Increased paternal corticosterone exposure preconception shifts offspring social behaviours and expression of urinary pheromones

Lucas B. Hoffmann[1,2], Evangeline A. McVicar[1,3], Rebekah V. Harris[1,3], Coralina Collar-Fernández[1,2], Michael B. Clark[4], Anthony J. Hannan[1,2,3] *, Terence Y. Pang [1,2,3] *

[1] The Florey Institute of Neuroscience and Mental Health, Parkville, VIC, Australia

[2] Florey Department of Neuroscience and Mental Health, Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, VIC, Australia

[3] Department of Anatomy and Physiology, University of Melbourne, Parkville, VIC, Australia

[4] Centre for Stem Cell Systems, Department of Anatomy and Physiology, University of Melbourne, Parkville, VIC, Australia

* Joint last authors

Corresponding author: terence.pang@florey.edu.au

Abstract

Studies have shown that paternal stress prior to conception can influence the innate behaviours of their offspring. The evolutionary impacts of such intergenerational effects are therefore of considerable interest. Our group previously showed that glucocorticoid treatment of adult male mouse breeders prior to conception leads to increased anxiety-related behaviours in male offspring. Here, we aimed to understand the transgenerational effects of paternal stress exposure on the social behaviour of progeny and its potential influence on reproductive success. We assessed social parameters including social reward, male attractiveness and social dominance, in the offspring (F_1) and grand-offspring (F_2). We report that paternal

corticosterone-treatment was associated with increased display of subordination towards other male mice. Those mice were unexpectedly more attractive to female mice while expressing reduced levels of the key rodent pheromone Darcin, contrary to its conventional purpose. We investigated the epigenetic regulation of major urinary protein (*Mup*) expression by performing the first Oxford Nanopore direct methylation of sperm DNA in a mouse model of stress, but found no differences in *Mup* genes that could be attributed to corticosterone-treatment. Furthermore, no overt differences of the prefrontal cortex transcriptome were found in F_1 offspring, implying that peripheral mechanisms are likely contributing to the phenotypic differences. Interestingly, no phenotypic differences were observed in the F_2 grand-offspring. Overall, our findings highlight the potential of moderate paternal stress to affect intergenerational (mal)adaptive responses, informing future studies of adaptiveness in rodents, humans and other species.

Keywords: paternal stress, epigenetic inheritance, social dominance, mate choice, major urinary protein, reproductive success

Background

Recent studies have demonstrated that the accumulation of paternal experiences before conception indirectly influence offspring behavioural phenotypes, and are largely attributed to epigenetic inheritance (1–5). This phenomenon has been studied in various animal models, with altered offspring phenotypes linked to paternal stress exposures persisting until the third generation of offspring (6). One common finding of studies to-date is a selective impact on offspring stress-relevant behaviours e.g. anxiety-like behaviour and social withdrawal. Studies of the potential mechanisms underlying these intergenerational effects have identified

51 contributions of distinct subpopulations of non-coding RNAs. For example, through
52 microinjection studies of fertilised oocytes and zygotes, paternal preconception stress-
53 associated changes to sperm small non-coding RNAs in mice were found to influence anxiety-
54 related behaviours and the stress-induced corticosterone response of adult F_1 offspring (7,8).
55 There is also emerging evidence of a specific contribution of sperm long non-coding RNAs to
56 influence adult F_1 offspring metabolic phenotypes (9). Thus, paternal stress-driven
57 intergenerational adaptations could be a contributor to the 'missing heritability' problem
58 associated with anxiety, depression and other psychiatric disorders in humans (10).
59 Additionally, some speculate about the evolutionary advantages that such heritability could
60 confer, such as adding phenotypic variation (11,12).

61 Our previous work on the paternal corticosterone-supplementation model of generalised
62 daily stress had reported elevated anxiety-like behaviours of male F_1 offspring (PatCort) and
63 the emergence of depressive-like behaviours in male F_2 grand-offspring (GPCort) (13). We
64 subsequently found that PatCort mice were resistant to the anxiolytic effects of environmental
65 enrichment (routinely reported in the wider literature) and had reduced sensitivity to the
66 selective serotonin reuptake inhibitor sertraline (14). Other independent preclinical studies of
67 distinct mouse models of stress have also found defects in sociality and social recognition
68 accompanying impaired serotonergic signalling (15), as well as dysregulation of the
69 physiological stress response (8). In rodents, appropriate social behaviour is particularly
70 important for reproduction and survival, and thus influences individual fitness. Given the
71 increasing evidence that epigenetic inheritance influences behavioural endophenotypes, it is
72 possible that epigenetic inheritance also underlies social behaviours relevant to successful
73 reproduction, with consequences for adaptivity and species evolution (16,17).

74 Here, we embarked on a transgenerational study of rodent social behaviours highly
75 relevant to their reproductive success. We investigated social dominance and male

76 attractiveness across two generations of progeny in the paternal corticosterone-
77 supplementation model of paternal stress. We hypothesised that a paternal history of stress
78 would result in offspring displaying lower preference for social reward, increased subordinate
79 behaviour during social interaction, and reduced preference from potential female mates. We
80 followed up on these findings by investigating the expression of major urinary proteins
81 (MUPs), which are non-volatile pheromones (18). Here, we present evidence that paternal
82 stress exposure preconception can exert an intergenerational influence over behavioural
83 endophenotypes that determine reproductive success in offspring. Our initial molecular and
84 epigenetic studies also highlight the complex involvement and regulation of major urinary
85 proteins (MUPs) in rodent social behaviour, and the regulation of MUP expression by the
86 paternal exposure to corticosterone.
87
88 **Results**
89
90 **Corticosterone (Cort)-treatment diminishes adult male social dominance but does not**
91 **adversely affect mate attraction**
92
93 We first evaluated the effect of Cort-treatment on the social behaviours of adult male mice. 4
94 weeks of Cort-treatment significantly reduced the percentage of wins in the tube test (Figure
95 1A, χ^2=30.25, p<0.001), which did not correlate to their body weight (Supplementary Figure
96 S3). Based on this, we expected that Cort-treated males would be less preferred by potential
97 female mates. However, there were no differences across all three variations of the test we
98 conducted ('Standard' set up: Sum of Signed Ranks W=8.000, p=0.8603, Figure 1B; 'Mouse
99 only' set up: Sum of Signed Ranks W=32.00, p=0.4332, Figure 1C; 'Bedding only' set up: Sum
100 of Signed Ranks W=36.00, p=0.3755, Figure 1D). Based on interaction times, females did not

101 show preference for one group of males over the other (Figure 1E, 'Standard' set up:
102 χ^2=0.2500, p=0.6171; Figure 1F, 'Mouse only' set up: χ^2= 1.000, p=0.3173; Figure 1G,
103 'Bedding only' set up: χ^2=0.2500, p=0.6171). Therefore, we found that Cort-treatment reduces
104 social dominance and does not affect male attractiveness.
105
106 **Paternal Cort-treatment affects social dominance and mate attraction of adult male**
107 **offspring**
108
109 Juvenile (PND 35) F_1 male and female PatCort offspring displayed a clear preference for the
110 social-conditioned bedding (Figure 2A, Males: $F_{(1,72)}$=12.76, p<0.001; Figure 2C, Females:
111 $F_{(1,74)}$=8.227, p=0.0054). No effects of paternal treatment were found for males (Figure 2B,
112 U=601, p=0.3811) nor for females (Figure 2D, U=640.5, p=0.4035). Therefore, F_1 PatCort
113 offspring mice displayed normal preference for social reward, and it was independent of the
114 paternal corticosterone exposure.
115
116 In the tube test, adult F_1 male PatCort offspring recorded significantly fewer winning
117 interactions, indicative of a lower order of social dominance (Figure 2E, χ^2=15.75, p<0.001).
118 In the mate-choice test, *naïve* females investigated F_1 male PatCort offspring for significantly
119 longer periods than their PatCT counterparts (Sum of Signed Ranks W=241.0, p=0.0080), and
120 a significantly higher percentage of time (Figure 2F, χ^2=7.759, p=0.0053). No differences were
121 observed between the groups for the 'mouse only' nor the 'bedding only' set ups ('Mouse
122 only': Sum of Signed Ranks W=-40.00, p=0.3225; 'Bedding only': Sum of Signed Ranks W=-
123 18.00, p=0.5186), and the total percentage of time was comparatively similar between the
124 groups (Figure 2G, 'mouse only' set up, χ^2=1.000, p=0.3173; Figure 2H, 'bedding only' set
125 up, χ^2=1.333, p=0.2482). Additionally, analysis of the total time each male directly interacted

126 with the female when she approached them revealed no differences (Figure 2I, $t_{(26)}$=0.8106,
127 p=0.4249). Thus, paternal Cort-treatment was associated with intergenerational shifts in social-
128 relevant behaviours of adult F_1 male PatCort offspring.
129
130 **Paternal Cort-treatment effects do not transgenerationally alter grand-offspring social**
131 **behaviours**
132
133 Juvenile F_2 male and female grand-offspring showed a preference for the social-conditioned
134 bedding (Figure 3A, Males: $F_{(1,76)}$=35.14, p<0.001; Figure 3C, Females: $F_{(1,80)}$=12.09,
135 p<0.001). No differences in social reward were found between the groups for males (Figure
136 3B, U=714.5, p=0.6527) nor for females (Figure 3D, U=755, p=0.4547).
137
138 In the tube test, GPatCort and GpatCT groups recorded similar numbers of wins (Figure 3E,
139 χ^2=2.667, p=0.1025). In the mate-choice test, females spent similar amounts of time interacting
140 with both groups of mice across all of the set ups (Sum of Signed Ranks W=-10.00, p=0.8209;
141 'Mouse only' set up: Sum of Signed Ranks W=40.00, p=0.3160; 'Bedding only' set up: Sum
142 of Signed Ranks W=-40.00, p= 0.3225). The percentage time spent by the female interacting
143 with both groups was similar (Figure 3F, 'Standard' set up: χ^2= 1.000, p= 0.3173; Figure 3G,
144 'Mouse only' set up: χ^2= 1.000, p= 0.3173; Figure 3H, 'Bedding only' set up: χ^2= 0.2500, p=
145 0.6171). Therefore, no transgenerational effects on social-relevant behaviours of F_2 GPCort
146 grand-offspring were observed in association with paternal Cort-treatment.
147
148 **Paternal Cort-treatment alters the expression of a subpopulation of MUPs in F_1 offspring**
149 Since pheromones play an important role in rodent male social hierarchy and mate attraction,
150 we quantified the expression profiles of MUPs in the urine of F_1 male offspring.

151

152 We first assessed urinary creatinine content to account for urinary dilution, and determined that
153 it was not different between PatCT and PatCort male offspring (Figure 4A, $t_{(12.74)}$=1.704,
154 p=0.1126), which indicates that their urination volume did not differ (19). The relative levels
155 of total MUPs also did not differ between the groups (Figure 4B, $t_{(15.24)}$=1.792, p=0.0930).
156 Next, we differentiated between the three major MUP bands (Figure 4C), in agreement with
157 previous publications (18,20). Semi-quantification of the protein bands revealed that PatCort
158 males had reduced concentrations of the small MUP band (Figure 4D, $t_{(15.41)}$=2.252, p=0.0393)
159 and of Darcin (Figure 4F, U=53, p=0.0243). No difference in the big MUP band was observed
160 (Figure 4E, $t_{(14.09)}$=1.904, p=0.0775). Additionally, the variability of all three bands of MUP
161 proteins was higher in the PatCort mice (Small MUP band: $F_{(12,15)}$=5.693, p=0.0022; Big MUP
162 band: $F_{(12,15)}$=9.406, p=0.0001; Darcin: $F_{(12,15)}$=5.614, p=0.0024; F test to compare variances).
163 Therefore, there appears to be some selectivity in terms of the intergenerational impacts of
164 paternal Cort-treatment on offspring production of MUPs.

165

166 Darcin was proposed to be the key MUP subtype to act as a female attractant pheromone (18).
167 However, urinary Darcin concentrations of male mice of both PatCT and PatCort groups were
168 not significantly correlated with total time being investigated by the female in the mate-choice
169 test (Figure 4G, PatCT: Pearson's correlation R^2=0.1112, p=0.2068; PatCort: Pearson's
170 correlation R^2=0.0921, p=0.3134).

171

172 Since MUP proteins are produced and secreted by the liver (21), we further investigated hepatic
173 gene expression of various *Mup* genes and *Zhx2* (a transcript factor for *Mup* genes) (22).
174 However, *Mup* gene expression in PatCort mice did not differ from PatCT mice (Figure 4H,
175 *Mup3*: $t_{(21.88)}$=1.666, p=0.1100; Figure 4I, *Mup20*: U=107, p=0.4393; Figure 4J, *MupB*:

176 $t_{(30)}$=1.235, p=0.2264), despite the increased variability in the gene expression of *Mup3* (*Mup3*:
177 $F_{(15,15)}$=4.117, p=0.0094; *Mup20*: $F_{(15,15)}$=1.555, p=0.4020; *MupB*: $F_{(15,15)}$=2.444, p=0.0939; F
178 test to compare variances) in PatCort mice. There was also no difference in hepatic *Zhx2* gene
179 expression (Figure 4K, *Zhx2*: $t_{(30)}$=0.5436, p=0.5907).
180
181 *Mup* gene expression levels displayed a strong positive correlation with MUP protein
182 populations in the PatCort offspring. *Mup3* correlates very strongly with total MUP urinary
183 concentration in the urine of PatCort mice (Figure 4L, Pearson's correlation R^2=0.6988,
184 p<0.001). Interestingly, this correlation was not observed for PatCT mice (Figure 4L, Pearson's
185 correlation R^2= 0.0003, p=0.9498). *Mup20*, in its turn, correlates very strongly with the Darcin
186 band population of MUPs in the urine of PatCort mice (Figure 4M, Pearson's correlation
187 R^2=0.6823, p=0.0005), but did not correlate in PatCT mice (Figure 4M, Pearson's correlation
188 R^2=0.041, p=0.4521). The Darcin band has been attributed to the *Mup20* gene in previous
189 publications (18,19). These findings suggest that in PatCort mice, the high variability in the
190 gene expression of *Mup3* is likely causing the high variability in the protein level.
191
192 **MUP profile is not transgenerationally influenced by paternal Cort-treatment**
193
194 We also assessed urinary creatinine and MUP protein concentrations of F_2 male grand-
195 offspring mice. Both urinary creatinine (Figure 5A, U=101.5, p=0.8897) and total MUP
196 concentrations (Figure 5B, $t_{(27)}$=0.9832, p=0.3343) did not significantly differ between the
197 groups. No between-group differences were also detected for any of the three major MUP
198 bands, namely small MUPs (Figure 5C, $t_{(27)}$=0.4547, p=0.6529), big MUPs (Figure 5D,
199 $t_{(27)}$=0.7838, p=0.4400) and Darcin (Figure 5E, $t_{(27)}$=0.5951, p=0.5568). Additionally, there
200 were no differences in variability between the groups.

201

202 **Cort-treatment does not alter *Mup* gene DNA methylation**

203

204 Differential methylation of *Mup* genes has been reported, but the epigenetic regulation of *Mup*
205 gene expression remains unclear (23–25). To address this, we developed an optomised sperm
206 DNA extraction protocol that enabled us to conduct the first long-read nanopore sequencing
207 study of *Mup* gene methylation in sperm DNA harvested from Cort-treated and control F_0
208 males. Overall, CpG methylation indicated a very high methylation frequency across the
209 genome (not shown), which is expected for sperm since it is a transcriptionally quiescent cell
210 population. CpG methylation located within the *Mup* gene cluster was also found to be highly
211 methylated (Figure 6H), with no obvious differences between both groups (Figure 6A). We
212 noted a potential pattern of increased methylation associated with Cort-treatment at the
213 promoter region of *Mup20* and decreased methylation in the 3' downstream region of the gene
214 (Figure 6B); these would be consistent with decreased gene expression of *Mup20* and
215 decreased expression of Darcin protein we had found (26). Therefore, we assessed CpG
216 methylation at the promoters of the *Mup20* gene, whose locations were obtained from the
217 UCSC genome browser promoter track. However, no differences in methylation were found
218 (p=0.96 – Figure 6J). We further inspected methylation of the transcription factor *Zhx2* and
219 additional *Mup* genes of interest (*Mup3*, *Mup2*, *Mup15*, *Mup18* – Figures 6C – 6G,) identifying
220 no major differences. Consistent with this, an analysis of differentially methylated regions
221 (DMRs) using DSS revealed no significant differences between the groups at the *Mup* locus.
222 Thus, it appears that DNA methylation is not a major epigenetic regulator of *Mup* expression
223 in this rodent model.

224

225 **Adult male offspring prefrontal cortex gene expression is relatively unchanged by**
226 **paternal Cort-treatment**
227
228 The rodent prefrontal cortex is heavily implicated in displays of social dominance (27), as well
229 as anxiety-relevant behaviours that we have reported in this model (13,28). We therefore
230 conducted transcriptome profiling of this brain region to determine whether gene expression
231 differences underlie the F_1 offspring phenotypes we observed. Overall, we found that samples
232 were very similar, independent of their group (Figure 7A). Only 32 genes were found to have
233 $p < 0.05$ and log-fold change (LFC) threshold = 1, with 3 upregulated and 29 downregulated
234 in the PatCort male mice (Figure 7B, Table 2), although they were not statistically significant
235 after FDR correction. Similarly, Gene Set Enrichment Analysis (GSEA) was performed, but
236 no significant gene sets were found (Table 3).
237
238 **Discussion**
239
240 This study has uncovered novel evidence of the paternal influence over offspring social
241 behaviours. Paternal Cort-treatment was associated with reduced social dominance and
242 increased attractiveness of their male F_1 offspring, in addition to the elevated anxiety phenotype
243 we previously reported (13). Interestingly, these effects on social behaviours were restricted to
244 the immediate generation (F_1 offspring), with no significant transgenerational effects on the F_2
245 grand-offspring. Additionally, the male F_1 PatCort offspring also showed reduced and more
246 variable MUP protein output in their urine, in particular the band that corresponds to the male
247 pheromone Darcin. By performing the first sperm DNA methylome sequencing study in mice
248 treated with corticosterone, we established that the abnormal MUP expression is not a result of
249 dysregulated DNA methylation of the various *Mup* genes. Additionally, the absence of

250 differences in the PFC transcriptome strongly suggests that the altered social responses of F_1
251 PatCort offspring likely originate in other brain regions key to social interaction, such as the
252 anterior cingulate cortex or the ventromedial hypothalamus nucleus (29,30).
253
254 **F_0 male mice showed reduced dominance and no difference in attractiveness**
255
256 Male mice treated with corticosterone in their drinking water for 4 weeks showed a large
257 reduction in their social dominance, with no effects on their attractiveness to female mice.
258 There is extensive literature on dominance and social hierarchy priming response to stress (31),
259 as it is known that hierarchical rank modulates the hypothalamic-pituitary-adrenal (HPA)
260 system reactivity (32), possibly due to higher-order impacts of the prefrontal cortex on
261 hypothalamic function. Studies on stressful exposures modulating social dominance and
262 hierarchical rank are less studied, but it has been reported that a cortico-hypothalamic circuit
263 modulates social dominance (33) and maternal separation reduces adult dominance in a
264 competition for water in water-restricted mice (34). Furthermore, chronic restraint decreases
265 social dominance in the tube test (35) and severe immobilisation stress heavily reduces social
266 dominance in anxious mice (36). Another study reported the opposite effect of stress on
267 dominance, with maternal separation resulting in increased social dominance in the tube test in
268 rats (37). Therefore, dominance can both regulate the response to stress but also be modulated
269 by it, which indicates the complexity of the neural circuitry regulating this social behaviour, as
270 well as its reactivity to corticosterone.
271
272 **F_1 male and female offspring do not show changes in social conditioned-place preference**
273

Male and female offspring were tested as juveniles in the social conditioned-place preference, and they displayed expression of social reward, with no differential effects of the paternal corticosterone exposure. Social reward has been proposed to drive the approach towards socially relevant environments, as well as the avoidance from predicted social isolation, and is expressed during youth in social animals (38). Through the acquired social experiences resulting from this behaviour, the social reward would then influence the development of adult sociality (39). Stress can modulate social reward, as it has been shown that foot-shock stress exposure reduces social reward expression (40). Stress exposure has also been found to impair sociability (41), with increased aggression in males and social withdrawal in females (42). The effects of stress on adult sociability could be due to impaired acquisition or expression of social reward, although further studies are needed to investigate this hypothesis. Nevertheless, differences in social reward were not observed in our model, which indicates that the changes in adult social behaviour observed in these mice are not due to altered social developmental trajectories.

Male F_1 offspring show lower social dominance and increased attractiveness

Male offspring were tested as adults in the social dominance tube test and mate-choice test. PatCort F_1 offspring showed reduced social dominance in the tube test, like their fathers. This effect was expected, due to their increased anxiety-like behaviour, as studies have showed that anxiety affects social behaviours (43,44), although other studies did not show association between anxiety-like behaviour and social rank in the tube test (45). These mice also showed increased attractiveness, which was not expected. It is important to consider that the setup we used for the mate-choice test intentionally did not let the males display territoriality, compete over mating opportunities, or initiate an interaction with the female, rendering them passive to

299 female mouse discretion. Therefore, the results in attractiveness could have been different if a
300 competitive setup had been used where the males could display their dominance.

301 Both MUPs and ultrasonic vocalisations (USVs) have been shown to attract females
302 (46,47). When males are exposed to females, they emit USVs as part of a 'male song' (48).
303 Additionally, males excrete MUPs in their urine. These proteins have pockets that bind volatile
304 pheromones, but they also act as non-volatile pheromones. MUP expression is complex and
305 conveys a plethora of information, ranging from sex, health status, individuality, and
306 attractiveness (49). By using different setups in the mate-choice test, we tried to determine the
307 key features underpinning, and potentially driving, the increased attractiveness in the PatCort
308 mice. This may include the USVs being derived from the physical presence of the mice in the
309 apparatus, and the MUPs being derived from the presence of soiled bedding. For instance, in
310 our 'standard' setup, where we added both mice and their soiled bedding, we accounted for
311 both USV and MUPs.

312 USVs have been shown to travel short distances and therefore are more relevant for short-
313 distance communication (50). MUPs and other olfactory-based signals, on the other hand, can
314 travel long distances and have therefore been associated with long-distance communication
315 (46). In a natural setting, pheromone marks scattered around a certain area would indicate to a
316 female mouse an attractive socially relevant stimulus. If there was a male mouse nearby, he
317 would then vocalise once the female mouse approached him. Therefore, it is possible that in
318 the mate-choice test setups we used, olfactory marks devoid of pairing with USVs, or vice-
319 versa, could have not been enough to signal attractiveness, hence no differences were observed
320 in the 'bedding-only' and the 'mouse-only' setups. Additionally, we assessed the male
321 interaction response towards the approaching female in the 'standard' set up, to determine
322 whether a differential interaction could have accounted for the changes in attractiveness.
323 However, no differences were observed.

324

325 **F_2 male and female grand-offspring do not show changes in social conditioned-place**
326 **preference**

327

328 Following the same experimental design, male and female grand-offspring were tested
329 as juveniles in social conditioned-place preference, and male offspring were assessed as adults
330 in the social-dominance tube test and mate-choice test. However, despite the previously
331 observed increase in depressive-like behaviour in the adult male mice, no differences were
332 observed for any of the social behaviours tested. This shows the limited heritability of the
333 effects that the paternal corticosterone exposure has on social behaviours, which is also
334 observed in other studies, with phenotypes spanning across only one generation following the
335 environmental exposure (10).

336

337 **F_1 offspring mRNA sequencing does not show overt changes in gene expression**

338

339 Following the reduced social dominance in the PatCort male offspring, we investigated
340 gene expression in the prefrontal cortex of these mice, to investigate potential transcriptomic
341 regulation underpinning this behaviour. We chose the prefrontal cortex because of the
342 association between this brain region and social dominance. For instance, studies have shown
343 that the synaptic efficacy regulated by AMPA receptors in this region controls the expression
344 of social dominance (51), that the increase in social dominance as assessed in the tube test
345 accompanies modifications of the stable actin fraction in synaptic spikes in this region (37),
346 and that social dominance is followed by differential gene expression in this region (45). Lastly,
347 neuronal population activity in the mPFC predicts social rank and success in competitive
348 settings (33).

349 However, no differentially expressed mRNAs were detected after a rigorous False
350 Discovery Rate correction for multiple comparisons. This effect might be due to four
351 possibilities: 1) Animals were culled at baseline (without acute stress). Unpublished data from
352 our group showed that PatCort males do not differ from controls in baseline plasma
353 corticosterone, but only after a restraint stress. 2) Sequencing was done from bulk-tissue RNA.
354 When considering the heterogeneity of the cellular populations in the prefrontal cortex, it is
355 possible that cell-specific differences in gene expression are not detected (i.e. 'diluted out') by
356 bulk-mRNA sequencing. 3) Differential gene expression occurs during development, and we
357 only assessed adult PFC. 4) We only assessed adult PFC, and transcriptomic changes may have
358 been present in one or more other relevant brain areas. We hypothesise that these mice exhibit
359 behavioural changes in adulthood due to differential developmental trajectories that result in
360 subtle neuronal changes, such as modifications in synaptic architecture, spine density or
361 dendritic arborisation. Therefore, differences in gene expression may have been present during
362 critical periods of development, such as during embryonic, early postnatal and/or adolescent
363 stages.
364
365 **F_1 male offspring MUP protein analyses**
366
367 Urine was collected from adult male F_1 offspring for quantification of MUP proteins.
368 PatCort mice showed reduced levels of specific MUP bands observed after separation in the
369 SDS-PAGE. Three different bands with molecular weight of around ~17 kDa to ~23 kDa were
370 identified, which is similar result to what have been described before (18,20). The band with
371 the lowest molecular weight has been shown to be present in males only and to be the most
372 relevant to signal male attractiveness, and it has been named 'Darcin' (18). In our dataset we

373 have observed a reduction in the Darcin and the small band MUP populations (the band with
374 higher molecular weight).

375 Importantly, contrary to the current literature (18,52), in the mate-choice test we did not
376 observe a correlation between urinary Darcin intensity and male attractiveness. Mate choice is
377 a complex decision that depends on the integration of multiple sensory, affective and cognitive
378 systems (46). Therefore, we hypothesise that although PatCort males exhibit lower levels of
379 Darcin in their urine, other factors might be modulating their attractiveness. Indeed, the
380 different setups we used for the mate-choice test indicate that a combination of both olfactory
381 and auditory stimuli seems to be necessary to signal the differential attractiveness in these mice.
382 Additionally, the highly individual and complex urinary MUP pattern, which is pronounced in
383 wild mice, indicates that female mice use this information for additional profiling of the male
384 quality (53), and therefore could modulate the attractiveness signalled by Darcin alone. It is
385 also important to consider the role of volatile pheromones that bind to and are slowly-released
386 from MUPs, which can have their concentration proportionally altered by social status (54).
387 Due to the reduced social dominance in the PatCort mice, it would be interesting to profile the
388 volatile pheromone content in their urine.

389

390 **F_1 male offspring *Mup* mRNA expression analysis and correlation with MUP bands in**
391 **SDS-PAGE**

392

393 Due to the changes in MUP protein expression, we collected the liver from the adult male
394 offspring for *Mup* mRNA expression assessment. No differences were observed between the
395 groups for *Mup3*, *Mup20*, class-B *Mups* and *Zhx2*. However, there was a very strong correlation
396 between *Mup3* gene expression and urinary MUP output in the PatCort only, with no
397 correlation in the PatCT.

398 The MUP protein concentration normalised by creatinine output, as analysed in this
399 study, is determined by a range of factors. To begin with, the expression of *Mup* genes is
400 induced by many different factors, such as testosterone (55), pulsatile growth hormone (21),
401 and circadian glucocorticoid (56). Social factors also modify the expression of MUPs, with
402 social dominance proportionally affecting MUP production (57), possibly due to changes in
403 testosterone levels (55). The differential transcription of *Mup* gene paralogs results in the large
404 diversity of MUP proteins observed in the urine (58), and one of their known transcription
405 factors is *Zhx2* (22). There is no evidence of post-transcriptional processes regulating MUP
406 protein concentration, as the mRNA expression predicts the urinary protein output (58), and
407 MUPs are not reabsorbed in the kidney (59). Lastly, creatinine levels relate to the muscle mass
408 and are a marker of glomerular filtration (55), and were used in this study to normalise the
409 absolute MUP protein levels in the urine. This normalisation accounts for urine dilution, which
410 can also be changed by social status, with submissive mice reducing their urine production and
411 subsequently increasing their urinary creatinine concentration (57). Therefore, the urinary
412 MUP concentration normalised by creatinine output represents the instantaneous MUP
413 expression relative to the protein levels in the body.

414 The very strong correlation between *Mup* gene expression and protein output in the
415 PatCort indicates that the overall effect of the factors described above should be homogeneous
416 across this group, resulting in a deterministic association between *Mup* gene expression and its
417 protein output. However, despite no differences in gene expression between the groups, MUP
418 protein is lower in the PatCort. More studies would be necessary to determine the regulatory
419 mechanisms underpinning this result. It is interesting as well that most measures of MUP band
420 levels and *Mup* gene expression have higher variability in the PatCort group only, compared
421 to the PatCT counterpart, which suggests that paternal-Cort exposure affects the expression of
422 these genes, but not homogenously.

423 Additionally, the primers we used for detecting class-B *Mups* aligned with a range of
424 different *Mup* genes (*Mups 1*, *2*, *7*, *8*, *9*, *10*, *11*, *12*, *13*, *14*, *15*, *17*, *18*, *19* and *22*) to cover some
425 of the *Mup* genes that are evolutionary related (60). Therefore, we may have missed resolving
426 individual *Mups* that are also implicated as female attractants. However, it is important to
427 notice that Darcin (*Mup20*), *Mup2* and *Mup3* are highly expressed compared to the other *Mup*
428 genes (20).
429
430 **No differences in MUPs in male F_2 grand-offspring**
431
432 Despite no differences in attractiveness observed in the male grand-offspring, urine was
433 collected from these mice for quantification of MUP proteins. As expected, no differences were
434 observed in MUP protein concentration, and neither for the bands separated in the SDS-PAGE.
435 Whilst we know that F_0 paternal-corticosterone treatment can have effects that transmit to F_2
436 grand-offspring, particularly with respect to depression-like behaviour (13), it appears that this
437 transgenerational epigenetic inheritance is specific and does not generalise to the social
438 behaviours and MUP expression that we now report as changed in the F_1 offspring.
439
440 **No differences in methylation of *Mup* genes in male F_0 sperm**
441
442 Sperm DNA methylation was assessed through DNA long-read sequencing to determine
443 whether the increased male offspring attractiveness and reduced urinary MUP protein levels
444 could be due to the inheritance of Cort-treatment-induced altered DNA methylation. Although
445 most of the parental DNA marks get erased during early development due to the embryonic
446 reprogramming (61), it has been suggested that certain DNA marks can escape this process
447 (62), as it has been shown to occur in regulatory regions of glucocorticoid and estrogen

448 receptors (63). Additionally, a previous study have shown increases in the DNA methylation
449 of *Mup* genes in adult mice, induced by early-embryonic manipulations, which also resulted in
450 repression of *Mup* transcription (24). However, no overall differences in CpG methylation
451 spanning the *Mup* gene cluster were found in our study, which suggests that other regulatory
452 mechanisms underlie the decreased MUP protein levels in the PatCort male mice.
453
454 **Conclusions**
455
456 Overall, these new results have implications for our understanding of adaptiveness in the
457 context of the epigenetic inheritance, as social interactions are known to contribute to fitness
458 in mice (64). More specifically, social hierarchy can affect survival due to differential access
459 to resources (65) and modulation of the HPA axis responsiveness (66) and, together with male
460 attractiveness, they can impact access to mating opportunities (67,68). The sons (F_1 offspring)
461 of corticosterone-treated mice showed reduced dominance and, although they showed
462 increased attractiveness, it was only when they were in proximity to their urinary marks.
463 Therefore, although it is not possible to establish the causality between low urinary output and
464 dominance, we hypothesise that in a natural environment, due to their lower dominance, these
465 mice would have lower total urine production and success in marking territory, which has been
466 shown to affect reproductive success in wild mice (69). These male offspring (whose fathers
467 had elevated stress hormone levels) would also have a decline in their survival rates, with the
468 overall effect of reducing their survival.
469 Aspects of epigenetic inheritance in mammals are still met with some scepticism, with
470 one of the questions being why such inheritance evolved if its impact is rarely observed across
471 many generations (70). However, the hypothesised decrease in survival proposed above due to
472 the reduced social dominance observed in the PatCort mice suggests that by modulating

473 endophenotypes that determine fitness, epigenetic inheritance could impair reproduction and
474 survival, which could then heavily impact the generations to come, even though the changes in
475 behaviour are observed in only one generation. This 'trans-populational impact' has been
476 suggested in mice before (3), and has been shown to occur in *C. elegans* (71).

477 Another factor that can impact adaptiveness and needs to be considered is the mismatch
478 between the environments experienced by the fathers and the offspring/ grand-offspring. Some
479 studies indicate that the epigenetic inheritance could fit into the mismatch hypothesis of disease
480 (72), which posits that changes in the environment during development induce adaptive
481 changes that can prime the individual for that environment (within genetic constraints). For
482 instance, adult-generated neurons born during chronic social stress are uniquely adapted to
483 respond to subsequent chronic social stress (73). However, when an environmental mismatch
484 happens between the timepoints, it can result in maladaptation and disease. Similar
485 mechanisms might be at play behind how the epigenetic inheritance functions. For instance, it
486 has been suggested that when environmental cues are not reliable predictors of offspring
487 environment, in the case of environmental conditions changing between generations, the
488 epigenetic inheritance could instead reduce fitness (74,75).

489 Lastly, it has been suggested that male attractiveness could have evolved with the aid of
490 epigenetic mechanisms and female mate choice (76). The evolutionary expansion of mouse
491 *Mup* genes is recent (77), occurring due to multiple duplication events (60) that have led to the
492 emergence of many pseudo-genes (78). This indicates selective pressures shaping scent signals
493 relevant for social communication (79), and this is in accordance with olfactory signals that
494 mediate territorial behaviour and sexual selection being under evolutionary pressure (79).
495 Interestingly, one of the first reports on epigenetic inheritance in mice showed differential
496 methylation of *Mup* genes (25), and it has been shown that different sociality levels can have
497 transgenerational effects on MUP expression (20). These studies indicate that one of the

498 avenues to understand the evolutionary relevance of the epigenetic inheritance could be
499 through investigating its regulation of genes that are relevant for sexual selection. For instance,
500 epigenetic reprogramming of reproductive function, driven by early-environment, has been
501 shown in humans (80).

502 In conclusion, in this study we showed that epigenetic inheritance can modulate social
503 behaviours that are important for determining reproductive success, thus potentially impacting
504 many generations. The present findings, along with other studies investigating epigenetic
505 inheritance, help shed light on the evolutionary impact that this type of inheritance confers.
506 The observed phenotype does not necessarily accompany altered gene transcription in adult
507 offspring (in the limited tissues examined at a single adult stage), which suggests
508 developmental processes are at play. Therefore, it is essential that studies on gene expression
509 focus on the critical developmental timepoints and explore additional relevant tissues and cell
510 population in offspring. Lastly, epigenetic mechanisms underpinning this type of inheritance
511 need to be investigated further, including sperm epigenetics and post-conceptual transfer of
512 epigenetic information via developmental algorithms.

513

514 **Methods**

515

516 *Mice and husbandry*:

517 Unless indicated, all mice were housed in groups of 3 to 5 in open-top cages with Aspen
518 shaving bedding (Romania) and 2 sheets of paper tissue for nesting. Cages were changed
519 weekly, and food and water were provided *ad libitum*. 7-week-old *naïve* male and female
520 C57BL/6 breeders were obtained from the Animal Resources Centre (Murdoch, WA,
521 Australia). One week later, male breeders were single-housed and randomly assigned to the
522 control (CT) or corticosterone (Cort) group (total liquid consumption can be found on

523 Supplementary Figure S1). One week before the end of the corticosterone treatment described
524 below, male breeders were tested for the mate-choice and social-dominance tube tests at PND
525 77. After the designated corticosterone treatment period, CT and Cort male breeders were
526 individually and randomly assigned to *naïve* female breeders and paired for 5 days, after which
527 the males were culled. The females were left single-housed and undisturbed for 19 days, after
528 which they were checked daily for newborn litters. Pups from CT or Cort fathers (Paternal CT
529 – PatCT or Paternal Cort – PatCort groups) were weighed on post-natal day (PND) 7, 14 and
530 21, during which the boxes were changed. Pups were weaned at PND 28 and were group-
531 housed according to sex and to paternal treatment, with pups from multiple different litters
532 being housed together to avoid litter effects. Male and female offspring were tested for the
533 social conditioned-place preference test at PND 35, and the male offspring were tested for the
534 mate-choice and social-dominance tube tests at PND 77. When the behavioural testing was
535 complete, the male offspring were single-housed and paired with *naïve* females to generate the
536 grand-offspring: grand-paternal CT (GPCT) and grand-paternal Cort (GPCort); the procedure
537 was the same as described above (Supplementary Figure S2). All procedures were approved
538 by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committees (AEC),
539 complying with the Australian Code for the Responsible Conduct of Research and the
540 Australian Code for the Care and Use of Animals for Scientific Purposes. For each generation
541 at least 16 mating pairs were used per group, generating at least 9 litters per group. The n size
542 per group for the behavioural experiments was 16-29. The numbers can be found in Table 1.
543
544 *Corticosterone treatment:*
545 Corticosterone treatment was as per our published protocols (13,14). Briefly, the Cort group of
546 male mice was given 25 µg/mL corticosterone hemisuccinate (Steraloids Inc., Newport, RI,

547 USA) in their drinking water, changed twice a week, for 4 weeks. Control (CT) male mice
548 received the same drinking water, without corticosterone added.
549
550 *Behavioural experiments*:
551 **Social conditioned-place preference:** the protocol was adapted from Dölen et al., 2013;
552 Nardou et al., 2019. This test was used to assess social reward, which is the result of the
553 interaction between the approach towards socially salient stimuli, and the avoidance of cues
554 that predict social isolation, which is more easily observed in juvenile mice when social
555 interactions are not affected by sex-specific interests (38). Male and female offspring and
556 grand-offspring, from paternal and grand-paternal CT and Cort treatment respectively, were
557 tested on PND 35. In a locomotor chamber (ENV-510, Med Associates, Fairfax, VT, USA)
558 divided in two similar halves with a door connecting the two halves, two different types of
559 bedding were used to cover the floor in each half (CornCob or Alpha Dri), around 1 cm height.
560 Mice were first assessed in the locomotor chamber and had their activity recorded for 30 min,
561 and the time spent on each side was measured (pre-conditioning). Soon after the exploration,
562 all mice from the same cage were housed together in a new cage with one of the bedding types
563 for 24h, after which they were single housed with the other bedding type for 24h. Mice were
564 then assessed in the locomotor chamber once again (post-conditioning). The time and ratio of
565 the time spent in the social-conditioned bedding during post- and pre-conditioning were
566 evaluated.
567
568 **Mate-choice test:** the protocol was adapted from Hoffmann et al., 2020; Mitra and Sapolsky,
569 2012. This test was used to assess male quality or "attractiveness" as perceived by a fertile
570 female, which predicts the likelihood of first copulation and mating duration (68). Male quality
571 is determined by scent marking (84) and vocalisations (85). Using a 3-chamber interaction test,

572 a fertile female (assessed daily before the test by vaginal smear) explored the apparatus for 10
573 min. Then, one male from each experimental group was put inside a small cage on each side
574 of the apparatus, and the female explored the apparatus again for 10 min, during which the time
575 she spent interacting with each male mouse was measured. Different set ups were used for this
576 test in order to investigate the underlying factor for an eventual change in attractiveness. The
577 'standard' set up consisted of presenting mice and the respective soiled bedding from their
578 home cage (Figure 1B). The 'bedding only' set up consisted of presenting the soiled bedding
579 from their home cage, to assess if pheromones alone determine attractiveness (Figure 1C),
580 whereas the 'mouse only' set up consisted of presenting the mice alone, to assess if ultrasound
581 vocalisations or the male interaction *per se* determine attractiveness (Figure 1D). A different
582 female mouse was used for every round of assessment, including for mouse only and bedding
583 only sessions. Females were not tested because this test relies on behavioural responses linked
584 to the development of male secondary sexual characteristics. As follow up on the results found
585 for the F_1 male offspring, we manually analysed their Mate-Choice trial video recordings to
586 quantify each male's responsive interaction to the approaching female, to determine if there
587 were differences between the groups for this measure. For this analysis, we quantified the time
588 that each male spent with its snout directed towards the female when she approached the
589 interaction zone.
590
591 **Social-dominance tube test:** the protocol was adapted from Tada et al., 2016; Zhou et al.,
592 2016. This test was used to assess social dominance, which underlies agonistic behaviours (87)
593 and can be defined as the capacity to prevail in conflicts encompassing aggression, threats,
594 fights or submission (88,89). The apparatus consisted of a 30-cm long clear plastic tube. During
595 habituation each mouse was trained to go through the tube for 10 times. On the following day,
596 during testing, each mouse from a CT cage faced every mouse from a Cort cage, in a total of 4

interactions per mouse per cage. Each mouse was tested once every after 7 to 9 interactions, so as to allow resting time between the face offs. The number of wins by the Cort group was compared to the null hypothesis on a chi-square test to determine statistical significance. Females were not tested because this test relies on behavioural responses linked to the development of male secondary sexual characteristics.

Other procedures:

Urinary component assessments

Immediately prior to being culled, urine was collected from each male by scruffing, and frozen at -80 °C immediately. Mouse urine was thawed and diluted 1/4 in MilliQ water. Previous studies have determined that most of the mouse urinary protein content corresponds to MUP proteins (90). Therefore, MUP concentration was determined using the Quick Start™ Bradford Protein Assay, according to the manufacturer's instructions. Briefly, diluted urine was incubated with Bradford reagent at room temperature for 5 min, after which it was read at 595 nm in Epoch 2 Microplate Spectrophotometer (Biotek/Agilent). The standard curve was constructed using BSA dilutions ranging from 125 to 1,000 µg/mL (Quick Start Bovine Serum Albumin Standard, Cat. #5000206). Creatine concentration was determined using Creatine (urinary) Colorimetric Assay Kit Cayman Chemical Item No 500701 to account for urine dilution (49), according to the manufacturer's instructions.

SDS-PAGE for MUP protein analysis

621 The protocol was adapted from Lee et al., 2017; Nelson et al., 2015. Mouse urine was thawed
622 and diluted 1/50 in MilliQ water. Beta-mercaptoethanol and SDS loading buffer were added to
623 each sample and heated for 5 min at 95 °C. 10 µL of each sample was loaded into 4-15% gel
624 and run at 200 V for around 20 min. The bands were stained with 0.1% colloidal blue in ethanol
625 using the Coomassie R-250 staining protocol. A reference comprised of pooled urine from 8
626 mice was used for semi-quantification, which was run in every gel and used as a normaliser
627 across all gels, after measuring its band intensity with ImageJ (v2.1.0/1.53c).
628
629 **RT-qPCR of *Mup* genes**
630
631 Mice were killed by cervical dislocation and the right lobe of the liver was dissected, then
632 frozen at -80 °C. Liver RNA was extracted using QIAzol according to the manufacturer's
633 instructions and quantified in Nanodrop (2000c Thermo Scientific). 1000 ng of RNA was
634 reverse transcribed with SuperScript™ VILO™ cDNA Syntesis Kit (Invitrogen, Cat.
635 #11754050). cDNA was diluted 1/10 for qPCR gene expression studies (Table 4). Relative
636 expression was determined using the comparative ΔΔCt method, with ß-actin as the
637 endogenous control gene. The primers used in this study can be found in Table 4.
638
639 **Offspring prefrontal cortex mRNA sequencing**
640
641 Mouse prefrontal cortex (bregma +1.42 mm, interaural 5.22 mm) was dissected and snap frozen
642 in dry ice. The RNA was extracted using a standard QIAzol Lysis Reagent (QIAGEN, Cat #
643 79306) procedure, according to the manufacturer's instructions. RNA was purified from
644 potential DNA contamination with DNA-*free*™ Kit (Ambion, Cat # AM1906), according to
645 the manufacturer's instructions. RNA quality was assessed using the Agilent 4200 TapeStation
646 system. Samples with RIN value higher or equal to 7.5 were sent for sequencing at the

647 Australian Genome Research Facility (AGRF) in Parkville, VIC, Australia. Library preparation
648 was performed using Illumina Stranded mRNA protocol, and sequencing was done in the
649 Illumina Novaseq platform on a SP flowcell. 100 bp long reads were single-end sequenced at
650 a depth of 20M to 49M. Adapters were trimmed by the Casava software used by the Illumina
651 platform.
652
653 **mRNA Sequencing data analysis**
654
655 The Galaxy Australia (v1.0) platform was used for quality control, read alignment and
656 generation of count matrix. Read quality control was done with FastQC (v0.72). Alignment
657 was done with HISAT2 (v2.1.0) (92) using mm10 as the reference. Gene count matrix was
658 generated with HTSeq-count (v0.9.1) (93) with the comprehensive gene annotation of the
659 gencode M25 release (GRCm38.p6) as reference. Lowly expressed genes were filtered out
660 using the default filtering conditions from the edgeR package (v3.34.1) (94,95). Differential
661 analysis expression was done using edgeR, and the volcano plot was made with ggplot2
662 (v3.3.5) (96). Gene Set Enrichment Analysis was performed using GSEA (v4.2.3) (97,98), with
663 the c2.cp.v7.5.1 gene set database.
664
665 **Sperm collection**
666
667 For sperm collection, mice were culled by cervical dislocation for immediate dissection of both
668 epididymides. The caudal epididymis was bisected with a clean surgical blade, then immersed
669 into 1.0 mL mt-PBS that was pre-warmed to 37°C and incubated at that temperature for at least
670 30 mins. Sperm counts were determined, and samples were examined for the absence of tissue
671 debris under a light microscope. Samples were then centrifuged at 400g for 15 mins and excess

672 mt-PBS was carefully removed leaving approximately 100 µL as the final volume. Samples
673 were immediately frozen down and stored at -80°C until subsequent use.
674
675 **Long-read sperm DNA sequencing**
676
677 High molecular-weight sperm DNA collected from F_0 males was obtained using MagAttract
678 HMW DNA Kit (QIAGEN, Cat # 67563), following a modified version of the manufacturer's
679 instructions which consisted of replacing the digestion step with Proteinase K with 40 µL of
680 the reducing agent TCEP (Tris(2-carboxyethyl)phosphine hydrochloride, Sigma-Aldrich, St.
681 Louis, Missouri, United States). 5 µg of sperm DNA from 4 CT and 4 Cort F_0 mice were
682 fragmented by centrifugation for 60 s at 7200 rpm using g-TUBES (Covaris, Woburn, MA,
683 USA) to obtain 8 kb-long fragments. These fragments were prepared for sequencing with the
684 Ligation Sequencing Kit (Cat # SQK-LSK 109) and sequenced on the PromethION and
685 GridION platforms (Oxford Nanopore Technologies, Oxford, UK) with a mean genome
686 coverage of ~20X, to ensure methylation calling power.
687
688 **Sperm DNA methylation analysis**
689

690 Samples were base called using guppy (v4.2.2) and megalodon (v2.2.9) (Oxford Nanopore
691 Technologies Ltd.) against the configuration file
692 "res_dna_r941_prom_modbases_5mC_v001.cfg". NanoStat (99) was used for data inspection
693 and quality control. Fastq files obtained from megalodon were aligned to the mm10 genome
694 using minimap2 (v2.17-r941) (100), and sam files were sorted and transformed into bam files
695 using samtools (v1.10) (101). The tool f5c (v0.5) (102) was used to call CpG methylation per
696 read, as well as to calculate frequencies of methylation per CpG. A methylation matrix

corresponding to the location of the *Mup* gene cluster and its flanking genes Slc46a2 and Zfp37 was generated using the coordinates Chr4: 59,904,830-62,212,385, totalling 11,026 CpGs. Differentially methylated regions (DMRs) between CT and Cort were determined with the program DSS (Dispersion shrinkage for sequencing data) (v2.43.2) (103), as previously described for Oxford Nanopore (104,105). Specific methylation at the *Mup20* promoters was determined using genomic coordinates (Table 5) obtained from the UCSC genome browser promoter track (106). Methylation plots were generated using Nanomethviz (107), and density plots, dotplots and boxplots were generated using ggplot2 (v3.3.5) (96). Statistical analysis of methylation frequencies and data visualisation were performed using RStudio (v4.0.5). The code used for this analysis can be found in the GitHub page (https://github.com/Coracollar/mup_methylation).

Statistical analysis

Data was tested for normality with D'Agostino-Pearson tests. If data distribution was normal, it was tested with unpaired t-tests and, if variance differed between the groups, t-tests with Welch Correction were used. If data was not normally distributed, Mann-Whitney tests were used. The mate-choice test was analysed by quantifying the total time spent interacting with each male, which was analysed with the Wilcoxon Matched-Pairs Signed-Ranks test, in accordance with Mitra and Sapolsky, 2012. Additionally, the data was also analysed with the chi-square test for relative preference for each male. The social-dominance tube test was analysed using a Chi-square to assess difference from an expected chance of 50:50, in accordance with Zhou et al., 2016. Comparisons of variance for MUP protein and gene expression data were performed with F test. Statistical analysis was performed using GraphPad Prism 9 for MacOS (v9.3.1). Statistical significance was reached when $p < 0.05$. Graphs are

722 represented as mean ± standard error of the mean (SEM) for normally distributed data, or
723 median ± interquartile range for non-normally distributed data.
724
725 **DECLARATIONS**
726
727 **Ethics approval and consent to participate**
728
729 All procedures were approved by the Florey Institute of Neuroscience and Mental Health
730 Animal Ethics Committees (AEC) #19-064, complying with the Australian Code for the
731 Responsible Conduct of Research and the Australian Code for the Care and Use of Animals
732 for Scientific Purposes.
733
734 **Consent for publication**
735
736 Not applicable.
737
738 **Availability of data and materials**
739
740 The sequencing datasets generated and/or analysed during the current study have been
741 deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number
742 PRJEB60786 (https://www.ebi.ac.uk/ena/browser/view/PRJEB60786) and PRJEB60812
743 (https://www.ebi.ac.uk/ena/browser/view/PRJEB60812).
744
745 **Competing interests**
746

M.B.C has received support from Oxford Nanopore Technologies (ONT) to present their findings at scientific conferences. However, ONT played no role in study design, execution, analysis or publication. The other authors declare that they have no competing interests.

Funding

L.B.H. and C.C.F. are supported by the Melbourne Research Scholarship. A.J.H. and T.Y.P are funded by NHMRC project grants and a DHB Foundation (Equity Trustees) Grant to A.J.H, and an NHMRC Investigator grant [APP11968410] to M.B.C. A.J.H. is an NHMRC Principal Research Fellow whose laboratory is also supported by an NHMRC Ideas Grant and an ARC Discovery Project Grant. M.B.C. and T.Y.P. were co-recipients of a University of Melbourne Midcareer seeding grant.

Authors' contributions

L.B.H. planned and conducted the experiments, performed the data analysis and wrote the manuscript. E.A.M. assisted in the animal studies. R.V.H performed the sperm collection and DNA extractions. C.C.F. performed the sperm long-read DNA sequencing study. M.B.C. planned and supervised the sperm long-read sequencing study. T.Y.P. conceived the study, designed the study, supervised data collection and analysis. A.J.H. and T.Y.P. reviewed and edited the manuscript.

Acknowledgements

We thank Brett Purcell for his assistance with behavioural data collection, Craig Thomson and Helen Huckle for providing beddings for the Social Conditioned-Place Preference, Shanshan Li for assistance with MUP protein analysis, AGRF for sequencing the offspring mRNA, the LIEF HPC-GPGPU Facility for long-read sequencing the sperm samples, and Galaxy Australia and Spartan HPC at the University of Melbourne for providing the platform for bioinformatic analyses. This research was supported by The University of Melbourne's Research Computing Services and the Petascale Campus Initiative. This research was undertaken using the LIEF HPC-GPGPU Facility hosted at the University of Melbourne. This Facility was established with the assistance of LIEF Grant LE170100200.

REFERENCES

1. Gapp K, Soldado-Magraner S, Alvarez-Sánchez M, Bohacek J, Vernaz G, Shu H, et al. Early life stress in fathers improves behavioural flexibility in their offspring. Nat Commun [Internet]. 2014 Dec 18;5(1):5466. Available from: http://www.nature.com/articles/ncomms6466
2. Chan JC, Morgan CP, Adrian Leu N, Shetty A, Cisse YM, Nugent BM, et al. Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. Nat Commun [Internet]. 2020 Dec 20;11(1):1499. Available from: http://www.nature.com/articles/s41467-020-15305-w
3. Crews D, Gore AC, Hsu TS, Dangleben NL, Spinetta M, Schallert T, et al. Transgenerational epigenetic imprints on mate preference. Proc Natl Acad Sci [Internet]. 2007 Apr 3;104(14):5942–6. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.0610410104
4. Zhang Y, Zhang X, Shi J, Tuorto F, Li X, Liu Y, et al. Dnmt2 mediates intergenerational

796 transmission of paternally acquired metabolic disorders through sperm small non-coding
797 RNAs. Nat Cell Biol [Internet]. 2018 May 25;20(5):535–40. Available from:
798 http://dx.doi.org/10.1038/s41556-018-0087-2
799 5. Hoffmann LB, Rae M, Marianno P, Pang TY, Hannan AJ, Camarini R. Preconceptual
800 paternal environmental stimulation alters behavioural phenotypes and adaptive
801 responses intergenerationally in Swiss mice. Physiol Behav [Internet]. 2020 Sep 26
802 [cited 2020 Jun 4];223(April):112968. Available from:
803 https://linkinghub.elsevier.com/retrieve/pii/S0031938420302821
804 6. van Steenwyk G, Roszkowski M, Manuella F, Franklin TB, Mansuy IM.
805 Transgenerational inheritance of behavioral and metabolic effects of paternal exposure
806 to traumatic stress in early postnatal life: evidence in the 4th generation. Environ
807 Epigenetics. 2018;4(2):1–8.
808 7. Gapp K, Jawaid A, Sarkies P, Bohacek J, Pelczar P, Prados J, et al. Implication of sperm
809 RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat
810 Neurosci [Internet]. 2014 May 13;17(5):667–9. Available from:
811 http://www.nature.com/articles/nn.3695
812 8. Rodgers AB, Morgan CP, Leu NA, Bale TL. Transgenerational epigenetic programming
813 via sperm microRNA recapitulates effects of paternal stress. Proc Natl Acad Sci
814 [Internet]. 2015 Nov 3;112(44):13699–704. Available from:
815 http://www.pnas.org/lookup/doi/10.1073/pnas.1508347112
816 9. Gapp K, van Steenwyk G, Germain PL, Matsushima W, Rudolph KLMM, Manuella F,
817 et al. Alterations in sperm long RNA contribute to the epigenetic inheritance of the
818 effects of postnatal trauma. Mol Psychiatry [Internet]. 2020 Sep 30;25(9):2162–74.
819 Available from: http://dx.doi.org/10.1038/s41380-018-0271-6
820 10. Yeshurun S, Hannan AJ. Transgenerational epigenetic influences of paternal

821 environmental exposures on brain function and predisposition to psychiatric disorders.
822 Mol Psychiatry [Internet]. 2019 Apr 8;24(4):536–48. Available from:
823 http://dx.doi.org/10.1038/s41380-018-0039-z
824 11. Richards EJ. Inherited epigenetic variation - Revisiting soft inheritance. Nat Rev Genet.
825 2006;7(5):395–401.
826 12. Badyaev A V. Epigenetic resolution of the 'curse of complexity' in adaptive evolution
827 of complex traits. J Physiol [Internet]. 2014 Jun 1;592(11):2251–60. Available from:
828 http://doi.wiley.com/10.1113/jphysiol.2014.272625
829 13. Short AK, Fennell KA, Perreau VM, Fox A, O'bryan MK, Kim JH, et al. Elevated
830 paternal glucocorticoid exposure alters the small noncoding RNA profile in sperm and
831 modifies anxiety and depressive phenotypes in the offspring. Transl Psychiatry
832 [Internet]. 2016 Jun 14;6(6):e837–e837. Available from:
833 http://www.nature.com/articles/tp2016109
834 14. Rawat A, Guo J, Renoir T, Pang TY, Hannan AJ. Hypersensitivity to sertraline in the
835 absence of hippocampal 5-HT1AR and 5-HTT gene expression changes following
836 paternal corticosterone treatment. Environ Epigenetics [Internet]. 2018 Apr;4(2):1–10.
837 Available from:
838 https://academic.oup.com/eep/article/doi/10.1093/eep/dvy015/5056150
839 15. Franklin TB, Linder N, Russig H, Thöny B, Mansuy IM. Influence of Early Stress on
840 Social Abilities and Serotonergic Functions across Generations in Mice. Combs C,
841 editor. PLoS One [Internet]. 2011 Jul 25;6(7):e21842. Available from:
842 http://dx.plos.org/10.1371/journal.pone.0021842
843 16. Skinner MK. Environmental epigenetics and a unified theory of the molecular aspects
844 of evolution: A neo-Lamarckian concept that facilitates neo-Darwinian evolution.
845 Genome Biol Evol. 2015;7(5):1296–302.

846 17. Klironomos FD, Berg J, Collins S. How epigenetic mutations can affect genetic
847 evolution: Model and mechanism. BioEssays. 2013;35(6):571–8.
848 18. Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, McLean L, et
849 al. Darcin: A male pheromone that stimulates female memory and sexual attraction to
850 an individual male's odour. BMC Biol. 2010;8.
851 19. Lee W, Khan A, Curley JP. Major urinary protein levels are associated with social status
852 and context in mouse social hierarchies. Proc R Soc B Biol Sci [Internet]. 2017 Sep
853 27;284(1863):20171570. Available from:
854 https://royalsocietypublishing.org/doi/10.1098/rspb.2017.1570
855 20. Nelson AC, Cauceglia JW, Merkley SD, Youngson NA, Oler AJ, Nelson RJ, et al.
856 Reintroducing domesticated wild mice to sociality induces adaptive transgenerational
857 effects on MUP expression. Proc Natl Acad Sci [Internet]. 2013 Dec 3;110(49):19848–
858 53. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1310427110
859 21. Norstedt G, Palmiter R. Secretory rhythm of growth hormone regulates sexual
860 differentiation of mouse liver. Cell. 1984 Apr;36(4):805–12.
861 22. Jiang J, Creasy KT, Purnell J, Peterson ML, Spear BT. Zhx2 (zinc fingers and
862 homeoboxes 2) regulates major urinary protein gene expression in the mouse liver. J
863 Biol Chem [Internet]. 2017 Apr 21;292(16):6765–74. Available from:
864 http://www.jbc.org/lookup/doi/10.1074/jbc.M116.768275
865 23. Howlett SK, Reik W. Methylation levels of maternal and paternal genomes during
866 preimplantation development. Development [Internet]. 1991 Sep 1;113(1):119–27.
867 Available from:
868 https://journals.biologists.com/dev/article/113/1/119/37172/Methylation-levels-of-
869 maternal-and-paternal
870 24. Reik W, Romer I, Barton SC, Surani MA, Howlett SK, Klose J. Adult phenotype in the

mouse can be affected by epigenetic in the early embryo. Development. 1993;

25. Roemer I, Reik W, Dean W, Klose J. Epigenetic inheritance in the mouse. Curr Biol [Internet]. 1997 Apr;7(4):277–80. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0960982206001242

26. Tirado-Magallanes R, Rebbani K, Lim R, Pradhan S, Benoukraf T. Whole genome DNA methylation: beyond genes silencing. Oncotarget [Internet]. 2017 Jan 17;8(3):5629–37. Available from: http://link.springer.com/10.1007/s00432-017-2467-6

27. Zhou T, Zhu H, Fan Z, Wang F, Chen Y, Liang H, et al. History of winning remodels thalamo-PFC circuit to reinforce social dominance. Science (80-) [Internet]. 2017 Jul 14;357(6347):162–8. Available from: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L617413491%0Ahttp://dx.doi.org/10.1126/science.aak9726

28. Calhoon GG, Tye KM. Resolving the neural circuits of anxiety. Nat Neurosci [Internet]. 2015 Oct 25;18(10):1394–404. Available from: http://www.nature.com/articles/nn.4101

29. Chen P, Hong W. Neural Circuit Mechanisms of Social Behavior. Neuron [Internet]. 2018 Apr 4;98(1):16–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29621486

30. Xu S, Jiang M, Liu X, Sun Y, Yang L, Yang Q, et al. Neural Circuits for Social Interactions: From Microcircuits to Input-Output Circuits. Front Neural Circuits [Internet]. 2021 Oct 29;15:768294. Available from: http://www.ncbi.nlm.nih.gov/pubmed/34776877

31. Sapolsky RM. The Influence of Social Hierarchy on Primate Health. Science (80-) [Internet]. 2005 Apr 29;308(5722):648–52. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1106477

896 32. Yohn CN, Ashamalla SA, Bokka L, Gergues MM, Garino A, Samuels BA. Social
897 instability is an effective chronic stress paradigm for both male and female mice.
898 Neuropharmacology [Internet]. 2019 Dec;160(May):107780. Available from:
899 https://doi.org/10.1016/j.neuropharm.2019.107780
900 33. Padilla-Coreano N, Batra K, Patarino M, Chen Z, Rock RR, Zhang R, et al. Cortical
901 ensembles orchestrate social competition through hypothalamic outputs. Nature
902 [Internet]. 2022 Mar 24;603(7902):667–71. Available from:
903 https://www.nature.com/articles/s41586-022-04507-5
904 34. Benner S, Endo T, Endo N, Kakeyama M, Tohyama C. Early deprivation induces
905 competitive subordinance in C57BL/6 male mice. Physiol Behav [Internet]. 2014
906 Oct;137:42–52. Available from: http://dx.doi.org/10.1016/j.physbeh.2014.06.018
907 35. Park M-J, Seo BA, Lee B, Shin H-S, Kang M-G. Stress-induced changes in social
908 dominance are scaled by AMPA-type glutamate receptor phosphorylation in the medial
909 prefrontal cortex. Sci Rep [Internet]. 2018 Dec 9;8(1):15008. Available from:
910 http://www.nature.com/articles/s41598-018-33410-1
911 36. Lucion A, Vogel WH. Effects of stress on defensive aggression and dominance in a
912 water competition test. Integr Physiol Behav Sci [Internet]. 1994 Oct;29(4):415–22.
913 Available from: http://link.springer.com/10.1007/BF02691361
914 37. Tada H, Miyazaki T, Takemoto K, Takase K, Jitsuki S, Nakajima W, et al. Neonatal
915 isolation augments social dominance by altering actin dynamics in the medial prefrontal
916 cortex. Proc Natl Acad Sci [Internet]. 2016 Nov 8;113(45):E7097–105. Available from:
917 https://pnas.org/doi/full/10.1073/pnas.1606351113
918 38. Panksepp JB, Lahvis GP. Social reward among juvenile mice. Genes, Brain Behav.
919 2007;6(7):661–71.
920 39. Yamamuro K, Bicks LK, Leventhal MB, Kato D, Im S, Flanigan ME, et al. A prefrontal–

921 paraventricular thalamus circuit requires juvenile social experience to regulate adult
922 sociability in mice. Nat Neurosci [Internet]. 2020 Oct 31;23(10):1240–52. Available
923 from: http://dx.doi.org/10.1038/s41593-020-0695-6
924 40. Daniels S, Lemaire D, Lapointe T, Limebeer C, Parker L, Leri F. Effects of inescapable
925 stress on responses to social incentive stimuli and modulation by escitalopram.
926 Psychopharmacology (Berl) [Internet]. 2021 Nov 30;238(11):3239–47. Available from:
927 https://doi.org/10.1007/s00213-021-05940-6
928 41. Meyer MAA, Anstötz M, Ren LY, Fiske MP, Guedea AL, Grayson VS, et al. Stress-
929 related memories disrupt sociability and associated patterning of hippocampal activity:
930 a role of hilar oxytocin receptor-positive interneurons. Transl Psychiatry [Internet]. 2020
931 Dec 12;10(1):428. Available from: https://www.nature.com/articles/s41398-020-
932 01091-y
933 42. Tan T, Wang W, Liu T, Zhong P, Conrow-Graham M, Tian X, et al. Neural circuits and
934 activity dynamics underlying sex-specific effects of chronic social isolation stress. Cell
935 Rep [Internet]. 2021 Mar;34(12):108874. Available from:
936 https://linkinghub.elsevier.com/retrieve/pii/S2211124721001881
937 43. Zhang G-W, Shen L, Tao C, Jung A-H, Peng B, Li Z, et al. Medial preoptic area
938 antagonistically mediates stress-induced anxiety and parental behavior. Nat Neurosci
939 [Internet]. 2021 Apr 1;24(4):516–28. Available from: http://dx.doi.org/10.1038/s41593-
940 020-00784-3
941 44. Allsop SA, Vander Weele CM, Wichmann R, Tye KM. Optogenetic insights on the
942 relationship between anxiety-related behaviors and social deficits. Front Behav
943 Neurosci [Internet]. 2014 Jul 16;8(JULY):1–14. Available from:
944 http://journal.frontiersin.org/article/10.3389/fnbeh.2014.00241/abstract
945 45. Pallé A, Zorzo C, Luskey VE, Mcgreevy KR, Fernández S, Trejo JL. Social dominance

946 differentially alters gene expression in the medial prefrontal cortex without affecting
947 adult hippocampal neurogenesis or stress and anxiety-like behavior. FASEB J [Internet].
948 2019 Jun 11;33(6):6995–7008. Available from:
949 https://onlinelibrary.wiley.com/doi/abs/10.1096/fj.201801600R
950 46. Asaba A, Hattori T, Mogi K, Kikusui T. Sexual attractiveness of male chemicals and
951 vocalizations in mice. Front Neurosci [Internet]. 2014 Aug 5;8. Available from:
952 http://journal.frontiersin.org/article/10.3389/fnins.2014.00231/abstract
953 47. Egnor SR, Seagraves KM. The contribution of ultrasonic vocalizations to mouse
954 courtship. Curr Opin Neurobiol [Internet]. 2016 Jun;38(Figure 1):1–5. Available from:
955 http://dx.doi.org/10.1016/j.conb.2015.12.009
956 48. Yang M, Loureiro D, Kalikhman D, Crawley JN. Male mice emit distinct ultrasonic
957 vocalizations when the female leaves the social interaction arena. Front Behav Neurosci
958 [Internet]. 2013;7(NOV):1–13. Available from:
959 http://journal.frontiersin.org/article/10.3389/fnbeh.2013.00159/abstract
960 49. Beynon RJ, Hurst JL. Multiple roles of major urinary proteins in the house mouse, Mus
961 domesticus. Biochem Soc Trans [Internet]. 2003 Feb 1;31(1):142–6. Available from:
962 https://portlandpress.com/biochemsoctrans/article/31/1/142/63238/Multiple-roles-of-
963 major-urinary-proteins-in-the
964 50. Chabout J, Serreau P, Ey E, Bellier L, Aubin T, Bourgeron T, et al. Adult Male Mice
965 Emit Context-Specific Ultrasonic Vocalizations That Are Modulated by Prior Isolation
966 or Group Rearing Environment. Casadesus G, editor. PLoS One [Internet]. 2012 Jan
967 6;7(1):e29401. Available from: https://dx.plos.org/10.1371/journal.pone.0029401
968 51. Wang F, Zhu J, Zhu H, Zhang Q, Lin Z, Hu H. Bidirectional Control of Social Hierarchy
969 by Synaptic Efficacy in Medial Prefrontal Cortex. Science (80-) [Internet]. 2011 Nov
970 4;334(6056):693–7. Available from:

971 https://www.sciencemag.org/lookup/doi/10.1126/science.1209951
972 52. Kumar V, Vasudevan A, Soh LJT, Le Min C, Vyas A, Zewail-Foote M, et al. Sexual
973 Attractiveness in Male Rats Is Associated with Greater Concentration of Major Urinary
974 Proteins. Biol Reprod [Internet]. 2014 Dec 1;91(6):1–7. Available from:
975 https://academic.oup.com/biolreprod/article-
976 lookup/doi/10.1095/biolreprod.114.117903
977 53. Armstrong SD, Robertson DHL, Cheetham SA, Hurst JL, Beynon RJ. Structural and
978 functional differences in isoforms of mouse major urinary proteins: a male-specific
979 protein that preferentially binds a male pheromone. Biochem J [Internet]. 2005 Oct
980 15;391(2):343–50. Available from:
981 https://portlandpress.com/biochemj/article/391/2/343/41717/Structural-and-functional-
982 differences-in-isoforms
983 54. Thoß M, Luzynski KC, Enk VM, Razzazi-Fazeli E, Kwak J, Ortner I, et al. Regulation
984 of volatile and non-volatile pheromone attractants depends upon male social status. Sci
985 Rep [Internet]. 2019 Dec 24;9(1):489. Available from:
986 http://www.nature.com/articles/s41598-018-36887-y
987 55. Stopka P, Janotova K, Heyrovsky D. The advertisement role of major urinary proteins
988 in mice. Physiol Behav [Internet]. 2007 Aug;91(5):667–70. Available from:
989 https://linkinghub.elsevier.com/retrieve/pii/S0031938407001230
990 56. Cho Y-H, Kim D, Choi I, Bae K. Identification of transcriptional regulatory elements
991 required for the Mup2 expression in circadian clock mutant mice. Biochem Biophys Res
992 Commun [Internet]. 2011 Jul;410(4):834–40. Available from:
993 http://dx.doi.org/10.1016/j.bbrc.2011.06.074
994 57. Janotova K, Stopka P. The Level of Major Urinary Proteins is Socially Regulated in
995 Wild Mus musculus musculus. J Chem Ecol [Internet]. 2011 Jun 19;37(6):647–56.

996 Available from: http://link.springer.com/10.1007/s10886-011-9966-8
997 58. Sheehan MJ, Lee V, Corbett-Detig R, Bi K, Beynon RJ, Hurst JL, et al. Selection on
998 Coding and Regulatory Variation Maintains Individuality in Major Urinary Protein
999 Scent Marks in Wild Mice. Barsh GS, editor. PLOS Genet [Internet]. 2016 Mar
1000 3;12(3):e1005891. Available from: https://dx.plos.org/10.1371/journal.pgen.1005891
1001 59. Lehman-McKeeman LD, Caudill D. Biochemical basis for mouse resistance to hyaline
1002 droplet nephropathy: Lack of relevance of the α2u-globulin protein superfamily in this
1003 male rat-specific syndrome. Toxicol Appl Pharmacol [Internet]. 1992 Feb;112(2):214–
1004 21. Available from: https://linkinghub.elsevier.com/retrieve/pii/0041008X92901904
1005 60. Logan DW, Marton TF, Stowers L. Species Specificity in Major Urinary Proteins by
1006 Parallel Evolution. Vosshall LB, editor. PLoS One [Internet]. 2008 Sep 25;3(9):e3280.
1007 Available from: https://dx.plos.org/10.1371/journal.pone.0003280
1008 61. Cantone I, Fisher AG. Epigenetic programming and reprogramming during
1009 development. Nat Struct Mol Biol [Internet]. 2013 Mar 5;20(3):282–9. Available from:
1010 http://dx.doi.org/10.1038/nsmb.2489
1011 62. Bohacek J, Mansuy IM. Molecular insights into transgenerational non-genetic
1012 inheritance of acquired behaviours. Nat Rev Genet [Internet]. 2015;16(11):641–52.
1013 Available from: http://dx.doi.org/10.1038/nrg3964
1014 63. Petropoulos S, Matthews SG, Szyf M. Adult Glucocorticoid Exposure Leads to
1015 Transcriptional and DNA Methylation Changes in Nuclear Steroid Receptors in the
1016 Hippocampus and Kidney of Mouse Male Offspring1. Biol Reprod [Internet].
1017 2014;90(2):1–10. Available from: https://academic.oup.com/biolreprod/article-
1018 lookup/doi/10.1095/biolreprod.113.115899
1019 64. Evans JC, Liechti JI, Boatman B, König B. A natural catastrophic turnover event:
1020 individual sociality matters despite community resilience in wild house mice. Proc R

1021 Soc B Biol Sci [Internet]. 2020 May 13;287(1926):20192880. Available from:
1022 https://royalsocietypublishing.org/doi/10.1098/rspb.2019.2880
1023 65. Strauss ED, Holekamp KE. Social alliances improve rank and fitness in convention-
1024 based societies. Proc Natl Acad Sci [Internet]. 2019 Apr 30;116(18):8919–24. Available
1025 from: https://pnas.org/doi/full/10.1073/pnas.1810384116
1026 66. Sapolsky RM. Importance of a sense of control and the physiological benefits of
1027 leadership. Proc Natl Acad Sci [Internet]. 2012 Oct 30;109(44):17730–1. Available
1028 from: http://www.pnas.org/cgi/doi/10.1073/pnas.1215502109
1029 67. Zhou T, Sandi C, Hu H. Advances in understanding neural mechanisms of social
1030 dominance. Curr Opin Neurobiol [Internet]. 2018 Apr;49:99–107. Available from:
1031 https://doi.org/10.1016/j.conb.2018.01.006
1032 68. Thonhauser KE, Raffetzeder A, Penn DJ. Sexual experience has no effect on male
1033 mating or reproductive success in house mice. Sci Rep [Internet]. 2019 Dec 21;9(1):1–
1034 11. Available from: http://dx.doi.org/10.1038/s41598-019-48392-x
1035 69. Thonhauser KE, Raveh S, Hettyey A, Beissmann H, Penn DJ. Scent marking increases
1036 male reproductive success in wild house mice. Anim Behav [Internet]. 2013
1037 Nov;86(5):1013–21. Available from:
1038 https://linkinghub.elsevier.com/retrieve/pii/S0003347213003904
1039 70. Tuscher JJ, Day JJ. Multigenerational epigenetic inheritance: One step forward, two
1040 generations back. Neurobiol Dis [Internet]. 2019 Dec;132(July):104591. Available
1041 from: https://doi.org/10.1016/j.nbd.2019.104591
1042 71. Toker IA, Lev I, Mor Y, Gurevich Y, Fisher D, Houri-Zeevi L, et al. Transgenerational
1043 inheritance of sexual attractiveness via small RNAs enhances evolvability in C. elegans.
1044 Dev Cell [Internet]. 2022 Feb;57(3):298-309.e9. Available from:
1045 https://doi.org/10.1016/j.devcel.2022.01.005

1046 72. Schmidt M V. Animal models for depression and the mismatch hypothesis of disease.
1047 Psychoneuroendocrinology [Internet]. 2011;36(3):330–8. Available from:
1048 http://dx.doi.org/10.1016/j.psyneuen.2010.07.001
1049 73. De Miguel Z, Haditsch U, Palmer TD, Azpiroz A, Sapolsky RM. Adult-generated
1050 neurons born during chronic social stress are uniquely adapted to respond to subsequent
1051 chronic social stress. Mol Psychiatry [Internet]. 2019 Aug 8;24(8):1178–88. Available
1052 from: http://dx.doi.org/10.1038/s41380-017-0013-1
1053 74. Burton T, Metcalfe NB. Can environmental conditions experienced in early life
1054 influence future generations? Proc R Soc B Biol Sci [Internet]. 2014 Jun
1055 22;281(1785):20140311. Available from:
1056 https://royalsocietypublishing.org/doi/10.1098/rspb.2014.0311
1057 75. Nettle D, Bateson M. Adaptive developmental plasticity: what is it, how can we
1058 recognize it and when can it evolve? Proc R Soc B Biol Sci [Internet]. 2015 Aug
1059 7;282(1812):20151005. Available from:
1060 http://www.ncbi.nlm.nih.gov/pubmed/26203000
1061 76. Fang Q, Zhang YH, Shi YL, Zhang JHJX, Zhang JHJX. Individuality and
1062 Transgenerational Inheritance of Social Dominance and Sex Pheromones in Isogenic
1063 Male Mice. J Exp Zool Part B Mol Dev Evol. 2016;326(4):225–36.
1064 77. Charkoftaki G, Wang Y, McAndrews M, Bruford EA, Thompson DC, Vasiliou V, et al.
1065 Update on the human and mouse lipocalin (LCN) gene family, including evidence the
1066 mouse Mup cluster is result of an "evolutionary bloom." Hum Genomics.
1067 2019;13(1):11.
1068 78. Thoß M, Enk V, Yu H, Miller I, Luzynski KC, Balint B, et al. Diversity of major urinary
1069 proteins (MUPs) in wild house mice. Sci Rep [Internet]. 2016 Dec 6;6(1):38378.
1070 Available from: http://www.nature.com/articles/srep38378

1071 79. Sheehan MJ, Campbell P, Miller CH. Evolutionary patterns of major urinary protein
1072 scent signals in house mice and relatives. Mol Ecol [Internet]. 2019 Aug
1073 31;28(15):3587–601. Available from:
1074 https://onlinelibrary.wiley.com/doi/abs/10.1111/mec.15155
1075 80. Bar-Sadeh B, Rudnizky S, Pnueli L, Bentley GR, Stöger R, Kaplan A, et al. Unravelling
1076 the role of epigenetics in reproductive adaptations to early-life environment. Nat Rev
1077 Endocrinol [Internet]. 2020 Sep 15;16(9):519–33. Available from:
1078 http://dx.doi.org/10.1038/s41574-020-0370-8
1079 81. Dölen G, Darvishzadeh A, Huang KW, Malenka RC. Social reward requires coordinated
1080 activity of nucleus accumbens oxytocin and serotonin. Nature [Internet]. 2013 Sep
1081 11;501(7466):179–84. Available from: http://www.nature.com/articles/nature12518
1082 82. Nardou R, Lewis EM, Rothhaas R, Xu R, Yang A, Boyden E, et al. Oxytocin-dependent
1083 reopening of a social reward learning critical period with MDMA. Nature.
1084 2019;569(7754):116–20.
1085 83. Mitra R, Sapolsky RM. Short-Term Enrichment Makes Male Rats More Attractive,
1086 More Defensive and Alters Hypothalamic Neurons. Malmierca MS, editor. PLoS One
1087 [Internet]. 2012 May 2;7(5):e36092. Available from:
1088 https://dx.plos.org/10.1371/journal.pone.0036092
1089 84. Hurst JL. Female recognition and assessment of males through scent. Behav Brain Res
1090 [Internet]. 2009 Jun 25;200(2):295–303. Available from:
1091 https://linkinghub.elsevier.com/retrieve/pii/S016643280800716X
1092 85. NOMOTO K, IKUMI M, Otsuka M, Asaba A, KATO M, Koshida N, et al. Female mice
1093 exhibit both sexual and social partner preferences for vocalizing males. Integr Zool
1094 [Internet]. 2018 Nov;13(6):735–44. Available from:
1095 http://doi.wiley.com/10.1111/1749-4877.12357

1096 86. Zhou YY, Kaiser T, Monteiro P, Zhang X, Van der Goes MS, Wang D, et al. Mice with
1097 Shank3 Mutations Associated with ASD and Schizophrenia Display Both Shared and
1098 Distinct Defects. Neuron. 2016;89(1):147–62.
1099 87. Wang F, Kessels HW, Hu H. The mouse that roared: Neural mechanisms of social
1100 hierarchy. Trends Neurosci [Internet]. 2014;37(11):674–82. Available from:
1101 http://dx.doi.org/10.1016/j.tins.2014.07.005
1102 88. Drews C. The Concept and Definition of Dominance in Animal Behaviour. Behaviour
1103 [Internet]. 1993;125(3–4):283–313. Available from:
1104 https://brill.com/view/journals/beh/125/3-4/article-p283_9.xml
1105 89. Young C. Agonistic Behavior. In: Vonk J, Shackelford T, editors. Encyclopedia of
1106 Animal Cognition and Behavior [Internet]. Cham: Springer International Publishing;
1107 2019. p. 1–6. Available from: https://doi.org/10.1007/978-3-319-47829-6_320-1
1108 90. Beynon RJ, Veggerby C, Payne CE, Robertson DHL, Gaskell SJ, Humphries RE, et al.
1109 Polymorphism in major urinary proteins: molecular heterogeneity in a wild mouse
1110 population. J Chem Ecol. 2002 Jul;28(7):1429–46.
1111 91. Nelson AC, Cunningham CB, Ruff JS, Potts WK. Protein pheromone expression levels
1112 predict and respond to the formation of social dominance networks. J Evol Biol
1113 [Internet]. 2015 Jun;28(6):1213–24. Available from:
1114 http://doi.wiley.com/10.1111/jeb.12643
1115 92. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and
1116 genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol [Internet]. 2019 Aug
1117 2;37(8):907–15. Available from: http://dx.doi.org/10.1038/s41587-019-0201-4
1118 93. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput
1119 sequencing data. Bioinformatics [Internet]. 2015 Jan 15;31(2):166–9. Available from:
1120 https://academic.oup.com/bioinformatics/article-

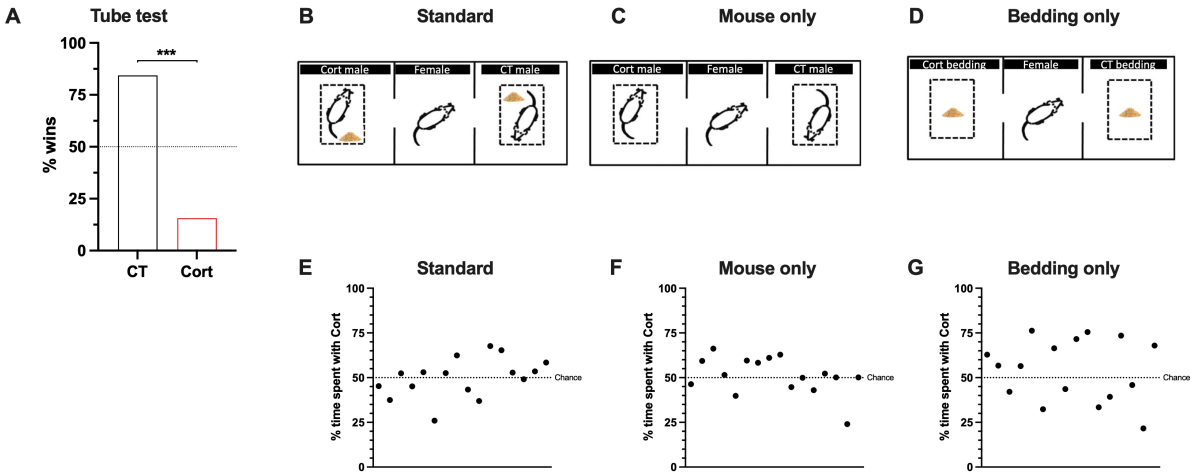
1121 lookup/doi/10.1093/bioinformatics/btu638
1122 94. Chen Y, Lun ATL, Smyth GK. From reads to genes to pathways: differential expression
1123 analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood
1124 pipeline. F1000Research [Internet]. 2016 Aug 2;5:1438. Available from:
1125 https://f1000research.com/articles/5-1438/v2
1126 95. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for
1127 differential expression analysis of digital gene expression data. Bioinformatics
1128 [Internet]. 2010 Jan 1;26(1):139–40. Available from:
1129 https://academic.oup.com/bioinformatics/article-
1130 lookup/doi/10.1093/bioinformatics/btp616
1131 96. Wickham H. ggplot2: Elegant Graphics for Data Analysis [Internet]. Springer-Verlag
1132 New York; 2016. Available from: https://ggplot2.tidyverse.org
1133 97. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al.
1134 Gene set enrichment analysis: A knowledge-based approach for interpreting genome-
1135 wide expression profiles. Proc Natl Acad Sci [Internet]. 2005 Oct 25;102(43):15545–
1136 50. Available from: https://pnas.org/doi/full/10.1073/pnas.0506580102
1137 98. Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J, et al. PGC-
1138 1α-responsive genes involved in oxidative phosphorylation are coordinately
1139 downregulated in human diabetes. Nat Genet [Internet]. 2003 Jul 15;34(3):267–73.
1140 Available from: http://www.nature.com/articles/ng1180
1141 99. De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. NanoPack:
1142 visualizing and processing long-read sequencing data. Berger B, editor. Bioinformatics
1143 [Internet]. 2018 Aug 1;34(15):2666–9. Available from:
1144 https://academic.oup.com/bioinformatics/article/34/15/2666/4934939
1145 100. Li H. Minimap2: pairwise alignment for nucleotide sequences. Birol I, editor.

1146 Bioinformatics [Internet]. 2018 Sep 15;34(18):3094–100. Available from:
1147 https://academic.oup.com/bioinformatics/article/34/18/3094/4994778
1148 101. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence
1149 Alignment/Map format and SAMtools. Bioinformatics [Internet]. 2009 Aug
1150 15;25(16):2078–9. Available from: https://academic.oup.com/bioinformatics/article-
1151 lookup/doi/10.1093/bioinformatics/btp352
1152 102. Gamaarachchi H, Lam CW, Jayatilaka G, Samarakoon H, Simpson JT, Smith MA, et al.
1153 GPU accelerated adaptive banded event alignment for rapid comparative nanopore
1154 signal analysis. BMC Bioinformatics [Internet]. 2020 Dec 5;21(1):343. Available from:
1155 https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-020-03697-x
1156 103. Park Y, Wu H. Differential methylation analysis for BS-seq data under general
1157 experimental design. Bioinformatics [Internet]. 2016 May 15;32(10):1446–53.
1158 Available from: https://academic.oup.com/bioinformatics/article-
1159 lookup/doi/10.1093/bioinformatics/btw026
1160 104. Gigante S, Gouil Q, Lucattini A, Keniry A, Beck T, Tinning M, et al. Using long-read
1161 sequencing to detect imprinted DNA methylation. Nucleic Acids Res [Internet]. 2019
1162 May 7;47(8):e46–e46. Available from:
1163 https://academic.oup.com/nar/article/47/8/e46/5356940
1164 105. Goldsmith C, Rodríguez-Aguilera JR, El-Rifai I, Jarretier-Yuste A, Hervieu V,
1165 Raineteau O, et al. Low biological fluctuation of mitochondrial CpG and non-CpG
1166 methylation at the single-molecule level. Sci Rep [Internet]. 2021 Dec 13;11(1):8032.
1167 Available from: https://doi.org/10.1038/s41598-021-87457-8
1168 106. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The Human
1169 Genome Browser at UCSC. Genome Res [Internet]. 2002 Jun 1;12(6):996–1006.
1170 Available from: http://genome.cshlp.org/lookup/doi/10.1101/gr.229102

1171 107. Su S, Gouil Q, Blewitt ME, Cook D, Hickey PF, Ritchie ME. NanoMethViz: An
1172 R/Bioconductor package for visualizing long-read methylation data. Schneidman-
1173 Duhovny D, editor. PLOS Comput Biol [Internet]. 2021 Oct 25;17(10):e1009524.
1174 Available from: http://dx.doi.org/10.1371/journal.pcbi.1009524
1175 108. Toth I, Neumann ID. Animal models of social avoidance and social fear. Cell Tissue
1176 Res. 2013;354(1):107–18.
1177

1178 **FIGURES, TABLES AND ADDITIONAL FILES**

1179



1180

1181 **Figure 1. Assessing effects of Cort-treatment on social dominance and mate-choice**
1182 **attractiveness.** (A), corticosterone treatment reduces male dominance as assessed by the
1183 percentage of wins in the tube test. % wins calculated as the number of wins per group in the
1184 total number of interactions. Total of 64 unique interactions. (B – D), figures adapted from
1185 Toth and Neumann, 2013. In all of them, a female mouse is represented in the centre of a 2-
1186 chamber apparatus. In figure B, male mice from CT and Cort group are located at each end of
1187 the apparatus, alongside soiled bedding from their home cage. In figure C, male mice from CT
1188 and Cort group are presented without bedding. In figure D, only soiled bedding from CT and
1189 Cort cages is presented. (E – G), male attractiveness was not affected by corticosterone-
1190 treatment. Each point represents the results from one female mouse. A, E – G: one-sample chi-
1191 squared test. *** $p<0.001$.

1192

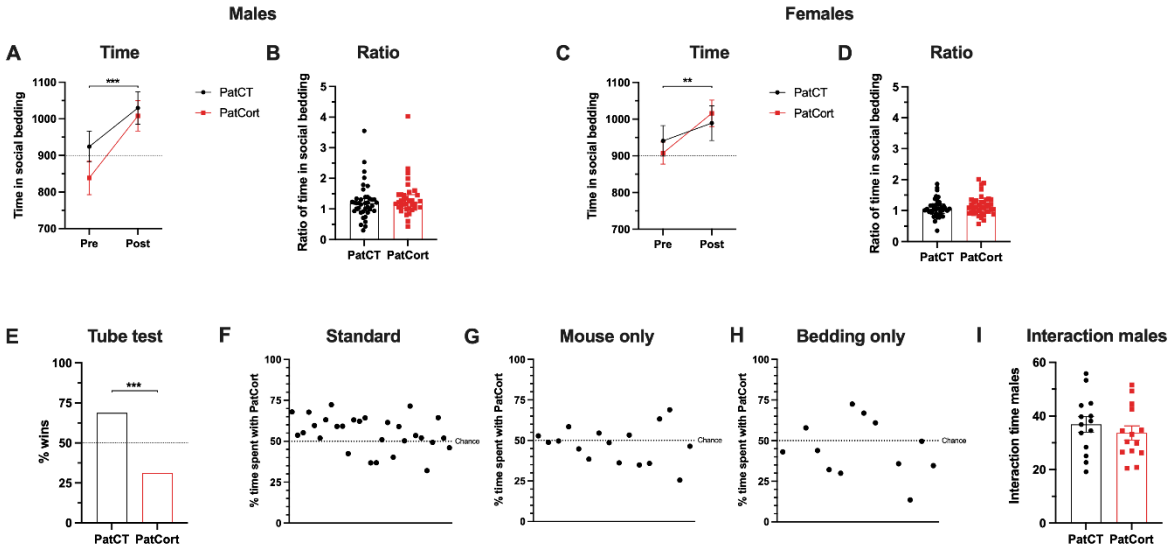


Figure 2. Assessing effects of paternal Cort-treatment on offspring social behaviour. (A – D), the degree of social reward in male or female offspring was not affected by paternal Cort-treatment. Time and ratio of the time spent in the social-conditioned bedding. (E), paternal Cort-treatment reduces male dominance in the tube test. % wins calculated as the number of wins per group in the total number of interactions. Total of 112 unique interactions. (F – H), paternal corticosterone treatment increases male attractiveness in the 'standard' set up only. Each point represents the results from one female mouse. (I), PatCort mice do not interact more with female mice, compared to PatCT. A and C: 2-way ANOVA, data represented as mean ± SEM. B and D: Mann-Whitney test, data represented as median ± interquartile range. E – H: one-sample chi-square test. I: unpaired t-test, data represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$.

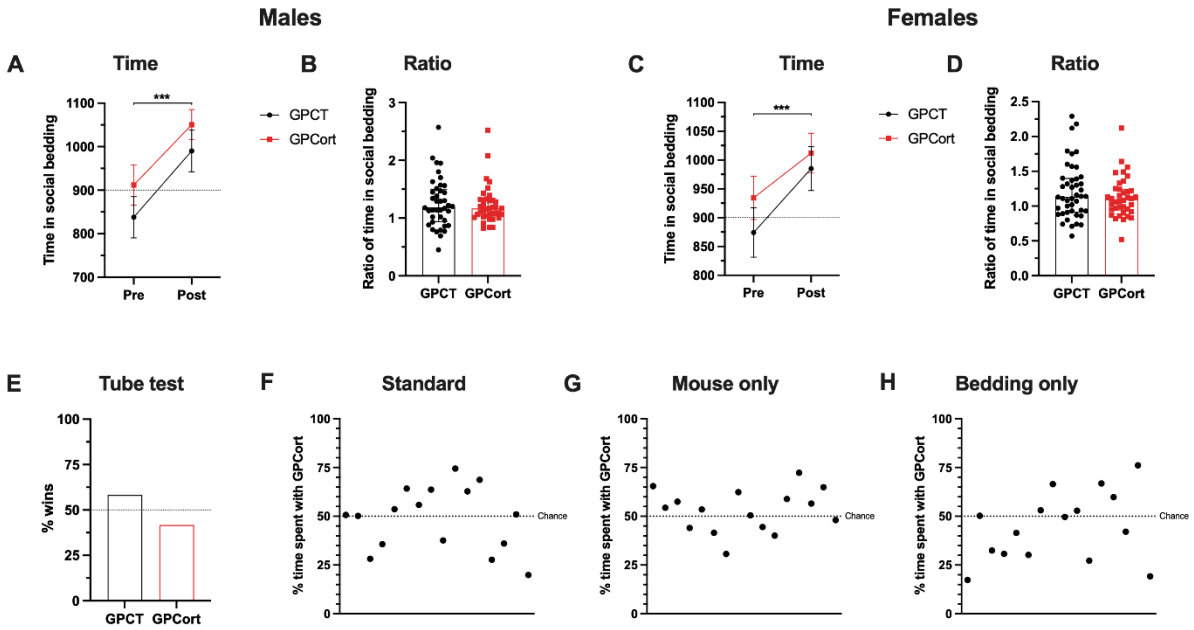
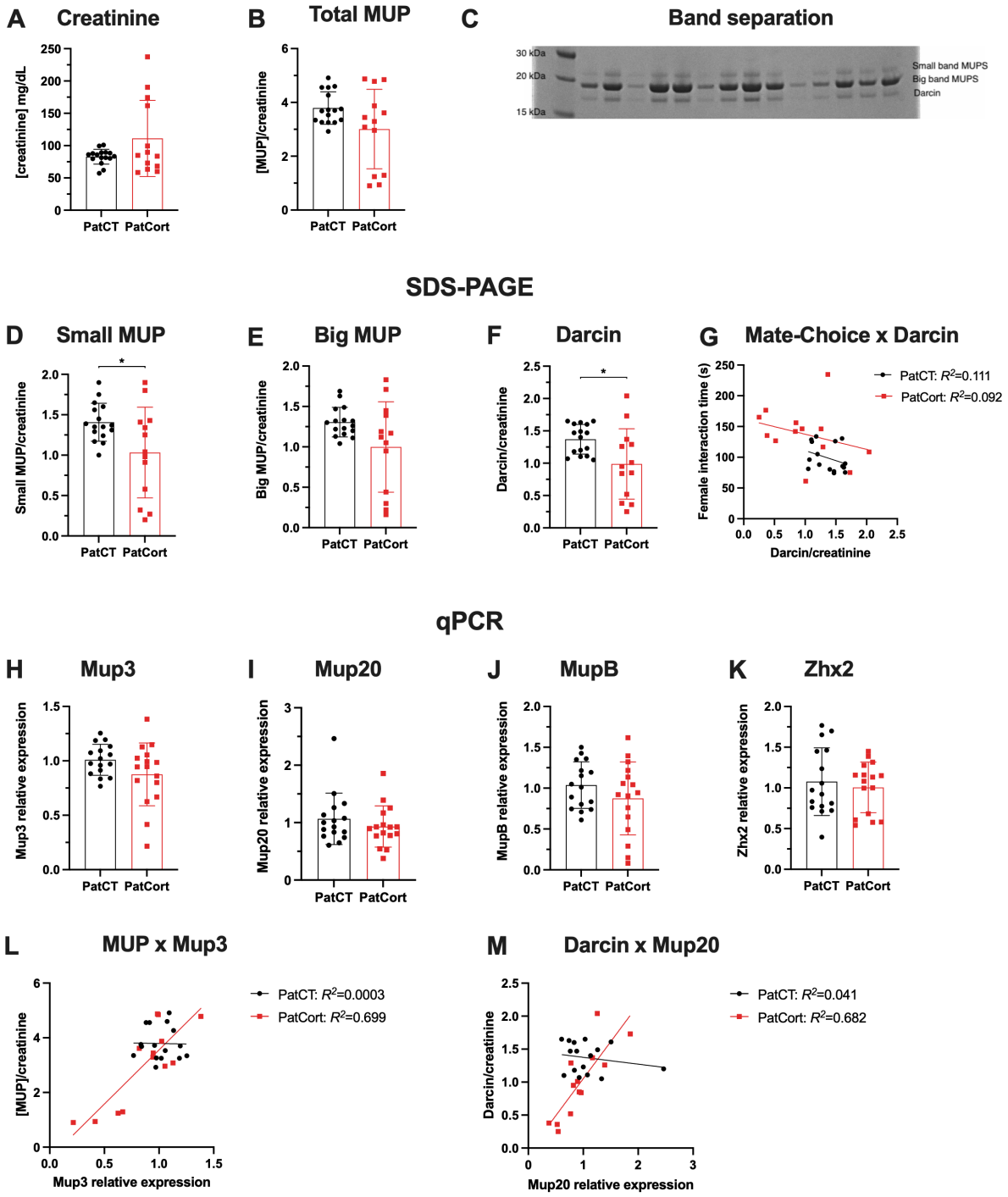


Figure 3. Assessing effects of grand-paternal Cort-treatment on grand-offspring social behaviour. (A – D), the degree of social reward in male or female grand-offspring was not affected by grand-paternal Cort-treatment. Time and ratio of the time spent in the social-conditioned bedding. (E), social dominance in male grand-offspring was not affected by grand-paternal Cort-treatment. % wins calculated as the number of wins per group in the total number of interactions. Total of 64 unique interactions. (F – H), male attractiveness in male grand-offspring was not affected by grand-paternal Cort-treatment. A and C: 2-way ANOVA, data represented as mean ± SEM. B and D: Mann-Whitney test, data represented as median ± interquartile range. E – H: one-sample chi-squared test. *** $p<0.001$.

1218



1219

1220 **Figure 4. Assessing effects of paternal Cort-treatment on offspring urinary MUP levels**

1221 **and liver gene expression.** (A – B), urinary creatinine or total MUP were not affected by

1222 paternal Cort-treatment. (C), after SDS-PAGE of mouse urine, three different MUP bands can

1223 be seen, whose molecular weight correspond to previously published literature. Heavier band

1224 = ‘Small MUP’, middle band = ‘Big MUP’, lighter band = ‘Darcin’. (D – F), paternal Cort-
1225 treatment induces reduced specific MUP populations in the urine. (G), time being investigated
1226 by the female does not correlate with urinary Darcin concentration. (H – K), *Mup* gene
1227 expression in the liver was not affected by paternal Cort-treatment. (L – M), *Mup* genes
1228 correlate with specific MUP populations in PatCort, but not in PatCT. A – B: unpaired t-test
1229 with Welch correction. D and E: unpaired t-test with Welch correction. F: Mann-Whitney test.
1230 G: correlation of Pearson and simple linear regression. H: unpaired t-test with Welch
1231 correction. I: Mann-Whitney test. J and K: unpaired t-test. L – M: correlation of Pearson and
1232 simple linear regression. Data represented as mean ± SD. * $p<0.05$.
1233

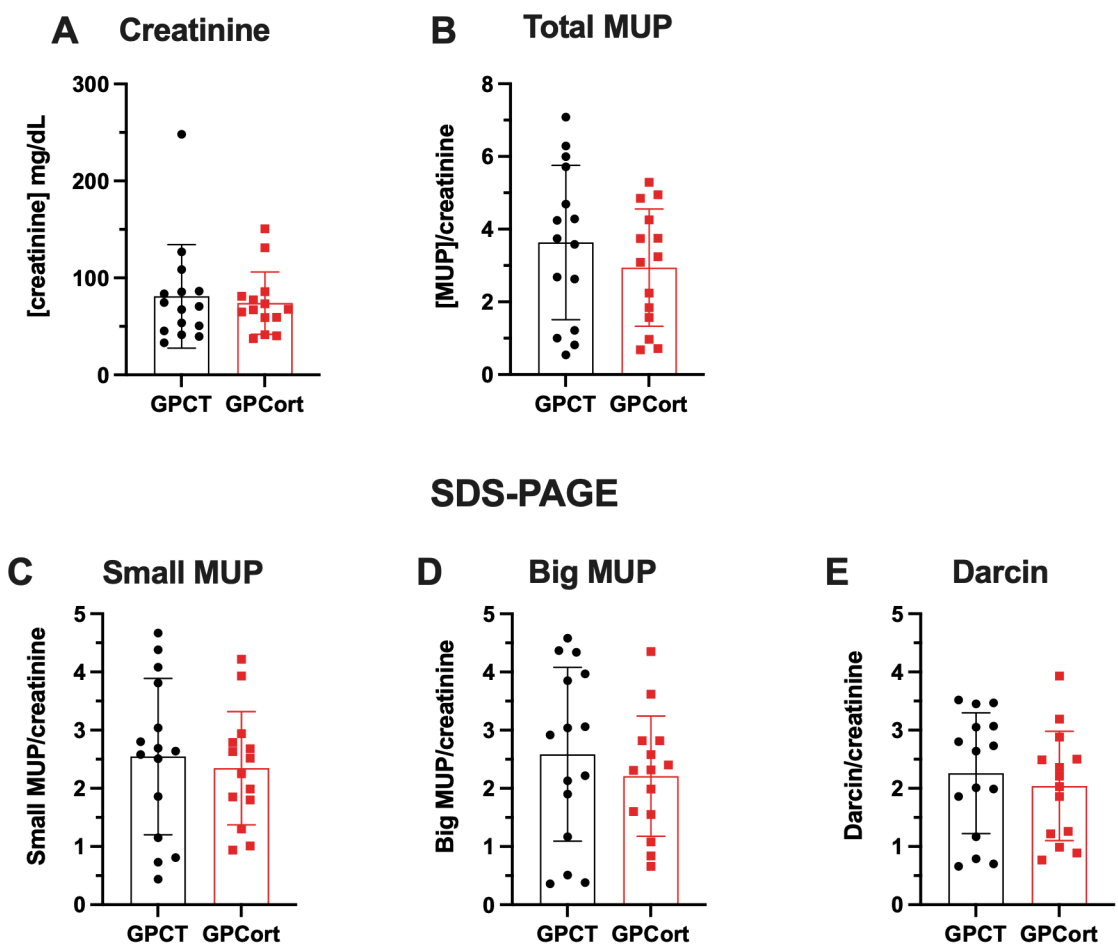


Figure 5. Assessing effects of grand-paternal Cort-treatment on grand-offspring urinary MUP levels. (A – B), urinary creatinine or total MUP were not affected by grand-paternal Cort-treatment. (C – E), specific MUP populations in the urine were not affected by grand-paternal Cort-treatment. A: Mann-Whitney test. B – E: unpaired t-test. Data represented as mean ± SD.

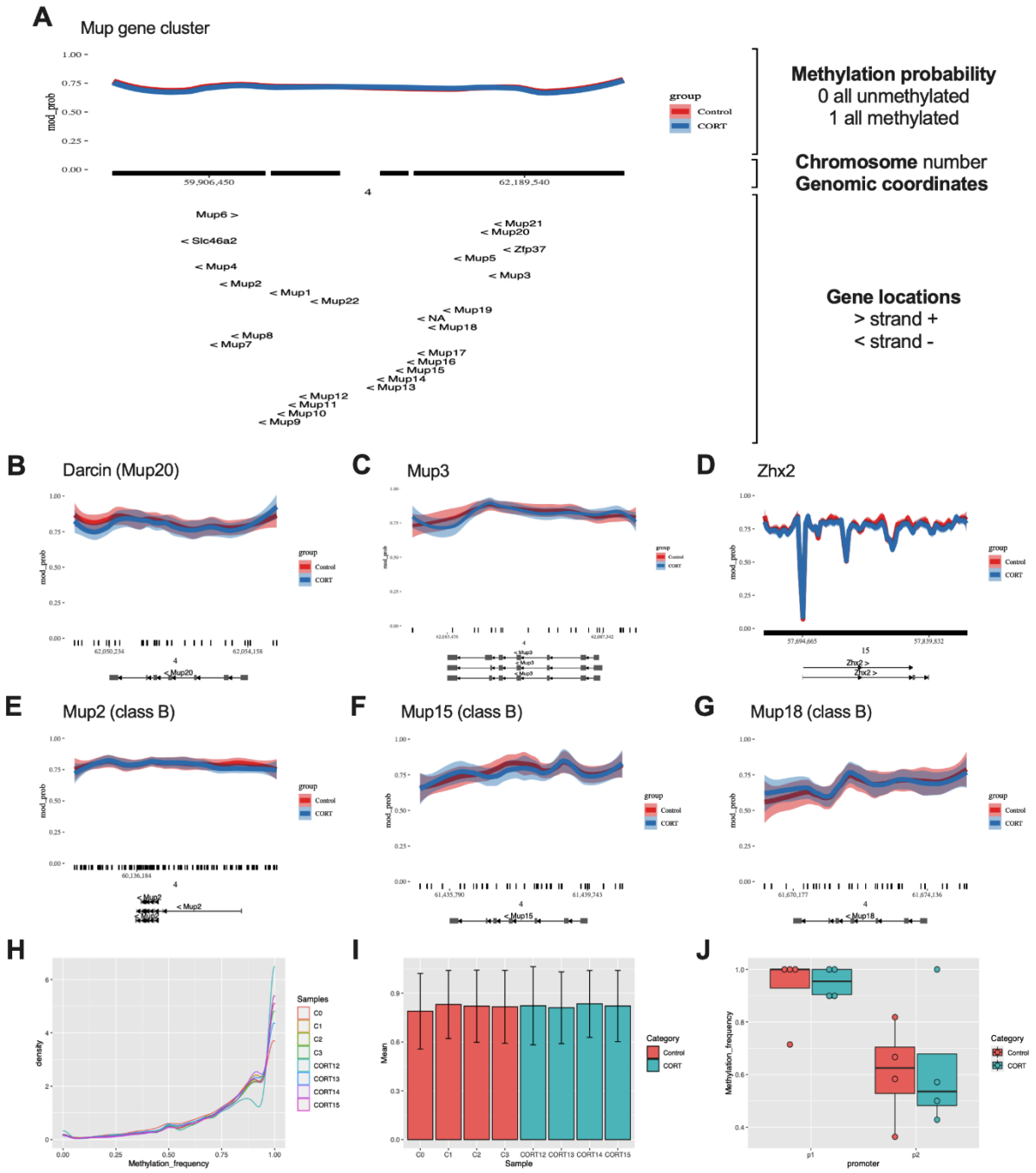
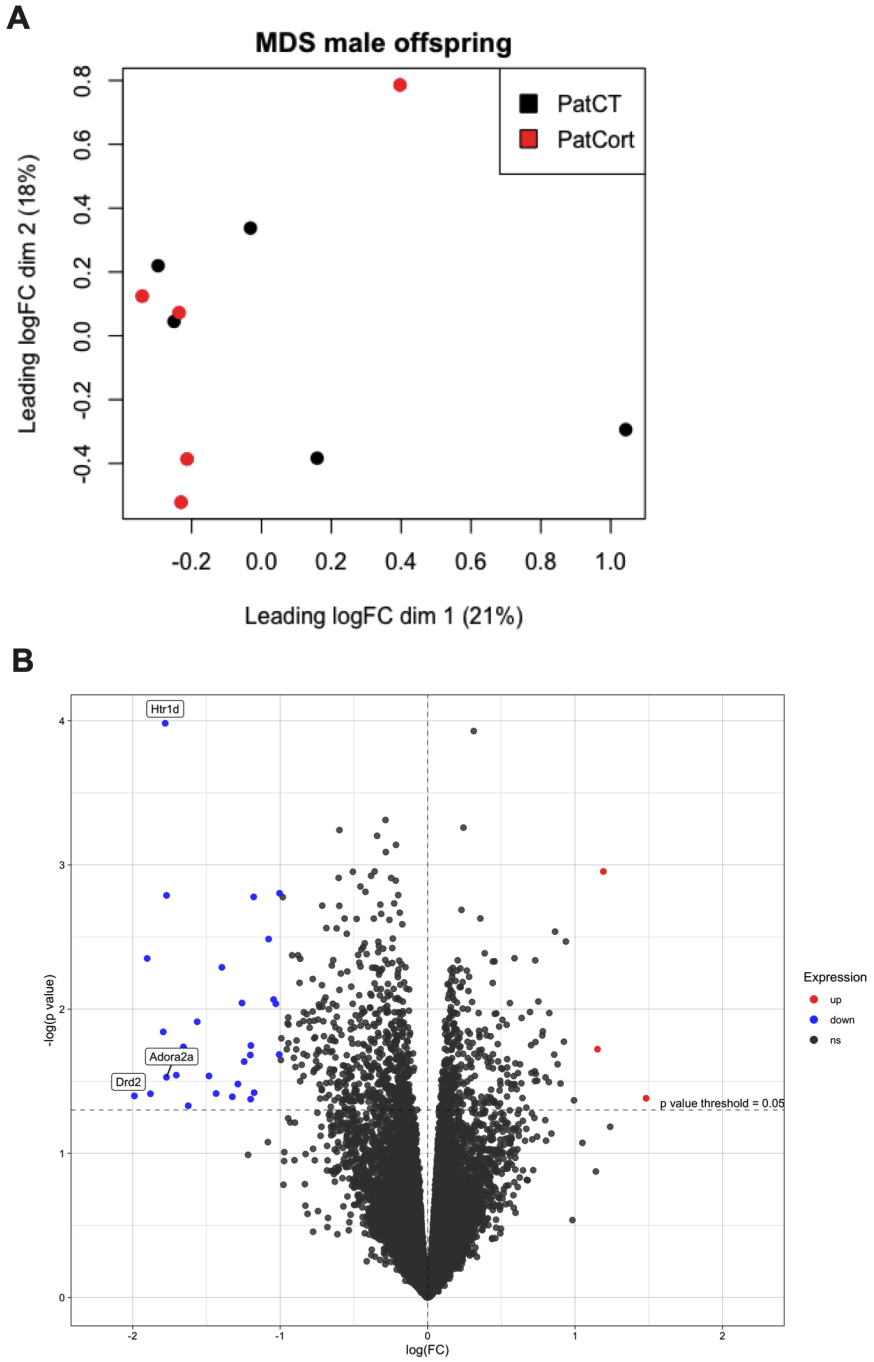


Figure 6. DNA methylation profile of *Mup* genes. (A), whole *Mup* cluster genomic region. (B), *Mup20*. (C), *Mup3*. (D), *Zhx2*. (E), *Mup2*. (F), *Mup15*. (G), *Mup18*. (H), *Mup* cluster methylation density plot. (I), *Mup20* gene methylation frequency per sample. (J), *Mup20* promoters 1 and 2 methylation frequency. Methylation frequency=1: Methylated CpG. Methylation frequency=0: Unmethylated CpG.



1250

1251 **Figure 7. Assessing effects of paternal Cort-treatment on offspring prefrontal cortex gene**

1252 **expression.** (A), multidimensional scaling plot of distances between the male F_1 offspring

1253 prefrontal cortex gene expression profiles. (B), volcano plot showing genes with $p<0.05$ and

1254 log-fold change threshold of 1 prior to FDR correction. Red: genes upregulated in PatCort.

1255 Blue: genes downregulated in PatCort, compared to PatCT.

1256