Sexual selection against natural hybrids may contribute to reinforcement in a house mouse hybrid zone

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1   **Last version**

2   **FULL TITLE:** Sexual selection against natural hybrids may contribute to reinforcement in a

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23 **ABSTRACT:** Sexual selection may hinder gene flow across contact zones when hybrid

24 recognition signals are discriminated against. We tested this hypothesis in a unimodal hybrid

25 zone between *Mus musculus musculus* and *M. m. domesticus* where a pattern of reinforcement

26 was described and lower hybrid fitness documented. We presented mice from the border of

27 the hybrid zone with a choice between opposite sex urine from same subspecies versus

28 hybrids sampled in different locations across the zone. While no preference was evidenced in

29 *domesticus* mice, *musculus* males discriminated in favour of *musculus* signals and against

30 hybrid signals. Remarkably, the pattern of hybrid unattractiveness did not vary across the

31 hybrid zone. Moreover, allopatric populations tested in the same conditions did not

32 discriminate against hybrid signals, indicating character displacement for signal perception or

33 preference. Finally, habituation-discrimination tests assessing similarities between signals

34 pointed out that hybrid signals differed from the parental ones. Overall, our results suggest

35 that perception of hybrids as unattractive has evolved in border populations of *musculus* after

36 the secondary contact with *domesticus*. We discuss the mechanisms involved in hybrid

37 unattractiveness, and the potential impact of asymmetric sexual selection on gene flow and

38 isolation between the two subspecies.

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40 **KEY WORDS:** hybrid signal, mate recognition, urinary cues, reproductive character

41 displacement, speciation.

42

INTRODUCTION

Hybridisation in nature is mostly observed at the limits of species or population range. Its presence in the wild can either facilitate or impede speciation [1-3]. If hybridisation is maladaptive, selection is expected to favour the evolution of assortative mate preference in the two hybridising populations, a process called reinforcement [4-7]. This adaptation can be driven by the cost of hybridisation between parental populations [8-10], but not only. Interactions with the hybrids could also be selected against. In particular, if hybrid signals are less attractive, sexual selection could reduce gene flow across the hybrid zone [11, 12]. Mating signals evolve under sexual and ecological selection [13]. Their complexity depends on the number and characteristics of the genes involved in their determinism [14-16] as well as on environmental factors influencing their expression and their perception [13, 17]. Hybrid genotypes could produce unrecognised or unattractive signals when new associations of alleles are brought together by hybridisation [18], and sexual selection can occur against such signals (review in Electronic Supplementary Materials ESM1). Sexual selection is considered a powerful potential driver of speciation [19]. Nevertheless, it was recently argued that empirical demonstration of its importance is still insufficient, notably because most studies do not "relate variation and divergence in mating traits and preferences to gene flow and genetic population differentiation" [20]. Here we investigate mating traits divergence between hybrid populations with different level of genetic divergence from their parental populations. The rationale behind our study is that if individuals of parental populations seldom mate with hybrids, gene flow across the hybrid zone can be hindered and the established zone could act as a barrier between incipient species. In contrast, hybrids could form a bridge between parental genomes if fitness of crosses between neighbouring, genetically less divergent, populations allows step-by-step gene flow across the zone [21], but only if they are not discriminated against [22].

3

68   Our study model is a unimodal hybrid zone formed during a secondary contact between two

69   subspecies of the house mouse *Mus musculus musculus* and *M. m. domesticus* (thereafter

70   *musculus* and *domesticus*). The history of divergence between these subspecies is

71   characterised by a long initial period in allopatry (a few hundred thousand years ago [23, 24]),

72   which could account for the accumulation of numerous genetic incompatibilities resulting in

73   selection against hybridisation and underlying the formation of a tension zone crossing

74   Europe [25-29].

75   Olfactory signals present in mice urine were shown to play a central role in social and sexual

76   communication and to be shaped both by sexual and natural selection [30-32], and a mouse

77   nose can detect odour differences in less than a second [33].

78   Populations of both subspecies at the border of the hybrid zone display assortative mate

79   preference for signals present in the urine, and reproductive character displacement (for both

80   preference and signals) was documented between border and allopatric populations of the two

81   subspecies in male and female mice [16, 34, 35]. Wild hybrids show preference for

82   *domesticus* odour with a steep shift from *domesticus* to *musculus* odour preference roughly

83   10 km north of the genetic centre of the Danish hybrid zone [16], while in other parts of the

84   zone this shift coincides with the genetic centre of the zone [35].

85   The present study addresses the signal component of potential hybrid mates, by assessing how

86   such signals are perceived, mainly by the choosiest subspecies (*musculus*) [16, 34, 36].

87   The specific questions addressed here are:

88   1) Are signals of hybrid mice discriminated against by the two parental subspecies?

89   What is the generality of this pattern? Does it vary with the genetic characteristics (hybrid

90   index) across the hybrid zone? To address this series of questions we assessed: a) preference

91   of male and female mice of parental populations from the border of the hybrid zone during

92   two-way choice tests involving hybrid versus parental opposite sex urine as the stimuli; b) we

93 replicated this test changing the origin of parental urine; c) we replicated the test, presenting

94 against the parental urine hybrid urine from different locations across the hybrid zone. We

95 predicted that if discrimination occurred and preference was linked to genetic similarities

96 between the chooser and the stimuli, assortative preference would be more marked in

97 presence of distant as compared to neighbour hybrids.

98   2) Did sexual selection against hybrids evolve in the hybrid zone? We compared

99 patterns of preference in populations of mice distant versus at the borders of the hybrid zone

100 (map in ESM2).

101   3) What may be the causation of hybrid unattractiveness, i.e. how different are hybrid

102 and parental odours? We investigated odour similarities between hybrids and the two parental

103 subspecies and addressed whether they were distinctively different (i.e. transgressive), using

104 habituation-discrimination/generalisation tests [37].

105

106 MATERIAL & METHODS

107 **Biological material**

108 *Mice*

109 All mice involved in this study were either wild trapped in Jutland, Denmark, in commensal

110 indoor habitats, in October 2010 and June 2011, or descendants of these, bred in the lab (see

111 details in ESM3). We sampled mice in several farms at the northern and southern edges of the

112 hybrid zone ("border mice"), across the hybrid zone ("hybrid mice") and approximately 40

113 km north of *musculus* border of the zone where "pure" *musculus* individuals putatively occur

114 ("close allopatric mice", see ESM2). Hybrid mice used in this study were wild, and after

115 stalling in laboratory conditions for several months, were used as urine donors for subsequent

116 behavioural experiments (see below). Border and close allopatric mice were first and second

117 generation progeny of wild trapped mice, and provided test mice and urine stimuli.

118 *Genetic markers and hybrid index estimation*

119 We genotyped wild mice using loci with insertion/deletion polymorphisms of transposable

120 elements, determined to have contrasted allele frequencies (alternatively fixed or nearly so)

121 between the two subspecies in allopatry (markers design in ESM3). Eighteen of them are

122 autosomal, two are X-linked and one Y-linked (methods as in ref. [38], described in ESM4).

123 On this basis, population samples of the present study could be characterised by a multilocus

124 hybrid index (HI, defined throughout as the proportion of *musculus* ancestry) estimated by

125 maximum likelihood [e.g. 39] given the parental allele frequencies estimated in samples from

126 populations more than 200 km away from the hybrid zone.

127 The genetic cline of the mouse hybrid zone is geographically well structured [25]. Hence,

128 geographic location of a sample is considered a good predictor of its average genetic

129 composition. Genetic typing involved a relatively limited number of markers as it served to

130 check that none of the studied populations or individuals was an outlier as compared to its

131 geographic origin, which could happen as a result of accidental, recent long distance

132 migration. The HI estimates of wild samples and their support limits are reported in figure 1

133 and ESM5.

134 *Urinary stimuli*

135 Urine donors were either first-generation-laboratory born (border or close allopatric

136 populations) or wild hybrid mice maintained in same standardised conditions for at least 2

137 months after trapping. Urine was collected at different times of the day and over several days

138 to capture intra- and inter-day variations in urine composition, both upon handling of mice

139 and pipetted from a cleaned surface, and stored at -20°C. All stimuli were pools of urine from

140 3 to 4 mice of the same sex and of different populations or farms to account for genetic and

6

environmental individual variance within a given category. In the specific case of hybrids, categories were defined with regard to their geographical positions on the hybrid zone (figure 1), going from sites neighbouring *musculus* border (H1) to those closer to *domesticus* border (H7). We only trapped one male in the H5 category, so we combined its urine with those of males of the H6 category to match the minimum of 3 different urine donors in a pool. The hybrid categories were intended to capture potential variations with reference to the shift of the preference cline (see figure 1).

Behavioural tests

Mate preference was assessed from relative time spent by a mouse investigating two urinary stimuli deposited in two peripheral boxes connected to a Y maze during 5 minutes trials (two-way choice tests). Odour discrimination and ability to perceive differences between two odorant stimuli was addressed via habituation-discrimination (or generalisation) tests. The latter experiment is based on observations that mice investigate more novel stimuli than familiar ones. Practically, a mouse is first presented with a single odour long enough to induce familiarity (habituation), immediately after the same mouse is presented with two new stimuli, which will be investigated differently if one is more similar to the habituation stimulus than the other [review in 37, details in ESM3]. Preference tests involved one sex presented with urine pools of the opposite sex, and habituation tests involved males presented with female urine. We used protocols described by Smadja and Ganem [40, 41], with minor changes (see ESM3).

A total of 232 wild derived mice (*musculus* :122 males, 81 female; *domesticus* 20 males, 9 females) were involved in two-way choice experiments, and 39 male *musculus* in the habituation/discrimination or habituation/generalisation experiments ("the noses"). Female mice were tested when sexually receptive. All tested mice were unrelated to urine donors, and

165 pregnant females were excluded. Each different choice or discrimination test was conducted

166 with 8 to 17 unrelated mice.

Experimental designs

168 *1. Are signals of hybrid mice discriminated against by parental subspecies?*

169 1*a*. Discrimination against hybrids.

170 We first determined if males and females of border populations displayed homogamous

171 choice when presented with "*musculus* border" versus "*domesticus* border" stimuli. Mice

172 discriminating against heterosubspecific stimuli, i.e. male and female *musculus* mice and male

173 *domesticus* (see results), were involved in choice tests between a border conspecific stimulus

174 and a stimulus from the geographically closest hybrid category (H1 for *musculus* or H7 for

175 *domesticus*, see figure 1). We expected assortative preference if the stimuli differed and the

176 hybrid signal was discriminated against.

177 1*b*. Generality.

178 To test the generality of the assortative mate preference assessed in 1a, we performed a

179 control test presenting *musculus* border mice with a hybrid (H1) and a conspecific stimulus.

180 from close allopatry rather than from border (1a) populations. We predicted preference for the

181 allopatric signal if discrimination against the hybrid stimulus was independent of the

182 conspecific stimulus geographic origin..

183 1*c*. Variation.

184 We determined whether hybrid stimuli perception varied with their genetic or geographic

185 characteristics during choice tests where we diversified the origin of the hybrid stimulus

186 presented against the parental stimulus (hybrid categories described in figure 1). Only the

187 most discriminating subspecies, *musculus,* participated to these tests. If hybrid attractiveness

8

188 varied with geographical proximity or genetic similarity we expected higher discrimination

189 against hybrids more distant from the noses, Alternatively, all hybrids were perceived as

190 equally unattractive (or attractive).

191 *2. Did discrimination against hybrid signals evolve in the hybrid zone?*

192 To determine the origin of discrimination against hybrid signals (evolution in contact zone or

193 early divergence in allopatry), we assessed preference of close allopatric *musculus* mice

194 during choice tests between conspecific (*musculus* border) and H1 hybrid stimuli. We

195 expected mice from close allopatry to discriminate less between the stimuli than border mice

196 (test 1*a*) if discrimination against hybrid signals evolved in the contact zone.

197 *3. How similar are hybrid and parental odours?*

198 The two extreme hybrid categories (H1 and H7) odours were each compared to the

199 *domesticus* parent, via habituation-discrimination tests to ascertain that they differed from this

200 subspecies. A second test assessed whether H7 was more similar to *domesticus* than H1,

201 which is expected if odour determinism is additive and odour similarity parallels genetic

202 similarity. In line with what precedes, we tested whether similarity between H1 and

203 *domesticus* was higher than similarity between the two parental subspecies (ESM3). If the

204 hybrid stimulus shared similarities with *domesticus* we expected it to be less investigated than

205 the *musculus* stimulus, and if odour similarities with *domesticus* varied with genetic

206 similarities we expected H7 to be less investigated than H1.

207

Statistical analysis

209 Preference and discrimination were assessed by pair comparisons of time spent sniffing or

210 touching the proposed stimuli. Random choice or absence of discrimination was concluded

when there was no significant difference between the times spent in contact with either

stimulus.

To compare preference across tests, we used an index (R): time spent in contact with

homosubspecific stimulus divided by total time in contact with both stimuli. We performed a

mixed ANCOVA on this transformed variable (expR) with sex and hybrid category as fixed

factors, population as a random factor and motivation (i.e. the sum of time spent in the right

and left sides of the Y maze) as a covariable.

All statistical analyses were performed with R 2.15.0 software [42-44].

RESULTS

1. Are signals of hybrid mice discriminated against by parental subspecies?

1a. Discrimination against hybrids.

Male and female *musculus* mice from populations at the border of the hybrid zone showed

assortative preference when presented with *musculus* versus their most neighbour hybrid

stimuli (H1, figure 2A&B; males: n=17, median=5.91s, V=130, p=0.009; females: n=10,

median=0.81s, V=49, p=0.027). In contrast, although border *domesticus* males discriminated

in favour of their own subspecies during choice between *musculus* and *domesticus* stimuli

(figure 2B; n=10, median=2.65s, V=8, p=0.049), they did not show any directional choice

when the alternative to their own subspecies signal was their most neighbour hybrid category

(H7, figure 2B; n=10, median=2.07s, V=18, p=0.375). Female *domesticus* didn't show any

directional choice when presented with *musculus versus domesticus* stimuli (figure 2A; n=9,

median=-0.92, V=20, p=0.8203).

1b. Generality

234   When border *musculus* mice were presented with a choice between a *musculus* stimulus from

235   close allopatry (instead of border population in 1a) versus a hybrid stimulus, the allopatric

236   signal was investigated significantly more than the hybrid one (paired Wilcoxon test: n=10,

237   median=10.54, V=54, p=0.004).

238   1c. Variation.

239   Remarkably, preference did not vary with the hybrid category presented as an alternative to

240   the *musculus* stimulus (table 1; maximal model: df=138, F=1.18, p=0.319) nor interactively

241   with the sexes (table 1). However, male *musculus* was more choosy than the females (table 1;

242   minimal model: df=166, F=9.85, p=0.002), a pattern which did not vary with motivation

243   (table 1; maximal model: df=138, F=0.62, p=0.434), Considering direction of preference for

244   hybrid across the zone, a consistent trend was for hybrid stimuli to be less investigated than

245   *musculus* ones (figure 3; males: n=84, median=6.19s, V=3335, p=4.832e-12; females: n=61,

246   median=0.91s, V=1406, p=9.529e-4).

247   *2. Did discrimination against hybrid signals evolve in the hybrid zone?*

248   Allopatric *musculus* mice did not show a directional preference when presented with a choice

249   between *musculus* versus hybrid stimuli (table 2; males: n=17, median=0.74, V=94, p=0.431;

250   females: n=10, median=0.70, V=40, p=0.232), while border mice presented with the same

251   stimuli discriminated against the hybrid one (table 2; males: n=17, median=5.91s, V=130,

252   p=0.009; females: n=10, median=0.81s, V=49, p=0.027).

253   *3. How similar are hybrid and parental odours?*

254   Assortative preference displayed by border *musculus* mice (results above) indicated that

255   hybrids and *musculus* signals differed. We hence compared hybrid and *domesticus* signals.

256   The results of the habituation-discrimination experiment indicated that signals of hybrid

257   categories neighbouring (H7) and most distant from *domesticus* (H1) were perceived as

258 different from *domesticus* (respectively figure 4a & 4b: n=8, V=35, p=0.016, and n=8, V=36,

259 p=0.008). Moreover, despite its geographical proximity and genetic similarity, H7 was not

260 perceived as more similar to *domesticus* than H1 (figure 4c: n=13, V=62, p=0.273). Finally,

261 unlike predicted if hybrid signals shared similarities with the two parental subspecies, when

262 *musculus* and H1 stimuli were presented simultaneously after habituation to *domesticus,* H1

263 signals did not show more similarities with *domesticus* than did *musculus* signals (figure 4d:

264 n=10, V=28 , p=1).

265

266 DISCUSSION

267 Selection against hybrids that carry less attractive signals could be an important mechanism

268 hindering gene flow between parapatric or sympatric populations [12, 19, 45, 46]. However,

269 this process remains scarcely demonstrated in nature. We investigated this process in a house

270 mouse hybrid zone where hybrid populations with highly recombined genomes are

271 established between the parental ones. We made the hypothesis that sexual selection could

272 facilitate the reinforcement process described between *musculus* and *domesticus* [40, 41] by

273 decreasing hybrid mating success and hence impeding step by step gene flow through the

274 zone. Our results indicate that hybrids are perceived as less attractive than own subspecies

275 stimuli only in *musculus* populations from the border of the hybrid zone, pointing out

276 character displacement of preference between allopatric and border populations of this

277 subspecies, and a potential for sexual selection against hybrids in the *musculus* side of the

278 zone. Studies involving other models e.g. flycatchers [47] and chorus frog [48], evaluated that

279 sexual selection could account, respectively, for 3/4 and 4/5 of reduction in hybrid male

280 fitness, suggesting that sexual selection could be an important force in evolution of

281 reproductive isolation in contact zones. Here, asymmetric sexual selection against hybrids

282 could act simultaneously to the reinforcement process.

283 Our results also indicate that unattractiveness of hybrid did not vary with genetic similarities

284 between hybrid and parental populations, suggesting that the hybrid signal may not

285 significantly vary across the zone and/or that it could share an unattractive component

286 sufficient to trigger a negative response (see below). This study also points out that

287 asymmetry exists between the sexes, as choosiness was higher in males as compared to

288 females

289

290 **Lower choosiness of females**

291 The extent to which males and females contribute to behavioural isolation varies between taxa

292 [e.g. 49, 50] and findings, like our study, that male mate choice plays an important role are

293 not an exception [51-55]. In the house mouse, both males and females may be involved in

294 mate choice [56-58].

295 Cost of heterospecific mating is expected to be similar in the two subspecies [59] but higher

296 in females. First, because female physiological investment in each reproduction event is

297 supposed to be higher than male investment [60] but see [52]. Second, in our biological

298 system sterility affects hybrid males more than hybrid females [26, 28, 61, 62]. This should

299 result in higher selection on females than males to discriminate against hybrid mates, which

300 our results partially contradict. The patterns of lower female choosiness pointed out in our

301 study could be explained if postmating-prezygotic mechanisms preventing the production of

302 costly hybrid zygotes occur [63]. Indeed, in vitro laboratory investigations involving the two

303 house mice subspecies [64] suggest that sperm competition may favour homogamous

304 fecundation; such a mechanism could possibly put less pressure on the evolution of female

305 premating choosiness, explaining the patterns evidenced in our study.

306 Notwithstanding, we cannot completely exclude that lower preference of females in our study

307 could be caused by the experimental design being less appropriate to assess female than male

308 mate choice. Indeed, it may be that simultaneous presentation of two male stimuli to a female

309 induces anxiety, while for males simultaneous presentation of two female stimuli may induce

310 higher pressure to properly allocate their energy [52, 65].

Gaining insight into hybrid signal unattractiveness

312 Hybrid unattractiveness for border population mice did not vary significantly with the origin

313 of hybrid stimuli, suggesting that different categories of hybrids were perceived as equally

314 unattractive.

315 The theory of odour-genes covariance proposes that odour similarity may parallel genetic

316 similarity [37]. If this was true we would have expected odour similarity between parental and

317 hybrid stimuli to vary with the hybrid index HI, which did not seem to be the case since

318 stimuli of the most different hybrids were discriminated against to the same extent and

319 perceived as equally different from *domesticus*.

320 A study assessing preference of wild hybrids when presented with a choice between stimuli of

321 *musculus* versus *domesticus* [16] revealed dominance of preference for *domesticus* and a shift

322 in favour of higher choosiness and preference for *musculus* 10 km north of the genetic centre

323 of the Danish hybrid zone (figure 1), in a location where hybrid genomes were very similar to

324 *musculus*. Our study concerns the same hybrid zone, hence our expectation that, if hybrid

325 preference followed a self-matching scheme, hybrid odours would be dominantly *domesticus*

326 with a shift in the hybrid signal nature roughly at the same location as the preference shift.

327 Precisely, we expected H7, neighbouring the *domesticus* border of the hybrid zone, to be more

328 similar to *domesticus* than H1, which does not seem to be the case. Further, if the hybrid

329 signals were intermediate (i.e. carry similarities with both parents), we would have expected

330 H1 to be perceived as more similar to *domesticus* than *musculus*. Our study indicates that all

331 hybrid signals tested were rejected by *musculus* noses (during choice tests), but not because

332 they had similarities with the *domesticus* signal, leading us to propose that hybrid signals may

333 share an unattractive transgressive component, i.e. out of the range of variation of the two

334 subspecies [see also 66].

335

336 **Evolution of sexual selection against hybrids**

337 The fact that, unlike border populations, mice from neighbour allopatry did not discriminate

338 against hybrid stimuli suggests that hybridisation may not produce signals that are not

339 recognised by *musculus* or unattractive *per se,* and that perception of hybrid signals as

340 unattractive has evolved in the border of the hybrid zone. Discrimination against hybrid

341 signals could be the consequence of changes in the olfactory receptors leading to non-

342 recognition of hybrid signals in the contact zone, or, possibly, changes in the neuronal

343 integration of the message transmitted by the receptor (discrimination leading to negative

344 behavioural response). Evolution of hybrid perception by *musculus* receiver could be a

345 pleiotropic consequence of narrowing the range of acceptable signals during evolution of

346 assortative mating (i.e. preference for alike) in response to selection against maladaptive

347 hybridisation with *domesticus*. Alternatively, natural selection against possibly maladaptive

348 backcross hybridisation [26, 59, 61] could have directly influenced the pattern evidenced in

349 this study. In both cases, if after selection in secondary contact *musculus* receiver is finely

350 tuned to the extent that it is able to reject any signal different to its "own" (i.e. self-matching),

351 one may consider that strong signal divergence is not necessary to induce premating isolation

352 in a hybrid zone, which might be sometimes the case [e.g. 67]. As far as the mouse hybrid

353 zone is concerned our results suggest that both *musculus* receiver component has diverged and

354 the hybrid signals are different from the parental ones.

355

356 **Sexual selection against hybrids and the hybrid zone dynamics**

357　Sexual selection against hybrids, as our study suggests, can contribute to strengthen

358　reproductive isolation between diverging taxa [11, 19, 45]. Further, asymmetry in premating

359　divergence between parental populations has been reported in many taxa [68-72] and can

360　impact the dynamics of hybrid zones [73]. Here, asymmetric sexual selection against hybrids

361　could have similar consequences. Particularly, given that *domesticus*, unlike *musculus,* tends

362　not to discriminate against hybrids (this study) and that hybrids tend not to reject *domesticus*

363　[16], we could expect back-crossing to occur more frequently on *domesticus* side of the

364　hybrid zone, facilitating some *domesticus* genes flow into the zone. Further, several studies

365　suggest that *domesticus* is dominant over *musculus* and more aggressive [review in 74], a

366　behavioural trait that might be an advantage when dispersing [75]. In the absence of

367　geographical or ecological constraints (e.g. absence of favourable habitats), aggressiveness

368　might facilitate *domesticus* progression across the zone. Nevertheless, about 10 km north of

369　the genetic centre of the zone, this progression could be hindered by lower attractiveness of

370　*domesticus* mice to hybrids in this region [16], and strong assortative mating of *musculus*

371　border population mice would further slow down *domesticus* advance. Still, whether

372　behavioural evolution in the hybrid zone actually drive a movement of the hybrid zone is

373　difficult to assess on the sole basis of behavioural data, and our scenario could be further

374　tested with both genomics and theoretical approaches. We also need a better knowledge of

375　demographic parameters, dispersal characteristics, population growth rate etc., which are

376　scarce for both subspecies in the context of the hybrid zone.

377

378　**Conclusion**

379　This study provides arguments in favour of a role for sexual selection against hybrids in

380　shaping mate recognition patterns and limiting gene flow in this house mouse hybrid zone.

381　Together with natural selection against hybrids, it could significantly contribute to

382   reproductive isolation between the two European subspecies of the house mouse. New

383   insights into the neurophysiology, chemistry and genetic bases of discrimination and

384   signalling components will further extend our understanding of the evolutionary forces in

385   action in this study model for speciation with gene flow.

386

396

397   DATA ACCESSIBILITY

398   Final DNA sequence assembly uploaded as online supplemental material

399   Raw Behavioural data will be archived on the Dryad Data Repository website.

400

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Table 1: A summary of the results of a mixed ANCOVA testing the influence of sex, stimulus category, the nose population, and motivation*, on variation of preference (expR) displayed by border *musculus* mice, during choice tests between *musculus* versus a hybrid or a *domesticus* stimulus.

model		explanatory variable	fixed / random	n.d.f	d.d.f.	F	P-value
maximal model:	ExpR ~	Sex	F	1	138	9.23	**0.003**
AIC: 64.1		Motivation	F	1	138	0.62	0.434
		stimulus category[1]	F	6	138	1.18	0.319
		sex **x** motivation	F	1	138	0.13	0.724
		sex **x** stimulus category1	F	6	138	0.9	0.499
		motivation **x** stimulus category[1]	F	6	138	1.26	0.279
		sex **x** motivation **x** stimulus category[1]	F	6	138	1.05	0.395
		Population	R			0.823	0.365
minimal model:	ExpR ~	Sex	F	1	166	9.85	**0.002**
AIC: 39.5							

*Motivation is the total time spent in either the right or left part of the maze (by opposition to the neutral central part), a measure of the mice willingness to participate to the test; [1]hybrids plus *domesticus*; [2]H5 and H6 were grouped for this analysis, see text; [3]Likelihood Ratio

Table 2: Results of preference tests assessing whether *musculus* discrimination against hybrids evolved in allopatry or in the contact zone. Preference is time spent in contact with *musculus* border stimulus minus with H1 neighbour hybrid stimulus(positive values means homogamous preferences). Sample sizes (n) are given alongside statistics of paired Wilcoxon tests. Bold p-values highlight significant deviance from the null hypothesis (H0: noses spent the same time in contact with the two stimuli).

	Border of hybrid zone					allopatry				
	median	*1st / 3rd quartiles*	*n*	*V*	*p*	*median*	*1st / 3rd quartiles*	*n*	*V*	*p*
females	0.805	-0.0300 / 2.66	10	49	**0.027**	0.695	-0.220 / 1.32	10	40	0.232
males	5.910	-0.320 / 9.33	17	130	**0.009**	0.740	-3.89 / 5.10	17	94	0.431

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FIGURE CAPTIONS

Figure 1: Trapping sites and their genetic characteristics plotted on a transect orthogonal to the genetic centre (bold line) of the Danish hybrid zone defined in [25]. The thick light grey line describes the general shape of the cline from [25]. Trapping sites are represented by white circles with reference to their HI estimated in this study (detailed in table ESM5). Rectangles show grouping of hybrid sites into categories; from left (light) to right (dark) across the hybrid zone respectively: border *domesticus*, hybrids H7, H6, H5, H4, H3, H2, H1 and border *musculus*. Circles' size is proportional to the sample size used to estimate the site HI. Dashed lines refer to an earlier study [16] testing wild hybrids preference for parental stimuli and showing an abrupt transition (behavioural centre) from preference for *domesticus,* left of the dashed lines, to preference for *musculus* right of these lines.

Figure 2: Results of two-ways choice tests assessing preference of *musculus* and *domesticus* mice from the border of the hybrid zone when one of the stimuli represents their geographically closest hybrid neighbours. The figure shows preferences of A) females and B) males (left: *musculus*, right *domesticus* mice) when the homosubspecific stimulus is presented against a heterosubspecific (white) or a hybrid (grey) stimulus. Identity of stimuli is indicated above (homosubspecific) and below (heterosubspecific or hybrid) each plot. Preference is calculated as the difference in time spent with one or the other stimulus (positive when assortative). The horizontal dotted line represents random choice (preference = 0). Box-plots show preference median (bold line), 1st and 3rd quartiles, and whiskers represent the entire data range. Sample sizes (n) and p-values of paired Wilcoxon rank tests (p) are given below each plot. Preference values were tested against random choice ("*" $p < 0.05$, "**" $p < 0.01$).

Figure 3: Results of preference assessing attractiveness of urinary hybrid stimuli categories sampled across the hybrid zone. Preferences of *musculus* individuals from the border of the hybrid zone (A: female noses; B: male noses) are shown as the difference in time spent in contact with the homosubspecific stimulus (*musculus* border, indicated as "mus" above the plot) versus a more distant hybrid stimulus ("H1", "H2"… specified below the plot). Preference data is as in Figure 2. The plot colour code follows that described in Figure 1 (from light to dark grey: from South to North of the hybrid zone). The estimated hybrid indexes (HI) of the hybrid stimuli presented against *musculus* stimulus, and sample sizes (n: number of noses involved in each test) are given below each plot. The plots on the right indicate preference results when data for all hybrid categories were considered as a block after mixed ANCOVA analysis revealed no significant difference between the stimulus categories; preference values were tested against random choice (Wilcoxon tests "***" $p < 0.001$)..

Figure 4: Results of the discrimination (a and b) and generalisation (c and d) tests assessing similarities between hybrid signals and parental odours. The noses were *musculus* border males for all experiments. The stimulus used for the habituation step was *domesticus* and the stimuli used during the discrimination (or generalisation) step are indicated below each plot. Results are presented as the average ± standard error of time spent in contact with each of the test stimuli after habituation to *domesticus*. The plot colour code refers to the geographical origins of the stimuli as in figure 1 and 3 (light for the *domesticus* side and dark for the *musculus* side of the zone). Results of paired comparisons: "NS" $p > 0.05$, "*" $p < 0.05$, "**" $p < 0.01$.