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

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► **To cite this version:**

Yasmin Latour, Marco Perriat-Sanguinet, Pierre Caminade, Pierre Boursot, Carole Smadja, et al.. Sexual selection against natural hybrids may contribute to reinforcement in a house mouse hybrid zone. *Proceedings of the Royal Society B: Biological Sciences*, 2014, 281 (1776), pp.20132733. 10.1098/rspb.2013.2733 . hal-02347496

HAL Id: hal-02347496

<https://hal.umontpellier.fr/hal-02347496>

Submitted on 14 Jan 2021

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1 **Last version**

2 **FULL TITLE:** Sexual selection against natural hybrids may contribute to reinforcement in a
3 house mouse hybrid zone

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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.

39

40 **KEY WORDS:** hybrid signal, mate recognition, urinary cues, reproductive character
41 displacement, speciation.

42

43 INTRODUCTION

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

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

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

148 **Behavioural tests**

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

161 A total of 232 wild derived mice (*musculus* :122 males, 81 female; *domesticus* 20 males, 9
162 females) were involved in two-way choice experiments, and 39 male *musculus* in the
163 habituation/discrimination or habituation/generalisation experiments (“the noses”). Female
164 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.

167 **Experimental designs**

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

169 *1a. 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 *1b. 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 *1c. 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

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 1a) 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

208 **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

211 when there was no significant difference between the times spent in contact with either
212 stimulus.

213 To compare preference across tests, we used an index (R): time spent in contact with
214 homosubspecific stimulus divided by total time in contact with both stimuli. We performed a
215 mixed ANCOVA on this transformed variable (expR) with sex and hybrid category as fixed
216 factors, population as a random factor and motivation (i.e. the sum of time spent in the right
217 and left sides of the Y maze) as a covariable.

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

219

220 RESULTS

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

222 1a. Discrimination against hybrids.

223 Male and female *musculus* mice from populations at the border of the hybrid zone showed
224 assortative preference when presented with *musculus* versus their most neighbour hybrid
225 stimuli (H1, figure 2A&B; males: n=17, median=5.91s, V=130, p=0.009; females: n=10,
226 median=0.81s, V=49, p=0.027). In contrast, although border *domesticus* males discriminated
227 in favour of their own subspecies during choice between *musculus* and *domesticus* stimuli
228 (figure 2B; n=10, median=2.65s, V=8, p=0.049), they did not show any directional choice
229 when the alternative to their own subspecies signal was their most neighbour hybrid category
230 (H7, figure 2B; n=10, median=2.07s, V=18, p=0.375). Female *domesticus* didn't show any
231 directional choice when presented with *musculus* versus *domesticus* stimuli (figure 2A; n=9,
232 median=-0.92, V=20, p=0.8203).

233 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 pre-mating 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].

311 **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

387 ACKNOWLEDGEMENTS

388 We are grateful to the 2010 field trapping team, to support from Givskud Zoo (particularly R.
389 Osterbald and K. Rasmussen) and to the hospitality of Jutland farmers. T. Lenormand
390 contributed with helpful discussions while designing the experiments. The transposon
391 insertion markers were developed by PB, B. Dod, P. Munclinger and V. Janoušek (Charles
392 University, Prague). T. Audoux and M. Boyaval helped with the behavioural study performed
393 under the permission of the French authorities to GG (authorization n°C34-265). This work
394 was funded by the French national research agency (ANR, N°2010BLAN171401-
395 AssortMate). ISEM/xxxxx.

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: AIC: 64.1	ExpR ~ Sex	F	1	138	9.23	0.003
	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 category ¹	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: AIC: 39.5	ExpR ~ Sex	F	1	166	9.85	0.002

*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; ¹hybrids plus *domesticus*; ²H5 and H6 were grouped for this analysis, see text; ³Likelihood 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				
	<i>median</i>	<i>1st / 3rd quartiles</i>	<i>n</i>	<i>V</i>	<i>p</i>	<i>median</i>	<i>1st / 3rd quartiles</i>	<i>n</i>	<i>V</i>	<i>p</i>
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

570
571

572 **FIGURE CAPTIONS**

573

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

582

583 **Figure 2:** Results of two-ways choice tests assessing preference of *musculus* and *domesticus* mice from the border of the
584 hybrid zone when one of the stimuli represents their geographically closest hybrid neighbours. The figure shows preferences
585 of A) females and B) males (left: *musculus*, right *domesticus* mice) when the homosubspecific stimulus is presented against a
586 heterosubspecific (white) or a hybrid (grey) stimulus. Identity of stimuli is indicated above (homosubspecific) and below
587 (heterosubspecific or hybrid) each plot. Preference is calculated as the difference in time spent with one or the other stimulus
588 (positive when assortative). The horizontal dotted line represents random choice (preference = 0). Box-plots show preference
589 median (bold line), 1st and 3rd quartiles, and whiskers represent the entire data range. Sample sizes (n) and p-values of paired
590 Wilcoxon rank tests (p) are given below each plot. Preference values were tested against random choice (“**” p < 0.05, “***” p
591 < 0.01).

592

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

602

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