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### ► To cite this version:

Jeffrey D Roth, F. Stephen Dobson, Peter Neuhaus, Asheber Abebe, Thibaut Barra, et al.. Territorial scent-marking effects on vigilance behavior, space use, and stress in female Columbian ground squirrels. *Hormones and Behavior*, 2022, 139, pp.105111. 10.1016/j.yhbeh.2022.105111 . hal-03538717

**HAL Id: hal-03538717**

**<https://hal.science/hal-03538717>**

Submitted on 21 Jan 2022

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1 *Published in "Hormones and Behavior"*

2 **Territorial scent-marking effects on vigilance behavior, space use,**  
3 **and stress in female Columbian ground squirrels**

4 Jeffrey D Roth<sup>1</sup>, F Stephen Dobson<sup>1,2,3</sup>, Peter Neuhaus<sup>4</sup>, Asheber Abebe<sup>5</sup>, Thibaut Barra<sup>2</sup>,  
5 Rudy Boonstra<sup>6</sup>, Phoebe D Edwards<sup>4</sup>, Manuel A Gonzalez<sup>4</sup>, Tracey L Hammer<sup>2,4</sup>, Erwan  
6 Harscouet<sup>2</sup>, Laura K McCaw<sup>6</sup>, Maria Mann<sup>4</sup>, Rupert Palme<sup>7</sup>, Mathilde Tissier<sup>2</sup>, Pierre  
7 Uhlich<sup>2</sup>, Claire Saraux<sup>2</sup> & Vincent A Viblanc<sup>2\*</sup>

8  
9 <sup>1</sup>Department of Biological Sciences, Auburn University, Auburn, AL, USA

10 <sup>2</sup>Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France

11 <sup>3</sup>University of Strasbourg, Institute of Advanced Sciences (USIAS), 5 allée du Général  
12 Rouvillois, 67083 Strasbourg, France

13 <sup>4</sup>Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4, Canada

14 <sup>5</sup>Department of Mathematics and Statistics, Auburn University, Auburn, AL, USA

15 <sup>6</sup>Department of Biological Sciences, University of Toronto Scarborough, ON M1C 1A4,  
16 Canada

17 <sup>7</sup>Department of Biological Sciences, University of Veterinary Medicine, Vienna, Austria

18  
19 \*Correspondance [vincent.viblanc@iphc.cnrs.fr](mailto:vincent.viblanc@iphc.cnrs.fr)

20  
21 **Author contributions**

22 Designed the study: VAV, PN and FSD; collected the data: VAV, JDR, PN, TB, FC, MAG,  
23 TLH, EH, MM, CS, MT, PU and FSD; did the laboratory work: PDE, LMC and RB;  
24 provided antibodies and expertise on FCM measurement: RP; analyzed the data: VAV, JDR,

25 AA, CS, and FSD; wrote the manuscript: VAV, JDR and FSD; all authors commented on the  
26 manuscript.

## 27 **Abstract**

28 Social environments can profoundly affect the behavior and stress physiology of group-living  
29 animals. In many territorial species, territory owners advertise territorial boundaries to  
30 conspecifics by scent marking. Several studies have investigated the information that scent  
31 marks convey about donors' characteristics (e.g., dominance, age, sex, reproductive status),  
32 but less is known about whether scents affect the behavior and stress of recipients. We  
33 experimentally tested the hypothesis that scent marking may be a potent source of social  
34 stress in territorial species. We tested this hypothesis for Columbian ground squirrels  
35 (*Urocitellus columbianus*) during lactation, when territorial females defend individual nest-  
36 burrows against conspecifics. We exposed resident lactating females, on their territory, to the  
37 scent of other lactating females. Scents were either from unfamiliar females, kin relatives (a  
38 mother, daughter, or sister), or their own scent (control condition). We expected resident  
39 females to react strongly to novel scents from other females on their territory, displaying  
40 increased vigilance, and higher cortisol levels, indicative of behavioral and physiological  
41 stress. We further expected females to be more sensitive to unfamiliar female scents than to  
42 kin scents, given the matrilineal social structure of this species and known benefits of co-  
43 breeding in female kin groups. Females were highly sensitive to intruder (both unfamiliar and  
44 kin) scents, but not to their own scent. Surprisingly, females reacted more strongly to the  
45 scent of close kin than to the scent of unfamiliar females. Vigilance behavior increased  
46 sharply in the presence of scents; this increase was more marked for kin than unfamiliar  
47 female scents, and was mirrored by a marked 131% increase in free plasma cortisol levels in  
48 the presence of kin (but not unfamiliar female) scents. Among kin scents, lactating females  
49 were more vigilant to the scent of sisters of equal age, but showed a marked 318% increase in

50 plasma free cortisol levels only in response to the scent of older and more dominant mothers.

51 These results suggest that scent marks convey detailed information on the identity of

52 intruders, directly affecting the stress axis of territory holders.

53

54 **Keywords: glucocorticoids, HPA axis, kin selection, olfaction, resource holding**

55 **potential, territoriality**

56

57

58

## 59 INTRODUCTION

60 Many species use scent as a form of chemosensory communication in a variety of social  
61 contexts (Ralls 1971; Johnson 1973; Bel et al. 1999). Scent-marking occurs via the deposition  
62 of secretions from exocrine scent glands or via odorous marks deposited in urine and feces,  
63 on strategic substrate locations. Both time and energy investments are needed to produce  
64 olfactory secretions and maintain volatile compounds active in the environment (Radwan et  
65 al. 2006). However, this form of social communication allows territory owners, in their  
66 absence, to advertise their quality to tentative mates (Johansson and Jones 2007; Boulet et al.  
67 2010), and/or resource holding potential to tentative competitors, while minimizing active  
68 resource-defense. This circumvents escalation of risky contests over resources, such as  
69 territories or mates (Hurst and Rich 1999; Gosling and Roberts 2001; Stockley et al. 2013).

70 As scent-marking is thought to convey honest information on the identity, condition,  
71 and social status of the bearer, numerous studies have focused on unraveling the attributes  
72 signaled by odorous secretions. Scents reflect information on individual sex and age (Kean et  
73 al. 2011; Vaglio et al. 2016), familiarity (Hare 1994), genetic similarity and kinship (Mateo  
74 2003; Leclaire et al. 2013), genetic dissimilarity or compatibility (Wedekind et al. 1995;  
75 Charpentier et al. 2008), reproductive status (Harris and Murie 1984; Scordato and Drea  
76 2007), health and immune status (Zala et al. 2004; López and Martín 2005), hormonal status  
77 and stress levels (Lumley et al. 1999; Yamaguchi et al. 2005; Shimozuru et al. 2006), and  
78 social rank (Scordato and Drea 2007; Vaglio et al. 2016). The complexity of odor signals has  
79 also been found to increase with social complexity (*e.g.* in *Eulemur* species; delBarco-Trillo  
80 et al. 2012), indicating the importance of scent communication in social species.

81 Despite this wealth of studies on the physiological/individual determinants of scent-  
82 marking, fewer studies have considered the effects that scent-marks have on the behavior and  
83 physiology of recipients. Those that have done so have mostly focused on inter-specific

84 relationships, *e.g.*, the effect of predator scents on prey stress responses via modulation of the  
85 hypothalamo-pituitary-adrenal (HPA) axis function and secretion of glucocorticoid (stress)  
86 hormones by the adrenal glands (Apfelbach et al. 2005; Fletcher and Boonstra 2006; Monclús  
87 et al. 2006; but see Zuri et al. 1998). However, if scent-marking is used to effectively  
88 advertise social dominance or ownership of resources, one might expect scent-marks to have  
89 potent effects on the receiver's behavior and stress physiology in social contexts (Roberts  
90 1998; Zuri et al. 1998). This should especially be the case in species where social systems are  
91 characterized by shifting spatio-temporal territories that require both active defense and  
92 regular up-keep of ownership advertisement by scent-marks. For instance, many sciurids rely  
93 on scents to mark the boundaries of territories defended during critical time periods of the  
94 annual cycle of mating, gestation, lactation, and weaning of offspring (Steiner 1974; Ouellet  
95 and Ferron 1988; Ferron and Ouellet 1989; Brady and Armitage 1999). The boundaries of  
96 those territories are likely to shift both within and over seasons, depending on the age,  
97 dominance rank, and reproductive status of the territory holder.

98         We tested the hypothesis that scent marking constitutes a source of social stress in  
99 territorial species, using Columbian ground squirrels (*Urocitellus columbianus*) as a model  
100 system. Columbian ground squirrels are sciurid rodents characterized by a matrilineal social  
101 system where related females share adjacent and overlapping territories over generations  
102 (King and Murie 1985; King 1989; Arnaud et al. 2012) and males are the dispersing sex  
103 (Wiggett and Boag 1989; Neuhaus 2006). Resident males show strong patterns of  
104 territoriality during the mating season (Murie and Harris 1978), allowing preferential access  
105 to females that share their territory (Manno and Dobson 2008). After reproduction, male  
106 territoriality subsides and females become more territorial during the subsequent gestation  
107 and lactation periods (Festa-Bianchet and Boag 1982; Murie and Harris 1988). Both males  
108 and females display scent-marking behavior, particularly during mating, gestation, and

109 lactation (Steiner 1974; Betts 1976). Scents are deposited by rubbing apocrine glands located  
110 at the oral angles of the mouth (Kivett et al. 1976; Kivett 1978), the anogenital area and a  
111 dorsal-glandular area of the body (Steiner 1974) against the substrate. There is clear evidence  
112 that scent is an important means of social communication in this species, either related to  
113 mating behavior (determination of female reproductive status from vaginal scent marks;  
114 Harris and Murie 1984); fear (anal gland pulsating during hostile encounters; Steiner 1970;  
115 Steiner 1974); or the discrimination of familiar and unfamiliar conspecifics (oral scents;  
116 Harris and Murie 1982; Hare 1994). In an experimental study of Columbian ground squirrels,  
117 Raynaud & Dobson (2011) found that females were more attentive to the scent of other  
118 females than males: Presented with conspecific scents, females invested more time re-  
119 marking female than male scent marks. These authors hypothesized that this behavior  
120 reflected female territory advertisement and protection of the litter, which may be especially  
121 important during lactation. During this period, infanticide by other, mostly unrelated,  
122 lactating females is known to occur (Balfour 1983; Dobson 1990; Hare 1991; Stevens 1998).

123         Here, we exposed lactating focal females on their territories to the scents of other  
124 lactating females, and tested the consequences of this exposure on the behavior and stress  
125 physiology of territory owners. We collected lactating female scents on acrylic cubes (by  
126 rubbing the cubes on the angulo-oral glands) (see Harris and Murie 1982; Raynaud and  
127 Dobson 2011). The scented cubes were then placed around the nest burrow of a focal female,  
128 testing her behavioral and physiological (HPA) response to the scent of either (i) an  
129 unfamiliar female, (ii) a kin female, or (iii) herself. If scent marking indeed constitutes a  
130 source of social stress, we expected to observe marked behavioral and HPA responses of  
131 females to the presence of novel scents on their territory. After exposure to conspecific  
132 scents, compared to her own scent or no scent conditions, we expected focal females to  
133 devote an increased proportion of time to vigilance behavior, and to show heightened HPA

134 activity measured through increased levels of blood cortisol and fecal cortisol metabolites  
135 (Bosson et al. 2009). We also expected females to display increasing amounts of scent  
136 marking behavior on their territory, to interact more with scented cubes, and to spend more  
137 time exploring their territorial boundaries seeking out the intruder. Because female  
138 Columbian ground squirrels are more tolerant of close female kin (King & Murie 1985; King  
139 1989; Viblanc et al. 2016), we expected the effects of scent marks to be stronger for  
140 unfamiliar than for kin scents. However, because mothers appear to be dominant over  
141 daughters in this species (Harris & Murie 1984; Wigget & Boag 1992; Viblanc et al. 2016;  
142 Sosa et al. 2020), we also expected females' behavioral and physiological stress responses to  
143 be stronger when exposed to the scent of their mother than to the scent of a daughter or  
144 littermate sister. Differences in terms both of behavioral and physiological reactions to  
145 different scent marks would indicate that subtle social information on identity was conveyed  
146 by chemical signals.

147

## 148 **MATERIAL AND METHODS**

### 149 **Data collection and study site**

150 The study was conducted during the 2017 and 2018 breeding seasons. We studied lactating  
151 females in three neighboring colonies of Columbian ground squirrels (Meadow B, Meadow C  
152 and DOT) that were actively monitored as part of long-term studies on the behavior and  
153 ecology of those animals. The colonies are located in the Sheep River Provincial Park,  
154 Alberta, Canada (Meadow B: 50° 38' 10.73" N; 114° 39' 56.52" W; 1524 m; 2.0 ha; Meadow  
155 C: 50° 37' 44.2" N; 114° 41' 18.4" W; 1555 m; 1.5 ha; and DOT: 50° 38' 59.74" N; 114° 39'  
156 41.79" W; 1545 m; 3.0 ha). In each year, females were followed throughout reproduction  
157 from emergence from hibernation in early April to the weaning of offspring in early to mid-  
158 July (Neuhaus 2000; Dobson et al. 2020). Female mating dates were determined from visual



159 observations of above ground consortships with males and inspection of genitalia upon  
160 trapping (presence of a copulatory plug or sperm in vaginal smears or fur; Murie 1995; Raveh  
161 et al. 2010). Gestation lasts ~24 days, upon which females give birth to an average of three  
162 (one to seven) altricial offspring in a specially constructed nest burrow (Dobson & Murie  
163 1987; Murie 1995). Lactation lasts ~27 days, after which, weaned offspring first emerge  
164 above ground (Murie & Harris 1982; Dobson et al. 1992).

165

### 166 **Scent collection**

167 We collected female ground squirrel scents following the protocol developed through  
168 previous investigations on Columbian ground squirrels (Harris & Murie 1982; Raynaud &  
169 Dobson 2011). We used 3.7 cm<sup>3</sup> acrylic cubes to collect oral gland scents by rubbing the  
170 cubes (3 times per side) on the oral angles of ground squirrels that were either female kin  
171 (littermate sister, daughter, or mother), an unfamiliar female (from other monitored  
172 populations), or the focal female being tested. We focused on collecting only female scents in  
173 this study, since previous results had indicated that females are more attentive to the scent of  
174 other females than males during gestation and lactation (Raynaud & Dobson 2011). We  
175 ensured that each face of the cube was properly marked, confirmed by the presence of an oily  
176 streak mark and characteristic smell. Scent cubes were prepared in the afternoon preceding  
177 the experimental trial (see below) and kept in an airtight Ziploc® bag at 4°C overnight until  
178 use. After each trial, the cubes were washed with soap and water, rinsed with boiling water,  
179 and rinsed a final time with 90% ethanol, before being air dried and stored in an airtight  
180 Ziploc® bag until further scent collection.

181

### 182 **Experimental trial**

183 Nest burrows were identified during gestation by field observations of females stocking them  
184 with dry grass material from the meadow (McLean 1978). The experimental trial proceeded  
185 in 3 phases:

186 *Phase 1, 'Habituation'*: On the date of expected parturition (day 0), 3 unscented cubes  
187 were deployed around a focal female's nest burrow in an equilateral triangle at a 30-cm  
188 distance from the nest entrance. Cubes were deployed on small metal pins at ~5-cm height off  
189 the ground and left for a period of 4 days to allow the female to habituate to the presence of  
190 these novel objects.

191 *Phase 2 'Control = unscented cubes'*: On day 4, an observer arrived at the colony  
192 early in the day (~6:00 am) before the female had emerged from her nest burrow. Cubes were  
193 replaced with fresh unscented cubes and the observer took position on a 3-m tall observation  
194 bench, which were generally located some 10 to 30 m from the nest burrows of interest.  
195 Replacing the cubes by fresh unscented cubes insured that we captured the behavior of  
196 individual females before they were exposed to a given scent, each female thus serving as her  
197 own control during the experiment. Upon morning emergence of the female from her nest  
198 burrow, the observer scored her behavior and location for a period of 30 minutes (see below).  
199 The observer was unaware of the type of scent provided, so that observations were blind.  
200 Depending on the year, either the female was captured ~7 hours following her emergence  
201 from the nest burrow and a fecal sample collected (in 2017, see below), or the female was  
202 captured immediately following the 30-min observation period and a blood sample collected  
203 usually within 3 minutes of capture (in 2018, see below).

204 *Phase 3 'Treatment = scented cubes'*: On day 5, the procedure for day 4 was repeated  
205 but this time replacement cubes were either marked with the scent of a kin female, the scent  
206 of an unfamiliar female, or the focal female's own scent (control).

207

208           Our sample size for the different groups were 32 focal females tested for kin scents  
209 (15 in 2017 and 17 in 2018), 35 focal females tested for unfamiliar female scents (14 in 2017  
210 and 21 in 2018), and 29 females tested for their own scent (13 in 2017 and 16 in 2018). All  
211 females were tested only once (*i.e.*, with one type of scent) within a given year, but some  
212 females were tested repeatedly in different years, and so we controlled for female identity in  
213 statistical analyses (see below). On average, the age distribution of females was fairly  
214 balanced between groups (female age in kin scent group: mean ( $\pm$  SD) =  $4.1 \pm 1.9$  y.o., range  
215 = 2 – 9 y.o; female age in unfamiliar scent group: mean =  $4.3 \pm 2.0$  y.o, range = 2 – 9 y.o;  
216 female age in own scent group: mean =  $4.1 \pm 1.8$ , range = 1 – 8). Within the kin group,  
217 females exposed to the scent of a daughter were slightly older (mean =  $6.3 \pm 1.3$  y.o., range =  
218 4 – 9 y.o.) than those exposed to the scent of a mother (mean =  $3.0 \pm 0.9$  y.o., range = 2 – 5  
219 y.o.) or a sister (mean  $\pm$  SD =  $3.7 \pm 1.6$  y.o., range = 2 – 7 y.o.). We thus controlled for  
220 female age as a covariate in subsequent analyses to account for potential age effects on  
221 behavioral and physiological variables.

222

### 223 **Behavioral observations**

224 Behavioral observations were carried out during lactation, from the 18<sup>th</sup> of May to the 8<sup>th</sup> of  
225 June in 2017, and from the from the 23<sup>rd</sup> of May to the 7<sup>th</sup> of June in 2018. Each female was  
226 observed by the same observer during the entire experimental trial (phases 2, and 3). This  
227 required 1-7 observers daily in 2017 and 1-8 observers in 2018. We used fixed-interval point  
228 sampling of behaviors (Martin & Bateson 2021). Females were observed every minute, for  
229 30-min periods. Only the behavior on the minute mark was checked on a pre-defined  
230 behavioral spread sheet. Each minute, the observer also recorded the exact position of the  
231 animal on a cartesian 10 x 10 m grid, delimited by colored flags placed throughout the study  
232 site. Thus, the position of the animal could be estimated to within about a 1-m resolution

233 during the observation period. Scored behaviors included: vigilance, locomotion, foraging,  
234 grooming, resting, alarm calling, aggressive (chases and fights) and amicable (sniffing)  
235 interactions. Vigilance behavior was scored whenever the animal was sitting still on its hind  
236 legs or on all four paws, its head pointing away from the ground, scanning the environment.  
237 If an animal was temporarily out of sight (*e.g.*, behind a tree or rise in the ground, in a  
238 burrow, etc.), an “out of sight” category was scored. In cases where the animal was lost for an  
239 extended period of time, observations were discarded. In addition to the above behaviors  
240 scored on the minute mark, we recorded and summed all occurrences of (1) cube interactions  
241 (licking, biting, scratching or sniffing a cube) and (2) scent marking (scratching and/or  
242 rubbing the ground with the cheek or the lateral side of the body, rubbing the cubes with the  
243 cheek) during the 30-minute observation period. Because cube interactions were very rare  
244 (Appendix 1), we pooled our observations into a binomial variable (interaction *vs.* no  
245 interaction with cubes) for further analyses (see below).

246

### 247 **Feces and blood sampling**

248 In 2017, we collected fecal samples to measure fecal cortisol metabolites (FCMs) as  
249 an integrative measure of female stress. Females were captured approximately 7 hours  
250 following control observations (phase 2) or first exposure to the scent (phase 3),  
251 corresponding to the time required for glucocorticoid plasma changes to be reflected in FCM  
252 changes (Bosson et al. 2009). Fecal samples were collected into 2-mL sterile vials as females  
253 defecated during handling, or from the floor of the trap. In the latter case, the female was  
254 always observed defecating in the trap and the feces collected immediately. The traps were  
255 systematically cleaned before being deployed to ensure fecal samples corresponded to  
256 targeted individuals. We insured that no fecal sample was contaminated by urine. Samples

257 were immediately stored on ice packs in the field and transferred to a -20°C freezer within 30  
258 min of collection.

259 In 2018, we collected blood samples to measure female's blood cortisol levels  
260 immediately after the 30 min of control (phase 2) and exposure to a scent (phase 3). A few  
261 females (N = 12) were also bled in 2017, in which case they were not sampled for feces, so as  
262 to not bias results with stress from the capture. Following the 30-min observation period,  
263 females were trapped and a 0.5 mL blood sample was collected from the saphenous vein  
264 using a 27-G needle fitted to a 1 mL heparinized syringe. An observer would set a trap on the  
265 female's territory, and keep watch until the targeted female entered the trap, in general within  
266 minutes of deployment and within 30 minutes maximum. As soon as the trap-door shut, a  
267 stop watch was started and the time until the animal bled was completed was timed. We  
268 aimed to sample blood within 3-min of trapping (mean  $\pm$  SD = 2.75  $\pm$  0.97 minutes, min =  
269 1.40, max = 7.00) to capture baseline CORT levels. Although 20 samples out of 93 were  
270 acquired after 3 min (on average at 4.44  $\pm$  1.12 minutes, min = 3.05, max = 7.00), we decided  
271 to keep those in our analyses, as total (Pearson's  $r$  =0.11,  $P$  = 0.33) and free ( $r$  =0.03,  $P$  =  
272 0.80) cortisol levels were not significantly correlated to sampling time in our data (Appendix  
273 2). Syringes were kept on ice packs in a cooler box in the field. Blood was centrifuged  
274 (3,000g for 10 min) within 30 min of collection, plasma and blood cells were separated and  
275 frozen at -20°C until the end of the field season.

276 Both fecal and blood samples were shipped at the end of the season on dry ice to the  
277 University of Toronto Scarborough (Canada), and were kept frozen at -80°C until analyses.

278

## 279 **Stress hormone analyses**

280 *Fecal cortisol metabolites (FCMs)*

281 FCMs represent the fraction of metabolized GCs that are excreted in the feces and reflect  
282 biologically active free levels of plasma GCs (Bosson et al. 2009; Sheriff et al. 2010; Fauteux  
283 et al. 2017). Lyophilized fecal samples were frozen in liquid nitrogen, and pulverized with a  
284 small grinding pestle. We extracted FCMs from  $0.054 \pm$  (s.d.)  $0.003$  g of pulverized-sample  
285 by vortexing it (30 min at 1450 rpm) in 1 mL of 80% methanol (v/v). FCMs (ng/g dried  
286 feces) were determined with a  $5\alpha$ -pregnane- $3\beta$ , $11\beta$ , $21$ -triol- $20$ -one enzyme immunoassay  
287 (EIA), designed to measure metabolites with a  $5\alpha$ - $3\beta$ , $11\beta$ -diol structure (see Touma et al.  
288 2003 for cross-reactivities of the antibody), which has been previously validated for use in  
289 Columbian ground squirrels (Bosson et al. 2009). We ran all samples in duplicate. Sample  
290 pools of low (~ 70% binding) and high (~ 30% binding) values were used as controls and run  
291 on all plates. Intra-assay coefficients of variation based on the pools were 17.38 % (low pool)  
292 and 8.03 % (high pool), and the mean inter-assay coefficient of variation based on the pools  
293 was 13.93 % (low pool) and 3.94 % (high pool). All sample duplicates had a coefficient of  
294 variation of 15% or less.

295

#### 296 *Plasma total cortisol*

297 We measured plasma total cortisol levels by radioimmunoassay (RIA) using a commercially  
298 available kit (ImmuChem<sup>TM</sup> Coated Tube Cortisol  $^{125}$ I RIA Kit; MP Biomedicals, LLC,  
299 Orangeburg, NY, USA). The antibody has a cross-reactivity of 5.5% to corticosterone and  
300 less than 3% to other naturally occurring steroids. Preliminary validation to ensure  
301 parallelism was done using different cortisol concentrations in CGS plasma (Bosson &  
302 Boonstra; unpublished data). We used the following modifications compared to the kit  
303 protocol. Each plasma sample was analyzed in duplicate with 10  $\mu$ l of plasma being added  
304 per RIA tube along with 1 mL of Cortisol- $^{125}$ I, 20  $\mu$ l  $\text{NH}_4\text{OH}$  (to saponify triglycerides), and  
305 40 $\mu$ l double-distilled  $\text{H}_2\text{O}$  prior to incubation. Samples were run against a 6-point standard

306 curve (0-100 ng/mL) on an automatic gamma counter (Wizard<sup>2</sup> 2470, PerkinElmer, Waltham,  
307 MA, USA). This method has a mean recovery of  $102.4 \pm 2.54\%$  (N=10, range 91–117%) and  
308 a detection limit of 17 pg/10  $\mu$ l. The mean intra-assay coefficient of variation was 12.55%  
309 (low pool) and 11.40% (high pool), and the mean inter-assay coefficient of variation was  
310 7.20% (low pool), and 9.72% (high pool). All duplicate samples had a coefficient of variation  
311 of 8% or less.

312 We determined how much plasma cortisol was free and not bound to its main carrier  
313 protein, corticosteroid-binding globulin (CBG). For this, we measured CBG for each sample  
314 as the maximum corticosteroid-binding capacity (MCBC) with the saturated ligand method  
315 described in McDonald et al. (1981) and then calculated the free cortisol. We followed the  
316 MCBC protocol outlined in Delehanty and Boonstra (2009) with slight modifications.  
317 Cortisol (Sigma Aldrich, St. Louis, MO, USA) was diluted to 20 ng/10  $\mu$ l EtOH, and 10  $\mu$ l  
318 was added to 12 x 75 mm polypropylene tubes and dried under filtered air. Once dry,  
319 duplicate 10  $\mu$ l of plasma were added to the tubes, followed by 100  $\mu$ l of cortisol [1,2,6,7-  
320 <sup>3</sup>H(N)] (7.21 pg/tube; PerkinElmer). Next, 400 $\mu$ l phosphate buffer (pH 7) was added to each  
321 tube and allowed to equilibrate at room temperature for 30 minutes, and left overnight at 4°C.  
322 The following morning, 200  $\mu$ l of dextran-coated charcoal was added to separate bound and  
323 free hormone. After a 10-minute incubation at 4°C, the samples were centrifuged at 2800 rpm  
324 for 10 minutes, and 500  $\mu$ l of supernatant was added to 3.5 mL of scintillation fluid (Gold  
325 Star, Meridian Biotechnologies Ltd., Surrey, ENG, UK), vortexed, and left to equilibrate in  
326 the dark for at least 4 hours before being read in a scintillation counter (Tri-Carb 3110 TR,  
327 PerkinElmer). The intra- and inter-assay coefficients of variation based on the pools were  
328 7.57% and 9.67%, respectively. All duplicate samples had a coefficient of variation of 11%  
329 or less.

330 We calculated free cortisol following Tait and Burstein (1964), using the obtained  
331 MCBC values, a value for albumin, and the CBG equilibrium dissociation constant ( $K_d$ ). We  
332 assumed the albumin concentration and proportion of cortisol bound to albumin were  
333 comparable to the values calculated for Arctic ground squirrels (2.54 g albumin/100 mL  
334 plasma; proportion bound = 0.19) in Boonstra and McColl (2000), and we used a  $K_d$  value of  
335 5.1 nM calculated in Delahanty et al. (2015).

336

### 337 **Statistical analyses**

338 All statistical analyses were performed using R v. 4.0.2 (R Core Team 2020). To understand  
339 how exposure to scents might have influenced female stress, we focused on behaviors  
340 relevant to territoriality including: (1) the time devoted to vigilance vs. foraging or vs. other  
341 behaviors, (2) the amount of territorial marking performed (3) the occurrence of interactions  
342 with scented cubes, and (4) exploration behavior; and on two aspects relevant to the HPA  
343 axis functioning: (5) FCM levels, and (6) plasma cortisol levels.

344

#### 345 *Vigilance behavior*

346 Female vigilance behavior in response to the scents was analyzed using multinomial logistic  
347 regression (MLR; package ‘mlogit’ in R), accounting for repeated measures on females in  
348 separate years. The multinomial response outcomes (dependent variable) included  
349 ‘vigilance’, ‘foraging’, and ‘other’ behaviors (<7.5% of total observations). We ran separate  
350 regressions for each scent category (unfamiliar, kin or own scents – and within the kin  
351 category: for mother, daughter, or sister scents) and specified treatment (unscented vs.  
352 scented cubes) as the independent variable. Thus, we tested how female vigilance behavior  
353 changed in proportion relative to foraging or other behaviors when females were exposed to a  
354 scent compared to the no scent situation (no scent fixed as reference level). Results are given



355 as odds ratios along with 95% confidence intervals. Significant odds ratios have confidence  
356 intervals not overlapping 1 and can be interpreted as the odds of increasing ( $>1$ ) or decreasing  
357 ( $<1$ ) vigilance relative to foraging or other behaviors for a transition from no-scent to scent  
358 condition.

359

### 360 *Territorial marking and interactions with cubes*

361 The number of occurrences recorded for territorial scent markings and cube interactions  
362 during the 30-min observations was heavily zero-inflated (Appendix 1). Thus, we first  
363 analyzed the likelihood to engage in scent marking or to interact with cubes as a binomial  
364 response (0/1). For this, we ran separate generalized linear mixed effects models (GLMM;  
365 binomial error structure, 'lme4' package in R) for unfamiliar, kin or own scents categories  
366 (and within the kin category: for mother, daughter, or sister scents) with the likelihood to  
367 engage (1) or not (0) in a scent marking or cube interactions specified as the dependent  
368 variable, and treatment (no-scent vs. scented cubes) as the independent variable. We  
369 originally included year and female ID as random effects in the models to account for year  
370 effects and repeated observations on individuals, but removed them if models did not  
371 converge and their associated variance could not be estimated.

372         Second, for scent marking only (too few occurrences of cube interactions, see  
373 Appendix 1), we focused on the number of scent marks actually performed for individuals  
374 that scent-marked (*i.e.*, all data  $> 0$ ). For this, we ran separate GLMMs (Poisson error  
375 distribution), with the number of scent marks as the dependent, and treatment (no-scent vs.  
376 scented cubes) as the independent variable for each of our scent treatments (unfamiliar, kin or  
377 own scents). Here also, we included year and female ID as random effects in the models, but  
378 removed them if models did not converge and their associated variance could not be  
379 estimated. When working at the kin level (mother, daughter, or sister scents), because of low

380 sample size  $N < 5$  in some of the categories, we tested the difference between control (no-  
381 scent cubes) and treated (scented cubes) using exact permutation tests ('lmp' function from  
382 the 'lmPerm' package in R; Wheeler & Torchiano 2016).

383

#### 384 *Exploration behavior*

385 To evaluate the effects of scent marks on female exploration behavior, we quantified: (1) the  
386 overall size of the area (in  $m^2$ ) used over the 30-min observation period, and (2) how far a  
387 female ventured from her nest burrow within the 30-min. First, we calculated the area used  
388 during the observations using Minimum Convex Polygons (MCP, 'adehabitatHR' package in  
389 R). Since our objective was to evaluate the maximal area covered by females over the 30-min  
390 observation period, we considered all observation coordinates (MCP 100%) corresponding to  
391 spatial coordinates recorded by the observers on the minute mark. We then ran separate linear  
392 mixed models (LMM) for own, kin and unfamiliar scent categories (and within kin for  
393 mother, sister or daughter scent categories) to test for the effects of treatment (no-scent vs.  
394 scented cubes) on area use (dependent variable). We included year and female ID as random  
395 effects in the models, but removed them if models did not converge and their associated  
396 variance could not be estimated.

397       Second, we calculated the distance a female ventured from her nest burrow (her  
398 starting location) at each minute of the 30-min observation period. We then ran separate  
399 Generalized Additive Mixed Models (GAMM, 'mgcv' and 'gaam4' packages in R) for own,  
400 kin and unfamiliar female scent categories (and within kin for mother, sister or daughter scent  
401 categories) to determine how this distance varied with time in control (no-scent cubes) and  
402 treatment (scented cubes) conditions. Differences between conditions were assessed using  
403 overlaps in 95% confidence intervals of the GAMMs. The estimated degree of freedom (edf)  
404 of GAMMs' smoothing function are reported.

405

406 *Physiological stress*

407 Female fecal cortisol metabolites (FCM; obtained in 2017; 7 hours after the exposure  
408 to unscented or scented cubes) and plasma cortisol levels (CORT; obtained in 2017 and 2018;  
409 immediately after the exposure to cubes) were analyzed using separate LMMs. FCM and  
410 CORT levels (either total CORT, free CORT or MCBC) were specified as dependent  
411 variables in the separate models for own, kin and unfamiliar scent categories and the  
412 treatment (no-scent vs. scented cubes) specified as an independent variable. Here also, female  
413 ID (and year for the plasma CORT data) were entered as random variables for LMMs, but  
414 removed if their associated variance could not be estimated. Again, when working at the kin  
415 level, because of low sample size of  $N < 5$  in some of the categories, we tested the difference  
416 between control (no-scent cubes) and treated (scented cubes) using exact permutation tests  
417 ('lmp' function from the 'lmpPerm' package in R; Wheeler & Torchiano 2016).

418

419 **Ethics**

420 This study was approved by the Auburn University Institutional Animal Care and Use  
421 Committee, with additional approval by the University of Calgary. Permits for conducting  
422 research and collecting samples in the Sheep River Provincial Park were obtained from  
423 Alberta Environment and Parks (research permits n° 58954, n° 58955) and Alberta Tourism,  
424 Parks, and Recreation (research and collection permit n° 17-046 and n°18-448)

425

426 **RESULTS**

427 **Vigilance behavior**

428 Controlling for age and compared to the no-scent condition, females exposed to either  
429 unfamiliar or kin scents, but not those exposed to their own scent, engaged in significantly

430 more vigilance than foraging or other behaviors over the 30-minute period (Fig 1A). For  
431 females exposed to unfamiliar scents, the odds of engaging into vigilance rather than foraging  
432 or other behaviors increased by 1.40 and 1.59, respectively, compared to no-scent conditions  
433 (multinomial;  $z = 2.98$  and  $3.77$ ,  $P = 0.003$  and  $P < 0.000$ ). For females exposed to kin scents,  
434 these odds increased by 1.82 and 1.91 ( $z = 4.63$  and  $4.54$ , both  $P < 0.000$ ) compared to no-  
435 scent conditions, whereas the odds did not change significantly (odds ratios = 1.24 and 1.00;  
436  $z = 1.60$  and  $0.02$ ,  $P = 0.11$  and  $0.99$ ) for females exposed to their own scents vs. no-scent  
437 conditions.

438         Controlling for age, significant increases in vigilance compared to foraging or other  
439 behaviors were evident among females exposed to scents of female kin (Fig 1B). For females  
440 exposed to the scent of a sister, the odds of engaging in vigilance rather than foraging or other  
441 behaviors increased by 1.86 and 2.99, respectively, compared to no-scent conditions ( $z = 3.12$   
442 and  $5.04$ ,  $P = 0.002$  and  $P < 0.000$ ). For females exposed to the scent of a daughter, these  
443 odds increased by 2.29 and 1.50, respectively, compared to no-scent conditions ( $z = 3.09$  and  
444  $1.35$ ,  $P = 0.002$  and  $0.18$ ). Finally, for females exposed to the scent of their mother these odds  
445 increased by 1.62 and 1.21, respectively, compared to no-scent conditions ( $z = 2.08$  and  $0.76$ ,  
446  $P = 0.04$  and  $0.45$ ).

447

## 448 **Territorial scent-marking and interactions with cubes**

### 449 *Territorial scent-marking*

450 We observed territorial scent-marking by resident females in 42% of our observation periods.  
451 Controlling for age, the probability for females to scent-mark (GLMM; binomial 1/0) during  
452 the 30-min observation period did not differ between scent (treatment) and no-scent (control)  
453 conditions, regardless of the scent considered (own scent: 27% vs. 34% for marking  
454 probability in the scent vs. no-scent condition, respectively; odds ratio = 0.72,  $CI_{95} = [0.23,$

455 2.24],  $z = -0.57$ ,  $P = 0.57$ ; kin scent: 57% vs. 38%, odds ratio = 2.16,  $CI_{95} = [0.69, 6.82]$ ,  $z =$   
456 1.32,  $P = 0.19$ ; unfamiliar scent: 44% vs. 40%, odds ratio = 1.18,  $CI_{95} = [0.38, 3.68]$ ,  $z =$   
457 0.29,  $P = 0.77$ ) (Fig 2A). Controlling for age, when considering the kin group only, females  
458 exposed to the scent of a sister had a higher probability of scent-marking in the presence than  
459 in the absence of scent (53% vs. 13%; odds ratio = 7.95,  $CI_{95} = [1.45, 66.28]$ ,  $z = 2.20$ ,  $P =$   
460 0.03). This was not the case, however, for females exposed to the scent of their mother (70%  
461 vs. 81%; odds ratio = 0.52,  $CI_{95} = [0.05, 5.24]$ ,  $z = -0.55$ ,  $P = 0.58$ ). For females exposed to  
462 the scent of a daughter, age was removed from the analysis since the model would not  
463 converge. Females exposed to the scent of a daughter had a similar probability of scent-  
464 marking when exposed to a scent or not (50% vs. 50%; odds ratio = 1.00,  $CI_{95} = [0.09,$   
465 11.30],  $z = 0.00$ ,  $P = 1.00$ ) (Fig 2B).

466 For females that did engage in scent-marking, controlling for age, the number of  
467 scent-marks performed was 48% lower in the treated condition (scent present) for the kin  
468 group (GLMM; Poisson, count data; estimate = -0.48;  $CI_{95} = [-0.83, -0.14]$ ,  $z = -2.72$ ,  $P =$   
469 0.007), but did not differ significantly between unscented and scented conditions for other  
470 groups (own scent: estimate = -0.13,  $CI_{95} = [-0.74, 0.42]$ ,  $z = -0.45$ ,  $P = 0.65$ ; unfamiliar  
471 scent: estimate = 0.24,  $CI_{95} = [-0.07, 0.56]$ ,  $z = 1.53$ ,  $P = 0.13$ ) (Fig 3A). However, this  
472 appeared to be the result of one female that spent a substantial amount of time scent marking.  
473 When this female was removed from the analysis, the effect was still negative (i.e., females  
474 exposed to a kin scent decreased territorial marking by 25%, on average, in the presence of  
475 the scent) but no longer significant (estimate = -0.23,  $CI_{95} = [-0.65, 0.18]$ ;  $z = -1.10$ ,  $P =$   
476 0.27). Within the kin group (Fig 3B), we found no significant difference in the number of  
477 scent marks performed regardless of whether females were exposed to the scent of a daughter  
478 (Exact permutation tests;  $P = 0.27$ ), a sister ( $P = 0.45$ ), or a mother ( $P = 0.69$ ).

479

480 *Interactions with cubes*

481 Resident females were observed interacting with cubes in 11% of our observation periods.  
482 Controlling for age, females exposed to unfamiliar scents interacted significantly more with  
483 cubes in the scent vs. no-scent condition (16% vs. 0.01%; odds ratio = 22.01,  $CI_{95} = [1.67,$   
484  $290.73]$ ,  $z = 2.35$ ,  $P = 0.02$ ) (Fig 4). Females also interacted significantly more with cubes  
485 when a kin scent was present vs. no-scent, though the predicted probabilities of interaction  
486 were, overall, extremely low (0.0005% vs. 0.000003%; odds ratio = 184.32,  $CI_{95} = [1.38,$   
487  $24699.21]$ ,  $z = 2.09$ ,  $P = 0.04$ ). In contrast, females did not interact significantly more with  
488 cubes in the 'own' condition, regardless of whether a scent was present or not (3% vs. 7%;  
489 odds ratio = 2.08,  $CI_{95} = [0.19, 46.23]$ ,  $z = 0.58$ ,  $P = 0.56$ ). The low number of cube  
490 interactions precluded us from meaningful analyses of the data within the kin category.

491

492 **Exploration behavior**

493 *Space use*

494 Controlling for age, no significant difference was found in the areas covered by females  
495 during the 30-min observation period between the no-scent and scent conditions for females  
496 exposed to unfamiliar scents (LMM; estimate = -17.85;  $CI_{95} = [-134.00, 98.30]$ ,  $t = -0.30$ ,  $P =$   
497  $0.76$ ), to kin scents (estimate = 59.46;  $CI_{95} = [-39.45, 158.37]$ ,  $t = 1.18$ ,  $P = 0.24$ ), or to their  
498 own scent (estimate = -75.06;  $CI_{95} = [-170.10, 19.98]$ ,  $t = -1.55$ ,  $P = 0.12$ ) (Fig 5a). On  
499 average, the area covered by females was similar for all 3 groups (unfamiliar female scent:  
500 area =  $260.32 \pm 30.34 \text{ m}^2$ ; kin scent:  $240.68 \pm 26.66 \text{ m}^2$ ; own scent:  $226.62 \pm 31.76 \text{ m}^2$ ), and  
501 not significantly different (LMM and Tukey HSD; all  $P > 0.73$ ). Similarly, when considering  
502 the kin group only, we found no significant differences in the areas covered by females  
503 during the 30-min observation between the no-scent and scent condition, regardless of  
504 whether the scent originated from a daughter (LMM; estimate = -47.03;  $CI_{95} = [-243.48,$

505 140.41],  $t = -0.49$ ,  $P = 0.62$ ), a mother (estimate = 32.79;  $CI_{95} = [-124.72, 190.31]$ ,  $t = 0.41$ ,  $P$   
506 = 0.68), or a littermate sister (estimate = 132.25;  $CI_{95} = [-24.47, 288.97]$ ,  $t = 1.65$ ,  $P = 0.10$ )  
507 (Fig 5b). Here also, on average, the area covered by females was similar for all 3 groups  
508 (daughter scent: area =  $232.52 \pm 52.21 \text{ m}^2$ ; mother scent:  $199.80 \pm 40.65 \text{ m}^2$ ; sister scent:  
509  $269.56 \pm 43.54 \text{ m}^2$ ), and not significantly different (LMM and Tukey HSD; all  $P > 0.62$ ).

510

### 511 *Distance to nest burrows*

512 Controlling for age, the distance a female travelled from her nest burrow generally increased  
513 in a non-linear fashion in all groups over the 30-minute observation period (GAMMs;  $2.30 <$   
514  $edf < 4.69$ ,  $10.00 < F < 46.80$ , all  $P < 0.001$ ; Fig 6). In all groups and treatments, females  
515 rapidly distanced themselves from their nest burrow upon emergence, reaching 10m within  
516 the first 9-10 minutes of observation. The distance from the nest increased more  
517 progressively (or plateaued out) after that. The overall overlap between 95% CI suggested no  
518 marked difference between experimental conditions (with or without scent) (Fig 6).  
519 Interestingly however, in the mother and sister scent group, females tended to travel further  
520 from their nest burrows towards the end of the observation period when the scent was present  
521 compared to the no-scent condition.

522

### 523 **Physiological stress**

#### 524 *Fecal cortisol metabolites (FCMs)*

525 In response to the scent application, controlling for age, females exhibited a significant 36%  
526 increase in FCM levels when exposed to their own scent compared to the no scent condition  
527 (LMM; estimate =  $747.5 \pm 301.0$ ,  $t = 2.48$ ,  $P = 0.03$ ; Fig 7A). No significant change was  
528 observed in the two other conditions (kin scent: estimate =  $513.7 \pm 327.6$ ,  $t = 1.57$ ,  $P = 0.14$ ;  
529 unfamiliar female scent: estimate =  $557.5 \pm 277.7$ ,  $t = 2.01$ ,  $P = 0.07$ ) (Fig 7A). Within kin

530 categories, females exhibited 118% higher FCM when exposed to the scent of their mother  
531 (Exact permutation test;  $P = 0.07$ ), but not when exposed to the scent of a sister ( $P = 0.78$ ) or  
532 their daughter ( $P = 0.34$ ) (Fig 7B).

533

#### 534 *Plasma cortisol*

535 In response to kin scents, controlling for age, females showed a significant 39% increase in  
536 total cortisol levels (LM; estimate =  $38.17 \pm 15.42$ ,  $t = 2.47$ ,  $P = 0.02$ , Fig 8A). Their MCBC  
537 levels (Fig 8B) did not differ significantly from the control levels (LM; estimate =  $1.54 \pm$   
538  $10.85$ ,  $t = 0.14$ ,  $P = 0.89$ ), resulting in a 35% (non-significant) increase in free cortisol levels  
539 (LM; estimate =  $0.89 \pm 1.10$ ,  $t = 0.81$ ,  $P = 0.43$ ). However, in this treatment, one female had  
540 inexplicably high free cortisol levels (over 4 standard deviations, sampling time = 2.82 min)  
541 (see Fig 8C). Once this data point was removed from the analyses, females showed a  
542 significant 131% increase in free cortisol levels in the presence of kin-scented cubes  
543 compared to the no scent control situation (LM; estimate =  $1.91 \pm 0.55$ ,  $t = 3.47$ ,  $P = 0.002$ ).  
544 Own-scent and unfamiliar female-scent categories did not differ significantly between control  
545 and scented cubes in terms of total cortisol (LMMs; own scent: estimate =  $12.84 \pm 15.33$ ,  $t =$   
546  $0.83$ ,  $P = 0.42$ ; unfamiliar female scent: estimate =  $2.30 \pm 9.59$ ,  $t = 0.24$ ,  $P = 0.81$ ), MCBC  
547 (LMMs; own scent: estimate =  $-0.83 \pm 12.43$ ,  $t = -0.07$ ,  $P = 0.95$ ; unfamiliar female scent:  
548 estimate =  $-23.63 \pm 14.64$ ,  $t = -1.61$ ,  $P = 0.12$ ), or free cortisol (LMMs; own scent: estimate =  
549  $0.69 \pm 0.60$ ,  $t = 1.17$ ,  $P = 0.26$ ; unfamiliar female scent: estimate =  $0.48 \pm 0.29$ ,  $t = 1.65$ ,  $P =$   
550  $0.12$ ).

551 Within kin (Fig 8, D-E), controlling for age, female total plasma cortisol levels  
552 increased by 76% when they were exposed to the scent of their mother (Exact permutation  
553 test;  $P = 0.03$ ), but not to the scent of a sister ( $P = 0.10$ ) or a daughter ( $P = 0.84$ ) (Fig 8D).  
554 MCBC levels did not differ significantly between control (unscented cubes) and treated



555 (scented cubes) situations for either group (Exact permutation tests;  $0.23 < P < 0.91$ ) (Fig  
556 8E). As a result, females exhibited a significant 318% increase in free cortisol levels in  
557 response to the scent of their mother (Exact permutation test;  $P = 0.002$ ) once the outlier was  
558 removed (24% with the outlier) (Fig 8F). Free cortisol levels did not differ significantly  
559 between control and treated cubes for daughter or sister scents (Exact permutation tests;  $0.09$   
560  $< P < 0.54$ ) (Fig 8E).

561

## 562 **DISCUSSION**

563 Resident female Columbian ground squirrels were highly sensitive to the scent marks of other  
564 lactating females deposited next to their nest burrows, exhibiting overall increases both in  
565 vigilance behavior and baseline glucocorticoid levels compared to control conditions (*i.e.*, the  
566 absence of scents, or the presence of the female's own scent). Our results suggest that scent  
567 marking of both familiar and unfamiliar animals around the nest burrows of focal females  
568 was stressful, leading to increased vigilance behavior and heightened HPA axis activity  
569 (higher baseline cortisol levels). Lactating females did not significantly increase or decrease  
570 space use in response to the treatment, suggesting they did not actively seek-out simulated  
571 intruders by increasing exploration behavior on their territories, nor did they remain close to  
572 their nest burrow to defend offspring. This is somewhat surprising since female ground  
573 squirrels exclude conspecifics forcefully from around their nest burrows, although daily  
574 foraging home ranges overlap substantially (*e.g.*, Murie and Harris 1988; King 1989; Arnaud  
575 et al. 2012). However, lactating females significantly increased the proportion of time spent  
576 in vigilance behavior compared to foraging or other behavioral categories, both when  
577 exposed to unfamiliar or kin scents, but not when exposed to their own scent. Contrary to our  
578 expectation, females were more vigilant in the presence of kin female scents on their  
579 territories than the scents of unfamiliar females. For example, females presented with kin

580 scents had 42% points higher odds of engaging in vigilance rather than foraging, than females  
581 presented with unfamiliar scents. The increase in vigilance towards kin was mirrored in the  
582 stress axis: females experienced a significant 131% increase in plasma free cortisol levels  
583 (but not FCMs) compared to the no-scent condition when exposed to kin scents, but not when  
584 exposed to unfamiliar or their own scents. The presence of an unfamiliar individual near a  
585 female's nest burrow should also be a stressful event, especially since female ground  
586 squirrels are more tolerant of kin than unrelated individuals (King 1989a; Viblanc et al.  
587 2016). Yet, neither FCM nor free cortisol levels were increased by our treatment that  
588 introduced the scents of potentially dangerous strangers. Given that vigilance was  
589 significantly increased by the same treatment, it seems that evidence of foreign individuals is  
590 acknowledged by increased observance, but without an associated increase in stress. The  
591 presence of close relatives, by comparison, is a more stressful situation.

592         The observation that kin scents elicited a stronger physiological stress response than  
593 unfamiliar female scents is surprising. The “dear-enemy” hypothesis proposes that territory  
594 holders should react more strongly to unknown tentative usurpers than to territorial neighbors  
595 of known resource holding potential (*e.g.*, Fisher 1954; Temeles 1994; Christiansen and  
596 Radford 2018). At first glance, our results might appear to reject the “dear enemy”  
597 hypothesis, since unfamiliar female scents were collected on neighboring meadows, and were  
598 therefore foreign and novel stimuli to resident females (Hare 1994). However, resident  
599 females also interacted more (licking, sniffing or attacking) with unfamiliar-scented than kin-  
600 scented cubes, suggesting that they were more responsive to novel than familiar scents (see  
601 also Raynaud & Dobson 2011), as would be predicted by the “dear-enemy” hypothesis. In  
602 addition, previous studies have found that female Columbian ground squirrels are less  
603 aggressive toward their female kin than unrelated individuals (King 1989a; Viblanc et al.  
604 2016). Thus, one explanation of our results is that lactating females may have a fine

605 knowledge of the territorial boundaries of their close female kin. They treat markings of  
606 relatives on their territories as territorial shifts, and evaluate those as a greater threat (as  
607 evidenced by increased glucocorticoid levels) than the scents of female squirrels not  
608 recognized as direct neighbors.

609         An alternative explanation may be that heightened HPA axis activity in response to  
610 close kin scents occurs as resident females prepare for increased metabolic activity associated  
611 with cooperation among relatives (Soares et al. 2010; but see Santema et al. 2013). Although  
612 Columbian ground squirrels are not known to engage into active cooperation, close kin are  
613 more tolerant of one-another (King 1989a; Viblanc et al. 2016), and kin females may engage  
614 in chasing intruders together at the border of adjacent territories (VAV, personal  
615 observations). In addition, our study used unfamiliar scents from females that originated in  
616 other populations. Yet, familiar scents from known non-kin female neighbors inhabiting the  
617 same colony may well prove more stressful than kin scents, a hypothesis that remains to be  
618 tested. In line with this idea, juvenile Columbian ground squirrels show similar levels of  
619 cohesive and agonistic behavior, and similar interindividual distances in staged dyadic  
620 interactions with conspecific juvenile colony members, but decreased cohesion, increased  
621 agonism, and greater inter-individual distances in staged interactions with juvenile  
622 conspecifics from neighbouring colonies (Hare 1992).

623         In the presence of artificial scent marks that mimicked territorial intrusions, the  
624 absence of territorial scent marking by lactating females might indicate that core territories of  
625 these females were already sufficiently saturated with their scent. Alternatively, if a female's  
626 scent were concentrated near her nest burrow, it might serve as an attractant to potential  
627 perpetrators of infanticide (Balfour 1983; Dobson 1990; Hare 1991; Stevens 1998). Females  
628 are indeed known to conceal their nest burrows by plugging them with soft soil and litter,  
629 possibly to deter infanticidal conspecifics (McLean 1978). This might explain why female

630 cortisol levels generally increased when their own scent was experimentally introduced  
631 around their nest burrows (compared to the no-scent situation), divulging their secretive  
632 location.

633         Interestingly, different kin scents elicited different reactions from resident females.  
634 Lactating resident females were most vigilant to the scent of their sisters, then daughters, and  
635 finally mothers. Surprisingly, however, the stress axis told a different story. Females reacted  
636 strongly to the scent of their mothers, but not to that of their daughters or sisters. They  
637 exhibited a significant 318% increase in free cortisol levels when exposed to their mother's  
638 scent compared to the no-scent condition, and a 118% ( $P = 0.06$ ) increase in FCM levels.  
639 Thus, whereas lactating females were more vigilant towards sisters' scents, their stress was  
640 actually higher when presented with mother scents. Mothers are known to behave cohesively  
641 to yearling daughters, but aggressively to yearling immigrants, when resources were not  
642 limiting (Wigget & Boag 1992). Relinquishment of breeding sites occurs in favor of yearling  
643 daughters (Harris & Murie 1984). Because mothers favor the establishment of yearling (but  
644 not older) daughters (Neuhaus et al. 2004), it is possible that the scent of a mother is  
645 perceived as a potential threat to an older daughter because of the possibility of a mother  
646 relinquishing a former territory to a yearling daughter. The glucocorticoid differences found  
647 here are consistent with our previous findings (Sosa et al. 2020): when compared to females  
648 that had no co-breeding kin present in the population, lactating female having only a daughter  
649 or a sister present in the population showed decreased FCM levels, whereas females having  
650 only their mother presented similarly elevated FCM levels as females having no close kin  
651 around.

652         To our knowledge, surprisingly few studies have investigated the effects of scent  
653 marking on receivers' stress physiology in territorial species. In solitary blind mole rats  
654 (*Spalax ehrenbergi*), long-term exposure to scent-marks of intruders resulted in

655 hypoglycemia, increased neutrophil/lymphocyte ratios, and partial ulceration of the liver and  
656 spleen, ultimately leading to death (Zuri et al. 1998). These effects were clearly indicative of  
657 over-activation of the adrenal function, impaired immunity, and chronic stress (Zuri et al.  
658 1998; see review by Davis et al. 2008). Interestingly, in solitary blind mole rats, it is  
659 specifically the long-term exposure to scents that was associated with increased mortality  
660 rates. This may indicate how profound the effects of scent-induced social stress may be when  
661 territory owners cannot retreat from scents, chase-away territory usurpers, or shift territories  
662 in response to intruders. Our study used an acute 30-min exposure to invader scents in a  
663 territorial species, and the stress response in terms of glucocorticoid secretion and HPA axis  
664 activation was substantial over this short time period. Whether longer exposure to scents  
665 might result in chronic stress in our species, or cause females to shift territories, remains to be  
666 tested. Nonetheless, it appears clear that, besides encoding specific information on the  
667 identity of the donor, social scent communication via territorial marking had pronounced  
668 effects on the receiver's behavior and physiology. We suggest that integrating the olfactory  
669 landscape related to social stress together with predation risk within "the ecology of fear"  
670 concept (Clinchy et al. 2013) should prove valuable to a proper understanding of behavioral  
671 and physiological consequences of habitat choice in territorial species.

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#### 674 **ACKNOWLEDGMENTS**

675 We are grateful to M. Holmes and P.D. Edwards for inviting us to contribute to this special  
676 issue, and to J.F. Hare and an anonymous reviewer for constructive and helpful comments on  
677 the paper. We are grateful to Alberta Parks, and Alberta Environment, Fish & Wildlife for  
678 granting us access to the study sites and support with the long-term research. The University  
679 of Calgary Biogeoscience Institute provided housing at the R. B. Miller field station during

680 data collection in Sheep River Provincial Park (AB, Canada). We are especially grateful to E.  
681 Johnson (Director), A. Cunnings (Station Manager) and K. Ruckstuhl (Faculty Responsible)  
682 for providing us with field camp and laboratory facilities, and to K. Ruckstuhl for her  
683 continued support in the field. We thank F. Criscuolo for his help and helpful comments on  
684 the manuscript. The IPHC-CNRS contributed to resources needed for data analysis. J. O.  
685 Murie initiated the long-term study on Columbian ground squirrels and provided helpful  
686 comments on the manuscript. As always, we are indebted for his continued advice and  
687 friendly support in our endeavors.

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#### 689 **COMPETING INTERESTS**

690 The authors declare no competing or financial interests.

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#### 692 **FUNDING**

693 The research was funded by a CNRS Projet International de Coopération Scientifique grant  
694 (PICS-07143) and a research grant from the Fondation Fyssen to V.A.V., by a USA National  
695 Science Foundation grant (DEB-0089473) to F.S.D., and a fellowship grant from the Institute  
696 of Advanced Studies of the University of Strasbourg to F.S.D. and V.A.V. F.S.D. thanks the  
697 Région Grand Est and the Eurométropole de Strasbourg for the award of a Gutenberg  
698 Excellence Chair.

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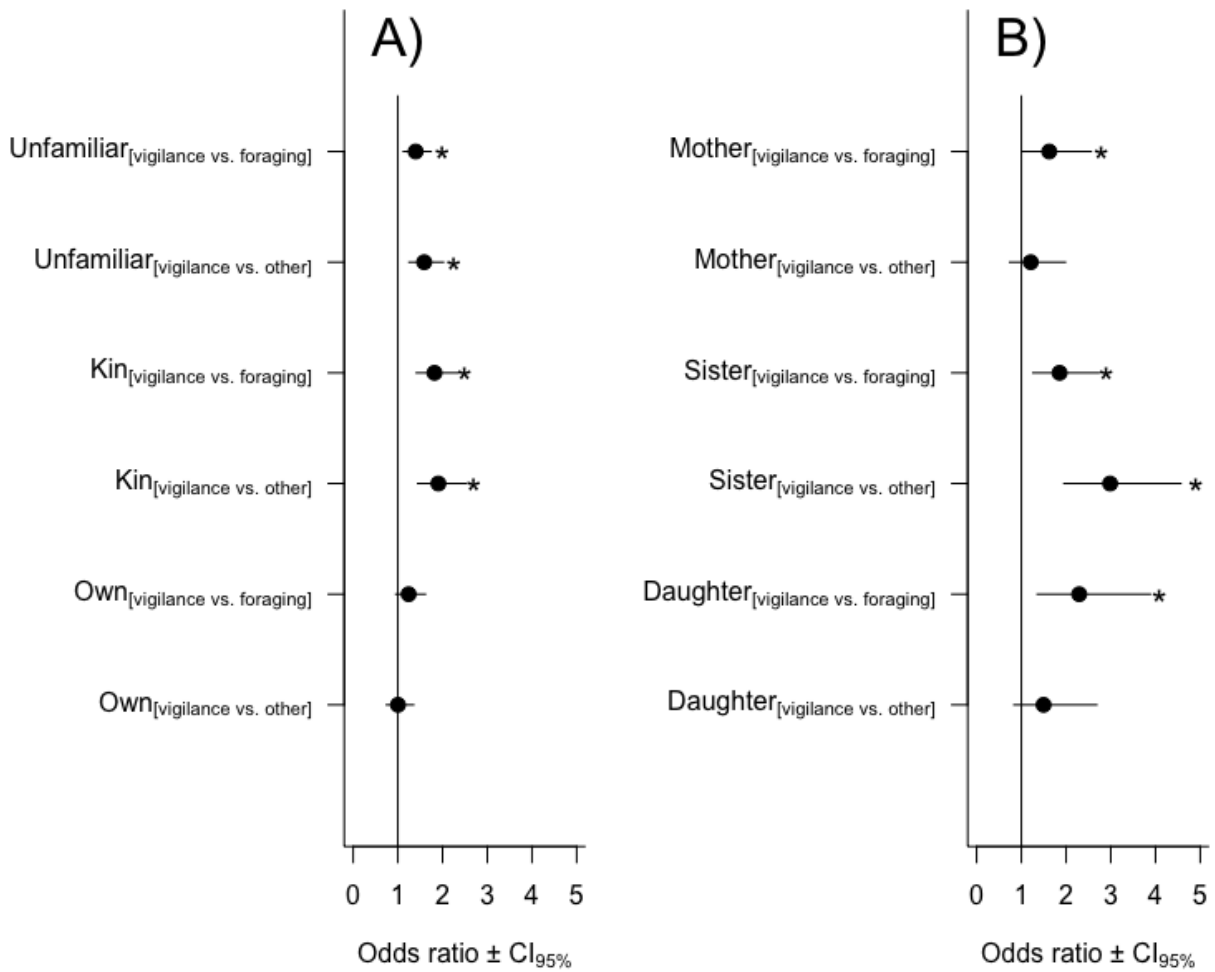
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881 **FIGURES**

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886 **Fig 1.** Changes in female vigilance behavior in reference to foraging or other behaviors  
887 during the 30-min observation period for female Columbian ground squirrels exposed to the  
888 scent of (A) an unfamiliar female, a kin female, or their own scent; and (B) within kin scents;  
889 the scent of their mother, a sister, or a daughter. Changes are expressed as odds ratio  $\pm$  95%  
890 CI. An odds ratio  $>$  (or  $<$ ) 1 indicates an increase (or a decrease) in vigilance behavior  
891 relative to foraging or other behaviors when a scent is presented. Significant changes occur  
892 for 95%CI not overlapping 1 and are indicated by an asterisk.  
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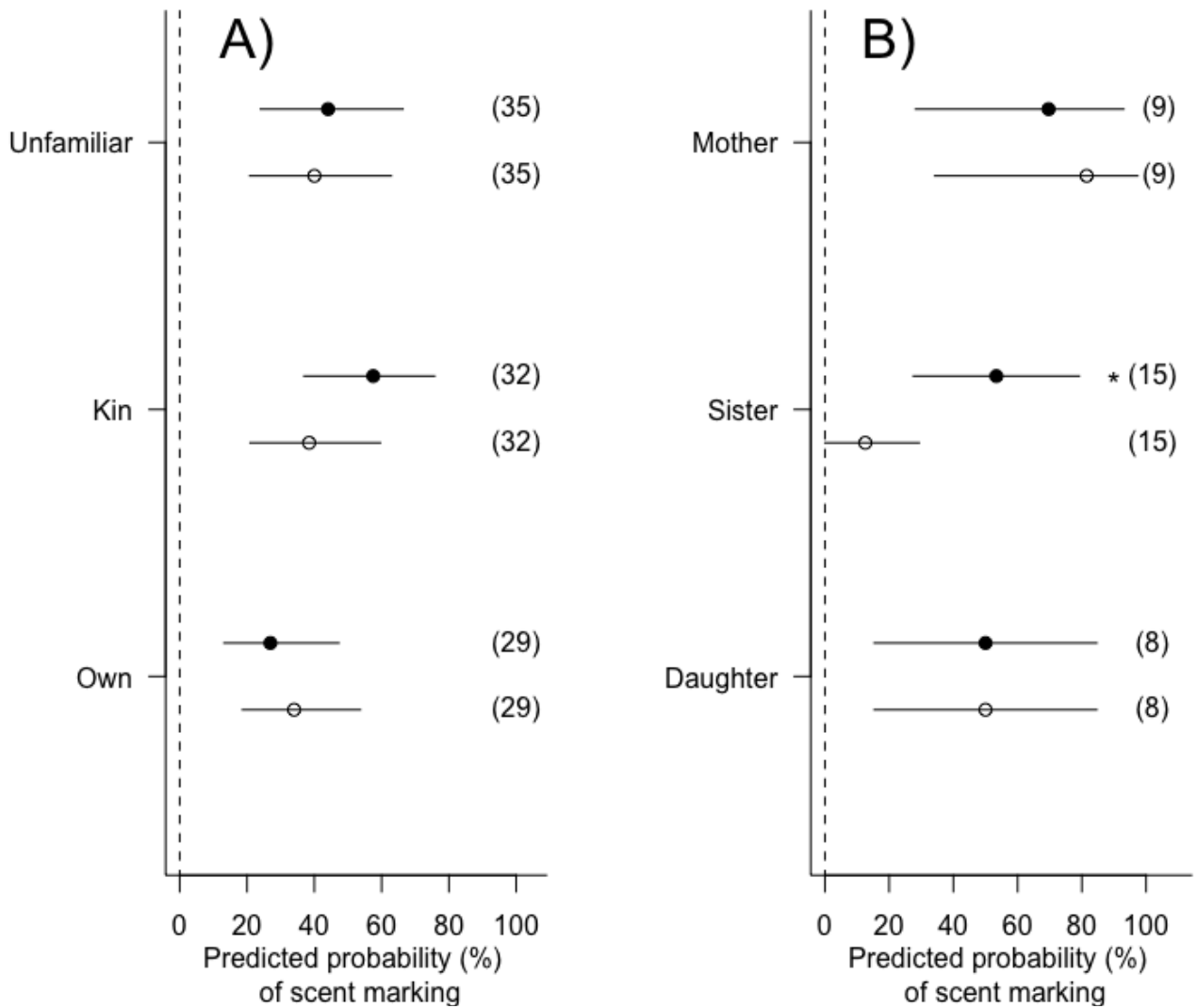
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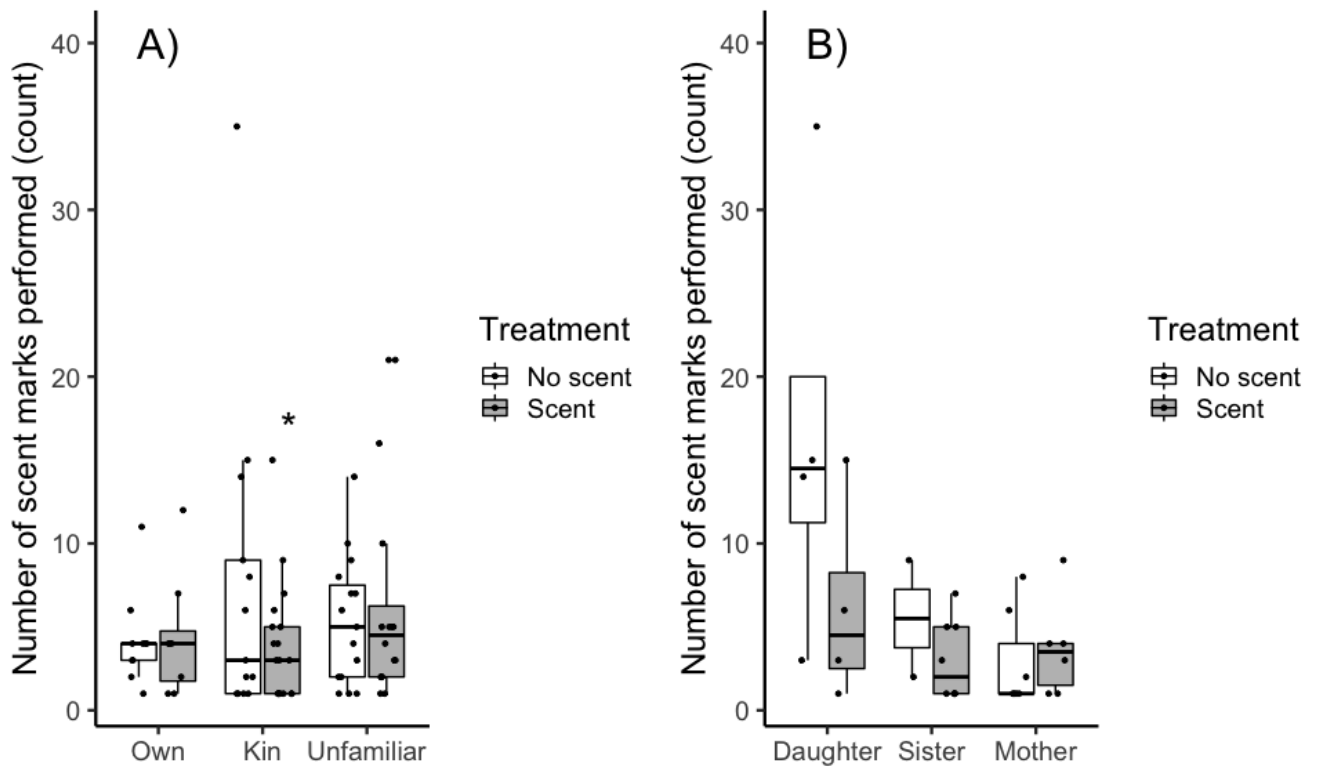


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**Fig 2.** Probability of a female ground squirrel engaging in scent marking during the 30-min observation period. Females were exposed to either no scent (○) or the scent (●) of (A) an unfamiliar female, a kin female, or their own scent. (B) within kin scents; the scent of their mother, a sister, or a daughter. Values are given as means  $\pm$  95% CI. Sample size is given in brackets. Significant differences ( $P < 0.05$ ) between (○) and (●) conditions are indicated by an asterisk.

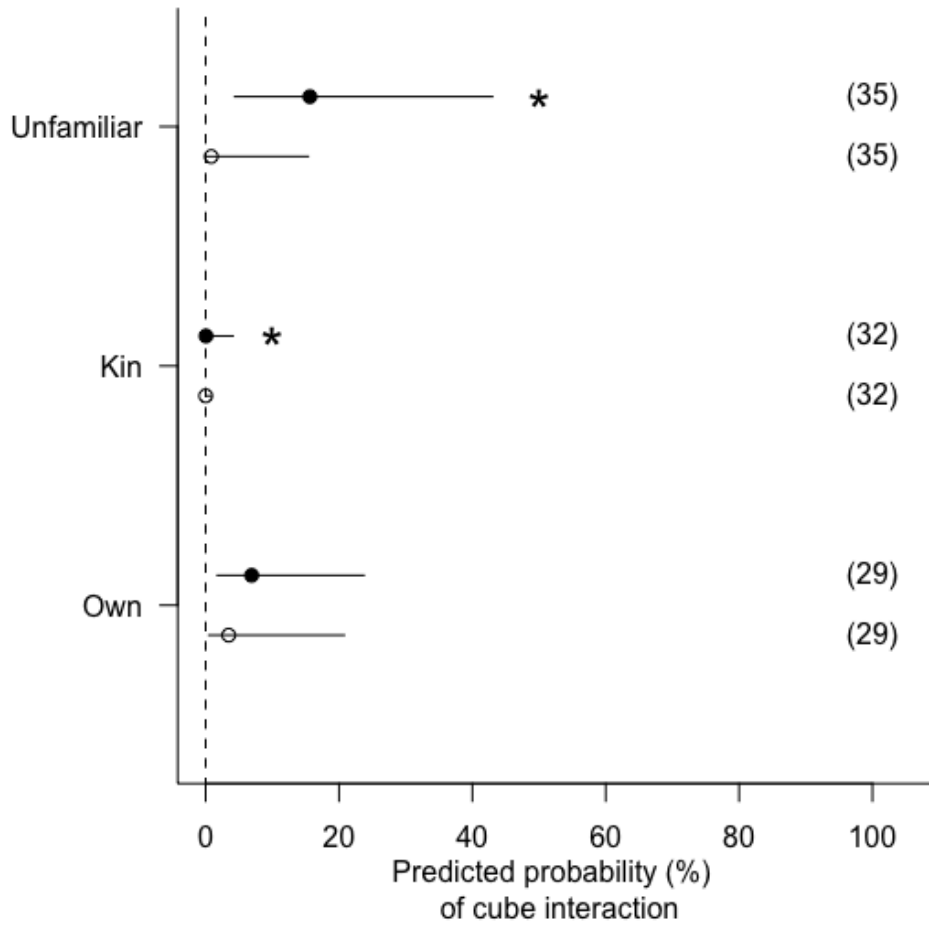
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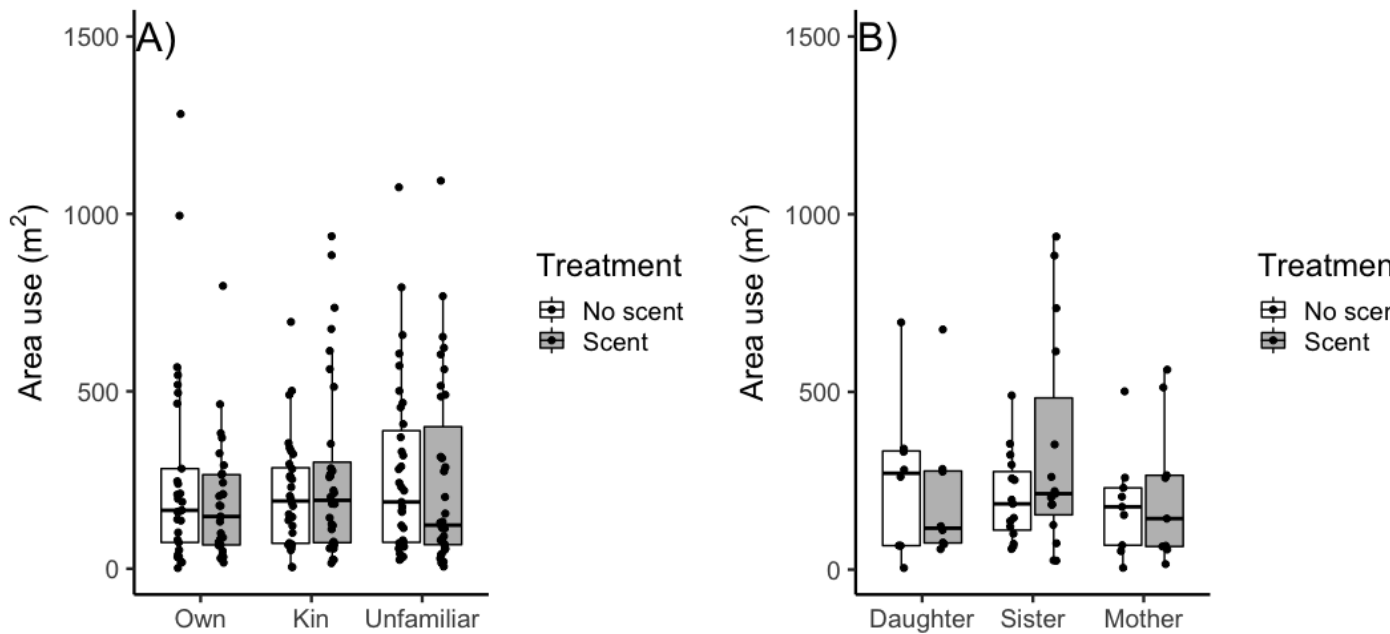
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 917 **Fig 3.** Number of scent marks deposited by a female ground squirrel during the 30-min  
 918 observation period. Females were exposed to either no scent (white boxplots) or the scent  
 919 (grey boxplots) of (A) an unfamiliar female, a kin female, or their own scent. (B) within kin  
 920 scents; the scent of their mother, a sister, or a daughter. Box plots show the median of the  
 921 data distribution (bold line) along with first and third quartiles (25<sup>th</sup> and 75<sup>th</sup> percentiles) of  
 922 the data distribution corresponding to the lower and upper hinges of the boxes. The upper and  
 923 lower whisker extend, respectively, to the largest and smallest value of the data set, no further  
 924 than 1.5 x IQR (where IQR is the inter-quartile range). Data beyond the end of the whiskers  
 925 are plotted individually. Significant differences ( $P < 0.05$ ) between treatments are indicated  
 926 by an asterisk. Note that this statistically significant difference disappears when an outlier in  
 927 the kin group is removed from the analyses (see text).

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**Fig 4.** Probability of a female ground squirrel interacting with a scent cube during the 30-min observation period. Females were exposed to either no scent (○) or the scent (●) of an unfamiliar female, a kin female, or their own scent. Values are given as means ± 95% CI. Significant differences ( $P < 0.05$ ) between (○) and (●) conditions are indicated by an asterisk. Sample size is given in brackets.



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945 **Fig 5.** Area (m<sup>2</sup>) covered over the 30-min observation period by female Columbian squirrels.

946 The area was calculated from Cartesian coordinates recorded every minute, starting as soon as a female emerged from her nest burrow in the morning. Females were exposed to either no

947 scent (white boxplots) or the scent (grey boxplots) of (A) an unfamiliar female, a kin female,

948 or their own scent. (B) within kin scents; the scent of their mother, a sister, or a daughter. Box

949 plots show the median of the data distribution (bold line) along with first and third quartiles

950 (25<sup>th</sup> and 75<sup>th</sup> percentiles) of the data distribution corresponding to the lower and upper hinges

951 of the boxes. The upper and lower whisker extend, respectively, to the largest and smallest

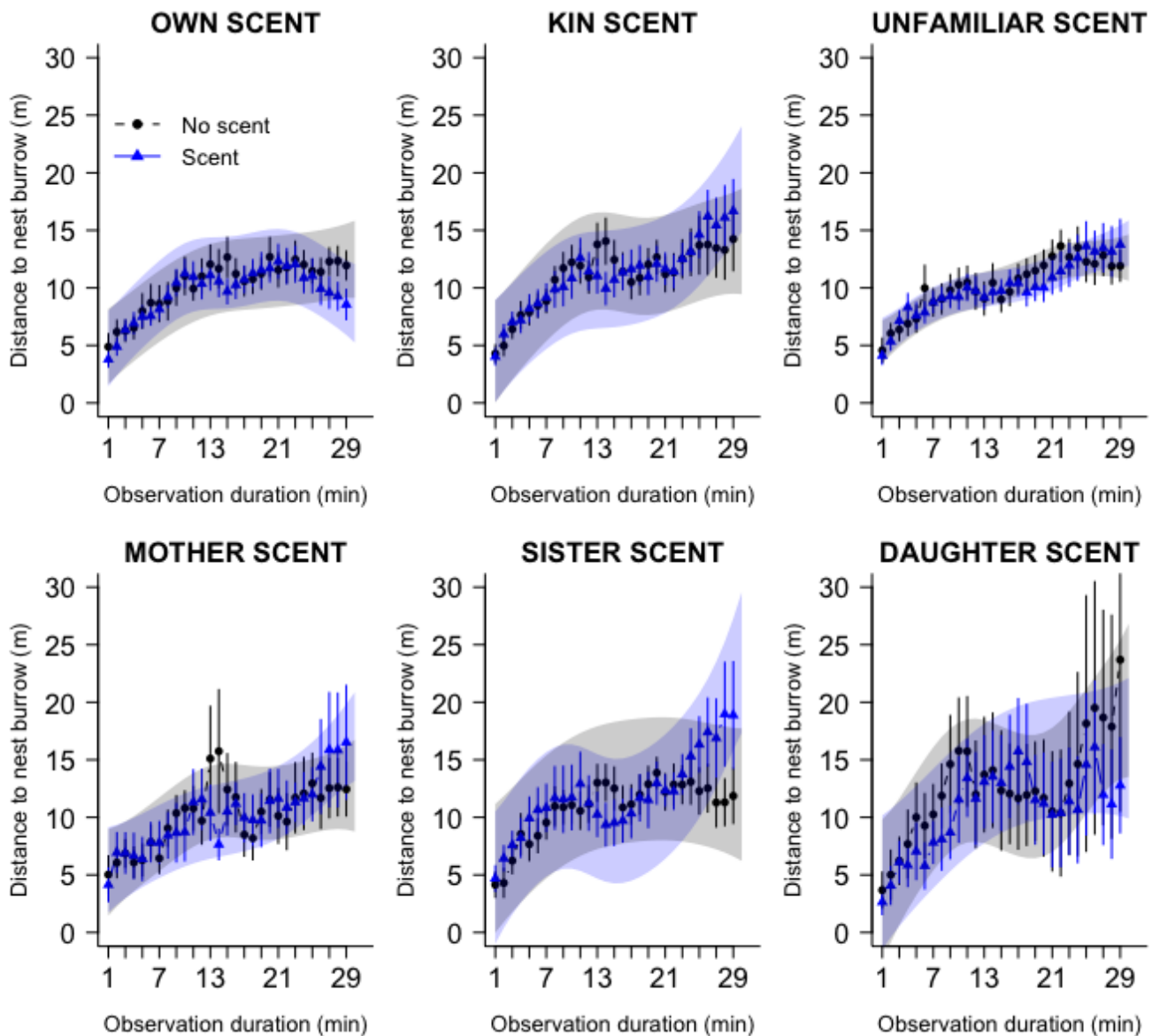
952 value of the data set, no further than 1.5 x IQR (where IQR is the inter-quartile range). Data

953 beyond the end of the whiskers are plotted individually.

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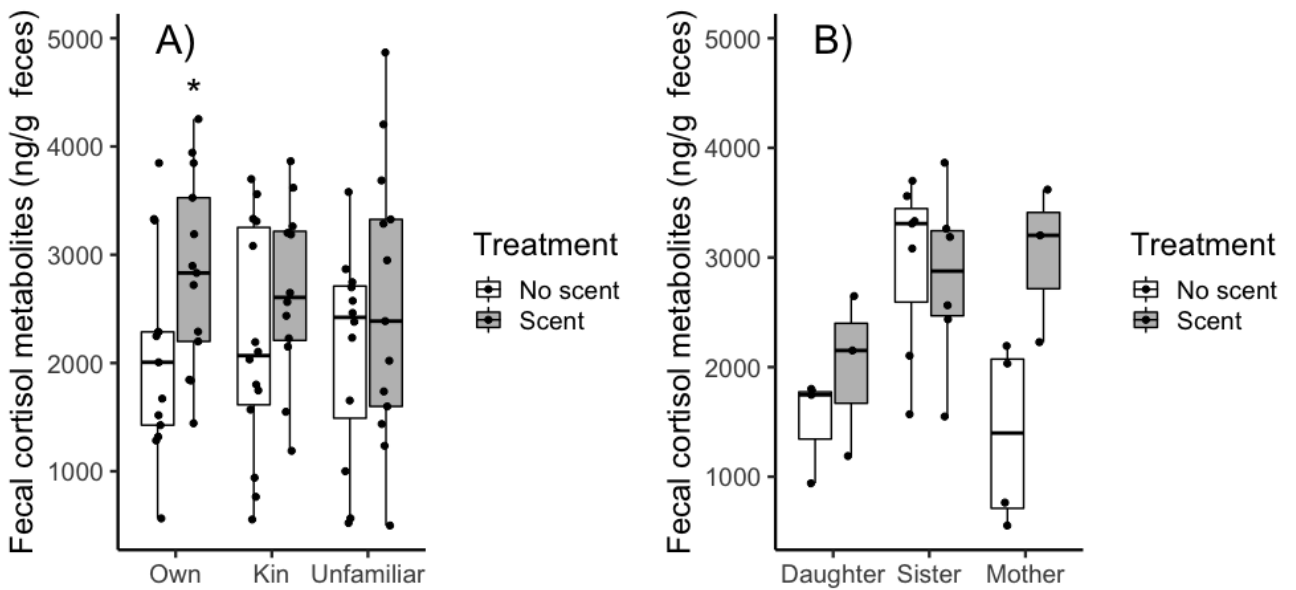
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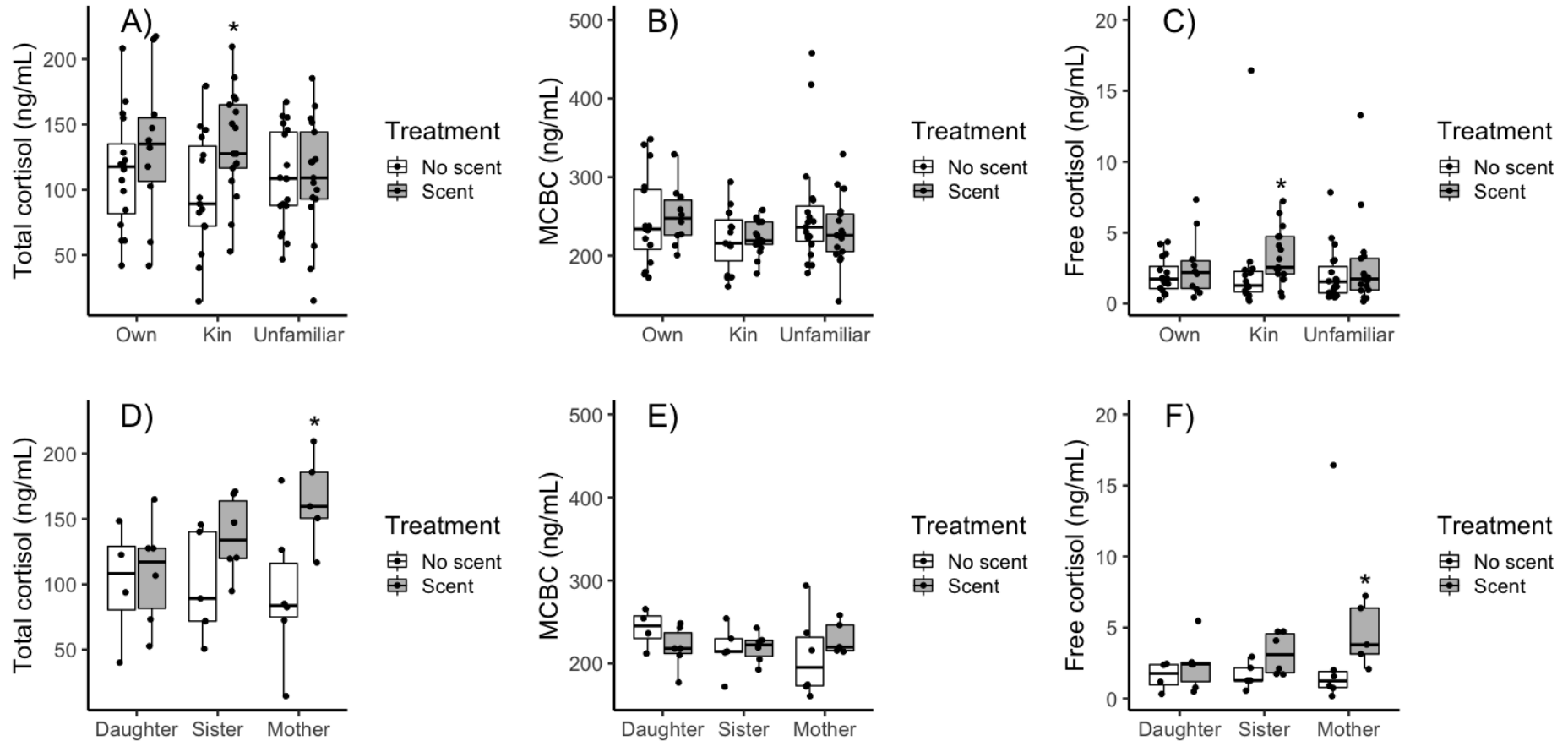
**Fig 6.** Distance of a female ground squirrel from her nest burrow at every minute of the 30-min observation period. The distance was calculated from Cartesian coordinates recorded every minute, starting as soon as a female emerged from her nest burrow in the morning. Values are presented from minute 1 through 29 (minute 0 was the emergence from nest burrow, and the distance by definition 0 m). Females were exposed to either no scent (black values) or the scent (blue values) of an unfamiliar female, a kin female, or their own scent (top row). Within the kin group, females were exposed to the scent of their mother, a littermate sister, or a daughter (bottom row). Values are given as means  $\pm$  SE. The grey and blue bands represent the 95% CI of the GAMMs.

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**Fig 7.** Fecal cortisol metabolite levels of female Columbian squirrels either exposed to no scent (white boxplots) or the scent (grey boxplots) of (A) an unfamiliar female, a kin female, or their own scent; (B) within kin scents; the scent of a daughter, a sister, or their mother. Box plots show the median of the data distribution (bold line) along with first and third quartiles (25<sup>th</sup> and 75<sup>th</sup> percentiles) of the data distribution corresponding to the lower and upper hinges of the boxes. The upper and lower whisker extend, respectively, to the largest and smallest value of the data set, no further than 1.5 x IQR (where IQR is the inter-quartile range). Data beyond the end of the whiskers are plotted individually. Significant differences ( $P < 0.05$ ) between the treatments are indicated by an asterisk.



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**Fig 8.** (A-C) Plasma levels of total cortisol (maximum cortisol binding capacity (MCBC) and free cortisol) of female Columbian squirrels exposed to no scent (white boxplots) or the scent (grey boxplots) of an unfamiliar female, a kin female or their own scent. (D-E) Within kin scents, plasma levels of total cortisol, MCBC and free cortisol after exposure to no scent or the scent of a daughter, a sister, or their mother. Box plots show the median of the data distribution (bold line) along with first and third quartiles (25<sup>th</sup> and 75<sup>th</sup> percentiles) of the data distribution corresponding to the lower and upper hinges of the boxes. The upper and lower whisker extend, respectively, to the largest and smallest value of

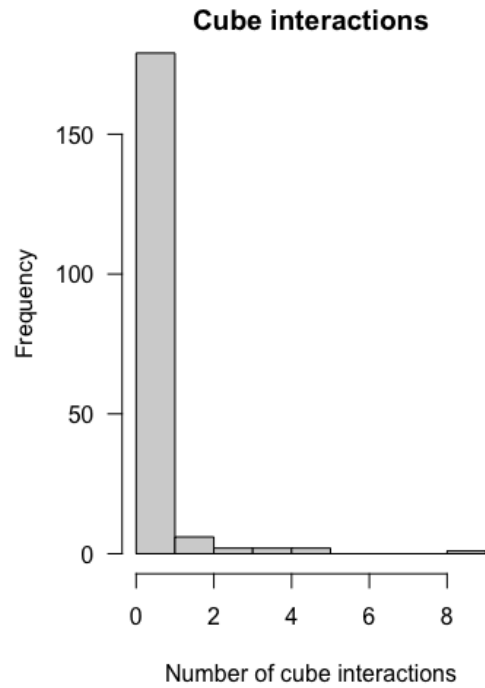
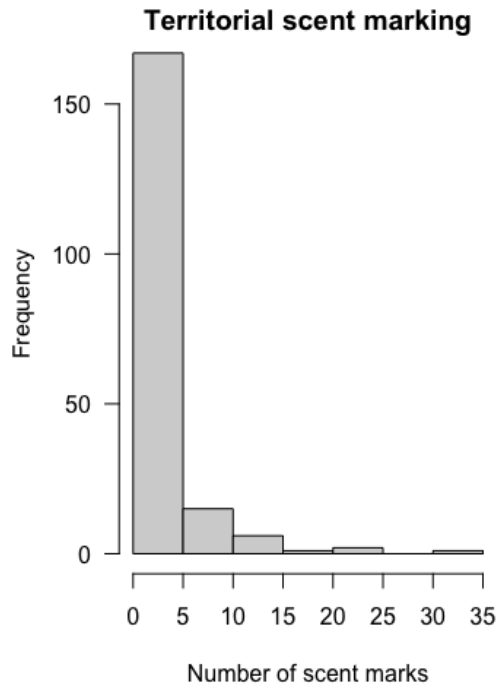
1009 the data set, no further than  $1.5 \times \text{IQR}$  (where IQR is the inter-quartile range). Data beyond the end of the whiskers are plotted individually.  
1010 Significant differences ( $P < 0.05$ ) between the treatments, once outliers removed, are indicated by an asterisk.  
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1013 **APPENDICES**

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1015 Appendix 1 : Distribution of territorial scent marking and interactions with scent cubes  
1016 during the 30 minute observation period for female Columbian ground squirrels

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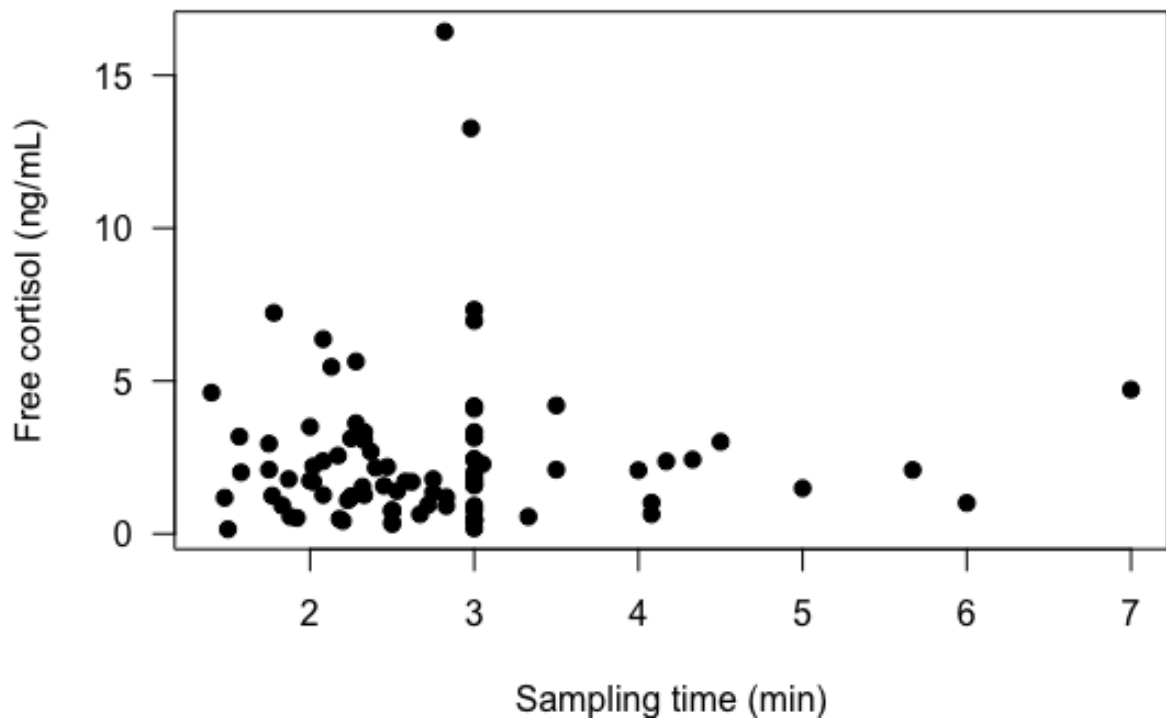
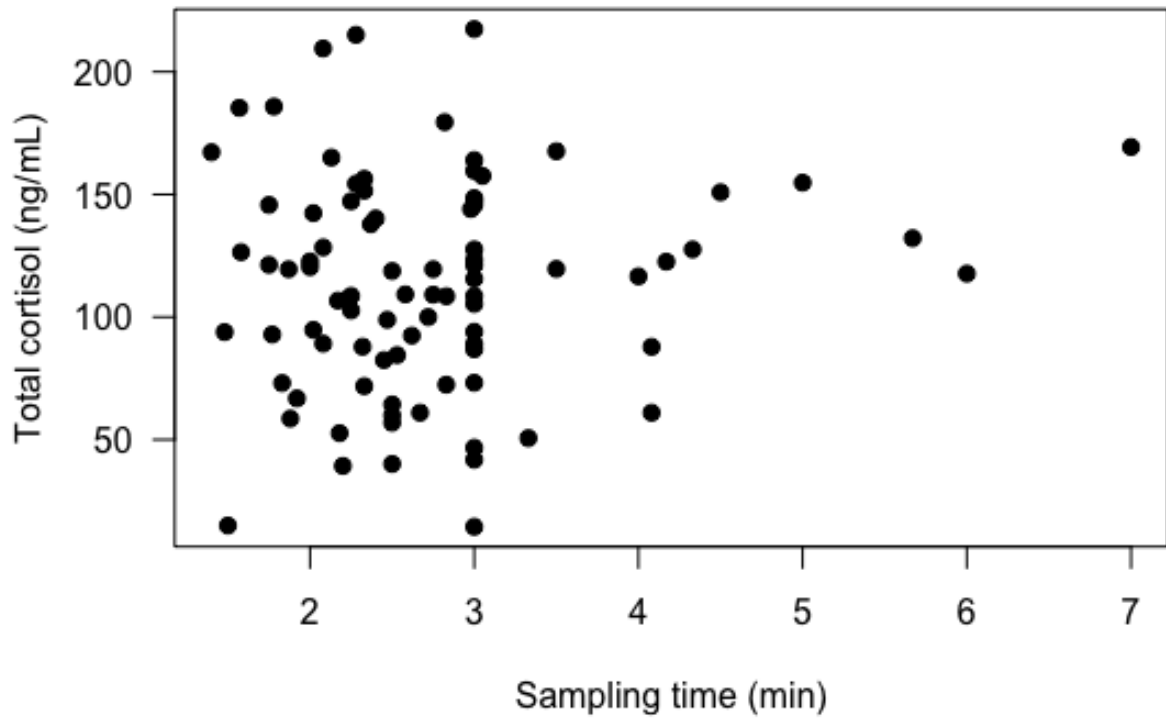


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1020 Appendix 2 : Relationships between plasma total cortisol levels (ng/mL) (top) and  
1021 plasma free cortisol levels (ng/mL) (bottom) and sampling time (min) in female  
1022 Columbian ground squirrels  
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