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1 **Relationships between play and responses to tickling in male juvenile rats**

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12

13 Declarations of competing interest: none

14

15 **Highlights**

- 16
- 17 • Solitary but not social play increased prior to and potentially in anticipation of tickling sessions
 - 18 • There were substantial differences between cohorts in their tickling responses and play
19 behaviour.
 - 20 • Taking account of cohort there was evidence that tickling showed rebound and emotional
21 contagion effects
 - 22 • Cohort effects may be explained by differences in physical condition prior to tickling.

23 **Abstract**

24 Play is a putatively positive experience and of key interest to the study of affective state in animals.
25 Rats produce 50kHz ultrasonic vocalisation (USVs) during positive experiences, including social play
26 and tickling. The tickling paradigm is intended to mimic social play resulting in positively valenced
27 ultrasonic vocalisation (USV) production. We tested two hypotheses on the relationship between
28 tickling and play: that tickling would increase play behaviour or that play behaviour would increase
29 in anticipation of tickling, and that tickling would share some specific properties of play (rebound
30 and emotional contagion of unexposed cage mates). Male Wistar rats (N=64, with 32 rats/cohort) of
31 28 days of age were housed in pairs with one rat assigned to be tickled and one as the non-tickled
32 control. Production of 50kHz USVs and hand-following behaviour was measured. Prior to handling,
33 solitary and social play was recorded for 5 minutes in the home cage. A two-day break in tickling was
34 used to assess a potential rebound increase in responses to tickling. Only one rat within each cage
35 was handled to assess emotional contagion through changes in the behaviour of the cage-mate.
36 Solitary but not social play increased prior to tickling relative to controls ($p = 0.01$). There were
37 marked differences between cohorts; tickled rats in C2 produced less 50kHz USVs than those in C1 (p
38 $= 0.04$) and overall, C2 rats played less than rats in C1 (social $p = 0.04$ and solitary $p < 0.001$) and had
39 a lighter start weight on arrival ($p = 0.009$) compared with cohort 1 (C1). In C1, there was evidence
40 of rebound in USV production ($p < 0.001$) and a contagious effect of tickling reflected by increased
41 hand-following in cage mates ($p = 0.02$). We found a positive relationship between start weight and
42 USV responses to tickling ($R_s = 0.43$, $p < 0.001$), suggesting that the divergence in USV production
43 may be due to developmental differences between cohorts. The results suggest that the
44 relationship between tickling and play is complex in that tickling only affected solitary and not social
45 play, and that tickling responses showed rebound and contagion effects on cage-mates which were
46 specific to cohort responses to tickling.

47

48 **Keywords** Tickling; Play; Male rats; Positive affective states; Positive animal welfare

49

50

51 **1. Introduction**

52 Play behaviour has been proposed as an indicator of positive affective states (Boissy et al., 2007). In
53 human children, play is important within developmental psychology, being a key indicator of cognitive
54 and physical development, as well as positive affect (Piaget, 1952). More recently, play has become
55 of key interest to the study of positive affective states in animals (for a recent review, see Graham and
56 Burghardt, 2010), a timely change as the vast majority of animal welfare research has been, and
57 continues to be, focused on more negative aspects of animals' lives (Lawrence et al., 2017). This focus
58 on negative affect has led to a relative lack of quantifiable models of positive affective state in animals.

59

60 Absence of play is widely recognised as an indicator of negative psychological and environmental
61 conditions (e.g. Ahloy-Dallaire et al., 2017, Bateson, 2014; Boissy et al., 2007; Burghardt, 2005; Held
62 and Špinka, 2011; Lawrence, 1987). This sensitivity of play to the environment, e.g. food availability,
63 social conditions and experiencing pain (reviewed in Held and Špinka, 2011), suggests that play only
64 occurs when primary survival needs are met and immediate fitness is secure (Boissy et al., 2007;
65 Lawrence, 1987). However, of more significance to positive animal welfare is the neurobiological and
66 behavioural evidence that play is thought to represent a positive psychological state (Siviy, 2016), and
67 is often used to reduce anxiety and negative emotion in children (Li et al., 2016).

68

69 Play behaviour is thought to be self-rewarding (Trezza et al., 2010), occurring spontaneously in all
70 mammalian species and perhaps also in other animal classes (Graham and Burghardt, 2010). Indeed,
71 animals place such value on social play that in a social discrimination task, they will show preference

72 for access to social interactions involving play (Humphreys and Einon, 1981). An increase in play
73 performance is also seen after a period of temporary social or locomotor deprivation (Hole, 1991).
74 This rebound effect occurs when a new opportunity for play is presented, either through increased
75 space or presentation of a play partner, with examples in rats (Ikemoto and Panksepp, 1992), as well
76 as pigs (Wood-Gush et al., 1990) and calves (Jensen, 1999). For example, calves under confinement
77 will conduct more locomotor-rotational play (bucks and leaps) than unconfined calves upon release
78 (Jensen, 1999) with social isolation commonly used to increase subsequent social play in rat studies
79 (e.g. Panksepp and Beatty, 1980). Play can also be stimulated in others by simply seeing another
80 animal play (Bekoff, 2001), with exposure to more playful partners increasing levels of play in rats (e.g.
81 Pellis and McKenna, 1992). As the behaviour of one rat was changed by the behaviour of another, it
82 can be assumed that emotional contagion has occurred, whereby the emotional state of one animal
83 is transferred to another (Held and Spinka, 2011). Although this contagious property of play has been
84 documented, it has received little attention despite having the potential to spread a positive
85 emotional state (Held and Spinka, 2011).

86

87 In rats, the production of 50kHz ultrasonic vocalisations (USVs) are associated with positive
88 behaviours, such as feeding and mating (Schwartz et al., 2007). Juvenile rats will also emit these
89 vocalisations during social play, as well as in anticipation of social play (Knutson et al., 1998). It is well
90 established that social behaviour (including social play) is regulated by the actions of endogenous
91 opioids (Vanderschuren, 2010). The play response to playback of 50kHz USVs can be negated by
92 administration of the opioid antagonist naloxone and enhanced by administration of the opioid
93 agonist morphine (Schwartz et al., 2007). These positive USVs may be one method by which play
94 facilitates emotional contagion in rats (Schwartz et al., 2007). Although the true function of play is
95 still unclear from an evolutionary perspective, play is likely to provide an animal with psychological

96 benefits through an opioid-mediated pleasurable experience (Vanderschuren et al., 1995) while
97 improving skills such as social interaction (Pellis and Pellis, 2007).

98

99 The heterospecific tickling model aims to mimic the 'rough and tumble' aspects of play without the
100 need to wait for the spontaneous occurrence of play (Panksepp and Burgdorf, 2003). During play, rats
101 partake in a series of chasing and wrestling movements, with rapid movement ceasing when one rat
102 allows the play partner to pin it onto its back with the play partner on top (Pellis and McKenna, 1992).
103 Tickling aims to mimic this interaction by stimulating areas which are contacted during play, as well as
104 replicating pinning behaviour by turning the rat onto its back (Panksepp and Burgdorf, 2003). Some
105 rats produce plentiful positive (50kHz) USVs during tickling and will actively seek interaction with the
106 experimenter (Burgdorf and Panksepp, 2001). As with play, tickling is thought to activate neural
107 pathways associated with positive affect, particularly the mesolimbic dopaminergic system or reward
108 system (Ishiyama and Brecht, 2016; Burgdorf and Panksepp, 2006). For example, Hori et al., 2013
109 found that tickling increased dopamine release in the nucleus accumbens from baseline levels, with
110 no increase found in rats which received light-touch stimulation. Activation of the mesolimbic
111 pathway is also induced by anticipation of a reward (e.g. Spruijt et al., 2001; Schultz, 1997). Following
112 the logic of Dudink et al. (2006), if tickling activates the mesolimbic dopaminergic system, rats should
113 learn to anticipate the rewarding experience of tickling leading to an increased expression of play
114 behaviour which is also known to depend on this system (Held and Spinka, 2011; Dudink et al., 2006).
115 Given this the aims of this study were: (a) To investigate whether the predicted positive experience of
116 tickling would increase play behaviour. Considering that tickling is intended to mimic social play and
117 that they share neural substrates, we hypothesised that a) tickled rats would show an increase in social
118 play prior to tickling and b) tickling responses, as with play, would show rebound following a period
119 without tickling and also have contagious effects on cage-mate vocalization production and approach
120 behaviour.

121

122 **2. Materials and Methods**

123 All work was carried out at the Roslin Institute, Edinburgh, U.K., in accordance with the U.K. Animals
124 (Scientific procedures) act 1986. Ethical approval was granted by the Roslin Institute Animal Welfare
125 and Ethical Review Body (AWERB) and the Royal (Dick) School of Veterinary Studies Veterinary Ethical
126 Review Committee (VERC).

127

128 **2.1 Subjects, housing and husbandry**

129 Male Wistar rats (N=64; 2 cohorts of 32) were obtained from Charles River Laboratories, Tranent,
130 Scotland. Adolescent males (28 days old) were used, as social play in rats is seen to peak between
131 30- and 40-days post-partum before declining until puberty at around 60 days, with males typically
132 expressing higher absolute frequencies of play fighting (Pellis and Pellis, 2013). Studies investigating
133 the tickling paradigm typically also use adolescent males with adolescents robustly showing stronger
134 tickling responses than adults (Burgdorf and Panksepp, 2001). The Wistar strain is the 3rd most
135 commonly used rat strain for tickling experiments (23%, n = 13) (LaFollette et al., 2017). On arrival,
136 rats were housed in pairs, randomly allocated to home cages, then left to acclimatise for 6 days prior
137 to handling. Home cages were made of clear plastic with a metal mesh open-top lid (l x d x h: 48cm x
138 26.3cm x 20.5cm; Techniplast, Italy) with aspen chip shavings topped with wood fibre bedding
139 (Estonia, England), a chewable wooden block (Datesand, England), and with *ad libitum* access to
140 food (Teklad Global Rodent Maintenance Diet (14% protein); Envigo, England) and tap water. Clean
141 bedding was provided on the morning of day 10 of the 32-day-long study, 3 days before the
142 experimental phase began. Cages were assigned to being tickled or neutrally handled controls with
143 one tickled cage and one control cage together as a “block”. Within the cage, one rat was randomly
144 allocated as the handled rat (to be tickled or neutrally handled), with their paired cage mate not

145 being handled until the final day (Section 2.2). Cages were distributed across four tiers of a standard
146 rodent rack (Techniplast, Italy) with lux levels, measured using an Isotech digital light meter Lux-
147 1337, varying across each tier due to the design of the rack and cages (top tier: 44 lx; 2nd tier: 25 lx;
148 3rd tier: 45 lx; bottom tier: 6 lx). To account for this variation in light, treatment and control cages
149 were balanced across all rows. Rats were kept under a 12:12 light: dark cycle (light from 7:00 a.m. to
150 7:00 p.m.), with an average room temperature of $22.4 \pm 0.3^{\circ}\text{C}$ and relative humidity of $43 \pm 5\%$. The
151 allocation of treatment or control of each cage was inverted for the second cohort of rats. This
152 aimed to control for effects of tier level and distance from human activity on behaviour (Cloutier and
153 Newberry, 2010). All handling was conducted by a single female experimenter (TH) to reduce
154 handling stress and create consistency in tickling.

155

156 2.2 *Handling procedures*

157 Habituation was conducted over 5 days prior to the experimental phase, aiming to expose the handled
158 rat gradually to test conditions, first as pairs (5 minutes exposure to the arena and handler; days 1, 2
159 and 3), then individually (5 minutes; days 4 and 5) (**Figure 1**).

160

161 **Figure 1.**

162

163 Handling took place every weekday for 2 weeks, with a 2 day break after 5 days to study rebound
164 effects (**Figure 1**; see below for details). Trial order was randomised for each day. All handling was
165 conducted in a procedure room away from the main holding room, during the light phase in the
166 afternoon (12.00 h to 17:00 h). Rats were transported to the procedure room on a trolley in the

167 home cage without being individually handled. During travel, the cage was covered by a large dark
168 towel to prevent exposure to bright light as the rats were tested in their dark photoperiod.

169 Red lighting produced a low-intensity illumination of the procedure room (4.5 lx). Rats, especially
170 albinos, prefer lower light intensities due to high visual sensitivity (see Burn, 2008, for a full review),
171 with positive USV production being reduced during exposure to bright light (Knutson et al., 1998).

172

173 The handling arena was a transparent, open top box (l x d x h: 51cm x 42cm x 23.2cm; VetTech
174 Solutions Ltd, England) with the base covered with disposable and absorbent non-slip bench liner
175 (LabMat, LabLogic Systems Ltd., England) secured with masking tape to provide a non-slip surface
176 which would not disturb vocalisation recordings by producing excessive background noise, as found
177 with litter during in-house pilot studies. At the end of each test day, the arena was cleaned with 70%
178 ethanol and new matting secured.

179 Tickling involved the experimenter using one hand, covered by soft knitted glove, to touch, tickle,
180 chase and pin the rat in a manner that mimics rough and tumble play (Bombail et al., 2019). Rats were
181 tickled in this way for repeated bouts of 20 seconds alternated with 20 second “pauses” lasting for a
182 total of 3 minutes (adapted from Panksepp and Burgdorf, 2010). This allowed for 4 periods of active
183 tickling (total 1min 20 secs) interspersed with 5 periods of pauses. For control animals, during the
184 active tickling phases the gloved hand rested in the centre of the arena. For all rats, during pauses the
185 hand moved in slow circles around the arena while wiggling the fingers to gain the attention of the rat
186 and to measure approach behaviour, as the duration and counts of hand-following events (Lampe et
187 al., 2017, Melotti et al., 2014) (see section 2.4).

188

189 2.3 *Recording and analysis of vocalisations*

190 Vocalisations produced during handling in the arena were recorded using a high-quality condenser
191 microphone designed for recording ultrasonic vocalisations produced by bats (Pettersson M500-384
192 USB Ultrasound microphone, Pettersson Elektronik; Sweden) and a free recording software (Audacity,
193 Version 2.1.3, Pennsylvania, United States of America). The microphone was placed over the centre
194 of the arena, pointing downwards 61.5 cm from the arena floor. Vocalisations were manually counted
195 from spectrograms produced using Audacity software (Version 2.1.3, Pennsylvania, United States of
196 America). Spectrograms were generated with a fast Fourier transform length of 512 points with a
197 Hanning window (50% overlap frame). Only 50kHz vocalisations (peak frequency between 30 and 80
198 kHz and a duration between 10–150 ms) were counted as the production of 50kHz USVs are associated
199 with positive behaviours and were used as an indicator of positive experience, (Brudzynski, 2009;
200 Wright et al., 2010; LaFollette et al., 2018). Overlapping calls were counted as one call (Wright et al.,
201 2010), with only clearly categorizable vocalisations counted.

202

203 2.4 *Recording and analysis of hand-following behaviour*

204 The behaviour of tickled and control rats in the arena was recorded using a second Sony HD camcorder
205 (HDR-CX405). Observer XT 11 software was used to analyse the duration of hand-following (HF) events
206 during the pause section of each handling session to gain information on approach behaviour (Lampe
207 et al., 2017). The duration (in seconds) and counts of hand-following events were calculated for each
208 rat using focal observation sampling with continuous recording. Rats were deemed to be hand-
209 following when the nose was oriented towards the hand and was actively moving towards the
210 experimenter's hand. The experimenter moved the hand in circles in the same manner for all rats,
211 standardised by counting the number of circles made by the hand, to help assess whether movement
212 towards the hand was intentional (Melotti et al., 2014).

213

214 2.5 *Recording and analysis of play behaviour in the home cage*

215 Prior to handling and immediately after being taken to the procedure room, behaviour in the home
216 cage was recorded using a Sony HD camcorder (HDR- CX405) for 5 minutes to measure anticipation of
217 interaction with the handler. Videos were analysed using Observer XT 11 software (Noldus
218 Information Technology, Wageningen, the Netherlands). The frequency of solitary play and the
219 frequency and direction of social play were scored using focal observation sampling with continuous
220 recording (see **Table 1** for full ethogram of behaviours scored). Solitary play was scored as a measure
221 of individual play with an event being defined as finished when the rat ceased movement or engaged
222 in behaviours other than the leaps and running described in **Table 1**, with the behavioural unit as the
223 number of solitary play events per 5 minutes. Initiation of social play was scored as a measure of the
224 appetitive motivation for social play. A bout of social play was deemed to be finished when rats had
225 no contact with each other for 2 or more seconds, with the behavioural unit as the number of
226 initiations of social play events per 5 minutes. Intra-observer reliability was high (Cohen's kappa =
227 0.93), with a percentage of agreement of 98.7% - only one observer scored all behaviours.

228

229 **Table 1.**

230

231 *2.6 Test of rebound effects*

232 To explore potential rebound effects, after 5 days of continuous handling all rats were given a 2-day
233 break, then handled for another 5 days. A rebound effect was defined as an increase in USV and hand
234 following responses to tickling (relative to day 5) on the first day after the 2-day break (day 6).

235

236 *2.7 Test of emotional contagion*

237 To explore whether there was a contagious effect of tickling, we investigated the behaviour of the
238 paired (previously unhandled) cage mate on the final test day (day 10) after being housed with a rat

239 which was tickled or control handled for 10 days. During the 10 days of handling, cage mates remained
240 in the home cage (fitted with a wire lid) and were placed on the bench next to handling arena. This
241 allowed the cage mate to have auditory and visual contact when their paired rat was handled. After
242 the handled rat had experienced tickling or control handling, all cage mates were subjected to the
243 same procedure and conditions as control rats to investigate whether treatment (i.e. being housed
244 with a tickled or neutrally handled rat) influenced USV production or hand-following of the cage mate.
245 Cage mates had 2 minutes of contact with their paired handled cage mate in the home cage prior to
246 being moved to the arena and tested. As such, emotional contagion could be facilitated by a long-
247 term mechanism in which the behaviour of the previously unhandled cage mate could be altered by
248 being exposed to another rat whom was routinely tickled. Responses were recorded and measured as
249 in Sections 2.3 and 2.4.

250

251 2.8 *Body weight measurement*

252 Following handling, body weight of both handled rats and cage mates was measured weekly by placing
253 the rat in a box on an electronic scale (OHAUS Adventurer Pro AV2101).

254

255 2.9 *Statistical analysis*

256 All data from the first and fifth day of consecutive handling were used in the analyses. Following a
257 two-day break from handling, data were also collected from the sixth and tenth day (see **Figure 1**).
258 Basic descriptive statistics and correlations were calculated using Minitab 18. For the majority of
259 analyses, Generalised Linear Mixed Models (containing both fixed and random effects) were fitted in
260 Genstat (16th Edition) using the REML algorithm with a log link function, a Poisson error distribution
261 and dispersion parameter fixed at one. To investigate differences in play and handling responses (USV
262 production and hand following) in handled rats between cohorts the fixed effects part of the model

263 comprised treatment, cohort, day, treatment X cohort x day interaction effects. The random effects
264 part of the model reflected the fact that cages were nested within pairs of cages and they were all
265 crossed with the two runs in which different cohorts were tested, as well as the fact that there were
266 four repeat observations over 2 weeks on each cage per cohort. As there was a significant interaction
267 between responses to tickling and cohort, each cohort was also analysed independently to investigate
268 rebound and contagion properties where effects may have been masked when fitted together. To
269 investigate a potential rebound effect, we looked at the difference between handling responses on
270 day 5 compared to day 6 within each cohort. Fixed effects within the REML model were treatment
271 and day and the interaction between treatment and day. The random effects part of the model
272 reflected the fact that cages were nested within pairs of cages. Cage mate handling responses (USVs
273 and hand following) during a single test were used to investigate contagion. When fitted for both
274 cohorts together, the fixed effects part of the model comprised treatment, cohort and a treatment X
275 cohort interaction effects. The random effects part of the model reflected the fact that cages were
276 nested within pairs of cages and they were all crossed with the two runs in which different cohorts
277 were tested. Due to the difference in cage mate responses between cohorts, responses were fitted
278 independently, with treatment as the only fixed effect. Predicted means and associated standard
279 errors of the mean (SEM) reported were back transformed to the original scale produced by the REML
280 output. As weight data met the assumptions of normality, two ANOVAs were used to investigate a
281 potential difference in start and final weight in all rats between cohorts, with cohort fitted as a factor.
282 To investigate the potential influence of physical condition on responses to tickling we ran a
283 Spearman's rank correlation on tickled rats in both cohorts between start weight and tickling
284 responses across the four test days. For all tests, the level of statistical significance was set at $p < 0.05$.

285

286 **3. Results**

287 *3.1 Cohort effects on USV production in response to tickling*

288 Overall, combining both cohorts, tickled rats produced more 50kHz USVs than control rats (tickled vs.
289 controls $M \pm SEM = 82.3$ vs. 32.8 ± 5.91 ; $F_{1,14} = 8.18$, $p = 0.013$; **Figure 2a**). Tickled rats also showed
290 increased HF compared with controls (tickled vs. controls $M \pm SEM = 5.9$ vs. 1.9 secs ± 1.22 ; $F_{1,14} =$
291 19.38 , $p = 0.004$; **Figure 2b**).

292 **Figure 2**

293 However, a significant interaction was observed between treatment and cohort in USV production;
294 tickled rats in cohort 1 performed more 50kHz USVs than those in cohort 2 (C1 vs. C2 $M \pm SEM = 125.9$
295 vs. 53.8 ± 4.59 ; $F_{1,14} = 5.18$, $p = 0.039$). There was also an interaction between cohort and day within
296 tickled rats ($F_{3,14} = 42.4$, $p = 0.003$), with USV production increasing across days in cohort 1 (day 1 vs.
297 day 10 $M \pm SEM = 114.6$ vs. 186.8 ± 5.9) and decreasing in cohort 2 (day 1 vs. day 10 $M \pm SEM = 92.5$
298 vs. 43.6 ± 5.9 ; **Figure 3**). This interaction was not seen in USV production of control rats, with no
299 significant difference between cohort (C1 vs. C2 $M \pm SEM = 85.1$ vs. 97.5 ± 4.68 ; $F_{3,14} = 0.6$, $p = 0.439$)
300 and no effect of day (day 1 vs. day 10 $M \pm SEM = 78.4$ vs. 112.6 ± 3.35 ; $F_{3,14} = 1.99$, $p = 0.121$; **Figure**
301 **3**). As such, the effect of tickling on USVs was dependent on cohort. There was no cohort effect in HF
302 duration ($F_{1,14} = 1.84$, $p = 0.202$).

303 **Figure 3**

304 3.2 *Tickling (and handling) induce play behaviour*

305 In handled rats in both cohorts, there was a significant effect of tickling on solitary play, with tickled
306 rats playing more than controls in the 15 minutes before handling (tickled vs. controls; $M \pm SEM = 1.01$
307 vs. 0.63 ± 0.18 ; $F_{1,31} = 6.86$, $p = 0.01$; **Figure 4**). In both cohorts, there was no effect of tickling observed
308 on social play ($F_{1,14} = 2.58$, $p = 0.11$). Across 4 days of testing, both tickled and control rats in cohort 1
309 consistently performed more play events than cohort 2 (solitary: C1 vs. C2 $M \pm SEM = 0.99$ vs. $0.47 \pm$
310 0.89 play events per 5 minutes, $F_{1,14} = 4.19$, $p = 0.042$; social play initiations: C1 vs. C2 $M \pm SEM = 2.62$
311 vs. 2.10 ± 1.08 play initiations per 5 minutes, $F_{1,14} = 14.79$, $p < 0.001$) with no significant effect of day
312 (solitary: $F_{1,14} = 3.18$, $p = 0.369$; social: $F_{1,14} = 0.73$, $p = 0.867$).

313 **Figure 4**

314 3.3 *Tickling responses show cohort-dependent evidence of rebound and contagion properties*

315 To investigate rebound, we examined the difference in handling responses (USVs and HF) before and
316 after a two-day break. Due to the previously observed cohort/day interaction (Section 3.2), cohorts
317 were tested independently to investigate rebound and contagion properties where effects may have
318 been masked when fitted together. In cohort 1, there was a significant interaction between day and
319 treatment in USV production ($F_{1,14} = 132.16, p < 0.001$), but not HF ($F_{1,14} = 2.95, p = 0.106$), with tickled
320 rats showing an increase in USV production on day 6 compared to day 5 (day 5 vs. day 6 tickled rats
321 $M \pm SEM = 105.5$ vs. 141.5 ± 5.91 ; **Figure 3**). This effect was not seen in cohort 2 in either USV
322 production ($F_{1,14} = 0.16, p = 0.69$; **Figure 3**) or HF ($F_{1,14} = 0.36, p = 0.55$).

323 Evidence of a contagious effect of tickling on cage mate behaviour was also specific to cohort.
324 Between cohorts there was a significant difference in cage mate performance of both 50kHz USVs (C1
325 vs. C2 $M \pm SEM = 131.97$ vs. 13.31 ± 1.17 ; $F_{1,14} = 16.71, p < 0.001$) and HF (C1 vs. C2 $M \pm SEM = 7.19$ vs.
326 154.69 secs ± 1.11 ; $F_{1,14} = 19.30, p < 0.001$) when tested on day 10. When cohorts were fitted
327 independently, cohort 1 cage mates of tickled rats showed increased HF compared with cage mates
328 of control rats (cage mates of tickled vs. controls $M \pm SEM = 14.51$ vs. 1.27 secs ± 1.17 ; $F_{1,14} = 0.59, p$
329 $= 0.023$; **Figure 5**). This was not seen in USV production ($F_{1,14} = 0.07, p = 0.79$). There was no treatment
330 effect on cage mate behaviour in cohort 2 (50kHz USVs: $F_{1,14} = 4.87, p = 0.90$; HF: $F_{1,14} = 0.04, p = 0.84$).

331 **Figure 5.**

332 3.4 *Potential influence of physical condition on response to tickling*

333 Differences in physical condition were observed between cohorts as rats in cohort 2 were lighter on
334 arrival (C1 vs. C2 $M \pm SEM = 103.0$ g vs. 89.54 g ± 3.40 ; $F_{1,63} = 7.83, p = 0.009$) and at the end of the
335 experiment (C1 vs. C2 $M \pm SEM = 264.87$ vs. 235.94 g ± 5.07 ; $F_{1,63} = 16.25, p < 0.001$). To investigate the
336 potential influence of physical condition on responses to tickling we ran a Spearman's rank correlation

337 on tickled rats in both cohorts between start and final weight and tickling responses across the four
338 test days. There was a statistically significant positive correlation between start weight and average
339 USV production across all days ($R_s = 0.43$, $p < 0.001$; **Figure 6**) with no relationship between start
340 weight and HF ($R_s = 0.19$, $p = 0.14$). No relationships were found between final weight and tickling
341 responses (USVS; $R_s = 0.23$, $p = 0.07$; HF; $R_s = (-) 0.04$, $p = 0.73$).

342 **Figure 6.**

343

344 **4. Discussion**

345 *4.1 General discussion*

346 Tickling aims to mimic rough and tumble social play between rats (Cloutier et al., 2018; Panksepp,
347 2000) with evidence suggesting that tickling activates the same reward mechanisms as play. However,
348 considering the proposed relationship between these hedonic experiences, there has been little
349 investigation into the relationship between tickling and play. We found that tickling male juvenile rats
350 increased solitary play but not social play before a predicted tickling experience. There were also
351 substantial differences between cohorts in their responses to tickling and play. Taking account of
352 these cohort effects, we found evidence that tickling and play share similar properties, inducing
353 rebound and having a contagious effect on cage mates. Differences between cohort responses may
354 be explained by the divergence in physical condition between cohorts on arrival and throughout the
355 study when taken as an indicator of early life experience.

356

357 *4.2 Vocalisations as an indicator of affective state*

358 Quantification of 50kHz USVs are the most commonly used measure to assess responses to tickling
359 and are often used to infer a positive affective state (e.g. Panksepp and Burgdorf, 2000). Overall, the
360 production of USVs were consistent with the idea that tickled rats in this study were in a more positive

361 state, with tickled rats producing over twice the average of 50kHz vocalisations of control rats. A
362 recent review supports these findings, with 94% (n=15/16) of all tickling experiments reporting that
363 tickled rats produced more 50kHz USVs than controls (LaFollette et al., 2017). However, we found
364 differences in USV production in response to tickling between cohorts; our first cohort produced
365 significantly more 50kHz USVs than the second cohort, with production increasing across days in
366 cohort 1 but decreasing in cohort 2.

367

368 4.3 *Approach behaviour as an indicator of motivation to be tickled*

369 In our other measure of response for tickling, we found no effect of cohort with tickled rats following
370 the experimenter's hand for longer than controls in both cohorts. This suggests that tickled rats in
371 both cohorts were as equally motivated to interact with the handler. Following the same protocol as
372 Lampe et al. (2017), approach behaviour was assessed by the duration and number of hand-following
373 events in the time gap between each handling stimulation. Approach tests in general have received
374 criticism for their lack of sensitivity in discriminating between emotions and arousal, for example,
375 enjoyment and curiosity (Waiblinger et al., 2006). Although supporting a treatment effect between
376 tickled and control rats, approach behaviour protocols such as the one used here may not be sensitive
377 enough to pick up cohort differences in responses to tickling. Supplementation with an evidenced
378 indicator of emotions during approach behaviour, such as use of facial indicators (Finlayson et al.,
379 2016; Sotocinal et al., 2011) would aid discrimination. This highlights the need to use multiple
380 measures to provide complementary information on emotional state, as well as, the need for a
381 comparison of different approaches of assessing how rewarding rats find tickling.

382

383 4.4 *The effect of tickling on play behaviour*

384 Tickling has been shown to be a positive experience for some rats (e.g. Panksepp and Burgdorf, 2003)
385 with evidence that lines bred for a high USV tickling response display more play behaviour (Panksepp
386 and Burgdorf, 2000). As tickling is intended to mimic social play (Panksepp and Burgdorf, 2003), and
387 both social play and tickling activate the mesolimbic dopaminergic system, we expected that tickled
388 rats would show increased social play behaviour prior to a scheduled handling experience compared
389 with controls. We found more solitary play being performed by tickled rats within the home cage prior
390 to treatment. However, in contrast to our prediction, we found that social play initiation was
391 unaffected by tickling. This distinction between types of play in rats was also noted by Melotti et al.
392 (2014), who found that solitary and social play were unrelated when measured in the home cage.
393 These results correspond with Burghardt's (2005) theory that different types of play may have evolved
394 independently, with solitary play perhaps reflecting anticipation for a rewarding positive experience,
395 in this case tickling.

396

397 Expectation of a reward, like play and tickling, activates the mesolimbic system and often results in
398 expression of anticipatory behaviours (e.g. Spruijt et al., 2001; Schultz, 1997). Anticipatory behaviour
399 has been suggested to reflect the value an animal places on a reward state (van der Harst and Spruijt,
400 2007). It has been suggested that spontaneous behavioural frequencies can be used to assess the
401 current affective state (van der Harst and Spruijt, 2007). We suggest that frequency of solitary play
402 could act as an indicator of anticipation, with solitary play reflecting the increase in reward stimulated
403 by expectation of tickling. Dudink et al. (2006) found that announcement of opportunity to access an
404 environmentally-enriched area facilitated locomotory solitary play behaviour in weaned pigs prior to
405 access. As such play behaviour could reflect anticipation for access to environmental enrichment.
406 Interestingly Dudink et al. (2006) only measured solitary play, suggesting that solitary and social play
407 are differently affected by the reward system.

408

409 4.5 *Evidence of a rebound effect in tickling*

410 Providing an opportunity for play following a period of deprivation induces an amplification of play
411 known as the rebound effect (Held and Špinka, 2011). Social isolation is commonly used to increase
412 the motivation for social play (e.g. Panksepp and Beatty, 1980) and to induce a behavioural rebound
413 in social play (Held and Špinka, 2011; Loranca et al., 1999; Ikemoto and Panksepp, 1992). Rebound
414 effects in social play in rats occur both with short (a few hours) (Siviy, 2016) and longer periods (up to
415 14 days) of deprivation (Ikemoto and Panksepp, 1992; Holloway and Suter, 2004), with Niesink and
416 Van Ree (1989) suggesting that 24h of social isolation is required to maximize rebound in social play.
417 We found that a two-day cessation of tickling led to a cohort-specific increase in USV production in
418 cohort 1. Considering the higher USV production by tickled rats in the first cohort and the presence
419 of a rebound effect in the first, but not second, cohort, this suggests that a rebound effect in tickling
420 is dependent on responsiveness to tickling as reflected by USV production. Further, this finding
421 suggests that isolation and the associated complete absence of play is not necessary to bring about
422 the rebound effect, as previously suggested because our rats were group housed (Holloway and Suter,
423 2004).

424

425 4.6 *Evidence of tickling-induced contagion*

426 We also found a cohort-specific effect of contagion related to tickling. Play behaviour is proposed as
427 a contagious activity in that the observation of animals playing can induce play in others (Bekoff,
428 2001). Further, play is also thought to represent an example of emotional contagion through the
429 transfer of a positive emotional state, assumed to be present during play, between play partners (Held
430 and Špinka, 2011). Play has specific cues and signals which may influence others (such as play bows
431 in canids; Rooney et al., 2001), with more playful individuals inducing play in another animal through
432 increased play cues (Pellis and McKenna, 1992). Along with the pinning, scampering and leaping
433 movements which characterize rat play (Pellis and Pellis, 1991), USV production may be a key signal

434 by which the transfer of positive emotions between individuals occurs. We expected that cage-mates
435 of tickled rats would show increased production of 50 kHz USVs and increased hand-following
436 behaviour when exposed in a single test to the control conditions of handled animals (i.e. placed in
437 the handling arena with human hand in the neutral position). This would indicate a more positive
438 emotional state transferred to them by their tickled cage-mates.

439 We found that in the first cohort, cage mates of tickled rats were more motivated to interact with the
440 experimenter than control rats. This discrepancy between cohorts provides further evidence that
441 additional effects of tickling (e.g. rebound and contagion) are reliant on responsiveness to tickling as
442 measured by USV production. Although there was no effect of treatment on USVs, cage mates in
443 cohort 1 produced almost ten times the number of USVs than cohort 2 which may have influenced
444 their cage mate. This is consistent with the idea that contagion is spread through greater USV
445 production from the tickled animal in the home cage (Saito et al., 2016). USVs have already been found
446 to evoke cognitive bias by Saito et al. (2016) with rats responding to ambiguous cues as positive after
447 hearing FM 50-kHz USVs and negative after 22-kHz USVs. Further investigation should investigate
448 what is signaling the change in behaviour in cage mates, whether it is USVs or some other signal.

449

450 4.7 *Potential influence of early life on play and tickling responses*

451 As well as an attenuated response to tickling in terms of USVs and HF, the second cohort of rats,
452 including handled rats and cage mates, also showed reduced overall play behaviour (both solitary and
453 social) compared to the first cohort. Play behaviour is known to be affected by early life conditions. In
454 animal models, prenatal stress has long been known to cause a number of long-term disturbances
455 including enhanced anxiety and a reduction in social play during adolescence (Ward and Stehm, 1991).
456 Further, post-natal manipulations, such as prolonged maternal separation (Arnold and Siviy, 2002),
457 and early fostering or handling (Maccari et al., 1995; Wakshlak and Weinstock, 1990) can directly
458 affect the interaction between mother and pup and consequently influence early life development

459 (Morley-Fletcher et al., 2003). There are also multiple lines of evidence that rats handled between
460 birth and weaning (approx. 0 – 28 days post-partum) exhibit less negative emotionality (i.e. anxiety)
461 than rats handled later in life (for a review see Hertenstein et al., 2006). As such, unknown early life
462 experiences may have resulted in a reduced propensity to partake in hedonic experiences in rats
463 within cohort two, indicated by reduced play and tickling responses.

464 In order to investigate these unexpected cohort effects on tickling, we used start and final weight as
465 indicators of development in early life. Rats in the second cohort were lighter on arrival and at the end
466 of the experiment. As an exploratory investigation, rats who were heavier on arrival across both
467 cohorts showed more pronounced responses to tickling as measured through 50kHz USV production.
468 As we found no relationship between final weight and tickling responses, this suggests there is an
469 influence of physical condition from before the experiment rather than concurrent with the
470 experiment. In several species, low birth weight shows an association with reduced total play (rats;
471 Morley-Fletcher et al., 2003, pigs; Brown et al., 2015; Litten et al., 2003 and horses; Cameron et al.,
472 2008). As also noted by Brown et al. (2015), our findings fit well with Burghardt's (2005) surplus
473 resource theory, which proposes that play evolved to occur only when juveniles were provided by the
474 parent(s) with periods of sufficient resource availability and protection. As such, the second cohort of
475 animals may have needed to allocate more resources towards growth, resulting in a decrease in the
476 motivation to play, both with another rat and with a human during tickling, as indicated by 50kHz USV
477 production.

478 Cohort effects are rarely discussed within animal behaviour literature. However, differences between
479 supposed replicates have important implications for many rodent studies using sensitive behavioural
480 assays. Although sourced from the same breeder, of the same age, sex and strain, we found
481 differences between cohorts in USV response to tickling and play behaviour. This is one of few studies
482 to report significant cohort-to-cohort differences in rat behaviour and physical condition. The one
483 other study known to the authors reported significant cohort variability in the acquisition and

484 performance of a skilled reaching task in Long-Evans rats (O'Bryant et al., 2011). The scarcity of
485 evidence may be due to a lack of replicates across cohorts or because of reporting bias towards
486 positive results (e.g. Dickersin, 1990). Ultimately, testing for differences between cohorts is an
487 important consideration in attempts to control for within experiment variability. It also has the
488 potential to yield understanding of mechanisms underlying behavioural responses in this case the
489 relationship between physical condition and responses to tickling.

490

491 **5. Conclusion**

492 In conclusion, our results show a previously unfound relationship between tickling and play. The
493 positive affect induced by tickling was specific to solitary play and may reflect a positively valenced
494 anticipation to be tickled. Like play, responses to tickling increased following a short break, with
495 tickling having a contagious effect on cage mate responses. However, rebound and contagion effects
496 were dependent on cohort, requiring augmented responses to tickling and a baseline level of play.
497 Overall, our results suggest that the effectiveness of tickling as a positive experience for rats may be
498 influenced by early life experience.

499

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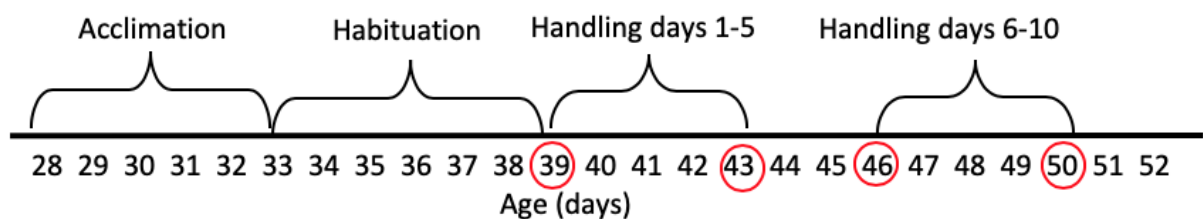
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Figure 1. Experimental timeline according to the age of the rats. Red circles indicate days from which data were collected and analysed.

724 **Table 1.** Ethogram describing play behaviour of pair-housed juvenile male Wistar rats (N =
725 64 split into 2 equal cohorts; aged between 39-50 days old) in the home cage 5 minutes prior
726 to experiencing either tickling or control handling.

Behaviour	Description
Solitary play	Seemingly spontaneous burst of motion involving at least two hops, where hops involve all four paws leaving the ground at the same time; can occur from stationary or during locomotor movement; not in the direction of a play partner during a play bout or as an evasion response to being chased by a play partner (adapted from Lampe et al., 2017)
Social play	One rat pounces or rubs on the partner, resulting in the partner either chasing the soliciting rat, rearing (in which pairs make rapid pawing movements at each other) or rotating to where one rat is pinned onto its back with the other standing over it (van Kerkhof et al., 2013)

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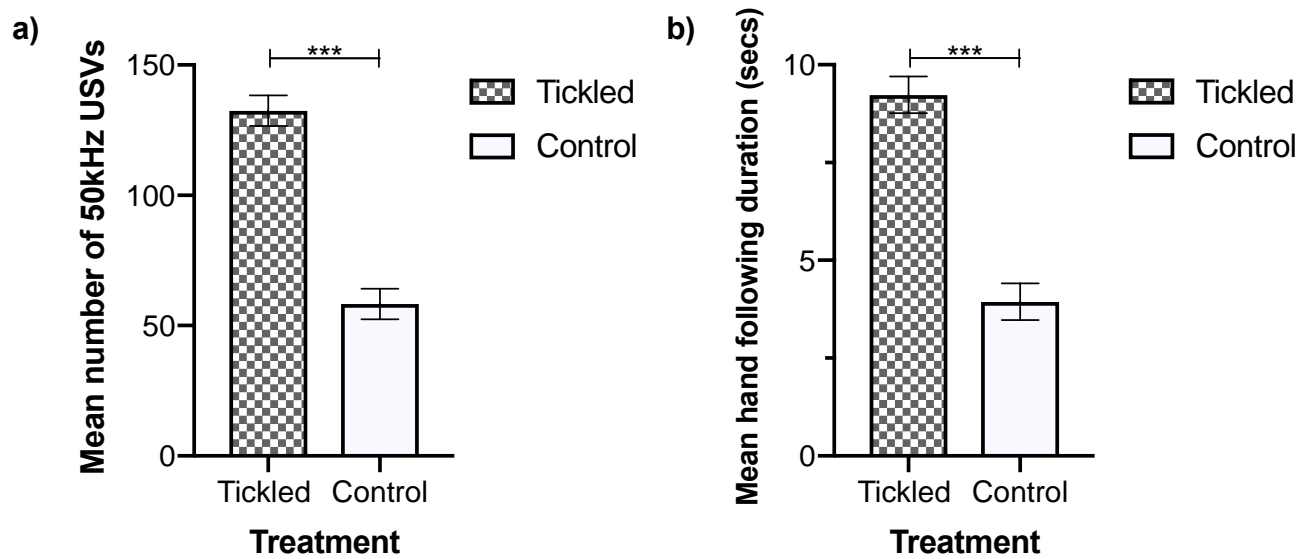
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737 **Figure 2.** Bar graphs showing the mean number of a) 50kHz USV production and the mean
 738 duration of b) hand following across two cohorts of juvenile male Wistar rats (N = 32 split
 739 into 2 equal cohorts; aged between 39-50 days old) when experiencing tickling or control
 740 handling. Tickled rats produced more 50kHz USVs and followed the experimenter's hand for
 741 a longer duration than control rats (USV production; tickled vs. controls; Mean \pm SED =
 742 132.45 vs. 58.31 \pm 5.91 and hand following duration; tickled vs. controls; Mean \pm SED =
 743 9.23 vs. 3.94 secs \pm 0.47). Data were analysed using a Generalised Linear Mixed Model in
 744 Genstat. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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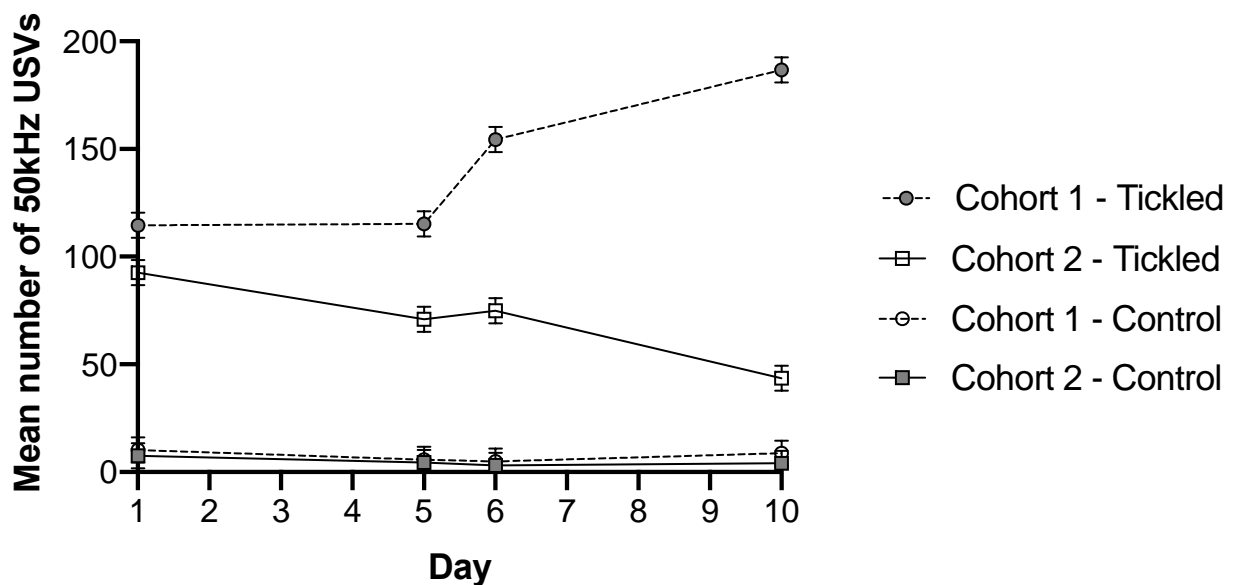
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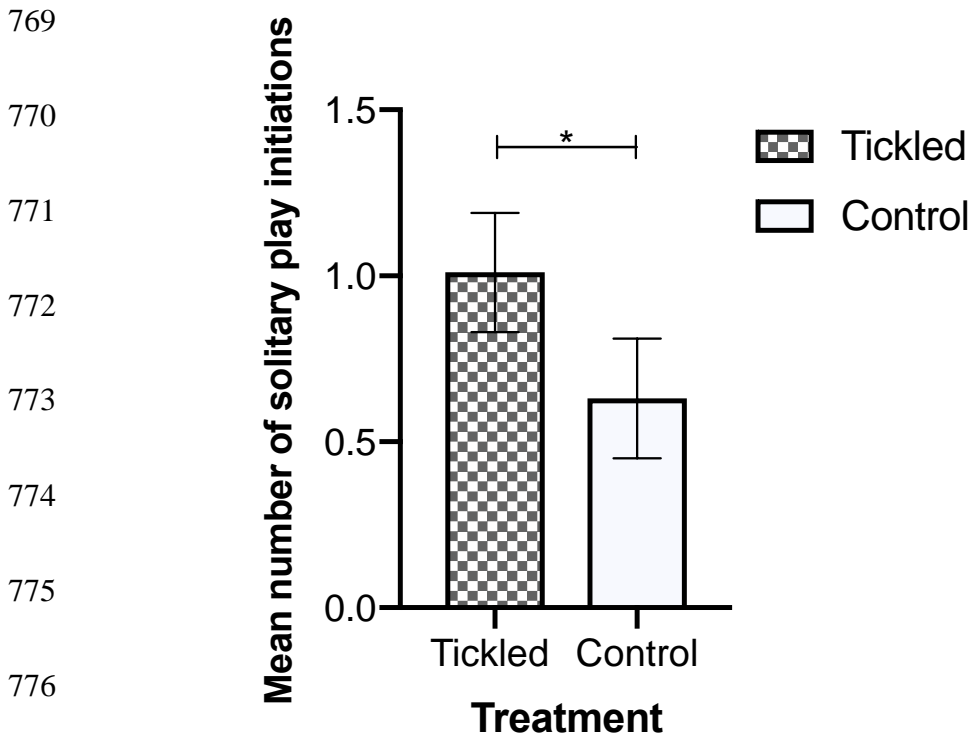
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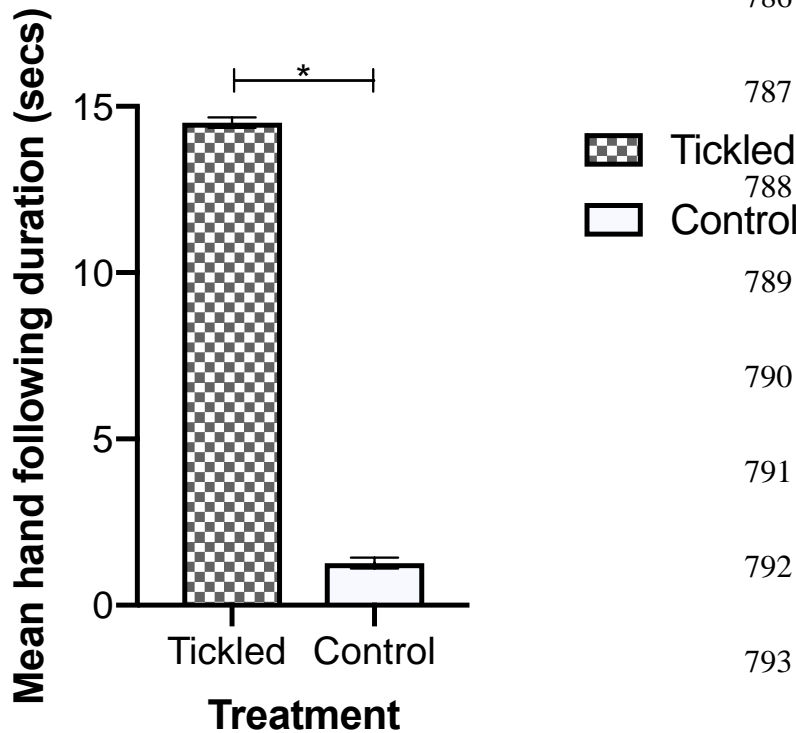
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756 **Figure 3.** The mean number of 50kHz USVs produced by two cohorts of juvenile male
757 Wistar rats (N = 32 split into 2 equal cohorts; aged between 39-50 days old) when
758 experiencing tickling or control handling across the 10-day experiment. Data were from the
759 first and fifth day of consecutive handling and following a two-day break from handling, data
760 were also collected from the sixth and tenth day. The legend indicates the symbols associated
761 with each cohort and treatment within cohort, with the top two lines showing 50kHz USV
762 production of tickled rats and the bottom two lines showing controls. 50kHz USV production
763 increased in cohort 1 (day 1 vs. day 10 $M \pm SEM = 114.6$ vs. 186.8 ± 5.9) and decreased in
764 cohort 2 (day 1 vs. day 10 $M \pm SEM = 92.5$ vs. 43.6 ± 5.9). In cohort 1 only, tickled rats
765 showed an increase in USV production on day 6 compared to day 5 (day 5 vs. day 6 tickled
766 rats $M \pm SED = 109.19$ vs. 146.33 ± 5.91): an indication of a rebound effect. Means and

767 standard errors are reported from analyses conducted using a Generalised Linear Mixed
768 Model in Genstat. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



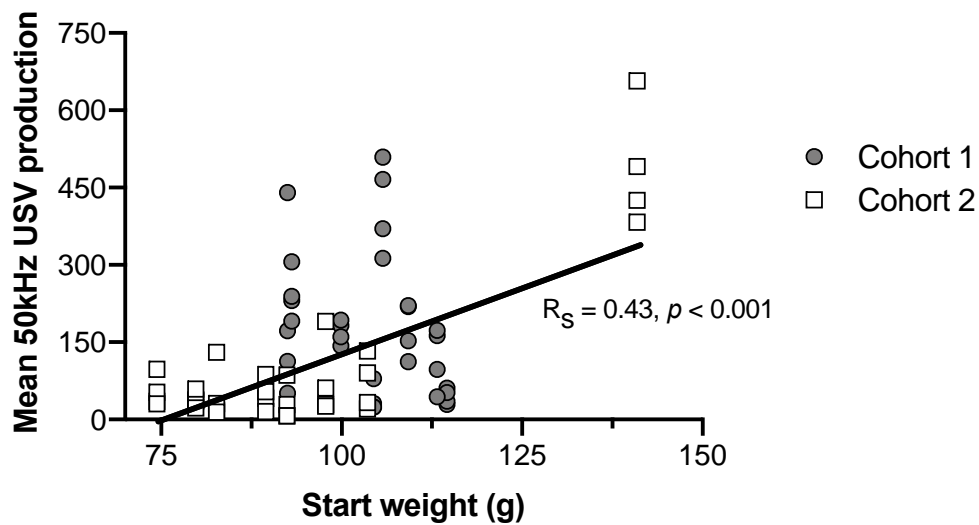
777 **Figure 4.** Bar graph showing the number of solitary play events by juvenile male Wistar rats
778 (N = 32 split into 2 equal cohorts; aged between 39-50 days old) in the home cage 5 minutes
779 prior to experiencing either tickling or control handling. Rats were pair housed with one rat
780 deemed as a handled rat and the other as an unhandled cage mate (total N = 64 split into
781 two equal cohorts). Only the handled rat play events are reported here. Solitary play
782 involved fast locomotor movement involving at least two hops; not in the direction of a play
783 partner. Tickled rats conducted more solitary play events than control rats (tickled vs.
784 controls; Mean \pm SED = 1.01 vs. 0.63 \pm 0.18). Data were analysed using a Generalised Linear
785 Mixed Model in Genstat. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



795 **Figure 5.** Hand following duration (secs) of cage mate juvenile male Wistar rats when placed
 796 the handling arena on day 10 (N = 32 split into 2 equal cohorts; aged between 39-50 days
 797 old). Rats were pair housed with one rat deemed as a handled rat and the other as an
 798 unhandled cage mate (total N = 64 split into 2 equal cohorts). Handled rats were either tickled
 799 or not-tickled (control). On the last day of the experiment, cage mates were placed into the
 800 arena and experienced the same conditions as control handled rats; for a total of 3 minutes,
 801 the experimenter's hand alternated each 20 seconds between resting motionless in the centre
 802 of the arena and moving in slow circles around the arena. Cage mates of tickled rats followed
 803 the hand for a longer duration than cage mates of control rats when the hand moved in slow
 804 circles around the arena (cage mates of tickled vs. cage mates of control rats; Mean \pm SED =
 805 14.51 vs. 1.27 secs \pm 0.16). Data were analysed using a Generalised Linear Mixed Model in
 806 Genstat. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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810 **Figure 6.** Relationship between start weight(g) and mean 50kHz USV produced across 4
811 days of tickling juvenile male Wistar rats (N = 32 split into 2 equal cohorts; aged between 39-
812 50 days old). Start weight was taken on the first day of handling (rats aged 39 days old).
813 50kHz USV production during tickling was recorded on first and fifth day of consecutive
814 handling and following a two-day break from handling, data were also collected from the
815 sixth and tenth day. Cohort 1 data points are filled grey circles and cohort 2 data points are in
816 clear filled squares. Data were analysed using Spearman's Rank Correlation in Minitab 17.
817 The line was fitted by Prism 8 (GraphPad) software using $R_s = 0.43$ as the slope.

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