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Olfactory attractants and parity affect prenatal androgens and territoriality of coyote breeding pairs



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HIGHLIGHTS

- Coyotes displayed higher androgens when given coyote odor attractants mid-gestation.
- Both males and females had higher androgens as first-time breeders during gestation.
- Coyotes demonstrated repeatability across years in hormones and scent-marking.
- Fecal androgens were positively associated with exploratory behaviors mid-gestation.
- Fecal glucocorticoid, androgen metabolites of both sexes decreased over gestation.

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ABSTRACT

Hormones are fundamental mediators of personality traits intimately linked with reproductive success. Hence, alterations to endocrine factors may dramatically affect individual behavior that has subsequent fitness consequences. Yet it is unclear how hormonal or behavioral traits change with environmental stressors or over multiple reproductive opportunities, particularly for biparental fauna. To simulate an environmental stressor, we exposed captive coyote (*Canis latrans*) pairs to novel coyote odor attractants (i.e. commercial scent lures) mid-gestation to influence territorial behaviors, fecal glucocorticoid (FGMs) and fecal androgen metabolites (FAMs). In addition, we observed coyote pairs as first-time and experienced breeders to assess the influence of parity on our measures. Treatment pairs received the odors four times over a 20-day period, while control pairs received water. Odor-treated pairs scent-marked (e.g. urinated, ground scratched) and investigated odors more frequently than control pairs, and had higher FAMs when odors were provided. Pairs had higher FAMs as first-time versus experienced breeders, indicating that parity also affected androgen production during gestation. Moreover, repeatability in scent-marking behaviors corresponded with FGMs and FAMs, implying that coyote territoriality during gestation is underpinned by individually-specific hormone profiles. Our results suggest coyote androgens during gestation are sensitive to conspecific olfactory stimuli and prior breeding experience. Consequently, fluctuations in social or other environmental stimuli as well as increasing parity may acutely affect coyote traits essential to reproductive success.

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1. Introduction

Organisms are constantly challenged by various external stimuli within their environment. Behavioral and morphological responses toward these environmental challenges are frequently initiated by neuroendocrine mechanisms [1–6]. For instance, glucocorticoids increase

gluconeogenesis to activate energy stores necessary to respond toward environmental stressors [7,8]. Glucocorticoids are also associated with individual social status [3,9–11], mate preference and choice [12,13], and individually consistent behavioral differences (i.e. personality) [14–17]. Reproductive hormones such as androgens represent another pervasive suite of physiological factors that are intimately involved in reproduction and the social environment [7]. For example, increased androgens are often associated with sexually-selected ornamentation that constitute an honest signal of both fitness and social rank [18–21]. In many instances, increased androgens also augment territorial

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and aggressive behaviors that facilitate the acquisition and maintenance of increased social status [11,22–25]. Further, individual differences in foraging [26], exploration [27], and territorial marking behaviors [28, 29] have demonstrated positive associations with androgens. Therefore, endocrine responses to environmentally-induced changes often have fitness consequences for the individual.

The role of endocrine factors in affecting personality may be particularly salient for social taxa. Social dynamics and structure are often regulated by the composition or diversity of personality types within a group [30–34]. For instance, increasingly aggressive individuals may secure more reproductive opportunities by maintaining higher social rank [33,35–37]. Bold or exploratory individuals may capitalize on rarely exploited food resources and subsequently affect overall group foraging success [31,38]. Further, the distribution of personalities within a group frequently dictates group success in competitive bouts against neighboring conspecifics for high-quality resources [34,39]. Hence, in many instances personality is linked to life-history productivity (e.g. fecundity, longevity) [40]. Previous literature has suggested that the stability of personality traits is determined by underlying endocrine correlates and alterations to endocrine factors can induce behavioral plasticity [41]. Determining the proximate factors that affect endocrine traits may therefore help to predict accompanying changes to personality traits or vice versa.

For social mammals in particular, variation in density and environmental characteristics are prominent proximate causes affecting individual hormones [3]. Increases in the number of conspecifics within a landscape are often followed by the constriction of territorial boundaries, increasing the likelihood of conflict and competition for resources among neighboring conspecifics [42,43]. Mammals routinely utilize olfaction as a primary mode of communication, regularly urinating or defecating to demarcate home range boundaries and deter intrusion from conspecifics [44]. Greater abundances and densities are thus frequently accompanied by parallel increases in olfactory cues that are known to elicit behavioral responses (e.g. increased scent-marking, investigation) [45,46] potentially associated with glucocorticoid or androgen responses. Indeed, the mere perception of social competition generates individual hormonal changes [3,47]. However, changes to environmental cues (e.g. odors) are not constant nor do they persist over time; rather, such cues vary with time and likely induce plasticity in behavioral and hormonal traits [48].

Phenotypic consequences of environmental and temporal variation become considerably more far-reaching when considering breeding individuals and their overall influence on group or population dynamics. Breeding individuals undergo significant hormonal and behavioral changes in preparation for parenting [49,50] that can affect both breeders and even non-breeding individuals in socially complex groups such as cooperative breeding systems [51–53]. Moreover, phenotypic changes during gestation may affect developing neonates epigenetically [54,55]. Gestation is therefore a critical period for study because density cues and temporal variation in such cues may likely have significant influences on personality and hormonal traits of an individual. Though several studies have quantified the effect of environmental stressors on gestating females, few studies to date have repeatedly measured individuals over successive breeding events to assess how temporal variation impacts endocrine profiles and behavior [56–58]. Even fewer have addressed the interplay among hormonal and personality correlates of individuals in biparental systems [59,60].

Coyotes (*Canis latrans*) are an excellent organism to examine the impact of environmental and temporal variation on gestational hormones and behavior. First, previous work has demonstrated increased serum testosterone and progesterone profiles over the mating season (December to February) that correspond with increased territorial behaviors such as urine-marking, ground scratching, and defecation [61–64]. Second, mated individuals frequently demarcate the boundaries of their territories via scent-marking and enforce home range limits using aggression against intruding conspecifics when necessary

[65–67]. Social territorial incursions may likely represent a prominent stressor to breeding pairs. Third, previous work has demonstrated consistent individual differences in behavior of the species [68–70], providing empirical foundation for the current study. Associations among consistent individual differences in behavior and hormones may suggest that behavioral profiles of the species are hormonally mediated. Finally, previous work has provided methodological foundation to quantify individual variation in fecal glucocorticoid metabolites for coyotes [71].

We observed coyote breeding pairs exposed to commercial scent lures (i.e. coyote odor attractants) mid-gestation (February to March) [72] to determine whether such odors could affect fecal glucocorticoid (FGM) and fecal androgen metabolites (FAM), as well as behaviors. Coyote odor attractants such as scent lures have previously been identified to elicit strong marking and investigative behaviors, and are typically used to attract the attention of neighboring coyotes by simulating odors from foreign or novel conspecifics [46,73]. Here, we specifically predicted that increased marking and investigative behaviors would be correlated with FGM and FAM concentrations mid-gestation. In addition, we examined breeding pairs over successive breeding events in 2011 and 2013 to assess whether hormonal or behavioral measures exhibited temporal plasticity. We also examined whether coyotes demonstrated repeatability in marking and investigative behaviors (i.e. personality), as well as in FGM and FAM concentrations (i.e. hormone profiles).

2. Methods

2.1. Subjects

We observed eight breeding coyote pairs in 2011 at the United States Department of Agriculture National Wildlife Research Center (NWRC) Predator Research Facility in Millville, UT. At the beginning of the study all pairs had no prior breeding experience and were 1 or 2 years of age (1.4 ± 0.1 years [$X \pm SD$]). Prior to breeding, animals at the facility were housed in multiple enclosure types ranging from large outdoor pens (1000–6000 m²) to raised kennels (3.3 m²). In December 2011, coyotes were randomly selected from the NWRC population, then moved to 1000 m² outdoor “clover” pens optimized for long-term behavioral observations [69,74]. Clover pens were grouped into housing blocks of three pens per block, with each individual pen containing a single breeding pair; hence, breeding pairs were adjacent to other study breeding pairs (Fig. 1). Pair relocation corresponded with the beginning of the breeding season [61,62,72]. To reduce potential effects of relocation stress on hormonal assays, each coyote pair was allowed one month to acclimate to their new pen before we collected fecal samples. From late December to January, each breeding pair was fed 1300 g of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, UT) daily and water was provided ad libitum. According to NWRC regulations, we doubled food rations in February to ensure that pregnant females were receiving adequate nutrition. We observed the same eight breeding pairs again in 2013 as experienced parents giving birth to their second litters.

Coyote parents were either wild-born and hand-reared (5 females, 5 males) or captive-born and coyote-reared (3 females, 3 males). Previous studies in multiple taxa have observed various differences in behavior and physiology attributed to hand-rearing (orange-winged Amazon parrots, *Amazona amazonica* [75]; red foxes, *Vulpes vulpes* [76]; gray wolves, *Canis lupus* [77]). Therefore, we considered rearing condition as a main effect in subsequent analyses (see Section 2.6 Statistical analyses) to quantify the effect of early rearing experience on gestational hormones and scent-marking behaviors. Hand- or coyote-reared individuals were randomly assigned for pairing and were not exclusively paired with individuals of the same rearing condition.

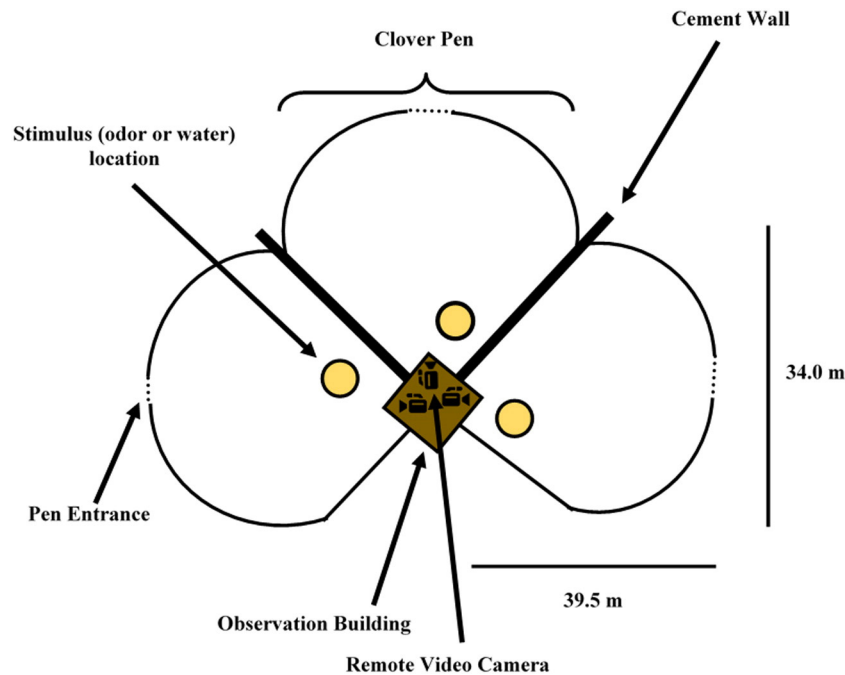


Fig. 1. Schematic depicting three of the 0.1 ha clover pens within a single housing block at the National Wildlife Research Center, Predator Research Facility (Millville, UT, USA), including the location of remote cameras during testing, and placement of the stimuli (odor versus water). Each clover pen contained a single breeding pair.

2.2. Coyote odor attractants

We administered coyote attractant odors to four pairs, and a control (i.e. water) to the other four. For the treatment group, we provided four different commercial odors developed by Russ Carman® (Canine Call®, Pro's Choice®, and two versions of Magna Gland®, New Milford, PA). The odors were a blend of fermented glandular materials, urines, and other volatile substances from other unrelated coyotes, known to increase marking and territorial behaviors in coyotes [46]. We manually administered odors to a treatment breeding pair territory (Fig. 1) every five days over a 20-day period (February 28th to March 15th) in 2011 (Table S1). We rotated the odor type provided to each breeding pair every five days to reduce potential presentation order effects. We provided control pairs with distilled water, and we provided all pairs with stimuli over the same timeframe. A single experimenter provided the stimuli and once administered, the tester and surrounding staff immediately vacated animal grounds to reduce species-typical caution to researcher presence [74]. The experimenter was inside each coyote pen for approximately 30 s. It took us approximately 55 min (54.88 ± 3.08 min [$X \pm SD$]) to provide all breeding pairs with their predetermined stimuli (i.e. either odor attractants or water). We video-recorded pairs beginning immediately before stimuli were provided and continued recording for approximately 140 min (137.75 ± 4.03 min [$X \pm SD$]) with one remote camera per pen (see Fig. 1). Cameras were stationed inside a closed environment in the center compound of three clover pens (Fig. 1). In sum, we recorded at least 2 h of video after exiting the enclosure to ensure that any impact of human presence in the enclosure was diminished.

We repeated these methods in 2013 with the same male-female pairs but reversed the odor treatment: 2011 control pairs became 2013 odor pairs and vice versa. We changed pen location for each pair in the second breeding year to reduce potential habituation effects to familiar surroundings witnessed in the first year. In both years, we noted several marking and investigative behaviors following Gese and Ruff [67] and Kimball et al. [46] (Table 1). These are critical behaviors regularly used to assert hierarchical status, maintain social bonds, and

demarcate territorial boundaries (Table 1) [67]. We began coding target behaviors at the moment of stimulus deposition using all-occurrence methods [78] for a 70-minute period. Note that coyotes had immediate access to odor cues at the moment each stimulus was provided, and were not restricted by any physical barriers.

In 2012, coyotes were individually housed over the breeding season to prevent breeding, then repaired mid-spring. Pairs were also equally and periodically rotated through different pen types (excluding testing areas) to accommodate concurrent research projects on other captive coyotes and per NWRC regulations. Our study animals were not on any other NWRC related projects in the interim between the 2011 and 2013 breeding seasons.

2.3. Fecal sample collection

In both 2011 and 2013, we collected fresh fecal samples twice weekly from February to April. We fed animals multi-colored glitter particles according to previous methodology [79–81] to separate samples and determine their freshness. Specifically, we mixed glitter with surplus

Table 1
Behavioral ethogram used during coyote odor attractants testing.

| Behavior | Description |
|----------------------|--|
| Aggression | Teeth baring, growling, and/or physical confrontation directed toward pairmate |
| Ground scratching | Digs and kicks down and backward; often follows urination |
| Urination | Discharges urine |
| Rubs | Descends head-first toward the ground and rakes, undulates body across the floor |
| Site sniffs | Individual directly placed its nose toward the stimulus site for ≥ 3 s |
| Site visits | Individual gets within ≤ 1 m from stimulus site |
| Site time (s) | Total time spent at the stimulus site |
| Latency to visit (s) | Length of time before individual gets ≤ 1 m from the stimulus site |

mink food, partitioned that food into small biscuits, froze them at -20°C , and then provided these mink food biscuits to each member of a breeding pair simultaneously the afternoon prior to sample collection. Glitter-marked samples retained their color once excreted the following morning. Each biscuit was mixed with a different color to identify sex within pairs. Certain individuals hesitated to approach mink food biscuits, allowing their mate enough time to eat both supplied biscuits. Individuals also tended to eat the biscuits while moving, which often resulted in crumbs spread for their mates to opportunistically eat. We therefore paired glitter biscuits (pre-excretion) with a previously established progesterone enzyme immunoassay (post-excretion: [82]). Females have significantly higher progesterone concentrations compared with males during gestation [61]. We hypothesized that we could confirm the sex of fecal samples via the progesterone assay (see Section 3 Results).

We assessed freshness by appearance, odor, and stiffness in response to freezing temperatures. We restricted sample collection to feces excreted between 0600 and 1000 h MST, as FGM content varies diurnally in coyotes [71]. Samples were stored at -20°C immediately to limit the amount of hormone metabolite degradation [83]. We collected multiple samples for each sampling period ($n = 4$ per day, per pair) to ensure suitable fresh samples were collected for each individual in a breeding pair. Feces contaminated by urine ($n = 56$) were not collected, and all animals were sampled over the same time period for each collection event. All samples were shipped overnight on dry ice to the Lincoln Park Zoo Endocrinology Laboratory (Chicago, IL, USA) for hormonal analyses.

2.4. Fecal sample processing

Fecal samples (2011: $n = 588$; 2013: $n = 689$) were freeze-dried on a lyophilizer (Thermo Modulyo Freeze Dryer; Thermo Scientific, Waltham, MA, USA) for 3 days and crushed to a fine powder before extraction [71]. Briefly, sample powder was weighed (0.2 ± 0.02 g), combined with 5.0 ml of 90% ethanol (ethanol:distilled water), and agitated on a mixer (Glas-col, Terre Haute, Indiana) for 30 min at setting 60. The samples were then centrifuged for 20 min at 1500 rpm and 10°C , and the supernatant was poured into clean glass tubes. The fecal pellets were re-suspended in 5.0 ml of 90% ethanol, vortexed for 30 s, and re-centrifuged for 15 min at 1500 rpm. The supernatant was poured into the corresponding glass tubes and the combined supernatants were dried under air and a hot-water bath (60°C). Dry samples were then reconstituted with 2.0 ml of phosphate-buffered saline (0.2 M NaH_2PO_4 , 0.2 M Na_2HPO_4 , NaCl), vortexed briefly, and sonicated for 20 min before analysis.

2.5. Enzyme immunoassays

We used a previously validated cortisol enzyme immunoassay [71] to measure coyote fecal glucocorticoid metabolites. Polyclonal cortisol antiserum (R4866) and horseradish peroxidase were provided by C. Munro (University of California, Davis, CA, USA). Cortisol antiserum and cortisol horseradish peroxidase were used at dilutions of 1:8500 and 1:20,000, respectively [71,84]. Assay sensitivity was 1.95 pg/well and intra- and interassay coefficient of variation was $<10\%$.

We also used a previously established testosterone enzyme immunoassay to measure coyote fecal androgen metabolites [85,86]. We biochemically validated the testosterone assay by (1) demonstrating parallelism between binding inhibition curves of fecal extract dilutions (1:2–1:8192) and hormonal standards (males: $R^2 = 0.990$; females: $R^2 = 0.993$) and (2) significant percent recovery ($>90\%$) of exogenous testosterone (2.3 – 600 pg/well) added to pooled fecal extracts ($1:3000$; $y = 0.8197x + 5.9562$; $R^2 = 0.9960$). Testosterone horseradish peroxidase and polyclonal antiserum were used at 1:30,000 and 1:10,000, respectively [84,85]. Assay sensitivity was 2.3 pg/well and

intra- and interassay coefficient of variation was $<10\%$ for the testosterone enzyme immunoassay.

Finally, the progesterone assay used to differentiate samples by sex was biochemically validated by (1) demonstrating parallelism between binding inhibition curves of fecal extract dilutions (1:2–1:8192) and hormonal standards (males: $R^2 = 0.968$; females: $R^2 = 0.995$), and (2) significant percent recovery ($>90\%$) of exogenous progesterone (0.78 – 200 pg/well) added to pooled fecal extracts (1:3000; $y = 0.9999x + 1.4882$; $R^2 = 0.9945$). We also biologically validated fecal progesterone in the species by comparing samples collected during and after gestation. Progesterone horseradish peroxidase and polyclonal antiserum were used at 1:10,000 and 1:40,000, respectively. Assay sensitivity for fecal progesterone metabolites was 0.78 pg/well and intra- and interassay coefficient of variation was $<10\%$. Cross-reactivities for all assays have been previously described [82].

2.6. Statistical analyses

To assess the impact of our coyote odor attractants on scent-marking and investigative behaviors, we used generalized linear mixed models (GLMMs) with a Poisson error distribution for behavioral count data. All behavioral data are reported as per 70 min of recorded video. Here, we observed treatment group and breeding year as main effects in our model, as well as the interaction term between the two factors. Coyote identity and test number (see Table S1) were set as random effects in the model. We partitioned our data by sex to observe odor treatment and breeding year differences within each sex. In addition, we determined whether coyote investigative behaviors were in fact influenced by test number (see Table S1) by using post-hoc Tukey contrasts in pair-wise comparisons.

We used linear mixed models (LMMs) to determine how odor treatment and breeding year were associated with hormonal outcomes. Prior to using LMMs, we binned hormonal values by weeks until birth as there were unknown differences in date of conception for each pair. Weeks until birth were therefore projected according to each female's date of parturition and the typical length of coyote gestation (63 days; see [72]). To date, no data exist that have characterized fecal glucocorticoids and androgens in the species. We therefore used Tukey contrasts to assess the general impact of weeks until birth on FGMs and FAMs. In subsequent LMMs, we set weeks until birth as a random effect to account for differences in the timing of conception that may have influenced FGM and FAM concentrations irrespective of their group identity (odor versus control).

We subsequently partitioned hormonal data into three descriptive categories: pre-test, test, and post-test. The test period specifically was the aforementioned 20-day period in which odors (treatment) and water (control) were provided (i.e. mid-gestation). The pre-test period (i.e. early gestation) comprised the 4 weeks of fecal collection before we provided odors, while the post-test period (i.e. late gestation) was the 3 weeks after. We set treatment group, breeding year, and test period as main effects for our LMMs. Mixed models were conducted separately for males and females, with coyote identity as the random effect term. We found a significant effect of period with LMMs focused within each period. Thus, within each separate test period we assessed the effect of odor treatment and breeding year as main effects. We tested hormonal data for normality using Shapiro-Wilk tests, and non-normally distributed data were log transformed [71].

To quantify consistent individual differences in behavioral and hormonal measures, we used variance components extracted from previously described GLMMs to estimate repeatability (R) as the proportion of total variation attributable to among-individual variation versus between-individual variation [87]. Both the test number and test period were confounding factors partially explaining variance within our behavioral and hormonal results, respectively. We therefore calculated adjusted repeatabilities (R_{adj}) which estimate individual differences while controlling for confounding effects by

including them in the model when calculating between-individual and residual variances. Adjusted repeatabilities were therefore calculated as:

$$R_{adj} = \sigma_{\alpha}^2 / (\sigma_{\alpha}^2 + \sigma_e^2 + 0.25)$$

in which σ_{α}^2 represents the between-individual variance, σ_e^2 represents the residual variance, and (+0.25) is the distribution-specific variance of a Poisson model with a square root link function and additive overdispersion [87]. Variances attributed to other random factors (test number and test period) were included in repeatability calculations, allowing us to determine whether consistent individual differences persisted despite confounding factors. We subsequently determined whether repeatability estimates were significant by examining whether each estimate's 95% confidence interval (CI) was pressed against zero, and if not we interpreted this as evidence of a significant repeatability estimate [49,88]. Repeatabilities for behavioral count and hormonal data were quantified using variance components extracted from GLMMs and LMMs, respectively. Individual measures were compared between the 2011 and 2013 seasons for our repeatability estimates. We then calculated best linear unbiased predictors (BLUPs) for each individual, which is the value fitted for the individual based on the intercept of the model and the standard deviation calculated for the individual random effect [89,90]. Hormonal BLUPs were later correlated with our marking and investigative behaviors using Spearman rank correlations. Finally, we examined Spearman correlations among maternal and paternal traits because marking behaviors of paired individuals are expected to affect the marking traits of their pairmate [67].

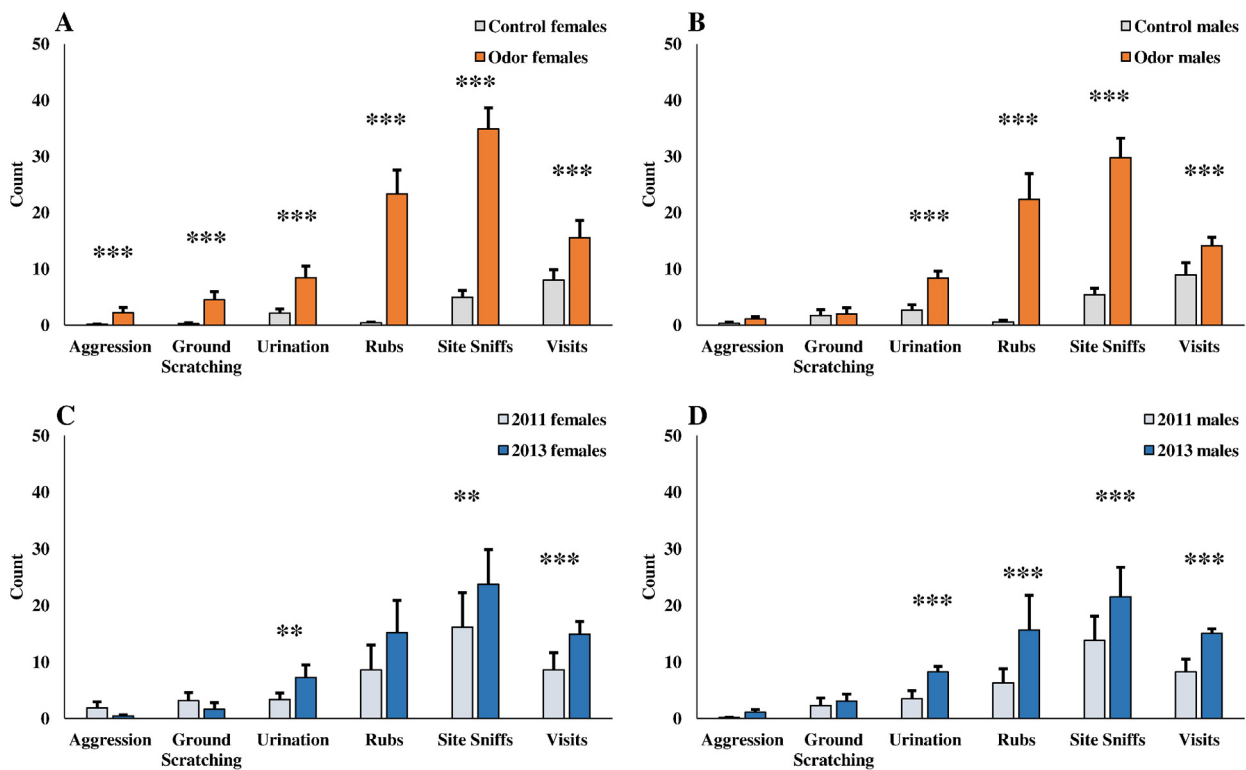
All analyses were conducted in R version 3.2.4 [91]. Linear mixed models were performed using the lmer function from 'lme4' [92] and

'lmerTest' [93] packages. Mixed models with a Poisson error distribution were performed using the glmer function from the 'nlme' package [94]. We used restricted estimation maximum likelihood (REML) with a diagonal covariance structure for all of our models, with Satterthwaite approximation for degrees of freedom. Repeatabilities were either calculated using the rpt.remlLMM.adj function from the 'rpt.R package' (Gaussian data) or from variance components of glmer model output (count data) [87]. Spearman correlations were performed using the corr function from the 'corrplot' package [95]. In all cases, we used two-tailed tests with alpha set to $P < 0.05$ and data reported as mean \pm SE and for all data we used Shapiro-Wilk to test for normality before analyses. None of our hormonal or behavioral measures demonstrated an effect of rearing condition. Rearing effects were therefore not addressed further. All research and methods described above were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago (protocol no. 72,184), the NWRC (protocol no. QA-1818), and the Lincoln Park Zoo Research Committee.

3. Results

3.1. Coyote odor attractants: behaviors

We first observed overall differences by treatment group and breeding year in each sex separately (Fig. 2, Table S2). We found that both odor-treated females and males urinated, rubbed, sniffed, and visited the affected site more frequently than control individuals (Fig. 2A–B, Table S2). Odor-treated females in particular exhibited greater within-pair aggressive behaviors and ground scratching compared to their control counterparts (Table S2). Both odor-treated females and males spent more time at the odor site, but only females differed by treatment group in latency to visit the odor site (Table S2). Female aggression demonstrated an interaction between odor treatment and year, with 2011



Marking and Investigative Behaviors

Fig. 2. Differences in marking and investigative behaviors (mean \pm SE) among odor and control females and males (A and B), and first-time (2011) versus experienced (2013) breeders (C and D). Asterisks indicate statistical differences (** $P < 0.01$, *** $P < 0.001$). All behavioral count data are reported as per 70 min of recorded video.

odor-treated females behaving equally as aggressive in 2013 as control females (Table S2). Males demonstrated similar interaction effects for ground scratching, urination, rubs, and site visits (Table S2).

We also found that pairs scent-marked and investigated the scented areas more as experienced breeders (Fig. 2C–D, Table S2). Specifically, both experienced males and females urinated, rubbed, sniffed, and visited the affected site more frequently than first-time breeders despite the treatment group membership (Fig. 2C–D, Table S2). Experienced breeders also spent more time at the affected area. However, only females differed in their latency to visit the site, in which experienced breeders approached the stimulus site quicker than first-time breeders.

To assess whether individuals adjusted their site time or latency to visit the stimulus site with each successive test (see Table S1), we used Tukey contrasts to compare coyotes within treatment groups in each of the four odor provisioning events (Fig. 3A–B). Again, we found that odor-treated males and females spent more time at the stimulus site within each test date (Fig. 3A–B). However, over each successive trial odor-treated females spent less time at the stimulus site ($F_{3,27} = 4.237, P = 0.014$) compared with control females ($F_{3,27} = 0.646, P = 0.592$). Males did not exhibit a significant decrease in time spent at the odor site for males as a function of treatment group (control: $F_{3,27} = 0.334, P = 0.801$; treatment: $F_{3,27} = 1.293, P = 0.297$; Fig. 3).

Finally, we determined whether coyotes demonstrated repeatability in our behavioral measures. Both females and males demonstrated repeatability in ground scratching, urination, and site visits across years (Table 2). We found that females were specifically repeatable in their aggression, whereas males demonstrated repeatability in their latency to visit the odor- or water-provisioned site (Table 2).

3.2. Coyote odor attractants: hormones

First, our progesterone assays were able to distinguish previously unidentified fecal samples by sex: females had consistently higher dilution rates compared to their male partners (females: 1:1500 to 1:15,000; males: 1:300), indicating higher progesterone concentrations for female samples. We therefore were able to successfully identify a total of 560 fecal samples for our 8 breeding pairs across the 2011 and 2013 seasons. Both FGMs and FAMS decreased toward parturition for both sexes (FGMs: females - $F_{10,257} = 2.945, P = 0.002$; males - $F_{10,254} = 1.986, P = 0.035$; FAMS: females - $F_{10,257} = 15.96, P < 0.001$; males - $F_{10,254} = 7.739, P < 0.001$). Post-hoc Tukey tests comparing

Table 2

Adjusted repeatabilities (R_{adj}) for marking and investigative behaviors across the odor attractant tests, as well as fecal glucocorticoid (FGMs) and androgen metabolites (FAMS) during gestation for females and males. Confidence intervals not pressed against zero (bolded) are considered statistically significant.

| Behavior/hormone | R_{adj} | 95% CI |
|-----------------------------------|--------------|--------------------|
| <i>Females</i> | | |
| Aggression ^a | 0.289 | 0.166–0.820 |
| Ground scratching ^a | 0.296 | 0.282–1.161 |
| Urination ^a | 0.320 | 0.238–0.929 |
| Rubs ^a | 0.130 | 0.000–1.291 |
| Site sniffs ^a | 0.088 | 0.000–1.026 |
| Site visits ^a | 0.357 | 0.374–1.320 |
| Site time (s) ^b | 0.140 | 0.000–0.162 |
| Latency to visit (s) ^b | 0.144 | 0.007–0.349 |
| FGMs ^b | 0.561 | 0.434–0.720 |
| FAMS ^b | 0.004 | 0.000–0.039 |
| <i>Males</i> | | |
| Aggression ^a | 0.193 | 0.000–0.461 |
| Ground scratching ^a | 0.544 | 0.539–1.668 |
| Urination ^a | 0.173 | 0.056–0.690 |
| Rubs ^a | 0.049 | 0.000–0.865 |
| Site sniffs ^a | 0.000 | 0.000–0.577 |
| Site visits ^a | 0.261 | 0.245–1.097 |
| Site time (s) ^b | 0.027 | 0.000–0.179 |
| Latency to visit (s) ^b | 0.224 | 0.198–0.434 |
| FGMs ^b | 0.300 | 0.043–0.524 |
| FAMS ^b | 0.204 | 0.060–0.468 |

Test number and test order were controlled for in estimating repeatabilities, and all behavioral data are reported as per 70 min of recorded video.

^a Repeatabilities for count data were quantified by comparing between-individual variance (σ^2_{ind}) and residual variance components (σ^2_{res}) of generalized linear mixed models (GLMMs) with a Poisson error distribution and square root link function.

^b Repeatabilities for Gaussian data were quantified using the rpt.remlMM.adj function from the 'rpt.R' package.

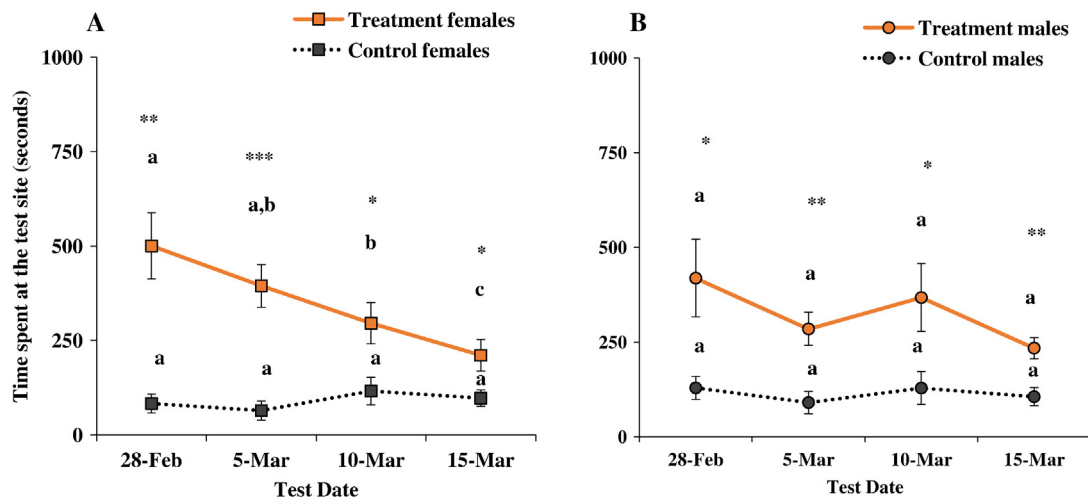


Fig. 3. Mean (\pm SE) time spent ≤ 1 m within the odor- (treatment) or water-treated (control) test site. Asterisks indicate differences between treatment groups within each test date ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Contrasts in subscript letters indicate statistical differences within treatment groups across test dates. Data are pooled among the 2011 and 2013 seasons. All behavioral data are reported as per 70 min of recorded video.

gestational weeks demonstrated lower FGMs and FAMS in the latter half of gestation compared to early gestation (Fig. 4).

When hormonal data were partitioned by testing period (i.e. pre-test, test, and post-test), we found that odor-treated females – but not odor-treated males – had lower FGM concentrations during the test period (females - $F_{1,67.2} = 10.77, P = 0.002$; males - $F_{1,69.3} = 0.84, P = 0.36$) compared with control pairs (Fig. 5A). Odor-treated females also had lower FGM concentrations during the pre-test period ($F_{1,115.1} = 4.19, P = 0.043$), but did not differ from controls in the post-test period

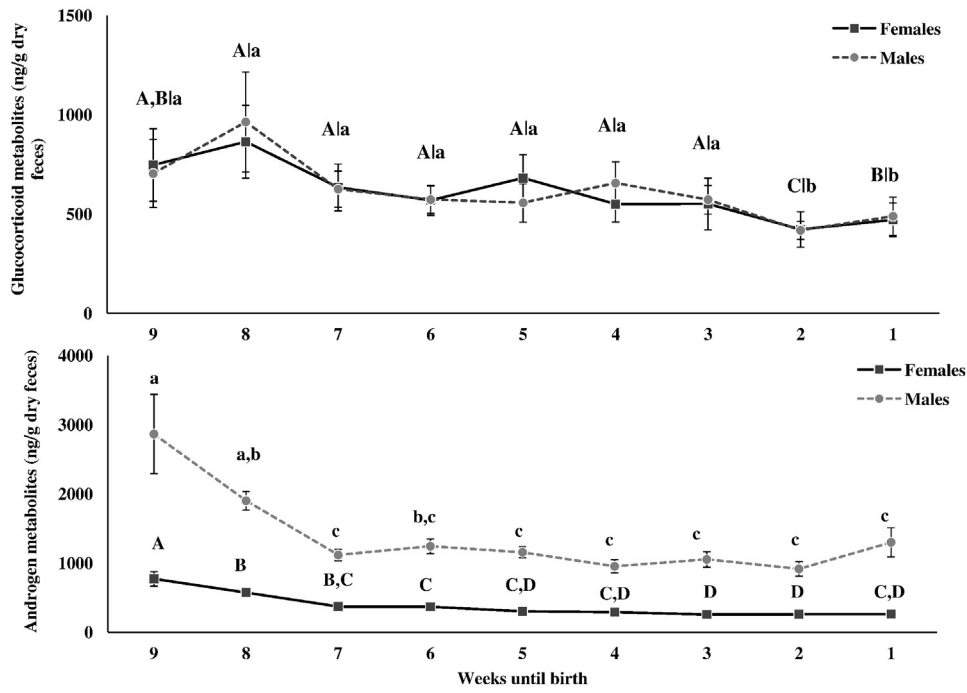


Fig. 4. Fecal glucocorticoid and androgen metabolite concentrations during gestation before parturition. Uppercase and lowercase superscripts correspond to within-maternal and within-paternal metabolites, respectively. Contrasts between uppercase letters and between lowercase indicate a statistical difference within each sex. Figures pool data from both treatment and control animals.

($F_{1,57.3} = 1.61, P = 0.21$), indicating that differences in FGMs may have existed prior to receiving odors (Fig. 5A). Odor-treated females generally had lower FGMs than their control counterparts (Table S2). Odor-treated males did not differ from controls in FGMs over the pre-test ($F_{1,110.4} = 0.59, P = 0.45$) or post-test periods ($F_{1,58.2} = 2.77, P = 0.10$), suggesting odor attractants did not influence male fecal

glucocorticoids (Fig. 5B). We did not find any significant FGM differences as a function of breeding experience in either males or females (Table S2).

We did find higher FAM concentrations during the test period for odor-treated pairs versus control pairs (females - $F_{1,68.6} = 6.11, P = 0.012$; males - $F_{1,72.0} = 6.18, P = 0.015$; Fig. 5C–D). Fecal androgen

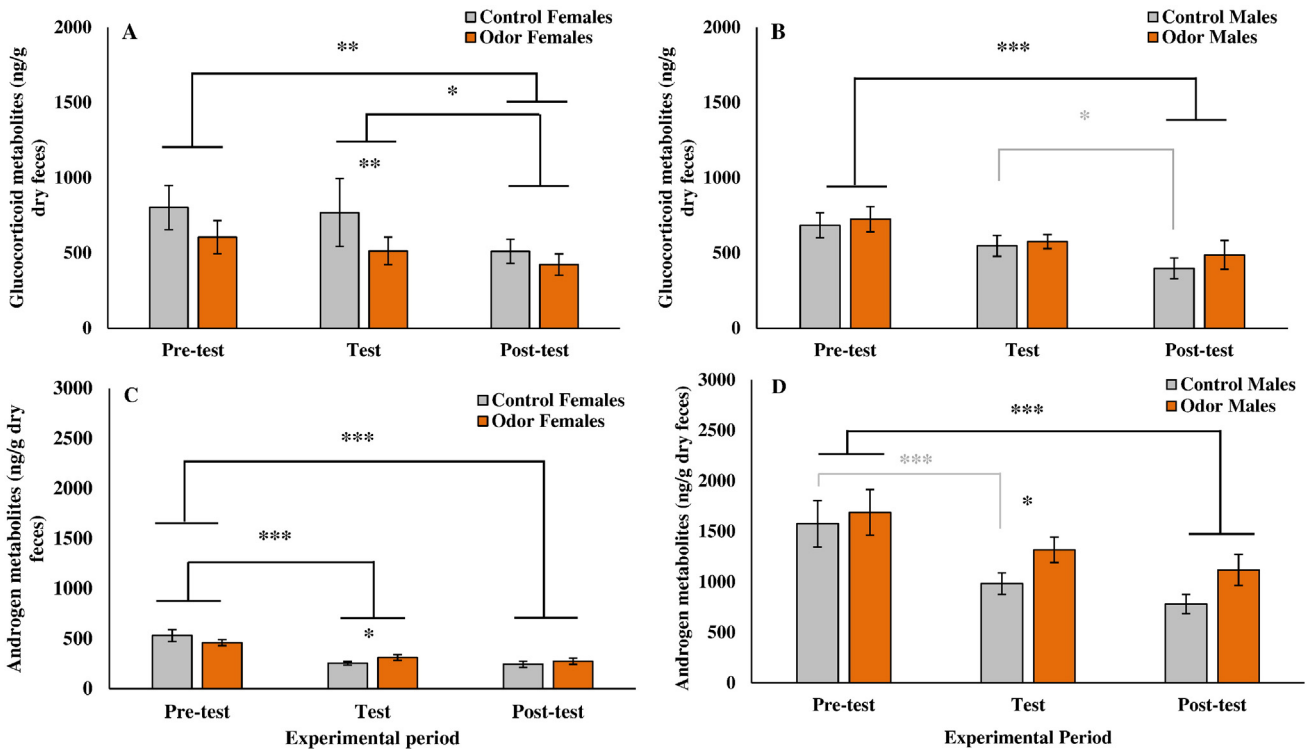


Fig. 5. Fecal glucocorticoid (A, B) and androgen metabolite concentrations (C, D) before (pre-test), during (test), and after (post-test) female (A, C) and male coyotes (B, D) received odor attractants or water as stimuli. Data represent means \pm SE ($*P < 0.05, **P < 0.01, ***P < 0.001$) and bars denote differences across experimental (i.e. testing) periods.

metabolites during the pre-test (females: $F_{1,117.5} = 0.62, P = 0.43$; males: $F_{1,112.8} = 1.29, P = 0.26$) and post-test periods (females: $F_{1,63.0} = 2.35, P = 0.13$; males: $F_{1,60.0} = 3.64, P = 0.061$) did not differ as a function of odor treatment for either sex (Fig. 5C–D). Within both sexes, we found that FAMs over the entirety of gestation were greater for first-time breeders versus experienced breeders (females: $F_{1,251.4} = 9.33, P = 0.003$; males: $F_{1,250.1} = 6.14, P = 0.014$). In addition, males had significantly greater FAMs during gestation compared with females ($F_{1,7} = 84.8, P < 0.001$).

Last, both female and male coyotes demonstrated repeatability in their FGMs across years (Table 2). Only males demonstrated consistency in their FAMs with successive breeding years (Table 2).

3.3. Correlations among behaviors and hormones

We found that both female ($r_s = 0.61, N = 16, P = 0.012$) and male ($r_s = 0.58, N = 16, P = 0.019$) androgen best linear unbiased predictors (BLUPs) were positively correlated with latency to visit the stimulus site (Table S3), indicating that individuals with higher androgen BLUPs took longer to investigate the odor or water-affected site. Male androgen BLUPs in particular were negatively associated with time at the odor site ($r_s = -0.51, N = 16, P = 0.044$), and number of site visits ($r_s = -0.69, N = 16, P = 0.003$), indicating that males with higher androgen BLUPs spent less time performing investigative behaviors (Table S3). Male androgen BLUPs were also negatively associated with the mean number of urinations ($r_s = -0.67, N = 16, P = 0.005$). Further, urination, body rubs, site sniffs, site visits, and time at the site all positively covaried (Table S4). Finally, we found positive correlations between identical male and female behaviors, in which high-marking and investigatory males were matched with high-marking and investigatory females (Table 3). Additional correlational data among BLUPs for glucocorticoid metabolites, androgen metabolites, and behaviors, as well as behavior-behavior relationships are in the supplementary materials (Tables 3, S3, and S4).

4. Discussion

We have demonstrated here that coyote odor attractants were both effective at eliciting strong territorial responses, as well as increasing FAMs of both sexes. In addition, coyote pairs had increased FAMs as first-time breeders, indicating that either parity or increasing age played a partial role in affecting FAM concentrations. We observed steady declines in both FGMs and FAMs toward parturition for both sexes, elucidating the temporal component of these hormones during gestation. Individual coyotes demonstrated repeatability for several territorial behaviors and hormones despite altering treatment conditions, highlighting the robustness of coyote personality and hormonal traits to novel disturbances. Moreover, pairs were consistent in marking behaviors and glucocorticoids suggesting that individual coyote responses to the

odor attractants were partially influenced by the behavior of their pairmate. Finally, though relatively minimal and sex-specific, we found that androgen BLUPs were correlated with a subset of observed investigative behaviors, suggesting that specific androgen profiles may be partially associated with coyote personality.

4.1. Odor attractants, parity, and behavioral responses

Scent-marking and investigative behaviors greatly increased for individuals that received novel odors. This is consistent with other odor studies in coyotes [46,73], as well as with other studies in Canidae (African wild dogs, *Lycaon pictus* [45]; Ethiopian wolves, *Canis simensis* [96]), highlighting the importance of olfactory cues in stimulating parallel territorial behaviors across the clade. What is unique to our study is that these behaviors exhibited plasticity as a function of odor presentation and either parity or increasing age (Table S2). From 2011 to 2013, both sexes increased the number of visits they made to the experimental site, despite treatment group. There was also an overall increase in the number of site sniffs and urine-marking events for all coyotes. Increased marking with age suggests that older individuals become more involved in demarcating territorial boundaries. In fact, older coyotes mark more frequently than yearlings or early-aged adults [67]. Our study also demonstrated that pair latency to visit the site dramatically decreased from 2011 to 2013, in which individuals in 2013 generally approached the affected site faster than they did in 2011. These results suggest several possibilities: (1) coyotes' prior experience with the mere process of stimulus application (i.e. an observer entering and placing a foreign substance in their pen) influenced their resultant visit latencies or (2) older coyotes are typically more investigative and therefore individuals were more likely to visit the site quickly in 2013 versus 2011. However, the covariance between coyote age and prior experience in our study does not allow us to definitively determine which factor is a better explanatory variable for visit latencies.

Multiple scent-marking and investigative behaviors were highly correlated with one another irrespective of the odor treatment (Table S4). For instance, ground scratching, urination, body rubs, and site sniffs all covaried. The multiple associations among these marking behaviors likely accentuate individual territoriality characteristic within this species [67,97]. Specifically, only particular individuals ascend to alpha pair status, and those individuals demarcate territorial boundaries more frequently than betas or transients [67]. Alpha individuals also maintain status via successful territorial defense from neighboring conspecifics and suppression of insurgency within a pack [65]. Because being an alpha coyote increases breeding opportunities for that individual [65,98], consistent individual differences in territoriality represent a tangible set of characteristics that can directly influence reproductive fitness. This interplay between rank and consistent individual differences may not be restricted to coyotes, but also found in African wild dogs [24], Ethiopian wolves [99], and gray wolves [28]. We temper our predictions as to how relevant these findings are to social dynamics of coyotes, however, because our design solely observed single pairs. However, our results provide a foundation for future research to closely examine pack systems and how individual differences in behavior shape the development of pack dynamics in Canidae.

Other studies have used similar manufactured and commercial odors that have elicited near identical behavioral responses from coyotes to varying degrees [46,73]. The use of coyote attractants that have trace odors from novel conspecifics suggest that our coyote attractants were a proxy for social cues. This is similar to Dantzer et al. [47], which presented conspecific audio playbacks toward pregnant red squirrel mothers as a proxy for social challenge and increased conspecific density. Because coyotes similarly respond to odors such as wolf urine (C. Schell, unpublished) it is not conclusive whether the behavioral responses observed were socially-motivated or if coyotes regularly follow a set marking protocol whenever the intensity of a scent is highly volatile. Nevertheless, it is known that coyotes are able to differentiate

Table 3

Spearman rank correlations among identical male and female behaviors and hormonal best linear unbiased predictors (BLUPs) during odor cue provisioning (i.e. mid-gestation; $N = 16$). Bolded values indicate statistical significance.

| Trait | r_s | P |
|-------------------|-------------|------------------|
| Aggression | 0.39 | 0.135 |
| Ground scratching | 0.35 | 0.184 |
| Urination | 0.79 | <0.001 |
| Body rubs | 0.85 | <0.001 |
| Site sniffs | 0.84 | <0.001 |
| Visits | 0.76 | <0.001 |
| Site time | 0.88 | <0.001 |
| Latency to visit | 0.88 | <0.001 |
| BLUP cortisol | -0.01 | 0.971 |
| BLUP testosterone | 0.63 | 0.009 |

All behavioral data are reported as per 70 min of recorded video.

between subtle differences in volatile odors and respond accordingly [46]. Indeed, individual coyotes have been shown to delineate among varying volatile odors by altering the degree of marking, biting, pulling, and rubbing behaviors they perform specific to the odor received. Water was therefore a suitable control in our study to compare against receiving coyote odor attractants, particularly given the profound behavioral differences we observed among our treatment and control breeding pairs. Further, the potential for indirect olfactory contact was negligible: though control and odor pairs were adjacent to each other, the stark behavioral differences between treatment groups indicate that having stimuli inside your pen was more salient than indirect detection of odors outside of your home pen. Our results support data from coyotes in the wild, as resident individuals are more responsive to foreign substances provided within their territories [67].

4.2. Odor attractants, parity, and hormonal responses

This study is the first to physiologically quantify gestational FGMs and FAMs in coyotes of both sexes, as previous studies were restricted to plasma samples and did not measure both hormones in each sex [61,64,100]. Our initial analyses to characterize FGM and FAM patterns demonstrated steady declines for both sexes as gestation progressed regardless of odor treatment. The observed hormonal patterns are in accord with previous findings on female progesterone [61] and male testosterone [64,100] in coyotes, and may be explained by several factors. First, glucocorticoids and androgens peak early during the breeding season, which corresponds with a peak in scent-marking behaviors of previous studies [63,72,101]. It is likely that hormonal physiology accompanies the onset and regression of marking behaviors, similar to urine-marking and testosterone in gray wolves [28]. Second, constant territorial maintenance over mating may require expectant pairs to have elevated glucocorticoids and androgens to cope with the stress of territorial intrusions. As competition wanes, however, it may be unnecessary to maintain elevated stress and reproductive hormone concentrations, especially as chronic activation of glucocorticoids can compromise maternal health and developing offspring [4]. Third, hormonal declines may also be evolutionarily conserved: related Canidae mothers demonstrate similar decreases in stress and reproductive hormones closer to parturition (domestic dog, *Canis lupus familiaris* [102]; Ethiopian wolves [99]). More distantly related mammals show the opposite trend (yellow baboons, *Papio cynocephalus* [103]; pygmy rabbits, *Brachylagus idahoensis* [104]), suggesting that decreases in reproductive and stress hormones over pregnancy are specific to Canidae.

Hormonal patterns of expectant coyote fathers closely followed maternal patterns over the entirety of gestation, suggesting that males are sensitive to maternal cues over pregnancy. Similarly, expectant cotton-top tamarin (*Saguinus oedipus*) fathers track glucocorticoid responses of paired pregnant partners [51]. The authors suggest that responsiveness of fathers is primarily explained by female deposition of periovulatory scents rather than increased rates of behavioral communication, as there were no observed interaction changes between mates. Expectant common marmoset (*Callithrix jacchus*) fathers show similar responsiveness, as males exhibited increased testosterone shortly after sniffing periovulatory scents of pregnant females [105]. Periovulatory scents may signal that the female is receptive to solicit copulation with the male and mate guarding from neighboring males, both of which are often accompanied by increased testosterone. Periovulatory scents may also signal maternal health status to expectant fathers, which can alter glucocorticoids and paternal behavior to assist the mother during gestation. Coincidentally, coyotes, cotton-top tamarins, and common marmosets are all socially and reproductively monogamous [51,72,98,105], which suggests that in monogamous biparental systems it is beneficial for fathers to be highly responsive toward maternal cues over pregnancy. Periovulatory scents may signal that the female is receptive to solicit copulation with the male and mate guarding from neighboring males, both of which are often accompanied by increased FAMs.

Periovulatory scents may also signal maternal health status to expectant fathers, which can alter glucocorticoids and paternal behavior to assist the mother during gestation. This study provides further evidence to suggest that hormonal patterns of expectant fathers are highly responsive to female stimuli.

Both sexes had increased FAMs when provided odor attractants, suggesting that novel odors were effective at soliciting a physiological response. Paired with increased scent-marking and investigatory behavior, it is likely that the coyote odor attractants were effective proxies for territorial incursion. These results support the challenge hypothesis previously described by Wingfield et al. [106] and revisited by Goymann et al. [107], in which individuals (specifically males) that are challenged for their social rank during the mating season exhibit increased androgens and aggression in response. Golden lion tamarin males (*Leontopithecus rosalia*) exhibit this trend, as dominant breeding males exhibit higher androgens during the mating season [22]. Similarly, in male chacma baboons (*Papio hamadryas ursinus*) testosterone concentrations and changes in rank are positively correlated, in which males rising in rank have higher testosterone than males falling in rank [23]. Here, our data suggest that the challenge hypothesis applies to both males and females, which may be due to the biparental nature of the coyote system. Future work should address the challenge hypothesis in socially monogamous systems to examine how androgens of both sexes are affected by artificial (i.e. odors) or actual challenges to social rank.

In addition to effects of the coyote attractants, we observed an effect of breeding experience on male and female androgens, in which pairs as first-time breeders had higher FAM concentrations. This is in contrast to cotton-top tamarins, in which FAM changes pre-partum are independent of breeding experience [58]. For coyotes, it is possible that as a young breeding pair, securing a territory and guarding against territorial intrusions may present a greater challenge than maintaining a territory is for experienced breeders. Consequently, increased FAMs during gestation may accompany increased territorial defense and maintenance. It is also possible that increased familiarity between individuals within a pair is related to decreased FAMs over time. Specifically, reduction of intra-pair aggression over time may result in decreased FAMs. An alternative explanation may be that unfamiliar physical changes such as pair relocation and first breeding event may have placed physical stress on the body that manifested as increased FAMs. In addition, this unfamiliarity may stem from novel experiences of young animals to captive conditions, and FAMs of experienced pairs merely reflect a perceived comfort or predictability of housing conditions. More data are necessary to examine these hypotheses on how age of pairs and familiarity within pairs impact FAMs.

4.3. Repeatability of behaviors and hormones

Both females and males demonstrated repeatability in urination and ground scratching behaviors across years despite differences in odor treatment condition (Table 2). Previous work has indicated that between-individual differences in wild coyote marking rates are primarily attributed to social organization (i.e. resident versus transient), social class (i.e. alpha versus beta), and season (i.e. breeding versus non-breeding), in which resident alpha adults have the highest rates of marking behaviors [67]. Our captive coyote pairs were individually housed, eliminating the potential for social hierarchies to develop among coyote packs. Our results therefore suggest marking rates are not merely determined by social organization or class but also by inherent differences in individual personalities. Consequently, personality type may be a credible predictor in determining which individual coyotes will establish home ranges and outcompete conspecifics to ascend in social rank.

Our results also demonstrated strong positive correlations between identical behaviors in male-female pairs except for aggression and ground scratching (see Table 3). Because most of these traits

demonstrated repeatability, these data suggest female personality may have affected male behaviors and vice versa. These data support findings from wild coyotes, in which one partner of a resident pair will urinate and the other partner subsequently responds by sniffing and urine-marking the same affected area [67]. Individuals within a breeding pair therefore have the potential to augment the expression of personality traits of their partner, indicating that pairmates are a potential source of plasticity in personality traits. Coalescing personalities between pairmates may provide a fitness benefit, as parents with relatively similar personality types may have greater reproductive success [108,109]. For coyotes, highly territorial pairs tend to be residents that can outcompete neighboring conspecifics and secure high-quality resources [66, 67]. If individuals with strong personalities in scent-marking and investigatory behaviors can augment the personality traits of their partner, this may positively affect their ability to secure and defend source habitats in which to rear offspring. Moreover, the significant correlation between male and female androgen BLUPs may indicate that individuals also partially influence the reproductive hormone profiles of their pairmate (Table 2). Hence, influences from the father during pregnancy may likely affect maternal androgen profiles that eventually affect developing offspring epigenetically, shaping pup developmental trajectories [54,55]. Our results therefore highlight the importance of pairmates in affecting both phenotypic outcomes for their partner and for subsequent offspring as well.

Both sexes demonstrated repeatability in their FGM concentrations over time, suggesting that coyotes either have stable stress physiology or distinct stress profiles. These stress profiles are reminiscent of the Hawk-Dove hypothesis proposed by Korte et al. [4], where individuals employ different behavioral strategies that are facilitated by underlying physiology. Korte et al. [4] originally delineated Hawk-Dove differences by levels of aggression and its association with the biological stress response of an individual, though the conceptual framework can be expanded to different behaviors and hormones. In contrast, only males demonstrated repeatability in FAMS (Table 2). Given that testosterone is generally higher in male mammals [110], it is likely that repeatability in coyote male FAMS reflect sex-linked traits important for reproduction.

Individually-consistent differences in hormones (i.e. BLUPs) were correlated with multiple scent-marking and investigative behaviors (Table S3). This is similar to male great tits (*Parus major*), in which testosterone levels were both repeatable over time and correlated with exploratory behaviors [27]. Likewise, individual white-eared hummingbirds (*Hylocharis leucotis*) show consistent individual differences in testosterone over time and those with higher testosterone concentrations are more risk-prone foragers (quantified by frequent visits to variable flowers) [26]. For this study, increased androgen BLUPs were positively associated with latency to visit the affected site for both sexes (Table S3). For male coyotes specifically, androgen BLUPs were also correlated with the number of visits made and the time spent at the affected site (Table S3), suggesting male coyote androgens are particularly salient for individually-specific investigative behaviors. Our results therefore suggest that individual differences in territoriality are partially mediated by androgens. However, the paradoxical negative association between androgen BLUPs and urine-marking may also suggest other environmental factors like pairmate behavior may mitigate behavioral expression of males. Indeed, we have provided evidence to suggest that coyote female marking behaviors may be met with matching behaviors from males (Table 3); hence, the potential of pairmates to influence behavioral profiles may be understated.

We have demonstrated that both conspecific odor attractants and parity affect behavior and hormones of breeding coyotes. Moreover, coyotes demonstrated personality for multiple territorial behaviors, and distinct hormone profiles for both glucocorticoid metabolites and androgen metabolites. Given the pervasive effects that parental influences may have pre-partum, it will be important to consider how individual differences in coyote traits and plasticity of those traits affect

both parents and offspring. Indeed, changes to parental glucocorticoid and androgen concentrations directly interact with offspring during gestation and greatly dictate offspring development in the process [47, 54,55,111]. Hence, a deeper understanding of the myriad effects environmental stressors and temporal variation have on pregnant parents and subsequent developmental trajectories of neonates will enrich our understanding of epigenetic processes [112–114]. This is particularly relevant for coyotes in the context of rapid adaptation to nonnative and urban habitats, which may suggest that environmental experiences of parents play some role in shaping offspring phenotypic traits integral for colonization and survival of novel environments.

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Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.physbeh.2016.06.038>.

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