

**Odour conditioning of positive affective states:  
Rats can learn to associate an odour with being tickled**

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28 **Abstract**

29 Most associative learning tests in rodents use negative stimuli, such as an electric shock. We  
30 investigated if young rats can learn to associate the presence of an odour with the experience of  
31 being tickled (i.e. using an experimenter's hand to mimic rough-and-tumble play), shown to elicit 50  
32 kHz ultrasonic vocalisations (USVs), which are indicative of positive affect. Male, pair-housed Wistar  
33 rats (N=24) were all exposed to two neutral odours (A and B) presented in a perforated container on  
34 alternate days in a test arena. Following 60s of exposure, the rats were either tickled on days when  
35 odour A (n=8) or odour B (n=8) was present, or never tickled (n=8). When tickled, rats produced  
36 significantly more 50 kHz USVs compared to the days when not being tickled, and compared to  
37 control rats. The level of anticipatory 50 kHz USVs in the 60s prior to tickling did not differ  
38 significantly between the tickled and control rats. Following the odour conditioning, rats were  
39 exposed successively in the same arena to three odours: an unknown neutral odour, extract of fox  
40 faeces, and either odours A or B. Compared to controls, 50 kHz USVs of tickled rats increased when  
41 exposed to the odour they had previously experienced when tickled, indicating that these rats had  
42 learned to associate the odour with the positive experience of being tickled. In a test with free access  
43 for 5 min to both arms of a T-maze, each containing one of the odours, rats tickled with odour A  
44 spent more time in the arm with this odour. This work is the first to test in a fully balanced design  
45 whether odours can be conditioned to tickling, and indicates that positive odour conditioning has  
46 potential to be used as an alternative to negative conditioning tests.

47  
48 **Key words:** conditioning, odours, positive affect, rats, tickling, ultrasonic vocalisation

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53 **Introduction**

54 Aversive conditioning, where a previously neutral stimulus or place is associated with an aversive  
55 stimulus, can be used to study memory and other brain functions in laboratory rodents (e.g. Ellis and  
56 Kesner, 1983). This paradigm is used in studies of learning, as odours can be associated with aversive  
57 states such as fear (Kroon and Carobrez, 2009; Moriceau et al., 2006) and malaise (Chapuis et al.,  
58 2007; Raineiki et al., 2009). The animals usually learn this association very quickly, making it a time-  
59 saving and efficient research method, which may be why only few attempts have been made to  
60 develop tests for this purpose using positive experiences. In studies where positive conditioning of  
61 odours has been applied, they involved pairing with psychostimulant drugs (Revillo et al., 2012;  
62 Caffrey and Febo, 2014; Lowen et al., 2015), alcohol (Deehan et al., 2012) or a food source (Shide and  
63 Blass, 1991; Sullivan et al., 2015; Torquet et al., 2014). However, using feed as the unconditioned  
64 stimulus is not always feasible in practice, and is likely to be associated with an increasing level of  
65 satiety.

66

67 We were therefore interested in finding an appropriate positive conditioning stimulus for use in an  
68 associative learning test for rats. This would ideally consist of a stimulus that was easy to use and  
69 which gave rise to the animal experiencing positive welfare (i.e. a positive affective state and not just  
70 absence of negative welfare; Lawrence et al., 2017). Despite the increasing interest in positive  
71 welfare indicators, the vast majority of animal welfare research has been and continues to be  
72 focused on more negative aspects (e.g. Boissy et al., 2007). One result of this is that there are few  
73 well-validated models of positive welfare in animals. One of the best candidates is the rat tickling  
74 model that was developed to mimic the effects of social play, a behaviour frequently displayed by  
75 young rats (see LaFollette et al. (2017) for a recent review). In this model, the human hand is used to  
76 mimic the tactile stimulation experienced during social play in rats. The model has been validated  
77 partly through the measurement of ultrasonic vocalisations (USVs) that rats produce under different  
78 emotional states. During tickling, rats produce many more frequency modulated USVs in the range of  
79 33-100 kHz (henceforth referred to as 50 kHz USVs); these are sometimes referred to as 'laughter'  
80 (Panksepp and Burgdorf, 2010), and have been shown to indicate a positive emotional state (Knutson  
81 et al., 2002). Tickled rats show shorter latencies to approach the human hand than controls, and  
82 express so-called optimistic biases when appraising environmental cues (Rygula et al., 2012). In  
83 addition, a number of pharmacological manipulations of rats using various psychotropes supports  
84 the notion that 50 kHz USVs are produced upon activation of the brain's reward pathways (Popik et  
85 al., 2014; Avvisati et al., 2016). Data thus support the interpretation that expressions of 50 kHz USVs  
86 indicate that tickling is a positive experience for the rat. However, USVs in the range of 22 kHz are  
87 emitted by rats under aversive situations (Tonoue et al., 1986; Blanchard et al., 1991; Choi and

88 Brown, 2003; Burgdorf et al., 2018). USVs have therefore been suggested to be a useful tool for  
89 inferring affective states of the rats (Burgdorf and Panksepp, 2006; Burgdorf et al., 2011; Wöhr and  
90 Schwarting, 2013; Barker, 2018).

91

92 In this paper, we present the results of an experiment which was designed to condition rats to  
93 associate the presence of an odour with the positive experience of tickling. We hypothesised that if  
94 rats learned to make the odour-tickling association, they would *i*) emit anticipatory USVs when  
95 exposed to the odour prior to being tickled, *ii*) emit more 50 kHz USVs than control rats when  
96 exposed to the conditioned odour following exposure to an aversive odour, and *iii*) would spend  
97 more time in the arm of a T-maze containing their tickling odour.

98

99

100 **Materials and Methods:**

101 Male Wistar rats (n=24) were used as subjects for odorant conditioning and subsequent behavioural  
102 testing. The rats were housed in pairs at 4 weeks of age in standard laboratory rodent cages (42.5 cm  
103 × 26.6 cm × 18.5 cm made from transparent polycarbonate; Techniplast 1291H) on a 4-tier rack. The  
104 lighting schedule of the room was inverse 12D:12L, with lights coming on at 19:00 hours. The cages  
105 had a metal grid lid with a dentation in which commercial rat pellets (Diet M25, Special Diet Services,  
106 Witham, Essex, United Kingdom) were placed for ad libitum access. Water was supplied via a drinking  
107 bottle with a metal spout, inverted and placed alongside the feed. The floor of the cage was covered  
108 by 2 cm of sawdust litter changed weekly, and wooden chew sticks (12 cm long) were supplied as  
109 enrichment. Individual rats were identified by marker pen lines on the tail. The rats were weighed  
110 once a week and, if needed, their tails were remarked.

111

112 The rats were handled daily by the same person, and all handling, conditioning and testing took place  
113 during the dark period. Over the course of 5 days, the rats were gradually habituated to being put  
114 into a transport box (identical to the home cage, but with no water and feed available), and  
115 transported within an opaque black sack to the conditioning room, which was illuminated by red  
116 incandescent bulbs. The rats were also habituated to the conditioning arena – initially in pairs and  
117 subsequently individually. The arena consisted of a Plexiglas tank (L x W x H: 66 cm x 41 cm x 41 cm),  
118 bedded with sawdust. At one end of the arena, a thin metal plate was fixed centrally at the bottom  
119 of the wall. During habituation, an empty stainless steel container (diameter 9.5 cm; height 3.7 cm;  
120 Grundtal IKEA) with a magnetic base and a screw-top lid with a perforated plastic inset was affixed  
121 vertically to the metal plate (Figure 1a).

122

123 In order to account for potential odour dependent effects, two different odours were used as the  
124 conditioning odours: **Odour A** (a 10% dilution of D-limonene; CAS no. 5989-27-5) and **Odour B** (a 5%  
125 dilution of 1-hexanol; CAS no. 111-27-3). Both were diluted in mineral oil (CAS no. 8042-47-5) at  
126 different concentrations to obtain similar levels of odour strength as assessed by the researchers,  
127 and 2 ml samples were transferred to a cotton pad in the container prior to the conditioning. All  
128 compounds were purchased from Sigma Aldrich (Saint-Quentin, Fallavier, France).

129

130 Each rat was allocated to one of three conditioning treatments, and all rats were placed in the arena  
131 on every conditioning day, where its conditioning treatment was applied: **Tickled A** rats were tickled  
132 when odour A was present in the container in the test arena; **Tickled B** rats were tickled when odour  
133 B was present in the container in the test arena; and **Control** rats were never tickled but still  
134 presented with either of the odours in the arena on alternate days (Figure 1b). When odour A was in  
135 the container, the Tickled B rats were treated as the Control rats, as were the A-tickled rats when  
136 odour B was in the container. Each pair of rats within a cage was randomly allocated to a treatment,  
137 whilst ensuring that rats housed on each tier of the rack received all three treatments. The order in  
138 which the rats were treated changed from one day to the next, starting with rats 1, 19, 13, and 7 on  
139 different days, respectively.

140

141 Conditioning began when the rats were 6 weeks of age. Only one of the two odours were used on  
142 each conditioning day to minimise the risk of cross-contamination. All conditioning sessions were  
143 video recorded (Sony 12.0 mega pixels HDR-XR-500 Handycam) and the USVs registered using a free-  
144 ware sound-recording programme (Audacity 2.1.3; [www.audacityteam.org](http://www.audacityteam.org)) via a USV sensitive  
145 microphone (M500-384, Pettersson Elektronik, Sweden) attached to the arena at an angle of 45°  
146 above the container (Figure 1a). A session began by moving the rat from its home cage in the  
147 transport box to the conditioning room and placing the rat in the arena 10 cm from and facing the  
148 container containing one of the two odours, A or B. The rat was left there for 1 min, and the rat was  
149 subsequently either **Tickled** (Tickled A rats on days when odour A was present, Tickled B rats on days  
150 when odour B was present) or **Not Tickled** (Control rats on all days, Tickled A rats when odour B was  
151 present, and Tickled B rats when odours A was present; Figure 1b) according to the following  
152 procedures:

153

154 **Tickled** consisted of the handler using one hand wearing a knitted glove to touch, tickle, and play  
155 with the rat for 20 second periods interspersed with 20 second pauses. During the active periods, the  
156 handler mimicked the rough-and-tumble play seen in adolescent rats, with the hand tickling, chasing  
157 and pinning the rat, depending on its response (Figure 1a.i). After 20 seconds the hand was placed

158 flat on the inside of the wall of the arena (Figure 1a.ii). It rested here for 20 seconds after which  
159 another tickling period was carried out. This was repeated for a total duration of 140 s, allowing 4  
160 periods of tickling interspersed with 3 periods of pauses. At the end of the tickling, the rat was  
161 moved in the transport box to a holding cage in a room separate from their home cage. The holding  
162 cage was identical to the home cage of the rat, placed in a similar 4-tier rack in the same position,  
163 and one holding cage was used for each pair of rats. The tickled rats were left in the holding cage for  
164 3-7 hours (depending on the test order of the day) to prevent emotional contagion by USVs of the  
165 yet un-tested rats in the home cages.

166

167 **Not Tickled** consisted of the handler placing the hand wearing a knitted glove flat on the inside of the  
168 wall of the arena. It rested here for 20 seconds after which the hand was moved to the adjacent wall  
169 for 20 seconds. This was repeated for a total duration of 140 s, allowing 4 and 3 periods, respectively,  
170 with the hand resting on each wall, the latter being identical to the pauses when the rats were being  
171 Tickled. When the Not Tickled procedure finished, the rat was moved in the transport box back to its  
172 home cage.

173

174 LaFollette et al. (2018) found that three tickling sessions sufficed to bring about a higher rate of 50  
175 kHz USVs in tickled rats, and in a pilot study using only one odour, we found a significant difference in  
176 50 kHz USVs emitted between tickled and control rats after four tickling days (Lam, 2017).  
177 Nevertheless, as two odours were used alternately in the present experiment, we chose to carry out  
178 a total of ten conditioning days, alternating between odours A and B (Figure 1b). This resulted in all  
179 tickled rats being exposed to the Tickled procedure five times and the Not Tickled procedure five  
180 times, and all the rats were exposed to both odours for the same amount of time over the course of  
181 the ten days, including the Control rats.

182

183 For subsequent data analyses, the 50 kHz USVs for each rat were counted for the first (days 1 and 2)  
184 and the fifth (days 9 and 10) conditioning sessions, using the method described by Brenes and  
185 Schwarting (2014). These counts were divided into 50 kHz USVs emitted during the four tickling  
186 periods (4 x 20s) and the three pauses (3 x 20s), as well as the 1-min habituation period prior to  
187 tickling to detect any differences in potential anticipatory USVs. This grouping of 50 kHz USVs was  
188 also done when the Not Tickled procedure was applied (Figure 1b). All counts were converted into  
189 USVs/min. Occurrences of 22 kHz USVs were rare, and not included in the data.

190

191 The behaviour of the rats was logged from the video recordings. This was done for the three 20s  
192 pauses only, as the hand did not move and the behaviour of the rats at this time was therefore

193 comparable within and among all rats and across treatments and procedures. The recorded  
194 behaviour consisted of hand seeking behaviour (the rat rears to sniff the motionless hand, see Figure  
195 1a.ii, or is facing and focusing on the hand), play jumping (jumping while running), sniffing the air,  
196 exploring the odour container, freezing (immobility, often sudden, with ears raised and eyes open)  
197 and other behaviour (locomotion, digging the litter, and self-grooming).

198

199 *Behavioural tests of conditioning*

200 On the two days following the last conditioning session, two behavioural tests were carried out to  
201 investigate the effects of the conditioning:

202

203 **T-maze test:** Without prior habituation to the T-maze, the rat was placed at one end of a large T-  
204 maze arena, which consisted of a rectangular open space (77 cm x 51 cm) with two accessible arms  
205 (WxL: 19 cm x 25 cm) extending from each side at one end of the rectangle, forming a broad T-shape.  
206 A ventilator fitted centrally at the other end of the rectangle extracted air from the T-maze, ensuring  
207 a simultaneous airflow from both arms. No litter was used, and a perforated metal tea-ball was  
208 placed in a pre-drilled hole at the end wall of each arm of the maze. The two tea-balls each contained  
209 a cotton pad imbibed with 2 ml of either odour A or B, with one odour in each arm, alternating  
210 between arms in a balanced way for each rat being tested. The test was video recorded from above.  
211 The rat was left in the arena for 3 min, and was free to explore both arms and the central arena.

212

213 **Triple Odour test:** The rat was placed in the same arena as used for the conditioning. The tests  
214 consisted of 30-sec periods with no odour container present in the arena, interspersed with three 1-  
215 min periods, where a container was positioned containing the following odours in said order: 1) a  
216 neutral odour unknown to the rat (**Novel odour**; a 5 % suspension of p-anisaldehyde, CAS no. 123-11-  
217 5, in mineral oil), which was assumed to have no aversive or attractive properties for the rats; 2) an  
218 extract in mineral oil of fox faeces (**Fox odour**; faecal pellets originating from several male foxes and  
219 soaked in mineral oil for 24h at 70°C, with extract diluted 1:6); this odour was expected to induce a  
220 level of fear in the rats, and 3) the **Tickling odour** with which the Tickled rats had been conditioned,  
221 and with half of the Control rats being exposed to odour A and the other half to odour B. The order  
222 of the three odours were chosen so as to measure the response of the rats to first an unknown, but  
223 neutral odour, then an unknown but fear-inducing odour, followed by the known conditioning odour.  
224 We hypothesised that if the Tickled rats had learned to associate their tickling odour with a positive  
225 experience, more 50 kHz USVs would be emitted by the Tickled rats compared to the Control group  
226 when exposed to the conditioning odour, the latter having been exposed to the odour for the same  
227 amount of time during conditioning but without being tickled. The tests were video recorded and the

228 amount of freezing displayed by the rats during exposure to the three different odours was scored.  
229 The USVs were registered in the same way as for the conditioning sessions.

230

231 *Statistical data analysis*

232 Data were analysed in MiniTab (ver. 17.1) using General Linear Models followed by post-hoc Tukey  
233 comparisons of significant effects. For the USVs emitted during conditioning, data from the four  
234 tickling periods were analysed fitting odour, treatment, and session no. with interaction. When  
235 relevant, Pearson's correlations were calculated. Anticipatory USVs were analysed for session 5 only,  
236 fitting odour, treatment and their interaction. Behaviour during pauses, expressed as percentage of  
237 time spent on each behaviour, was analysed by the non-parametric Kruskal-Wallis test, as data were  
238 not normally distributed.

239

240 **Results**

241 All tickled rats emitted significantly more 50 kHz USVs during the sessions with the Tickled procedure  
242 than during the sessions when Not Tickled ( $F_{2,89}=31.3$ ;  $P<0.001$ ), with the latter not differing in  
243 magnitude from that of the never tickled Control rats (Figure 2a). This was evident already during the  
244 very first tickling session, but with significantly more 50 kHz USVs emitted during the 5<sup>th</sup> compared to  
245 the 1<sup>st</sup> tickling session (233 vs 83 ( $\pm 8.4$ ) USVs/min;  $P < 0.001$ ), and these were significantly correlated  
246 (Pearson's  $r=0.53$ ;  $P = 0.036$ ), indicating that response level of 50 kHz USVs to tickling is a  
247 characteristic of the individual rat. No differences were found between rats tickled with Odours A  
248 and B, respectively. On days when the tickled rats were Not Tickled (i.e. A-tickled rats when odour B  
249 was present and vice versa), the 50 kHz USVs emitted per minute by the 5<sup>th</sup> tickling session did not  
250 differ significantly from the level observed during tickling in the 1<sup>st</sup> session, indicating a degree of  
251 place association had developed (Figure 2a).

252

253 Most rats also emitted 50 kHz USVs during the pauses between tickling periods. When rats were  
254 tickled, although numerically greater, the USVs during pauses did not differ significantly from those  
255 observed during the tickling in the 1<sup>st</sup> session (108 vs 83 ( $\pm 11.1$ ) USVs/min;  $P = 0.390$ ), whereas by the  
256 5<sup>th</sup> session, significantly more 50 kHz USVs were emitted during tickling than during the pauses (151  
257 vs 233 ( $\pm 11.1$ ) USVs/min for pauses and tickling periods, respectively;  $P < 0.001$ ; Figure 2b). For the  
258 Not Tickled rats, including Controls, the levels of 50 kHz USVs were similar between tickling periods  
259 and pauses because no actual tickling took place. When rats were tickled, 50 kHz USVs emitted  
260 during pauses and during tickling were correlated for the 1<sup>st</sup> (Pearson's  $r=0.76$ ;  $P = 0.001$ ) but not the  
261 5<sup>th</sup> session (Pearson's  $r=0.02$ ;  $P = 0.955$ ). Medians of the behaviour during pauses in the 5<sup>th</sup> session  
262 are shown in Table 1, with tickled rats showing significantly more hand seeking behaviour and play

263 jumping, with consequently less time spent in general locomotion, than when Not Tickled or  
264 compared to Control rats.

265

266 Even after five tickling sessions, rats did not emit more anticipatory USVs when exposed to their  
267 tickling odour, compared to when exposed to their non-tickling odour and compared to the control  
268 group ( $F_{2,42}=0.33$ ;  $P=0.718$ ; Figure 3). Figure 4 shows the frequency of 50 kHz USVs emitted by the  
269 rats as a function of their anticipatory 50 kHz USVs. The higher level of USVs during tickling is clearly  
270 visible, but with no clear correlation with anticipatory USVs for the tickled rats. However, for the  
271 sessions without tickling (Not Tickled and Control rats), the 50 kHz USVs emitted show a positive  
272 relationship with the 50 kHz USVs emitted during the pre-session (anticipatory) minute ( $R^2=53.1\%$ ;  
273  $T=4.99$ ;  $P < 0.001$ ; Figure 4), supporting the previous finding that rats may be characterised according  
274 to their level of vocalisation.

275

276 USVs emitted during the Triple Odour test are shown in Figure 5. During the first minute, where no  
277 odour was present, the frequencies of 50 kHz USVs were no different from those seen during the  
278 anticipatory period during odour condition (see Figure 3). Over the course of the Triple odour test,  
279 USV frequency decreased gradually, but a significant increase in 50 kHz USVs from the preceding  
280 pause was found for the tickled rats when exposed to their tickling odour (increase: 19, 31 and  $-3$   
281 ( $\pm 5.9$ ) USVs/min for Tickled A, Tickled B, and Control rats, respectively;  $F_{2,21}=8.7$ ;  $P = 0.002$ ), with the  
282 increase being significantly different for both A-tickled ( $P=0.032$ ) and B-tickled rats ( $P=0.001$ ) from  
283 that of the controls. The rats thus increase their USV frequency when their conditioning odour was  
284 presented indicating that the rats had learned to associate an odour with the positive experience of  
285 tickling.

286

287 Freezing was scored during the Triple Odour test, as exposure to fox odour was expected to induce  
288 more freezing as an indicator of fear. However, as shown in Figure 6, freezing did not increase when  
289 fox odour was in the arena. The Tickled rats showed the same low level of freezing throughout the  
290 test, independent of odour present. The Control rats, however, showed a significant increase in  
291 freezing when one of the conditioned odours were present, and this was mainly due to greatly  
292 elevated levels of freezing in the Control rats ( $n=4$ ) exposed to odour A ( $F_{3,19}=9.0$ ;  $P=0.001$ ; Figure 6).

293

294 Behaviour during the T-maze test showed that overall, more time was spent in the arm with odour A  
295 (Figure 7). This was significantly different from time spent in the arm with odour B for the Tickled A  
296 rats ( $F_{1,14}=5.0$ ;  $P = 0.041$ ) with a similar tendency for Control rats ( $P = 0.088$ ). The proportion of time

297 spent in the arm with Odour A was significantly different from chance (0.5) only for the rats tickled  
298 with odour A (0.60;  $T=2.51$ ;  $P = 0.041$ ).

299

300 **General discussion**

301 Using an appetitive conditioning method, we aimed to condition rats to learn to associate the  
302 presence of an odour with the positive experience of tickling. Our first hypothesis was that rats,  
303 which had learned to make the odour-tickling association would emit more anticipatory USVs when  
304 exposed to the odour prior to being tickled. This was not the case, as no differences in anticipatory  
305 USVs were found between the treatment groups. Indeed, our use of the term anticipatory can be  
306 questioned, given the findings. Heyse et al. (2015) found that rats would emit 50 kHz calls in  
307 anticipation of access to a running wheel. Others have found that individual rats vocalize more in a  
308 chamber associated with play than in a habituated control chamber (Knutson et al., 1998), indicative  
309 of anticipation of positive experiences. The absence of similar anticipatory vocalisations in the  
310 present experiment would indicate that, with respect to our first hypothesis, the conditioning  
311 paradigm was not successful. However, the levels of USVs emitted during the (anticipatory) pre-  
312 session minute appeared to predict the overall level of vocalisation for individual rats when these  
313 were not tickled, suggesting that rats can be categorised according to their USV frequency  
314 independent of any tickling occurring. This is in accordance with Burgdorf et al. (2005, 2013), who  
315 divergently selected rats based on their 50 kHz vocalisations. It has previously been found that  
316 tickling-induced 50 kHz ultrasonic vocalizations are individually stable and can predict behaviour in  
317 tests of anxiety and depression in rats (Schwarting et al. 2007; Mällo et al., 2007). Our finding that  
318 USVs produced when not being tickled show large inter-individual differences, but little intra-  
319 individual variation, is complementary to these results.

320

321 The second hypothesis was that more USVs would be emitted by the tickled than by control rats  
322 when exposed to the conditioned odour following exposure to an aversive odour. We found an  
323 increase in USVs produced by the tickled rats when their tickling odour was placed in the arena.  
324 Given that 50 kHz USVs are indicative of positive affect (e.g. Burgdorf and Panksepp, 2006), this  
325 would indicate that the tickled rats had learned to associate the odour with a positive experience.  
326 Ideally, we would have tested the conditioned rats with both odours, but the small number of  
327 animals made this statistically inappropriate. However, the increase in 50 kHz USVs by the Tickled A  
328 and Tickled B rats when exposed to their conditioned odour in the Triple Odour test was not simply  
329 because the odour was known compared to the two previous odours, as the Control rats showed no  
330 such increase in USV production. It was noted that exposure to the fox odour did not provoke neither

331 22 kHz vocalisations, nor freezing behaviour in the rats, indicating that this odour was less aversive  
332 than anticipated.

333

334 We also expected tickled rats to spend more time in the arm of a T-maze containing their tickling  
335 odour. This was found only for rats tickled in the presence of odour A. However, control rats  
336 appeared to be more attracted to odour A, and for the rats tickled while exposed to odour B, this  
337 preference for odour A was not evident. This could be an indication that they had developed an  
338 attraction to odour B, which was sufficiently strong to eliminate a potentially intrinsic preference for  
339 odour A. This corresponds to the findings from the Triple Odour test, where odour B appeared to  
340 have a stronger effect than odour A (see Figure 5). Control rats also showed more freezing when  
341 exposed to odour A in the Triple Odour test. Although freezing is often considered an indication of  
342 fear, the behaviour is but a display of increased alertness, and the interpretation is context specific.  
343 Exposure to oestrus odours can elicit freezing in rats (Nielsen et al., 2013, 2019) and this is enhanced  
344 by sexual experience (Nielsen et al., 2016). It may be that odour A induced freezing in control rats  
345 because they find it more interesting, as shown by their un-conditioned preference in the T-maze  
346 test. The two odours were chosen as being neutral to rats (Devore et al., 2013), and we struggle to  
347 explain why they affect the behaviour of the rats differently. One, speculative possibility is a  
348 potential sedative effect of inhaling limonene resulting in decreased locomotor activity, which has  
349 been found in mice (Carvalho-Freitas and Costa, 2002; Wolffebüttel et al., 2018), but no differences  
350 in activity of the rats were found among treatments during odour conditioning.

351

352 As mentioned in the introduction, aversive conditioning is a widely used technique in learning studies  
353 of memory and other brain functions in laboratory rodents (e.g. Ellis and Kesner, 1983; Chapuis et al.,  
354 2007; Raineiki et al., 2009). Pairing aversive stimuli such as electric shocks, with a neutral stimulus or  
355 situation usually give rise to associations learned within a few sessions (Kroon and Carobrez, 2009).  
356 In contrast, the application of appetitive conditioning regimes often require more pairing sessions to  
357 become effective. Although our positive conditioning was successful, as shown by the response of  
358 the rats to their conditioning odour in the Triple Odour test, this did not appear to be a very strong  
359 association, as no increase in anticipatory vocalisation was seen, nor a very convincing preference for  
360 the tickling odour in the T-maze test. Others have also struggled to demonstrate a link between  
361 increased 50 kHz USVs and reward-related stimuli: Brenes and Schwarting (2014) conditioned rats to  
362 associate a tone with a food reward, and measured the expression of reward anticipation as  
363 increases in USVs. However, when the rats were food-deprived, they showed only behavioural but  
364 not vocal anticipation and when sated, the reward cue continued to elicit 50 kHz USVs despite being  
365 devalued by pre-feeding. These findings may, in part, have been due to the large inter-individual

366 variability among rats, giving rise to different types of responders (Brenes and Schwarting, 2015). It is  
367 evident from these and the present results that appetitive conditioning is likely to be more complex  
368 and less effective than most aversive conditioning.

369  
370 One protocol of rat tickling has been described in detail by Cloutier et al. (2018). The benefit of this is  
371 that it allows comparisons to be made if the same method is used across experiments. However, we  
372 did not standardise the tickling method used in the present experiment, over and above the fixed  
373 alternating periods of tickling and pauses. This was a conscious choice on our part, as we had  
374 previously found a large individual variation in the response of the rats to tickling. Our experience  
375 indicated that this variation was reduced if the rats were tickled and played with whilst allowing the  
376 hand to react to the behavioural responses of the individual rat. In addition, as tickling is a playful  
377 experience, it should be varied and unpredictable to the rats. Although the lack of standardisation  
378 prevented us from comparing behaviour of the rats during the active tickling period, i.e. as the  
379 behaviour of the experimenter varied slightly across rats and across sessions, we were able to use  
380 the hand-seeking behaviour during the pauses to assess the likability of tickling for each rat. Tickled  
381 rats showed more hand seeking behaviour and play jumping with simultaneously more 50 kHz USVs  
382 emitted during the pauses between tickling, indicating that the tickling lead to a positive affective  
383 state.

384  
385 In conclusion, rats learned to associate an odour with the positive experience of being tickled, as they  
386 increased their 50 kHz USVs when exposed to this odour in a test situation without tickling,  
387 compared to control rats that had been exposed to the same odour for the same amount of time  
388 without being tickled. However, no increase was seen in anticipatory USVs when exposed to the  
389 conditioning odour prior to being tickled, and only one of the conditioning odours gave rise to a  
390 preference by the tickled rats in a T-maze test. These findings indicate that rats can learn to associate  
391 an odour with the positive experience of tickling, and positive odour conditioning may thus have  
392 potential to be developed further with a view to replacing negative odour conditioning tests.  
393 However, different odours may differ in their efficacy, and appetitive (positive) conditioning is clearly  
394 more difficult and slower to induce than aversive (negative) conditioning.

395  
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401

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526

527 **Table 1.** Median percentage of time spent in different behaviours by rats during pauses between  
528 tickling\* periods in the 5<sup>th</sup> conditioning session.

Behaviour (%)	Control rats	Not Tickled rats	Tickled rats	P ≤
Sniffing the air	34.2	30.2	28.3	0.198
Locomotion	29.2	32.5	<b>12.5</b>	<b>0.001</b>
Hand seeking behaviour	12.5	18.5	<b>35.0</b>	<b>0.001</b>
Exploring odour container	3.3	3.3	1.67	0.839
Play jumping	0.0	0.8	<b>10.8</b>	<b>0.001</b>
Freezing	0.0	1.7	4.2	0.168
Digging the litter	0.8	2.5	0.0	0.057
Self-grooming	0.8	0.0	0.0	0.060

529 \* Control rats were never tickled, and data for Not Tickled rats are from the session with the non-  
530 conditioning odour, when the Tickled rats were subjected to the Not Tickled procedure. Within rows,  
531 a median in bold font differ significantly from the other medians.

532

533

534

535 **Figure headings**

536

537 **Figure 1. a)** Screen-shots from the video recording of an odour conditioning session, showing **i)** the  
538 rat being tickled, and **ii)** the position of the hand flat against the arena wall during the pauses  
539 between tickling periods. The container with the odour source can be seen in the left of the pictures.  
540 The pictures have a green hue as the procedure was carried out under red lighting; **b)** Diagram of the  
541 odour conditioning schedule over days and within treatment. Control rats were never tickled and A-  
542 tickled rats were tickled when odour A was present in the container, and B-tickled rats were tickled  
543 when odour B was present in the container in the test arena. When odour A was in the container, the  
544 B-tickled rats were treated as the Control rats, as was the A-tickled rats when odour B was in the  
545 container.

546

547 **Figure 2a.** Data plot of 50 kHz ultrasonic vocalisations (USVs) per minute for individual rats during the  
548 1<sup>st</sup> and 5<sup>th</sup> sessions in the periods when tickling occurred for the Tickled rats; the Control rats and the  
549 Not Tickled rats were not tickled during these periods (red circles: Odour A; blue circles: Odour B).  
550 Means ( $\pm$  s.e.) are indicated with black plus symbols for each grouping, and different superscripts  
551 indicate significant difference ( $P < 0.001$ ).

552

553 **Figure 2b.** Data of 50 kHz ultrasonic vocalisations (USVs) per minute for individual rats during the  
554 pauses plotted against 50 kHz USVs per minute in the periods when tickling occurred for the Tickled  
555 rats for the **i)** 1<sup>st</sup> and **ii)** 5<sup>th</sup> sessions; the Control rats and the Not Tickled rats were not tickled during  
556 these periods. The dashed lines indicate where  $Y=X$ .

557

558 **Figure 3.** Data plot of 50 kHz ultrasonic vocalisations (USVs) per minute for individual rats during the  
559 5<sup>th</sup> session from the 60 s period (anticipatory) prior to tickling; the Control rats and the Non-tickled  
560 rats were not tickled during the sessions that followed (red circles: Odour A; blue circles: Odour B).  
561 Means ( $\pm$  s.e.) are indicated with black plus symbols for each grouping, and the y-axis scale is  
562 comparable to Figure 2a.

563

564 **Figure 4.** Data plot of 50 kHz ultrasonic vocalisations (USVs) per minute during the 5<sup>th</sup> session in the  
565 periods when tickling occurred for the Tickled rats plotted against the USVs per minute during the  
566 60-s period (anticipatory) prior to tickling. Each data point is an individual rat, with Control rats  
567 plotted for both Odours A and B. The regression equation, where data from Tickled rats have been  
568 excluded, is  $Y = 1 + 0.78X$  ( $R^2=58\%$ ;  $P < 0.001$ ).

569

570 **Figure 5.** Mean number of 50 kHz ultrasonic vocalisations (USVs  $\pm$  s.e.) per minute during the Triple  
571 Odour test for Tickled A, Tickled B, and Control rats. Tickled rats were exposed to their conditioning  
572 odour as the third odour, and for the Control rats, half were exposed to Odour A and half to Odour B.  
573 Asterisks indicate a significant increase in USVs (\* P = 0.032; \*\*\* P = 0.001).

574

575 **Figure 6.** Mean duration (s  $\pm$  s.e.) of freezing behaviour during exposure to the three odours in the  
576 Triple Odour test. Bars indicate the mean for each treatment group (n=8). For the third odour  
577 exposure (Odour A or B), the means for Control rats are also shown separately for rats exposed to  
578 Odour A (Con A; n=4) and to Odour B (Con B; n=4).

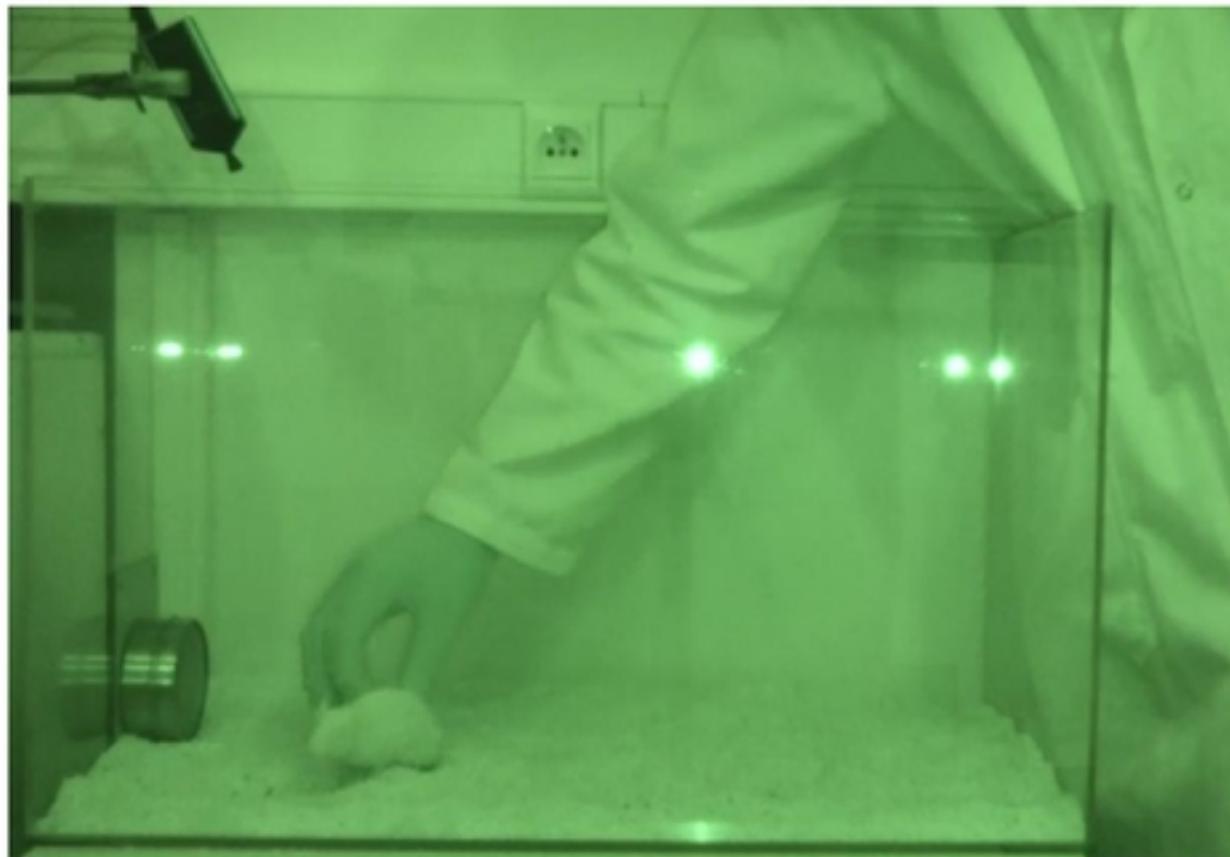
579

580 **Figure 7.** Time (s; mean  $\pm$  s.e.) spent in each arm of the T-maze for Tickled and Control rats. Asterisk  
581 indicate a significant difference between arms within treatment group (\* P = 0.041). Photo shows a  
582 screen-shots from the video recording of a test, with the rat in one of the two arms. Each arm was  
583 fitted with a tea-ball containing one of the two odours. The ventilator drawing air from both arms  
584 into the main area can be seen at the bottom of the photo.

585

Figure 1a

i)



ii)

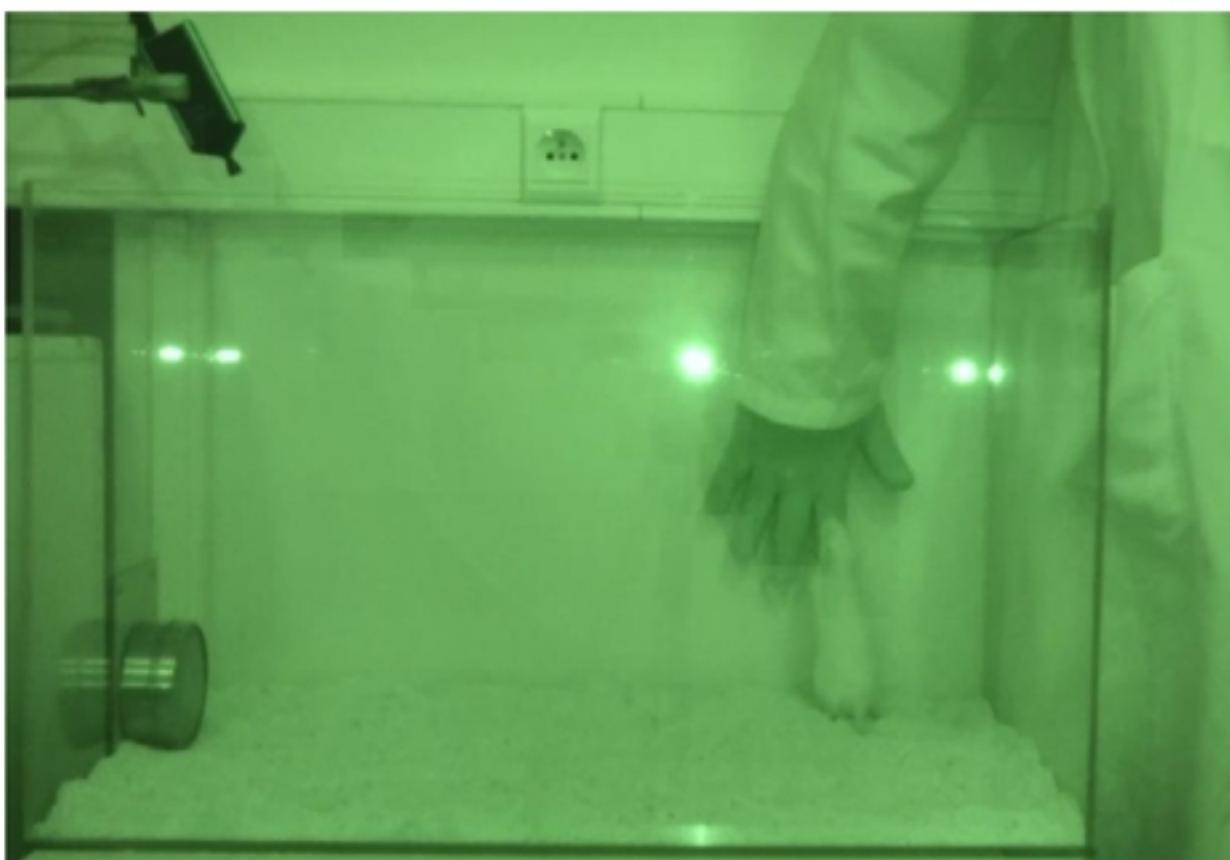


Figure 1b

<b>V</b> = Tickled (T) <b>X</b> = Not Tickled (NT)	Day 1	Day 2	...	Day 9	Day 10
Odour:	A	B	...	A	B
Control (n=8):	X	X		X	X
Tickled with A (n=8):	V	X	...	V	X
Tickled with B (n=8):	X	V		X	V
	Session 1		...	Session 5	
Odour conditioning treatment schedule:					
Anticipatory	T or NT	Pause	T or NT	Pause	T or NT
60 s	20 s	20 s	20 s	20 s	20 s

Figure 2a

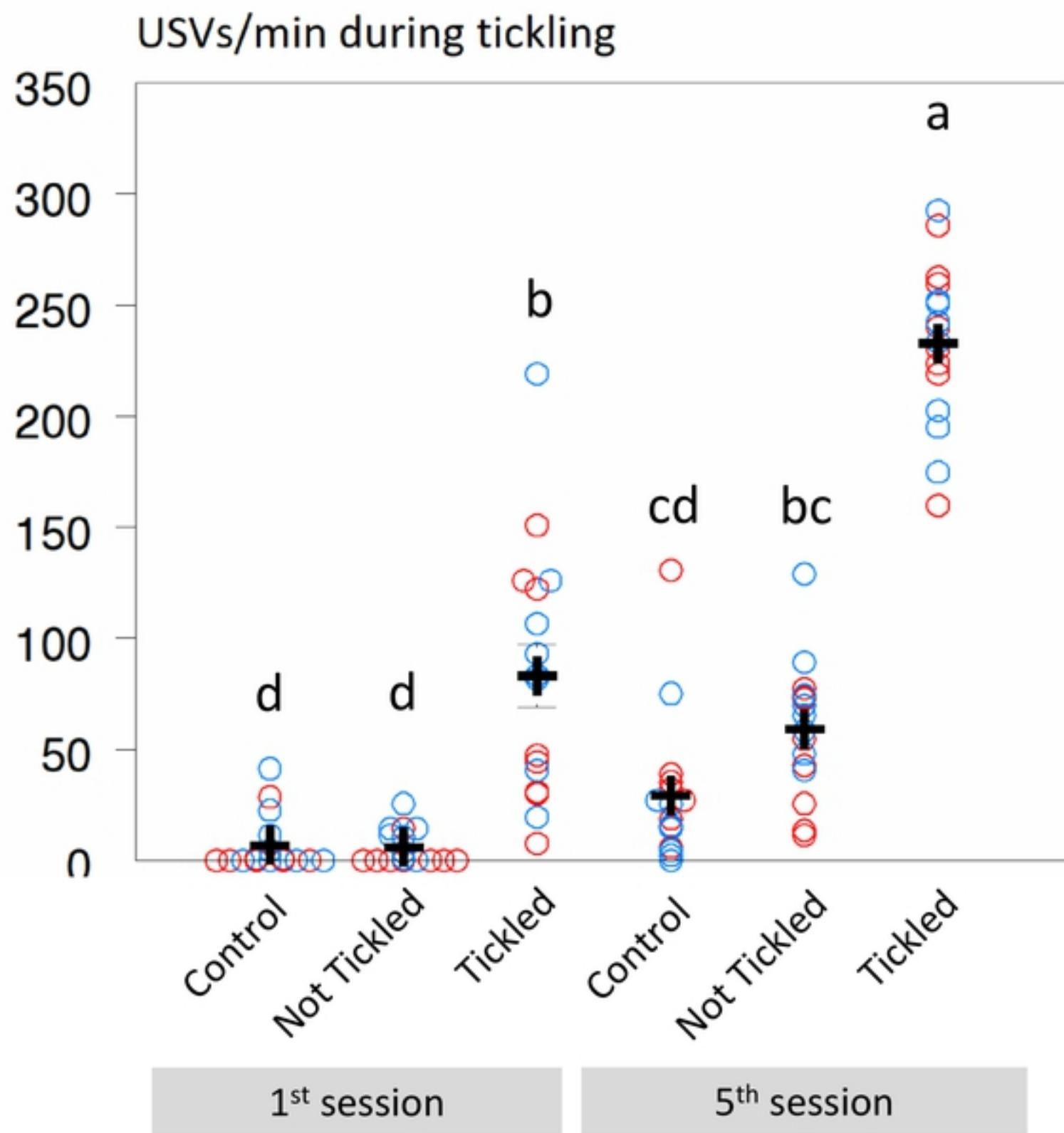


Figure 2b

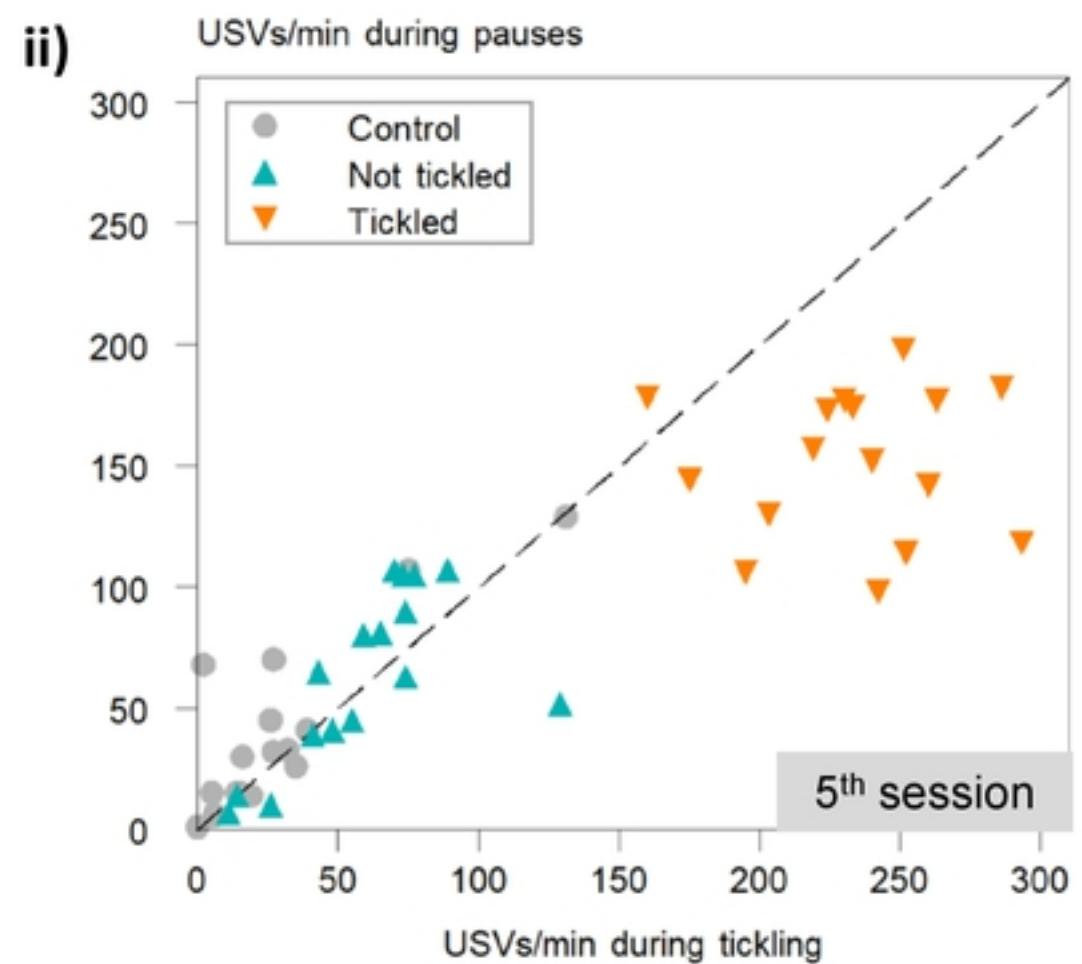
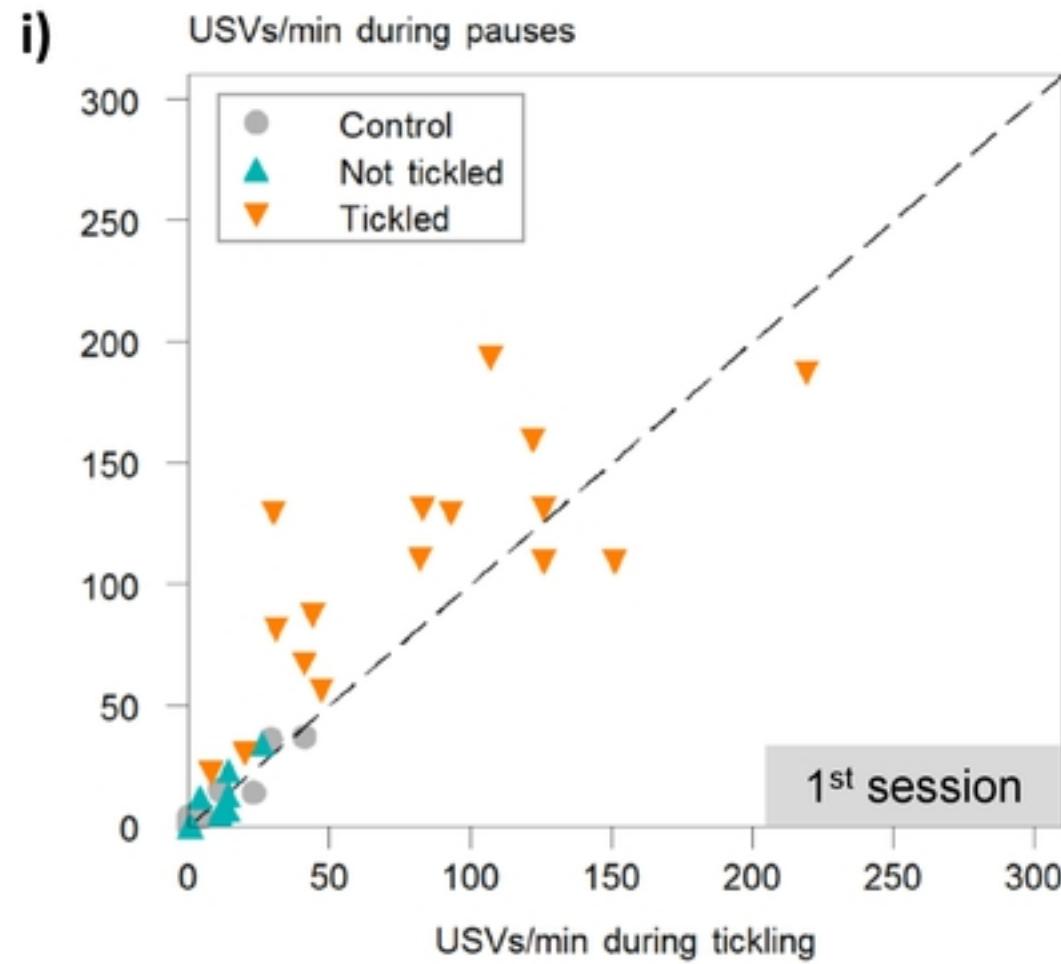


Figure 3

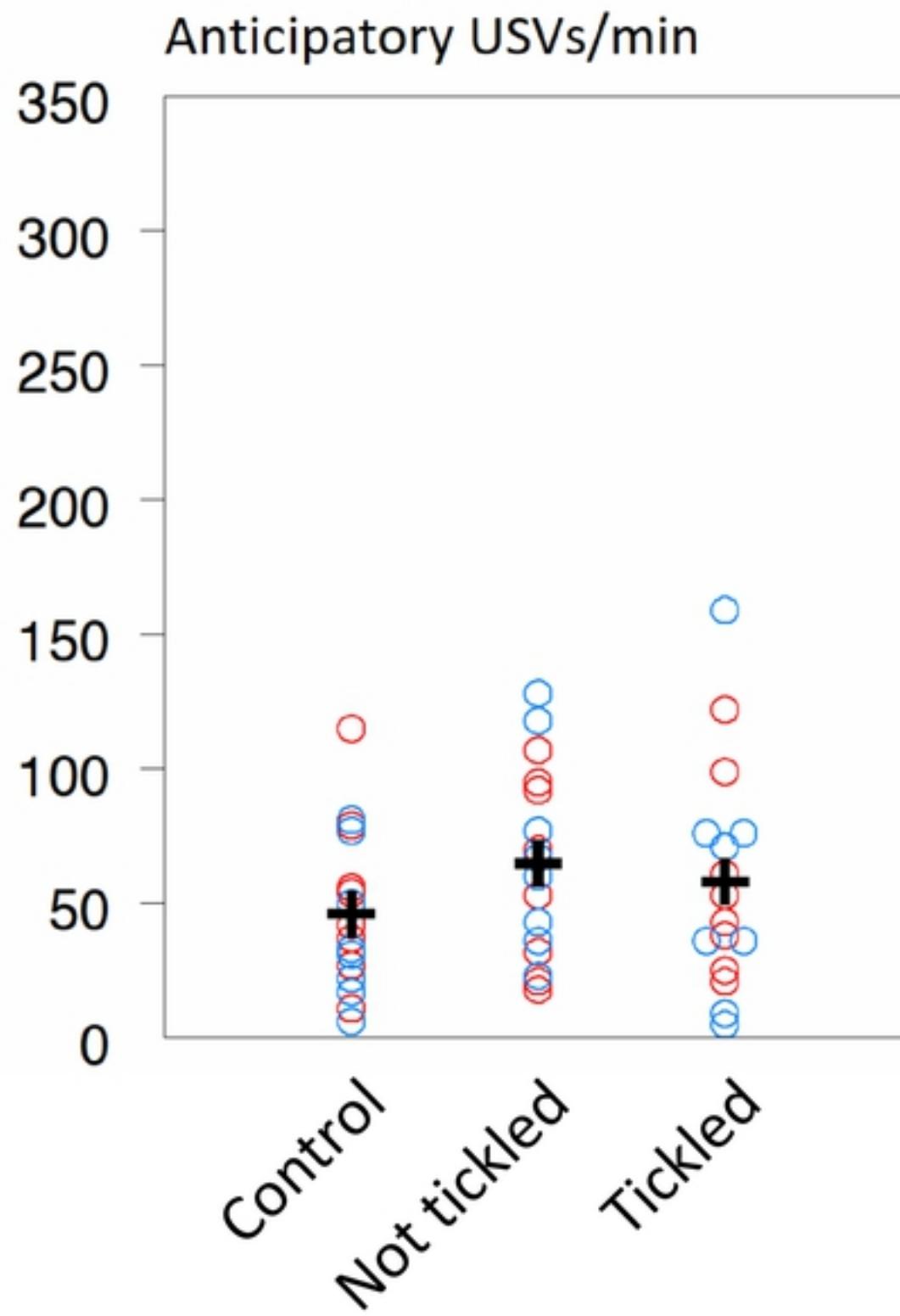


Figure 4

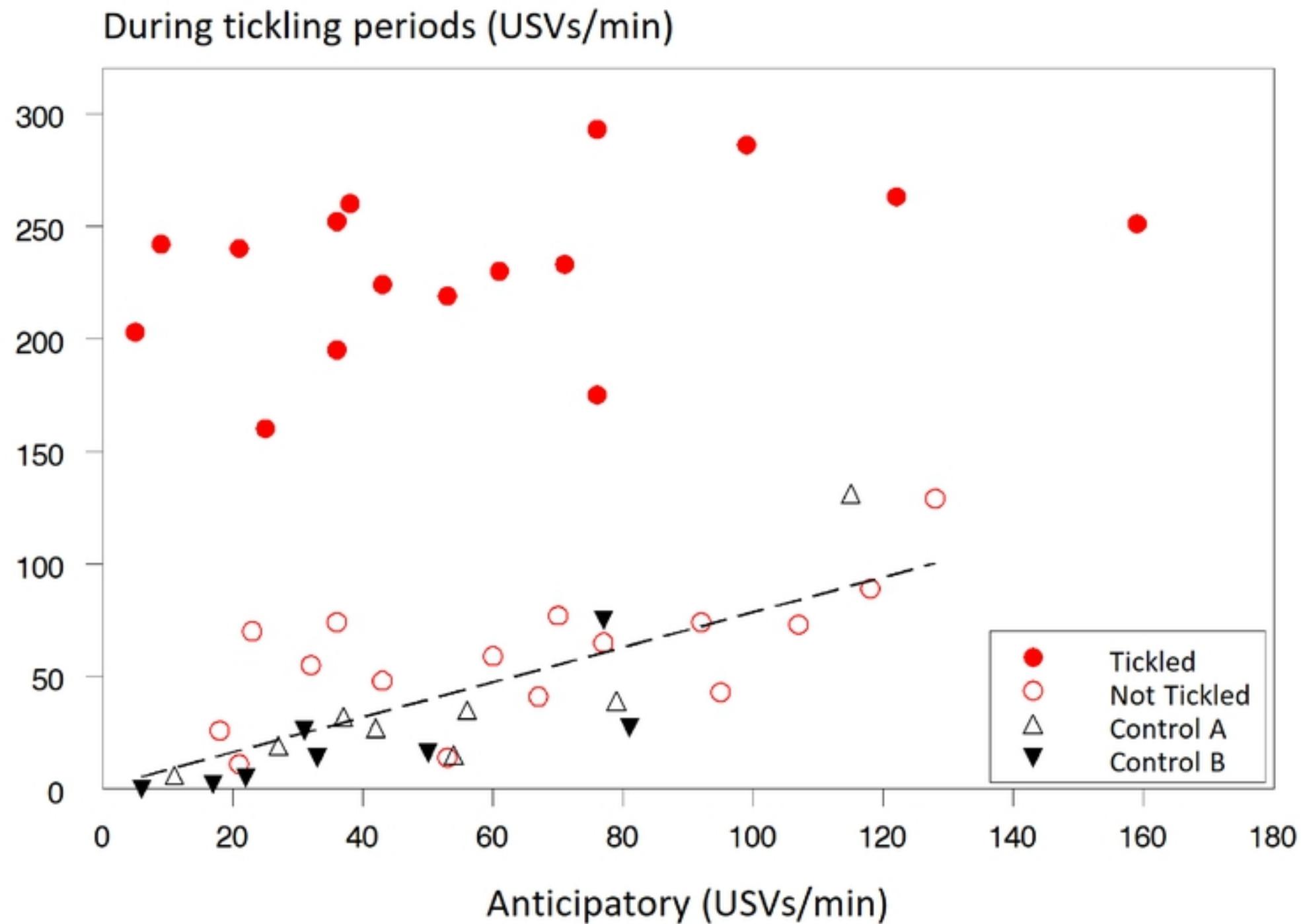


Figure 5

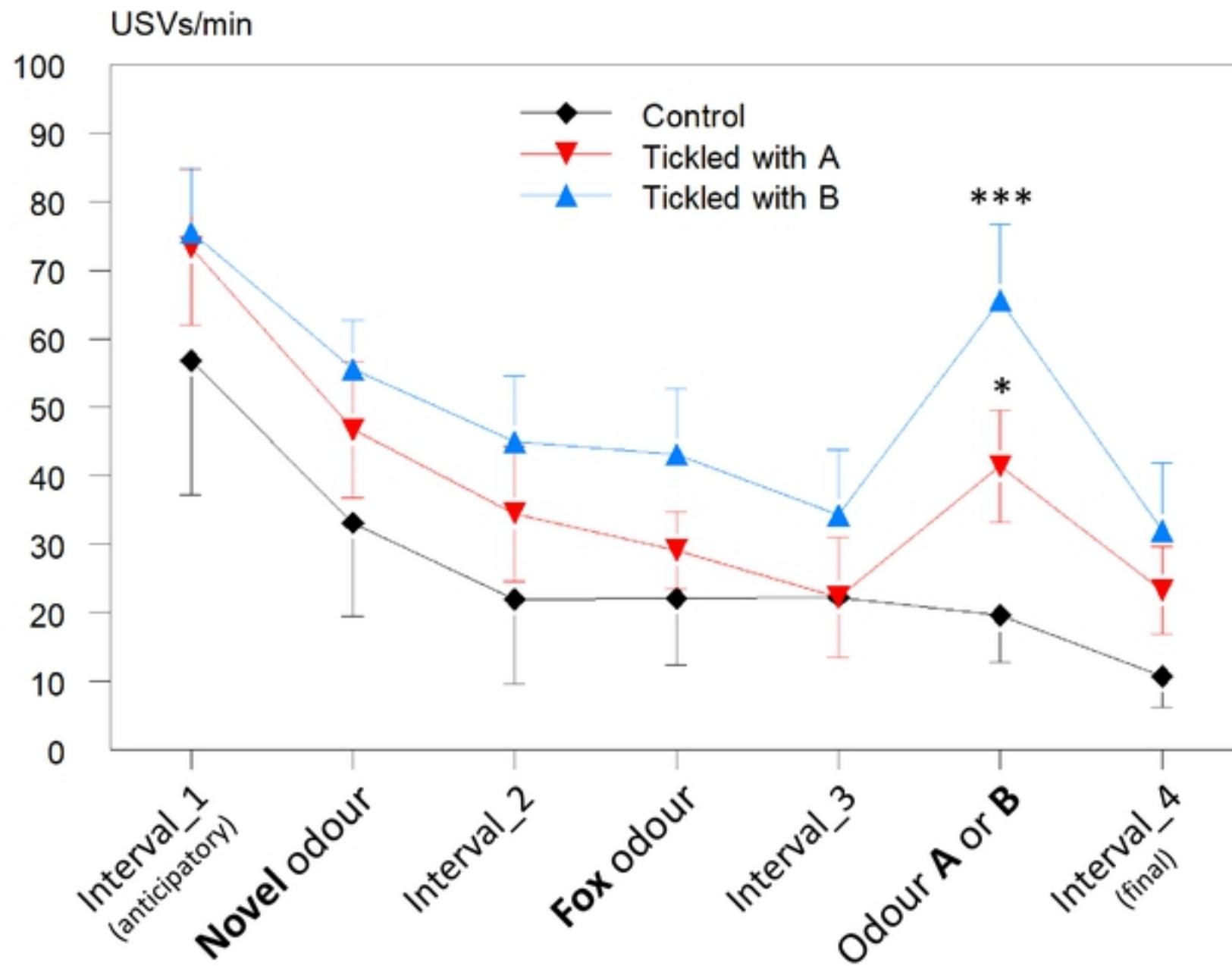


Figure 6

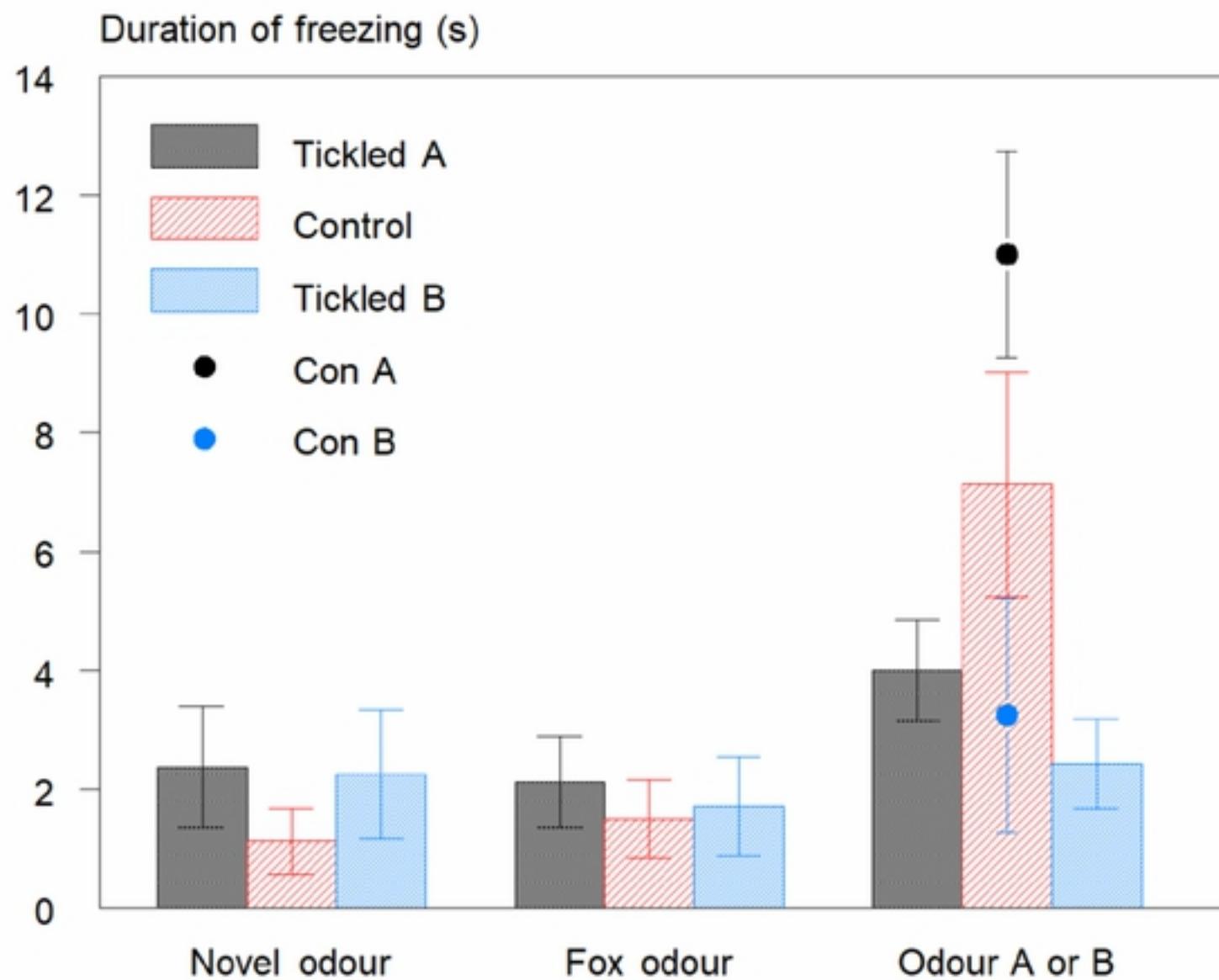


Figure 7

