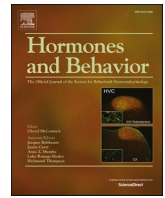




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## Territorial scent-marking effects on vigilance behavior, space use, and stress in female Columbian ground squirrels

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### ABSTRACT

Social environments can profoundly affect the behavior and stress physiology of group-living animals. In many territorial species, territory owners advertise territorial boundaries to conspecifics by scent marking. Several studies have investigated the information that scent marks convey about donors' characteristics (e.g., dominance, age, sex, reproductive status), but less is known about whether scents affect the behavior and stress of recipients. We experimentally tested the hypothesis that scent marking may be a potent source of social stress in territorial species. We tested this hypothesis for Columbian ground squirrels (*Urocyon columbianus*) during lactation, when territorial females defend individual nest-burrows against conspecifics. We exposed lactating females, on their territory, to the scent of other lactating females. Scents were either from unfamiliar females, kin relatives (a mother, daughter, or sister), or their own scent (control condition). We expected females to react strongly to novel scents from other females on their territory, displaying increased vigilance, and higher cortisol levels, indicative of behavioral and physiological stress. We further expected females to be more sensitive to unfamiliar female scents than to kin scents, given the matrilineal social structure of this species and known fitness benefits of co-breeding in female kin groups. Females were highly sensitive to intruder (both unfamiliar and kin) scents, but not to their own scent. Surprisingly, females reacted more strongly to the scent of close kin than to the scent of unfamiliar females. Vigilance behavior increased sharply in the presence of scents; this increase was more marked for kin than unfamiliar female scents, and was mirrored by a marked 131% increase in free plasma cortisol levels in the presence of kin (but not unfamiliar female) scents. Among kin scents, lactating females were more vigilant to the scent of sisters of equal age, but showed a marked 318% increase in plasma free cortisol levels in response to the scent of older and more dominant mothers. These results suggest that scent marks convey detailed information on the identity of intruders, directly affecting the stress axis of territory holders.

### 1. Introduction

Many species use scent as a form of chemosensory communication in a variety of social contexts (Ralls, 1971; Johnson, 1973; Bel et al., 1999). Scent-marking occurs via the deposition of secretions from exocrine

scent glands or via odorous marks deposited in urine and feces, on strategic substrate locations. Both time and energy investments are needed to produce olfactory secretions and maintain volatile compounds active in the environment (Radwan et al., 2006). However, this form of social communication allows territory owners, in their absence,

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to advertise their quality to tentative mates (Johansson and Jones, 2007; Boulet et al., 2010), and/or resource holding potential to tentative competitors, while minimizing active resource-defense. This circumvents escalation of risky contests over resources, such as territories or mates (Hurst and Rich, 1999; Gosling and Roberts, 2001; Stockley et al., 2013).

As scent-marking is thought to convey honest information on the identity, condition, and social status of the bearer, numerous studies have focused on unraveling the attributes signaled by odorous secretions. Scents reflect information on individual sex and age (Kean et al., 2011; Vaglio et al., 2016), familiarity (Hare, 1994), genetic similarity and kinship (Mateo, 2003; Leclaire et al., 2013), genetic dissimilarity or compatibility (Wedekind et al., 1995; Charpentier et al., 2008), reproductive status (Harris and Murie, 1984a, 1984b; Scordato and Drea, 2007; Zidat et al., 2018), health and immune status (Zala et al., 2004; López and Martín, 2005), hormonal status and stress levels (Lumley et al., 1999; Yamaguchi et al., 2005; Shimozuru et al., 2006), and social rank (Scordato and Drea, 2007; Vaglio et al., 2016). The complexity of odor signals has also been found to increase with social complexity (e.g. in *Eulemur* species; delBarco-Trillo et al., 2012), indicating the importance of scent communication in social species.

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

We tested the hypothesis that scent marking constitutes a source of social stress in territorial species, using Columbian ground squirrels (*Urocitellus columbianus*) as a model system. Columbian ground squirrels are sciurid rodents characterized by a matrilineal social system where related females share adjacent and overlapping territories over generations (King and Murie, 1985; King, 1989a, 1989b; Arnaud et al., 2012) and males are the dispersing sex (Wiggett and Boag, 1989; Neuhaus, 2006). Resident males show strong patterns of territoriality during the mating season (Murie and Harris, 1978), allowing preferential access to females that share their territory (Manno and Dobson, 2008). After reproduction, male territoriality subsides and females become more territorial during the subsequent gestation and lactation periods (Festa-Bianchet and Boag, 1982; Murie and Harris, 1988). Both males and females display scent-marking behavior, particularly during mating, gestation, and lactation (Steiner, 1974; Betts, 1976). Scents are deposited by rubbing apocrine glands located at the oral angles of the mouth (Kivett et al., 1976; Kivett, 1978), the anogenital area and a dorsal-glandular area of the body (Steiner, 1974) against the substrate. There is clear evidence that scent is an important means of social communication in this species, either related to mating behavior (determination of female reproductive status from vaginal scent marks; Harris and Murie, 1984a, 1984b); fear (anal gland pulsating during hostile encounters; Steiner, 1970; Steiner, 1974); or the discrimination of familiar

and unfamiliar conspecifics (oral scents; Harris and Murie, 1982; Hare, 1994). In an experimental study of Columbian ground squirrels, Raynaud and Dobson (2011) found that females were more attentive to the scent of other females than males: Presented with conspecific scents, females invested more time re-marking female than male scent marks. These authors hypothesized that this behavior reflected female territory advertisement and protection of the litter, which is especially important during lactation. During this period, infanticide by other, mostly unrelated, lactating females is known to occur (Balfour, 1983; Dobson, 1990; Hare, 1991; Stevens, 1998).

Here, we exposed lactating females on their territories to the scents of other lactating females, and tested the consequences of this exposure on the behavior and stress physiology of territory owners. We collected female scents on acrylic cubes (by rubbing the cubes on the angulo-oral glands) (see Harris and Murie, 1982; Raynaud and Dobson, 2011). The scented cubes were then placed around the nest burrow of a focal female, testing her behavioral and physiological (HPA) response to the scent of either (i) an unfamiliar female, (ii) a kin female, or (iii) herself. If scent marking indeed constitutes a source of social stress, we expected to observe marked behavioral and HPA responses of females to the presence of novel scents on their territory. After exposure to conspecific scents, compared to her own scent or no scent conditions, we expected focal females to devote an increased proportion of time to vigilance behavior, and to show heightened HPA activity measured through increased levels of blood cortisol and fecal cortisol metabolites (Bossion et al., 2009). We also expected females to display increasing amounts of scent marking behavior on their territory, to interact more with scented cubes, and to spend more time exploring their territorial boundaries seeking out the intruder. Because female Columbian ground squirrels are more tolerant of close female kin (King and Murie, 1985; King, 1989a, 1989b; Viblanc et al., 2016), we expected the effects of scent marks to be stronger for unfamiliar than for kin scents. However, because mothers appear to be dominant over daughters in this species (Harris and Murie, 1984a, 1984b; Wiggett and Boag, 1992; Viblanc et al., 2016; Sosa et al., 2020), we also expected females' behavioral and physiological stress responses to be stronger when exposed to the scent of their mother than to the scent of a daughter or littermate sister. Differences in terms both of behavioral and physiological reactions to different scent marks would indicate that subtle social information on identity was conveyed by chemical signals.

## 2. Material and methods

### 2.1. Data collection and study site

The study was conducted during the 2017 and 2018 breeding seasons. We studied lactating females in three neighboring colonies of Columbian ground squirrels (Meadow B, Meadow C and DOT) that were actively monitored as part of long-term studies on the behavior and ecology of those animals. The colonies are located in the Sheep River Provincial Park, Alberta, Canada (Meadow B: 50° 38' 10.73" N; 114° 39' 56.52" W; 1524 m; 2.0 ha; Meadow 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' 41.79" W; 1545 m; 3.0 ha). In each year, females were followed throughout reproduction, from emergence from hibernation in early April to the weaning of offspring in early to mid-July (Neuhaus, 2000; Dobson et al., 2020). Female mating dates were determined from visual observations of above ground consortships with males and inspection of genitalia upon trapping (presence of a copulatory plug or sperm in vaginal smears or fur; Murie, 1995; Raveh et al., 2010). Gestation lasts ~24 days, upon which females give birth to an average of three (one to seven) altricial offspring in a specially constructed nest burrow (Dobson and Murie, 1987; Murie, 1995). Lactation lasts ~27 days, after which, weaned offspring first emerge above ground (Murie and Harris, 1982; Dobson et al., 1992).

## 2.2. Scent collection

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

## 2.3. Experimental trial

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

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

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

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

Our sample size for the different groups were 32 focal females tested for kin scents (15 in 2017 and 17 in 2018), 35 focal females tested for unfamiliar female scents (14 in 2017 and 21 in 2018), and 29 females tested for their own scent (13 in 2017 and 16 in 2018). All females were tested only once (i.e., with one type of scent) within a given year, but some females were tested repeatedly in different years, and so we controlled for female identity in statistical analyses (see below). On average, the age distribution of females was fairly balanced between groups (female age in kin scent group: mean ( $\pm$ SD) = 4.1  $\pm$  1.9 y.o., range = 2–9 y.o.; female age in unfamiliar scent group: mean = 4.3  $\pm$  2.0 y.o., range = 2–9 y.o.; female age in own scent group: mean = 4.1  $\pm$  1.8 y.o., range = 1–8 y.o.). Within the kin group, females exposed to the scent of a daughter were slightly older (mean = 6.3  $\pm$  1.3 y.o., range = 4–9 y.o.) than those exposed to the scent of a mother (mean = 3.0  $\pm$  0.9 y.o.,

range = 2–5 y.o.) or a sister (mean  $\pm$  SD = 3.7  $\pm$  1.6 y.o., range = 2–7 y.o.). We thus controlled for female age as a covariate in subsequent analyses to account for potential age effects on behavioral and physiological variables.

## 2.4. Behavioral observations

Behavioral observations were carried out during lactation, from the 18th of May to the 8th of June in 2017, and from the 23rd of May to the 7th of June in 2018. Each female was observed by the same observer during the entire experimental trial (phases 2, and 3). This required 1–7 observers daily in 2017 and 1–8 observers in 2018. We used fixed-interval point sampling of behaviors (Bateson and Martin, 2021). Females were observed every minute, for 30-min periods. Only the behavior on the minute mark was checked on a pre-defined behavioral spread sheet. Each minute, the observer also recorded the exact position of the animal on a Cartesian 10  $\times$  10 m grid, delimited by colored flags placed throughout the study site. Thus, the position of the animal could be estimated to within about a 1-m resolution during the observation period. Scored behaviors included: vigilance, locomotion, foraging, grooming, resting, alarm calling, aggressive (chases and fights) and amicable (sniffing) interactions. Vigilance behavior was scored whenever the animal was sitting still on its hind legs or on all four paws, its head pointing away from the ground, scanning the environment. If an animal was temporarily out of sight (e.g., behind a tree or rise in the ground, in a burrow, etc.), an "out of sight" category was scored. In cases where the animal was lost for an extended period of time, observations were discarded. In addition to the above behaviors scored on the minute mark, we recorded and summed all occurrences of (1) cube interactions (licking, biting, scratching or sniffing a cube) and (2) scent marking (scratching and/or rubbing the ground with the cheek or the lateral side of the body, rubbing the cubes with the cheek) during the 30-minute observation period. Because cube interactions were very rare (Appendix 1), we pooled our observations into a binomial variable (interaction vs. no interaction with cubes) for further analyses (see below).

## 2.5. Feces and blood sampling

In 2017, we collected fecal samples to measure fecal cortisol metabolites (FCMs) as an integrative measure of female stress. Females were captured approximately 7 h following control observations (phase 2) or first exposure to the scent (phase 3), corresponding to the time required for glucocorticoid plasma changes to be reflected in FCM changes (Bosson et al., 2009). Fecal samples were collected into 2-mL sterile vials as females defecated during handling, or from the floor of the trap. In the latter case, the female was always observed defecating in the trap and the feces collected immediately. The traps were systematically cleaned before being deployed to ensure fecal samples corresponded to targeted individuals. We insured that no fecal sample was contaminated by urine. Samples were immediately stored on ice packs in the field and transferred to a –20 °C freezer within 30 min of collection.

In 2018, we collected blood samples to measure female's blood cortisol levels immediately after the 30 min of control (phase 2) and exposure to a scent (phase 3). A few females (N = 12) were also bled in 2017, in which case they were not sampled for feces, so as to not bias results with stress from the capture. Following the 30-min observation period, females were trapped and a 0.5 mL blood sample was collected from the saphenous vein using a 27-G needle fitted to a 1 mL heparinized syringe. An observer would set a trap on the female's territory, and keep watch until the targeted female entered the trap, in general within minutes of deployment and within 30 min maximum. As soon as the trap-door shut, a stop watch was started and the time until the animal bled was completed was timed. We aimed to sample blood within 3-min of trapping (mean  $\pm$  SD = 2.75  $\pm$  0.97 min, min = 1.40, max = 7.00) to capture baseline CORT levels. Although 20 samples out of 93 were acquired after 3 min (on average at 4.44  $\pm$  1.12 min, min = 3.05, max =

7.00), we decided to keep those in our analyses, as total (Pearson's  $r = 0.11$ ,  $P = 0.33$ ) and free ( $r = 0.03$ ,  $P = 0.80$ ) cortisol levels were not significantly correlated to sampling time in our data (Appendix 2). Syringes were kept on ice packs in a cooler box in the field. Blood was centrifuged (3000 g for 10 min) within 30 min of collection, plasma and blood cells were separated and frozen at  $-20^{\circ}\text{C}$  until the end of the field season.

Both fecal and blood samples were shipped at the end of the season on dry ice to the University of Toronto Scarborough (Canada), and were kept frozen at  $-80^{\circ}\text{C}$  until analyses.

## 2.6. Stress hormone analyses

### 2.6.1. Fecal cortisol metabolites (FCMs)

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

### 2.6.2. Plasma total cortisol

We measured plasma total cortisol levels by radioimmunoassay (RIA) using a commercially available kit (ImmuChem™ Coated Tube Cortisol  $^{125}\text{I}$  RIA Kit; MP Biomedicals, LLC, Orangeburg, NY, USA). The antibody has a cross-reactivity of 5.5% to corticosterone and less than 3% to other naturally occurring steroids. Preliminary validation to ensure parallelism was done using different cortisol concentrations in CGS plasma (Bosson & Boonstra; unpublished data). We used the following modifications compared to the kit protocol. Each plasma sample was analyzed in duplicate with 10  $\mu\text{L}$  of plasma being added per RIA tube along with 1 mL of Cortisol- $^{125}\text{I}$ , 20  $\mu\text{L}$   $\text{NH}_4\text{OH}$  (to saponify triglycerides), and 40 L double-distilled  $\text{H}_2\text{O}$  prior to incubation. Samples were run against a 6-point standard curve (0–100 ng/mL) on an automatic gamma counter (Wizard<sup>2</sup> 2470, PerkinElmer, Waltham, MA, USA). This method has a mean recovery of  $102.4 \pm 2.54\%$  ( $N = 10$ , range 91–117%) and a detection limit of 17 pg/10  $\mu\text{L}$ . The mean intra-assay coefficient of variation was 12.55% (low pool) and 11.40% (high pool), and the mean inter-assay coefficient of variation was 7.20% (low pool), and 9.72% (high pool). All duplicate samples had a coefficient of variation of 8% or less.

We determined how much plasma cortisol was free and not bound to its main carrier protein, corticosteroid-binding globulin (CBG). For this, we measured CBG for each sample as the maximum corticosteroid-binding capacity (MCBC) with the saturated ligand method described in McDonald et al. (1981) and then calculated the free cortisol. We followed the MCBC protocol outlined in Delehanty and Boonstra (2009) with slight modifications. Cortisol (Sigma Aldrich, St. Louis, MO, USA) was diluted to 20 ng/10  $\mu\text{L}$  EtOH, and 10  $\mu\text{L}$  was added to  $12 \times 75$  mm polypropylene tubes and dried under filtered air. Once dry, duplicate 10  $\mu\text{L}$  of plasma were added to the tubes, followed by 100  $\mu\text{L}$  of cortisol [ $1,2,6,7$ - $^3\text{H}(\text{N})$ ] (7.21 pg/tube; PerkinElmer). Next, 400  $\mu\text{L}$  phosphate buffer (pH 7) was added to each tube and allowed to equilibrate at room temperature for 30 min, and left overnight at  $4^{\circ}\text{C}$ . The following

morning, 200  $\mu\text{L}$  of dextran-coated charcoal was added to separate bound and free hormone. After a 10-minute incubation at  $4^{\circ}\text{C}$ , the samples were centrifuged at 2800 rpm for 10 min, and 500  $\mu\text{L}$  of supernatant was added to 3.5 mL of scintillation fluid (Gold Star, Meridian Biotechnologies Ltd., Surrey, ENG, UK), vortexed, and left to equilibrate in the dark for at least 4 h before being read in a scintillation counter (Tri-Carb 3110 TR, PerkinElmer). The intra- and inter-assay coefficients of variation based on the pools were 7.57% and 9.67%, respectively. All duplicate samples had a coefficient of variation of 11% or less.

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

## 2.7. Statistical analyses

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

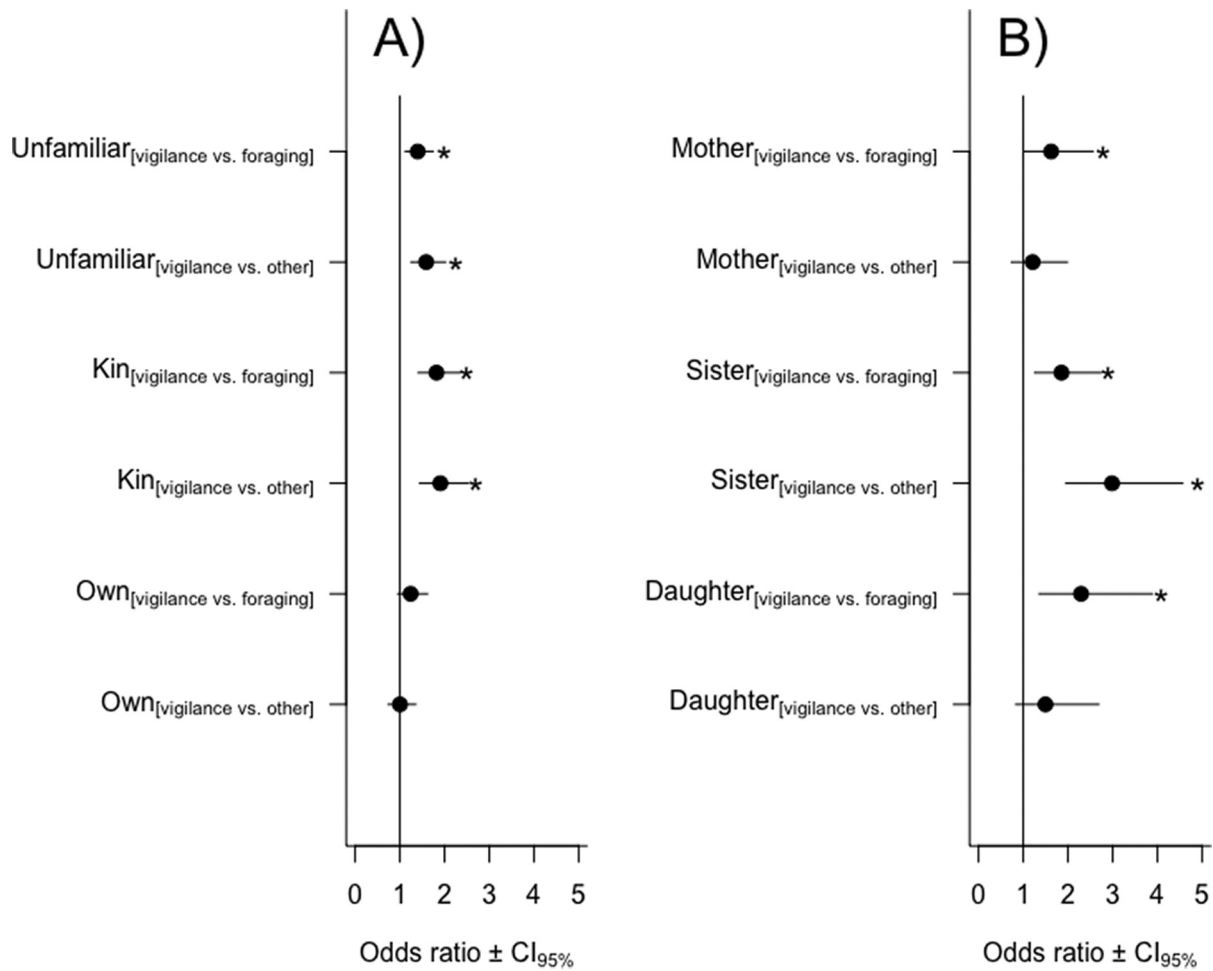
### 2.7.1. Vigilance behavior

Female vigilance behavior in response to the scents was analyzed using multinomial logistic regression (MLR; package ‘mlogit’ in R), accounting for repeated measures on females in separate years. The multinomial response outcomes (dependent variable) included ‘vigilance’, ‘foraging’, and ‘other’ behaviors ( $<7.5\%$  of total observations). We ran separate regressions for each scent category (unfamiliar, kin or own scents – and within the kin category: for mother, daughter, or sister scents) and specified treatment (unscented vs. scented cubes) as the independent variable. Thus, we tested how female vigilance behavior changed in proportion relative to foraging or other behaviors when females were exposed to a scent compared to the no scent situation (no scent fixed as reference level). Results are given as odds ratios along with 95% confidence intervals. Significant odds ratios have confidence intervals not overlapping 1 and can be interpreted as the odds of increasing ( $>1$ ) or decreasing ( $<1$ ) vigilance relative to foraging or other behaviors for a transition from no-scent to scent condition.

### 2.7.2. Territorial marking and interactions with cubes

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

Second, for scent marking only (there were too few occurrences of cube interactions, see Appendix 1), we focused on the number of scent marks actually performed for individuals that scent-marked (i.e., all data  $>0$ ). For this, we ran separate GLMMs (Poisson error distribution), with the number of scent marks as the dependent, and treatment (no-scent vs.



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

scented cubes) as the independent variable for each of our scent treatments (unfamiliar, kin or own scents). Here also, we included year and female ID as random effects in the models, but removed them if models did not converge and their associated variance could not be estimated. When working at the kin level (mother, daughter, or sister scents), because of low sample size  $N < 5$  in some of the categories, we tested the difference between control (no-scent cubes) and treated (scented cubes) conditions using exact permutation tests ('lmp' function from the 'lmpPerm' package in R; Wheeler and Torchiano, 2016).

### 2.7.3. Exploration behavior

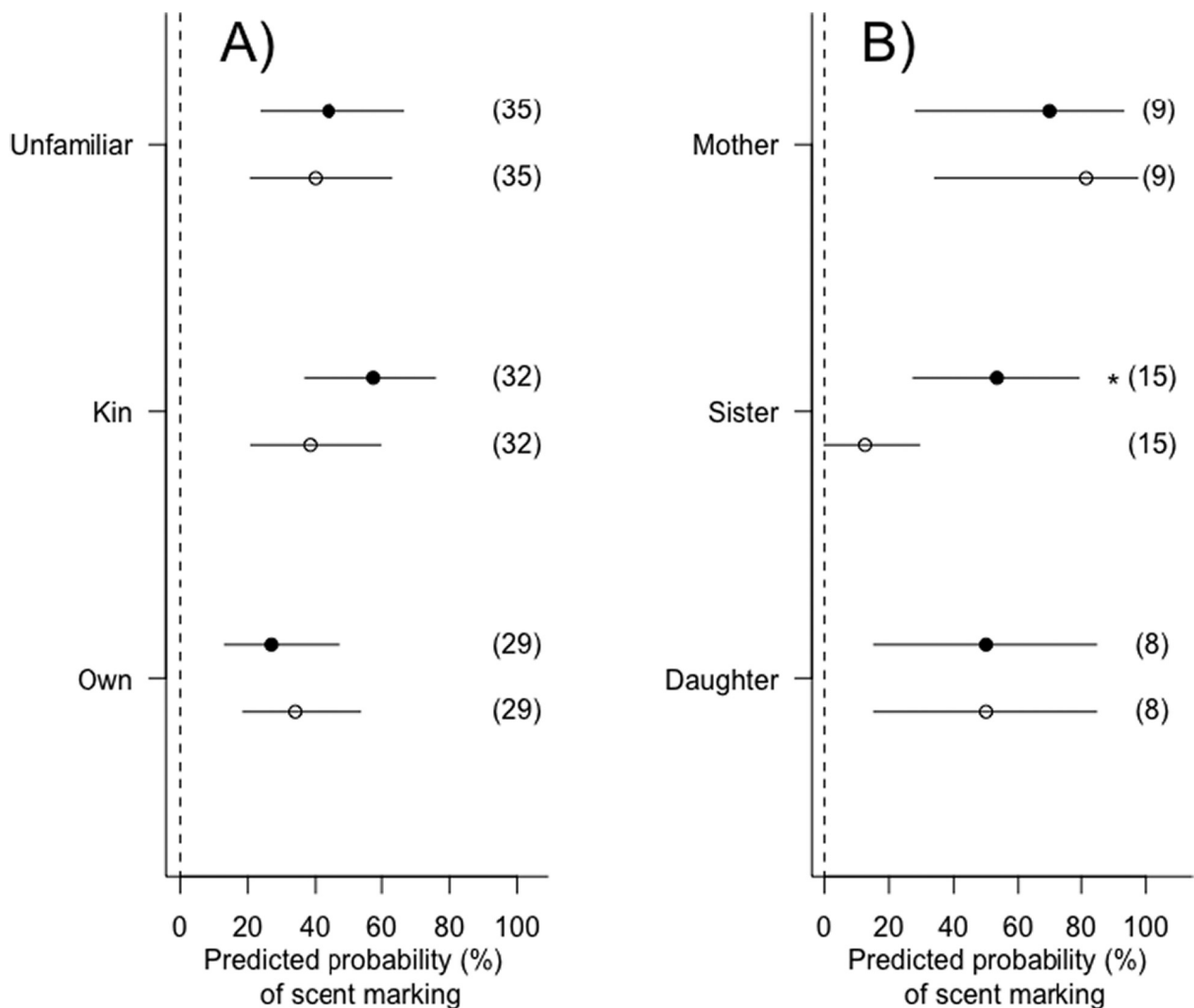
To evaluate the effects of scent marks on female exploration behavior, we quantified: (1) the overall size of the area (in  $m^2$ ) used over the 30-min observation period, and (2) how far a female ventured from her nest burrow within the 30-min. First, we calculated the area used during the observations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

converge and their associated variance could not be estimated.

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

### 2.7.4. Physiological stress

Female fecal cortisol metabolites (FCM; obtained in 2017; 7 h after the exposure to unscented or scented cubes) and plasma cortisol levels (CORT; obtained in 2017 and 2018; immediately after the exposure to cubes) were analyzed using separate LMMs. FCM and CORT levels (either total CORT, free CORT or MCBC) were specified as dependent variables in the separate models for own, kin and unfamiliar scent categories and the treatment (no-scent vs. scented cubes) specified as an independent variable. Here also, female ID (and year for the plasma CORT data) were entered as random variables for LMMs, but removed if their associated variance could not be estimated. Again, when working at the kin level, because of low sample size of  $N < 5$  in some of the categories, we tested the difference between control (no-scent cubes)



**Fig. 2.** Probability of a female ground squirrel engaging in scent marking during a 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 ±95% CI. Sample size is given in brackets. Significant differences ( $P < 0.05$ ) between (○) and (●) conditions are indicated by an asterisk.

and treated (scented cubes) using exact permutation tests ('lmp' function from the 'lmpPerm' package in R; [Wheeler and Torchiano, 2016](#)).

### 2.8. Ethics

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

## 3. Results

### 3.1. Vigilance behavior

Controlling for age and compared to the no-scent condition, females exposed to either unfamiliar or kin scents, but not those exposed to their own scent, engaged in significantly more vigilance than foraging or other behaviors over the 30-minute period (Fig. 1A). For females exposed to unfamiliar scents, the odds of engaging into vigilance rather than foraging or other behaviors increased by 1.40 and 1.59,

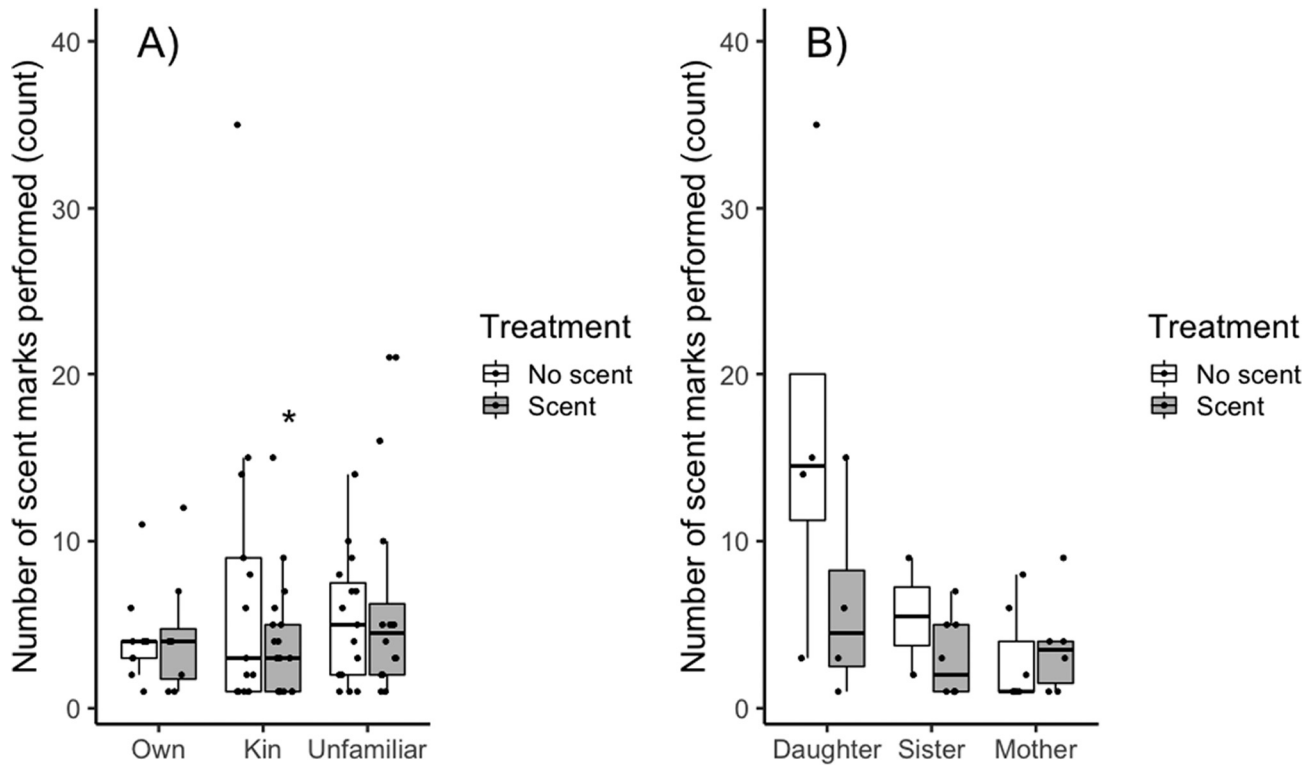
respectively, compared to no-scent conditions (multinomial;  $z = 2.98$  and  $3.77$ ,  $P = 0.003$  and  $P < 0.000$ ). For females exposed to kin scents, these odds increased by 1.82 and 1.91 ( $z = 4.63$  and  $4.54$ , both  $P < 0.000$ ) compared to no-scent conditions, whereas the odds did not change significantly (odds ratios = 1.24 and 1.00;  $z = 1.60$  and  $0.02$ ,  $P = 0.11$  and  $0.99$ ) for females exposed to their own scents vs. no-scent conditions.

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

### 3.2. Territorial scent-marking and interactions with cubes

#### 3.2.1. Territorial scent-marking

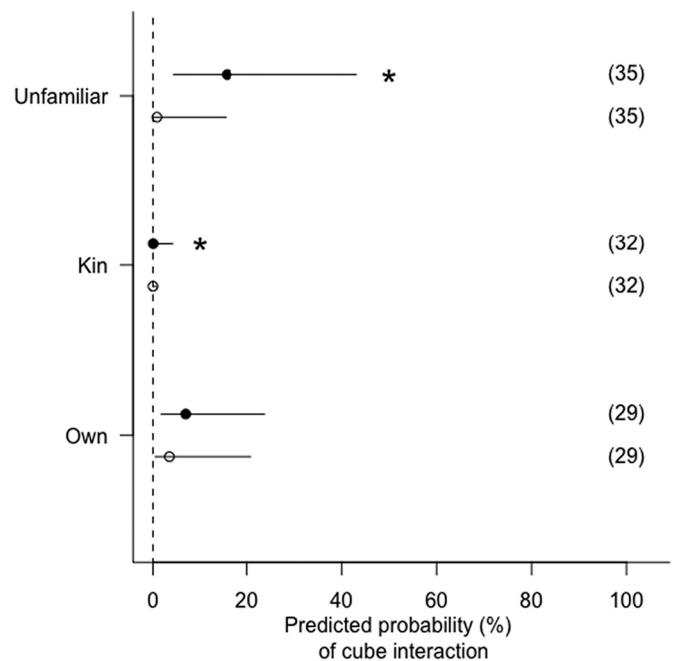
We observed territorial scent-marking by resident females in 42% of



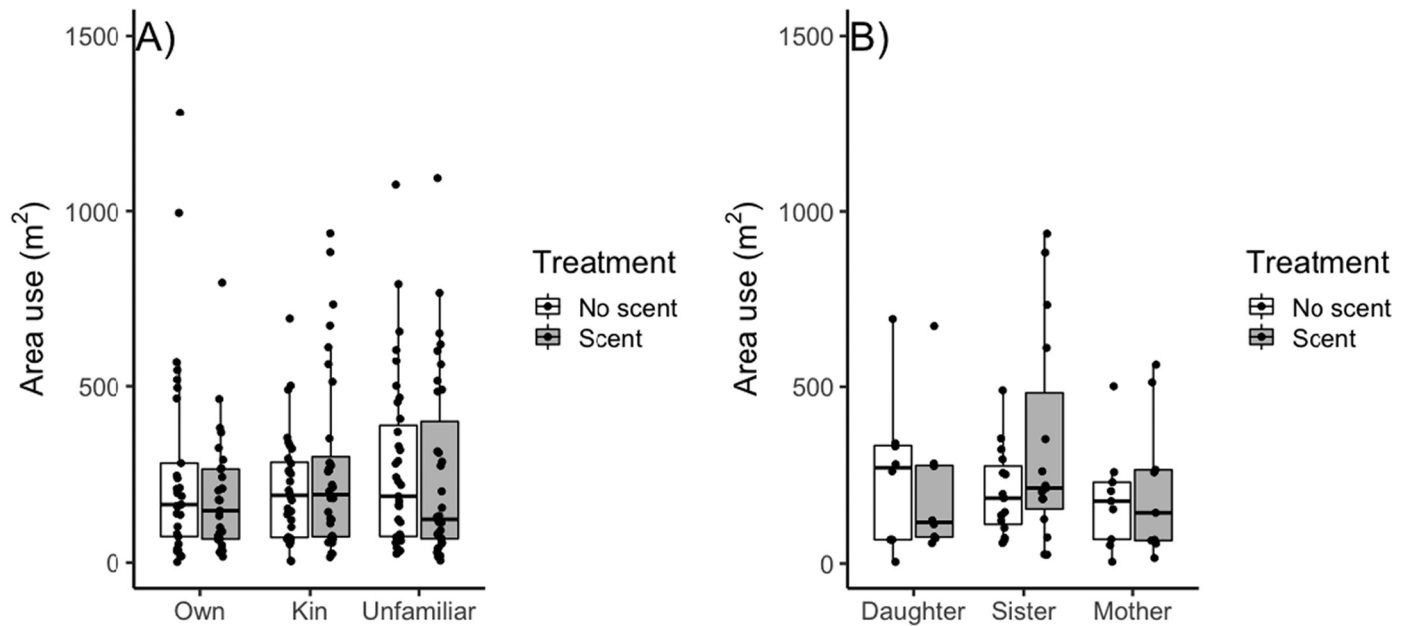
**Fig. 3.** Number of scent marks deposited by a female ground squirrel during a 30-min observation period. Females were exposed to either no scent (white boxplots) or the scent (grey boxplots) of (A) an unfamiliar female, a kin female, or their own scent. (B) within kin scents; the scent of their mother,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

our observation periods. Controlling for age, the probability for females to scent-mark (GLMM; binomial 1/0) during the 30-min observation period did not differ between scent (treatment) and no-scent (control) conditions, regardless of the scent considered (own scent: 27% vs. 34% for marking probability in the scent vs. no-scent condition, respectively; odds ratio = 0.72,  $CI_{95} = [0.23, 2.24]$ ,  $z = -0.57$ ,  $P = 0.57$ ; kin scent: 57% vs. 38%, odds ratio = 2.16,  $CI_{95} = [0.69, 6.82]$ ,  $z = 1.32$ ,  $P = 0.19$ ; unfamiliar scent: 44% vs. 40%, odds ratio = 1.18,  $CI_{95} = [0.38, 3.68]$ ,  $z = 0.29$ ,  $P = 0.77$ ) (Fig. 2A). Controlling for age, when considering the kin group only, females exposed to the scent of a sister had a higher probability of scent-marking in the presence than in the absence of scent (53% vs. 13%; odds ratio = 7.95,  $CI_{95} = [1.45, 66.28]$ ,  $z = 2.20$ ,  $P = 0.03$ ). This was not the case, however, for females exposed to the scent of their mother (70% vs. 81%; odds ratio = 0.52,  $CI_{95} = [0.05, 5.24]$ ,  $z = -0.55$ ,  $P = 0.58$ ). For females exposed to the scent of a daughter, age was removed from the analysis since the model would not converge. Females exposed to the scent of a daughter had a similar probability of scent-marking when exposed to a scent or not (50% vs. 50%; odds ratio = 1.00,  $CI_{95} = [0.09, 11.30]$ ,  $z = 0.00$ ,  $P = 1.00$ ) (Fig. 2B).

For females that did engage in scent-marking, controlling for age, the number of scent-marks performed was 48% lower in the treated condition (scent present) for the kin group (GLMM; Poisson, count data; estimate =  $-0.48$ ;  $CI_{95} = [-0.83, -0.14]$ ,  $z = -2.72$ ,  $P = 0.007$ ), but did not differ significantly between unscented and scented conditions for other groups (own scent: estimate =  $-0.13$ ,  $CI_{95} = [-0.74, 0.42]$ ,  $z = -0.45$ ,  $P = 0.65$ ; unfamiliar scent: estimate = 0.24,  $CI_{95} = [-0.07, 0.56]$ ,  $z = 1.53$ ,  $P = 0.13$ ) (Fig. 3A). However, this appeared to be the result of one female that spent a substantial amount of time scent marking. When this female was removed from the analysis, the effect was still negative (i.e., females exposed to a kin scent decreased territorial marking by 25%, on average, in the presence of the scent) but no



**Fig. 4.** Probability of a female ground squirrel interacting with a scent cube during a 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  $\pm$  95% CI. Significant differences ( $P < 0.05$ ) between (○) and (●) conditions are indicated by an asterisk. Sample size is given in brackets.



**Fig. 5.** Area ( $\text{m}^2$ ) covered over a 30-min observation period by female Columbian squirrels. 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 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 their mother, a sister, or a daughter. Box plots show the median of the data distribution (bold line) along with its first and third quartiles (25<sup>th</sup> and 75<sup>th</sup> percentiles), corresponding to the lower and upper hinges of the boxes. The upper and lower whiskers extend, respectively, to the largest and smallest value of 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.

longer significant (estimate =  $-0.23$ ,  $\text{CI}_{95} = [-0.65, 0.18]$ ;  $z = -1.10$ ,  $P = 0.27$ ). Within the kin group (Fig. 3B), we found no significant difference in the number of scent marks performed regardless of whether females were exposed to the scent of a daughter (Exact permutation tests;  $P = 0.27$ ), a sister ( $P = 0.45$ ), or a mother ( $P = 0.69$ ).

### 3.2.2. Interactions with cubes

Resident females were observed interacting with cubes in 11% of our observation periods. Controlling for age, females exposed to unfamiliar scents interacted significantly more with cubes in the scent vs. no-scent condition (16% vs. 0.01%; odds ratio = 22.01,  $\text{CI}_{95} = [1.67, 290.73]$ ,  $z = 2.35$ ,  $P = 0.02$ ) (Fig. 4). Females also interacted significantly more with cubes when a kin scent was present vs. no-scent, though the predicted probabilities of interaction were, overall, extremely low (0.0005% vs. 0.000003%; odds ratio = 184.32,  $\text{CI}_{95} = [1.38, 24,699.21]$ ,  $z = 2.09$ ,  $P = 0.04$ ). In contrast, females did not interact significantly more with cubes in the 'own' condition, regardless of whether a scent was present or not (3% vs. 7%; odds ratio = 2.08,  $\text{CI}_{95} = [0.19, 46.23]$ ,  $z = 0.58$ ,  $P = 0.56$ ). The low number of cube interactions precluded us from meaningful analyses of the data within the kin category.

## 3.3. Exploration behavior

### 3.3.1. Space use

Controlling for age, no significant difference was found in the areas covered by females during the 30-min observation period between the no-scent and scent conditions for females exposed to unfamiliar scents (LMM; estimate =  $-17.85$ ;  $\text{CI}_{95} = [-134.00, 98.30]$ ,  $t = -0.30$ ,  $P = 0.76$ ), to kin scents (estimate =  $59.46$ ;  $\text{CI}_{95} = [-39.45, 158.37]$ ,  $t = 1.18$ ,  $P = 0.24$ ), or to their own scent (estimate =  $-75.06$ ;  $\text{CI}_{95} = [-170.10, 19.98]$ ,  $t = -1.55$ ,  $P = 0.12$ ) (Fig. 5a). On average, the area covered by females was similar for all 3 groups (unfamiliar female scent: 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 not significantly different (LMM and Tukey HSD; all  $P > 0.73$ ). Similarly, when considering the kin group only, we

found no significant differences in the areas covered by females during the 30-min observation between the no-scent and scent condition, regardless of whether the scent originated from a daughter (LMM; estimate =  $-47.03$ ;  $\text{CI}_{95} = [-243.48, 140.41]$ ,  $t = -0.49$ ,  $P = 0.62$ ), a mother (estimate =  $32.79$ ;  $\text{CI}_{95} = [-124.72, 190.31]$ ,  $t = 0.41$ ,  $P = 0.68$ ), or a littermate sister (estimate =  $132.25$ ;  $\text{CI}_{95} = [-24.47, 288.97]$ ,  $t = 1.65$ ,  $P = 0.10$ ) (Fig. 5b). Here also, on average, the area covered by females was similar for all 3 groups (daughter scent: area =  $232.52 \pm 52.21 \text{ m}^2$ ; mother scent:  $199.80 \pm 40.65 \text{ m}^2$ ; sister scent:  $269.56 \pm 43.54 \text{ m}^2$ ), and not significantly different (LMM and Tukey HSD; all  $P > 0.62$ ).

### 3.3.2. Distance to nest burrows

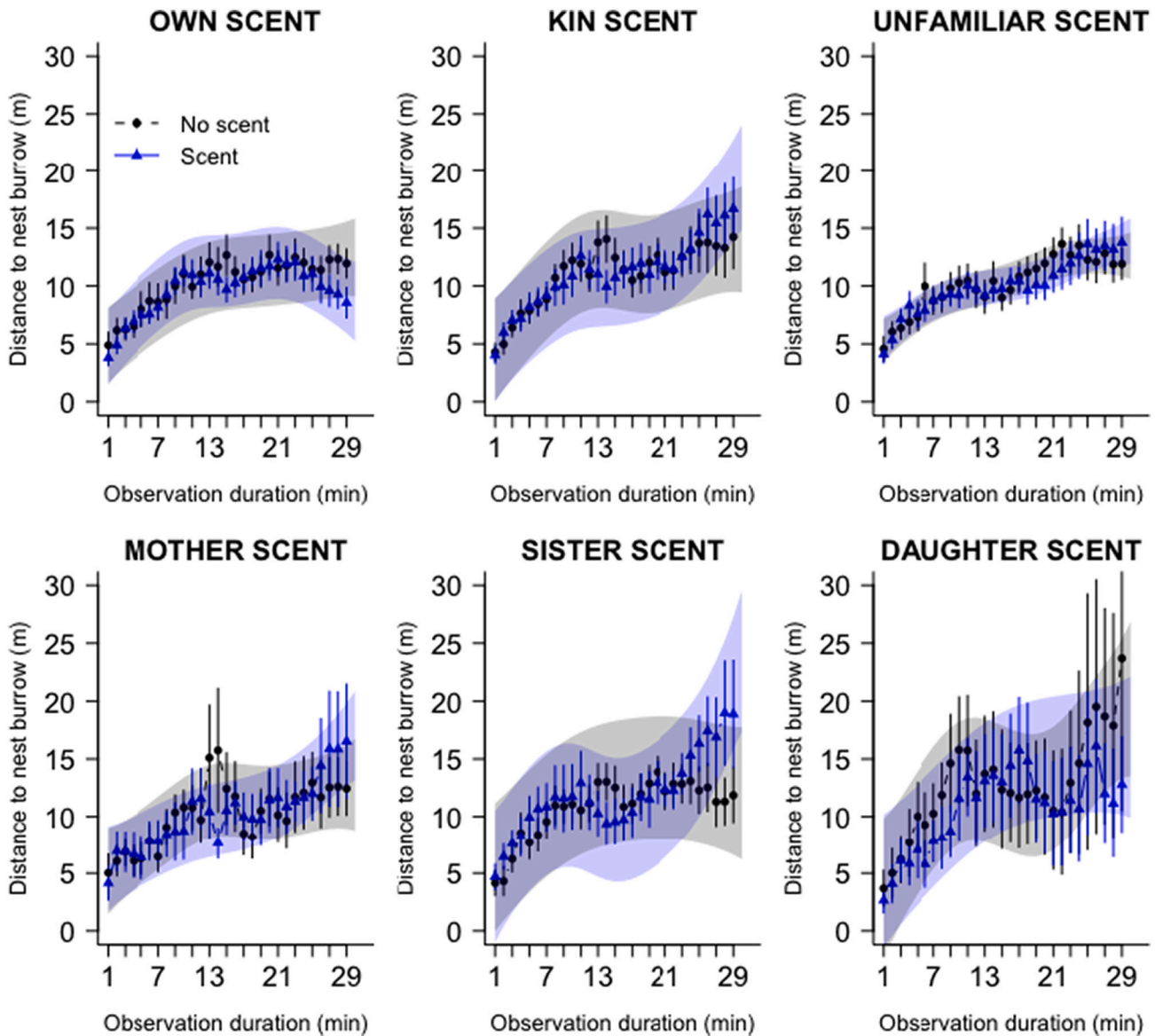
Controlling for age, the distance a female travelled from her nest burrow generally increased in a non-linear fashion in all groups over the 30-minute observation period (GAMMs;  $2.30 < \text{edf} < 4.69$ ,  $10.00 < F < 46.80$ , all  $P < 0.001$ ; Fig. 6). In all groups and treatments, females rapidly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

## 3.4. Physiological stress

### 3.4.1. Fecal cortisol metabolites (FCMs)

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





**Fig. 6.** Distance of a female ground squirrel from her nest burrow at every minute of a 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

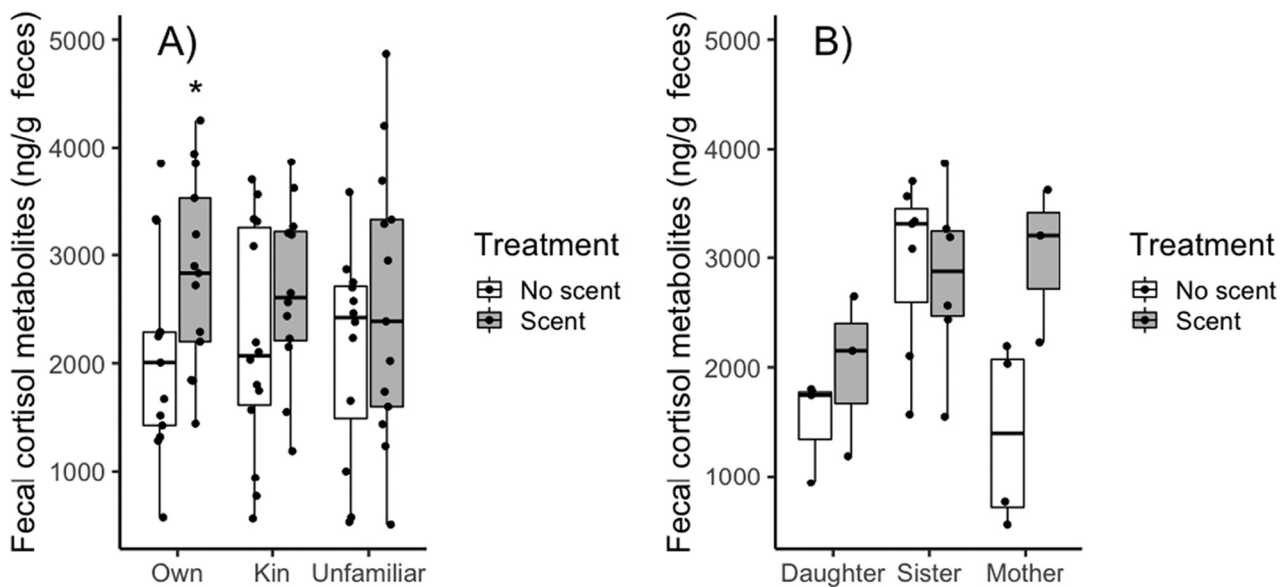
2.01,  $P = 0.07$ ) (Fig. 7A). Within kin categories, females exhibited 118% higher FCM when exposed to the scent of their mother (Exact permutation test;  $P = 0.07$ ), but not when exposed to the scent of a sister ( $P = 0.78$ ) or their daughter ( $P = 0.34$ ) (Fig. 7B).

### 3.4.2. Plasma cortisol

In response to kin scents, controlling for age, females showed a significant 39% increase in total cortisol levels (LM; estimate =  $38.17 \pm 15.42$ ,  $t = 2.47$ ,  $P = 0.02$ , Fig. 8A). Their MCBC levels (Fig. 8B) did not differ significantly from the control levels (LM; estimate =  $1.54 \pm 10.85$ ,  $t = 0.14$ ,  $P = 0.89$ ), resulting in a 35% (non-significant) increase in free cortisol levels (LM; estimate =  $0.89 \pm 1.10$ ,  $t = 0.81$ ,  $P = 0.43$ ). However, in this treatment, one female had inexplicably high free cortisol levels (over 4 standard deviations, sampling time = 2.82 min) (see Fig. 8C). Once this data point was removed from the analyses, females showed a significant 131% increase in free cortisol levels in the presence

of kin-scented cubes compared to the no scent control situation (LM; estimate =  $1.91 \pm 0.55$ ,  $t = 3.47$ ,  $P = 0.002$ ). Own-scent and unfamiliar female-scent categories did not differ significantly between control and scented cubes in terms of total cortisol (LMMs; own scent: estimate =  $12.84 \pm 15.33$ ,  $t = 0.83$ ,  $P = 0.42$ ; unfamiliar female scent: estimate =  $2.30 \pm 9.59$ ,  $t = 0.24$ ,  $P = 0.81$ ), MCBC (LMMs; own scent: estimate =  $-0.83 \pm 12.43$ ,  $t = -0.07$ ,  $P = 0.95$ ; unfamiliar female scent: estimate =  $-23.63 \pm 14.64$ ,  $t = -1.61$ ,  $P = 0.12$ ), or free cortisol (LMMs; own scent: estimate =  $0.69 \pm 0.60$ ,  $t = 1.17$ ,  $P = 0.26$ ; unfamiliar female scent: estimate =  $0.48 \pm 0.29$ ,  $t = 1.65$ ,  $P = 0.12$ ).

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



**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 its first and third quartiles (25<sup>th</sup> and 75<sup>th</sup> percentiles), corresponding to the lower and upper hinges of the boxes. The upper and lower whiskers extend, respectively, to the largest and smallest value of 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. Significant differences ( $P < 0.05$ ) between the treatments are indicated by an asterisk.

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

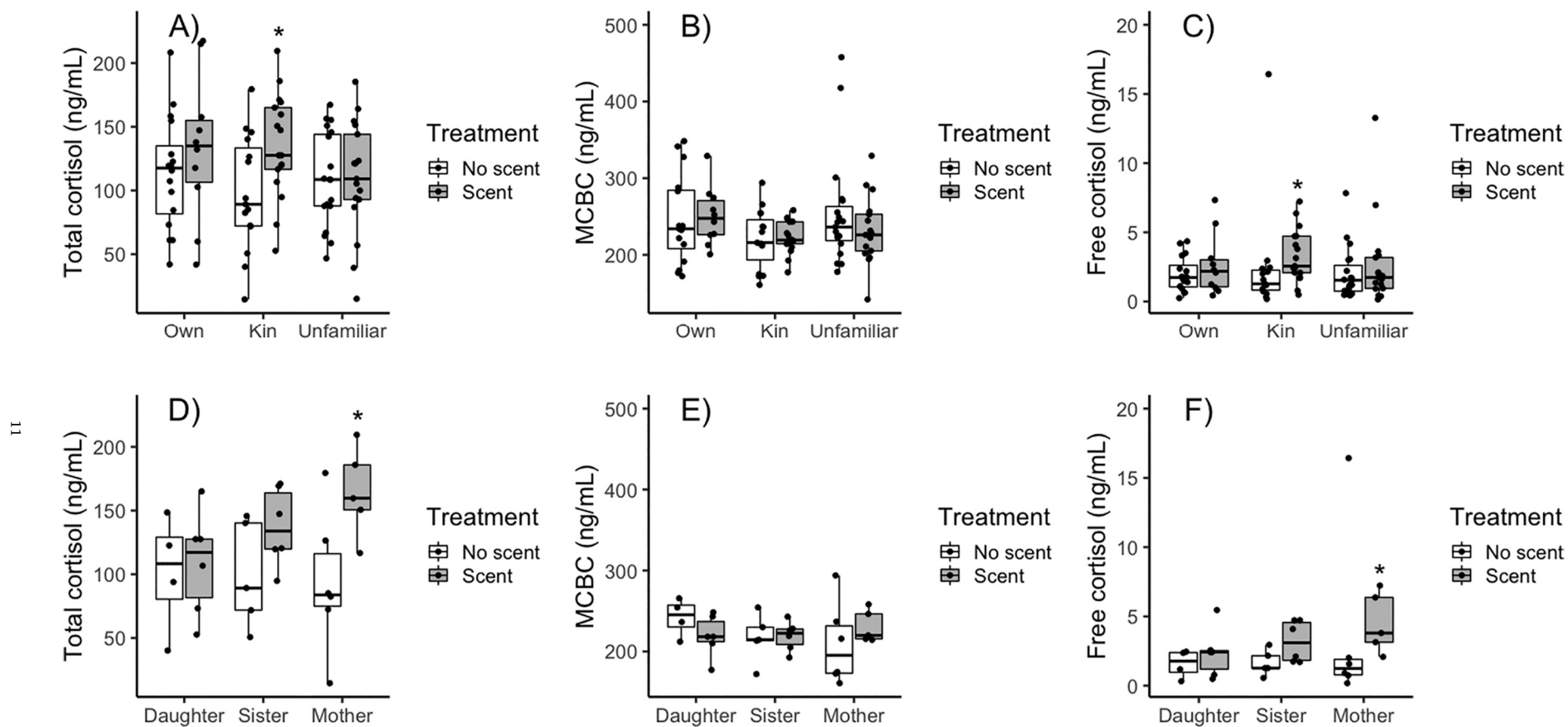
#### 4. Discussion

Resident female Columbian ground squirrels were highly sensitive to the scent marks of other lactating females deposited next to their nest burrows, exhibiting overall increases both in vigilance behavior and baseline glucocorticoid levels compared to control conditions (i.e., the absence of scents, or the presence of the female's own scent). Our results suggest that scent marking of both familiar and unfamiliar animals around the nest burrows of focal females was stressful, leading to increased vigilance behavior and heightened HPA axis activity (higher baseline cortisol levels). Lactating females did not significantly increase or decrease space use in response to the treatment, suggesting they did not actively seek-out simulated intruders by increasing exploration behavior on their territories, nor did they remain close to their nest burrow to defend offspring. This is somewhat surprising since female ground squirrels exclude conspecifics forcefully from around their nest burrows, although daily foraging home ranges overlap substantially (e.g., Murie and Harris, 1988; King, 1989a, 1989b; Arnaud et al., 2012). However, lactating females significantly increased the proportion of time spent in vigilance behavior compared to foraging or other behavioral categories, both when exposed to unfamiliar or kin scents, but not when exposed to their own scent. Contrary to our expectation, females were more vigilant in the presence of kin female scents on their territories than the scents of unfamiliar females. For example, females presented with kin scents had 42% points higher odds of engaging in vigilance rather than foraging, than females presented with unfamiliar scents. The increase in vigilance towards kin was mirrored in the stress axis: females experienced a significant 131% increase in plasma free cortisol levels (but not FCMs) compared to the no-scent condition when exposed to kin scents, but not when exposed to unfamiliar or their own

scents. The presence of an unfamiliar individual near a female's nest burrow should also be a stressful event, especially since female ground squirrels are more tolerant of kin than unrelated individuals (King, 1989a, 1989b; Viblanc et al., 2016). Yet, neither FCM nor free cortisol levels were increased by our treatment that introduced the scents of potentially dangerous strangers. Given that vigilance was significantly increased by the same treatment, it seems that evidence of foreign individuals is acknowledged by increased observance, but without an associated increase in stress. The presence of close relatives, by comparison, is a more stressful situation.

The observation that kin scents elicited a stronger physiological stress response than unfamiliar female scents is surprising. The “dear-enemy” hypothesis proposes that territory holders should react more strongly to unknown tentative usurpers than to territorial neighbors of known resource holding potential (e.g., Fisher, 1954; Temeles, 1994; Christensen and Radford, 2018). At first glance, our results might appear to reject the “dear enemy” hypothesis, since unfamiliar female scents were collected on neighboring meadows, and were therefore foreign and novel stimuli to resident females (Hare, 1994). However, resident females also interacted more (licking, sniffing or attacking) with unfamiliar-scented than kin-scented cubes, suggesting that they were more responsive to novel than familiar scents (see also Raynaud and Dobson, 2011), as would be predicted by the “dear-enemy” hypothesis. In addition, previous studies have found that female Columbian ground squirrels are less aggressive towards their female kin than unrelated individuals (King, 1989a, 1989b; Viblanc et al., 2016). Thus, one explanation of our results is that lactating females have a fine knowledge of the territorial boundaries of their close female kin. They treat markings of relatives on their territories as territorial shifts, and evaluate those as a greater threat (as evidenced by increased glucocorticoid levels) than the scents of female squirrels not recognized as direct neighbors.

An alternative explanation may be that heightened HPA axis activity in response to close kin scents occurs as resident females prepare for increased metabolic activity associated with cooperation among relatives (Soares et al., 2010; but see Santema et al., 2013). Although Columbian ground squirrels are not known to engage into active



**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–F) 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 its first and third quartiles (25<sup>th</sup> and 75<sup>th</sup> percentiles), corresponding to the lower and upper hinges of the boxes. The upper and lower whiskers extend, respectively, to the largest and smallest value of the data set, no further than  $1.5 \times$  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, once outliers removed, are indicated by an asterisk.

cooperation, close kin are more tolerant of one-another (King, 1989a, 1989b; Viblanc et al., 2016), and kin females may engage in chasing intruders together at the border of adjacent territories (VAV, PN, CS & FSD, personal observations). In addition, our study used unfamiliar scents from females that originated in other populations. Yet, familiar scents from known non-kin female neighbors inhabiting the same colony may well prove more stressful than kin scents, an expectation that remains to be tested. In line with this idea, juvenile Columbian ground squirrels show similar levels of cohesive and agonistic behavior, and similar interindividual distances in staged dyadic interactions with conspecific juvenile colony members, but decreased cohesion, increased agonism, and greater inter-individual distances in staged interactions with juvenile conspecifics from neighboring colonies (Hare, 1992).

In the presence of artificial scent marks that mimicked territorial intrusions, the absence of territorial scent marking by lactating females might indicate that core territories of these females were already sufficiently saturated with their scent. Alternatively, if a female's scent was concentrated near her nest burrow, it might serve as an attractant to potential perpetrators of infanticide (Balfour, 1983; Dobson, 1990; Hare, 1991; Stevens, 1998). Females are indeed known to conceal their nest burrows by plugging them with soft soil and litter, possibly to deter infanticidal conspecifics (McLean, 1978). This might explain why female cortisol levels generally increased when their own scent was experimentally introduced around their nest burrows (compared to the no-scent situation), divulging their secretive location.

Interestingly, different kin scents elicited different reactions from resident females. Lactating resident females were most vigilant to the scent of their sisters, then daughters, and finally mothers. Surprisingly, however, the stress axis told a different story. Females reacted strongly to the scent of their mothers, but not to that of their daughters or sisters. They exhibited a significant 318% increase in free cortisol levels when exposed to their mother's scent compared to the no-scent condition, and a 118% ( $P = 0.07$ ) increase in FCM levels. Thus, whereas lactating females were more vigilant towards sisters' scents, their stress was actually higher when presented with mother scents. Mothers are known to behave cohesively to yearling daughters, but aggressively to yearling immigrants, when resources are not limiting (Wigget and Boag, 1992). Relinquishment of breeding sites occurs in favor of yearling daughters (Harris and Murie, 1984a, 1984b). Because mothers favor the establishment of yearling (but not older) daughters (Neuhauser et al., 2004), it is possible that the scent of a mother is perceived as a potential threat to an older daughter because of the possibility of a mother relinquishing a former territory to a yearling daughter. The glucocorticoid differences found here are consistent with our previous findings (Sosa et al., 2020): when compared to females that had no co-breeding kin present in the population, lactating female having only a daughter or a sister present in the population showed decreased FCM levels, whereas females having only their mother presented similarly elevated FCM levels as females having no close kin around.

To our knowledge, surprisingly few studies have investigated the effects of scent marking on receivers' stress physiology in territorial species. In solitary blind mole rats (*Spalax ehrenbergi*), long-term exposure to scent-marks of intruders resulted in hypoglycemia, increased neutrophil/lymphocyte ratios, and partial ulceration of the liver and spleen, ultimately leading to death (Zuri et al., 1998). These effects were clearly indicative of over-activation of the adrenal function, impaired immunity, and chronic stress (see review by Davis et al., 2008). Interestingly, in solitary blind mole rats, it is specifically the long-term exposure to scents that was associated with increased mortality rates.

This indicates how profound the effects of scent-induced social stress may be when territory owners cannot retreat from scents, chase-away territory usurpers, or shift territories in response to intruders. Our study used an acute 30-min exposure to invader scents in a territorial species, and the stress response in terms of glucocorticoid secretion and HPA axis activation was substantial, even over this short time period. Whether longer exposure to scents might result in chronic stress in our species, or cause females to shift territories, remains to be tested. Nonetheless, it appears clear that, besides encoding specific information on the identity of the donor, social scent communication via territorial marking had pronounced effects on the receiver's behavior and physiology. We suggest that integrating the olfactory landscape related to social stress together with predation risk within "the ecology of fear" concept (Clinchy et al., 2013) should prove valuable to a proper understanding of behavioral and physiological consequences of habitat choice in territorial species.

#### Credit authorship contribution statement

Designed the study: VAV, PN and FSD; collected the data: VAV, JDR, PN, TB, MAG, TLH, EH, MM, CS, MT, PU and FSD; did the laboratory work: PDE, LMC and RB; provided antibodies and expertise on FCM measurement: RP; analyzed the data: VAV, JDR, AA, CS, and FSD; wrote the manuscript: VAV, JDR and FSD; all authors commented on the manuscript.

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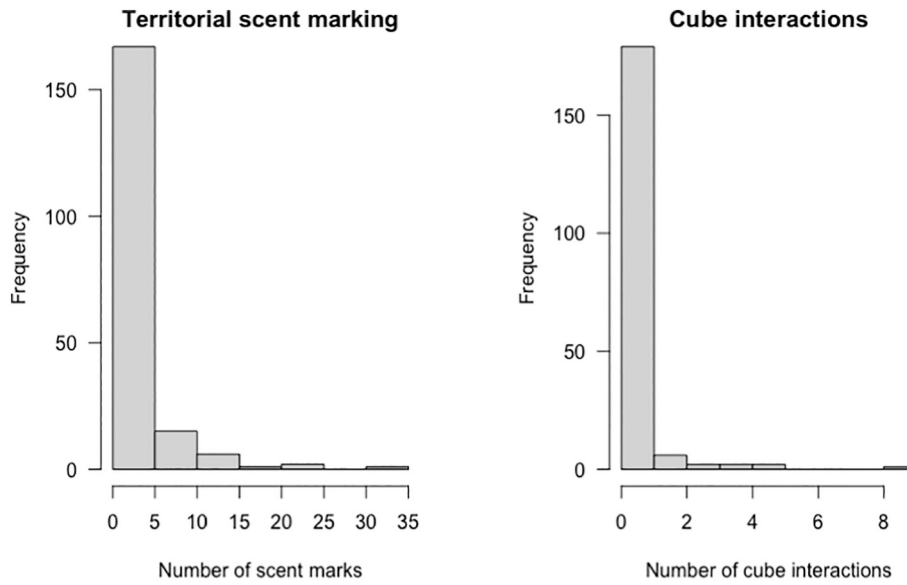
#### Declaration of competing interest

The authors declare no competing or financial interests.

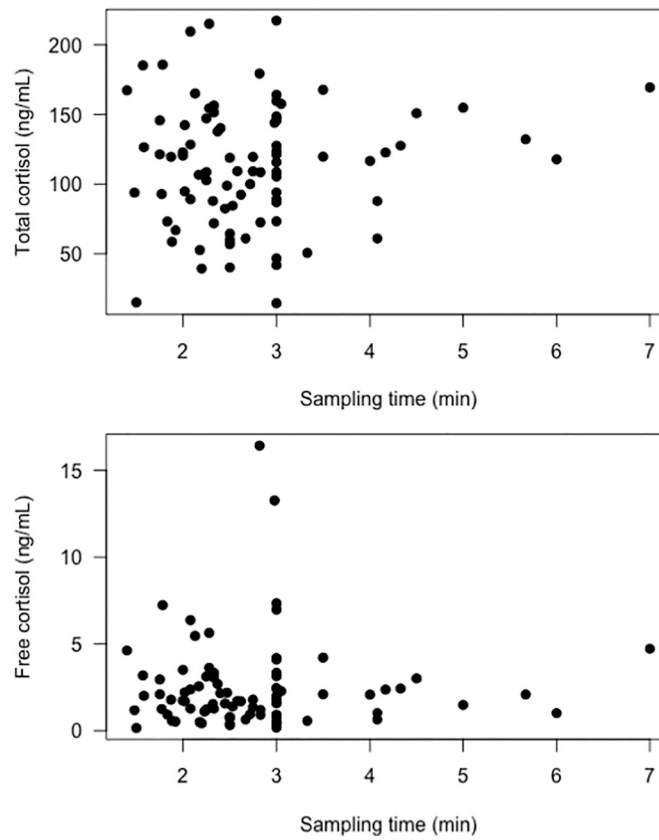
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**Appendix 1. Distribution of territorial scent marking and interactions with scent cubes during the 30 min observation period for female Columbian ground squirrels**



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



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