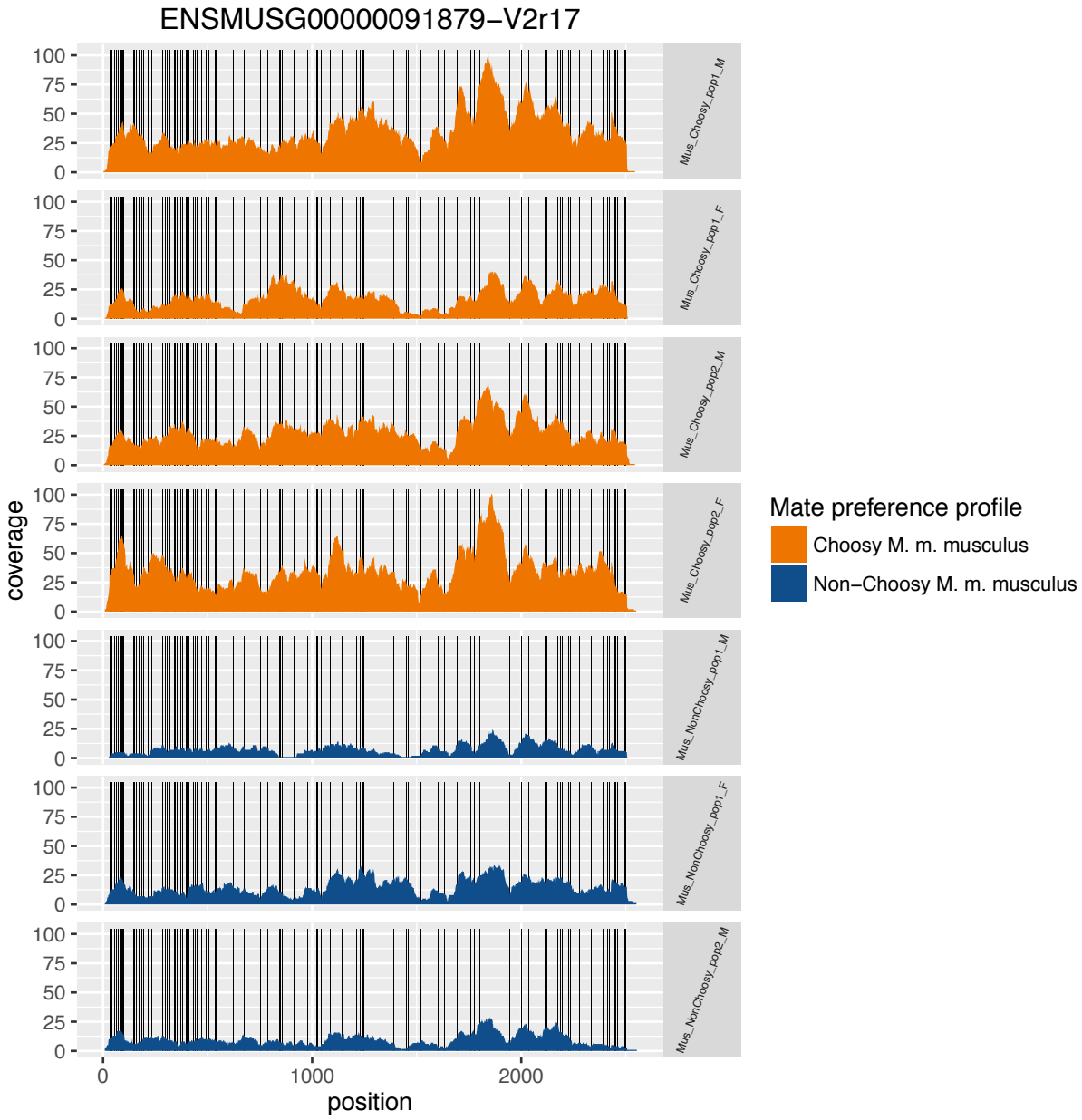


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