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DIFFERENTIAL AND LONG-TERM IMPACTS OF BIPARENTAL EFFECTS ON OFFSPRING PERSONALITY AND HORMONES IN COYOTES (*CANIS LATRANS*)

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CHRISTOPHER JOHN SCHELL

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I dedicate this to my parents and grandparents, my beautiful wife Danielle LaVeta Schell, and my future children. May they too be infected with the same curiosity, courage, and sense of adventure that my parents nurtured inside me.

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ABSTRACT

In response to unpredictable or stochastic environmental fluctuations, parents may alter their behavior, morphology, and physiology to cope with such changes. Consequently, these sudden changes can impact their offspring by shaping their phenotypic development beyond the influence exerted from inherited genes. This facet of parental effects theory – deemed parental programming – contributes to phenotypic variation within a population, as parents "prepare" their offspring for success by tailoring their phenotypes toward future environmental conditions. Thus, parental effects are partially responsible for generating the raw material by which natural selection operates. For mammalian and avian species in particular, endocrine factors are likely key components driving parental programming, as hormonal changes often precede or accompany phenotypic change (e.g. morphology, behavior, etc.). However, few studies have addressed this mechanism in biparental care systems, or determined whether offspring traits modified by parental effects are consistent into later life stages. In this dissertation, pre-partum hormones of captive coyote (*Canis latrans*) breeding pairs were assessed in response to environmental cues (i.e. novel conspecific odors) and prior breeding experiences (i.e. first-time versus experienced breeders). Resultant parenting behaviors, pup personality traits (i.e. boldness, activity, aggression), and pup hormonal outcomes (i.e. cortisol, testosterone) were examined to determine if pre-partum hormones of parents were associated with phenotypic outcomes of their offspring across multiple life stages.

First, I exposed captive coyote pairs to novel conspecific odors (i.e. mixture of fermented glandular oils, urine, etc.) mid-gestation as a proxy for increased conspecific density (Chapter 2). Additionally, breeding pairs were observed as first-time and experienced parents. Coyote pairs provided with odors had higher fecal androgen metabolites compared with those that received water as a control, implying coyote androgens were sensitive to social olfactory cues. Meanwhile, both males and females had lower pre-partum fecal androgen metabolites as experienced versus first-time breeders.

Second, parenting behaviors of coyote pairs were observed from 5 to 15 weeks of offspring age to assess whether pre-partum hormonal outcomes were associated with subsequent parenting behaviors (Chapter 3). Maternal (but not paternal) fecal androgen metabolites observed mid-gestation were negatively associated with contact and aggression, suggesting that mothers with higher androgen metabolites over gestation contacted and aggressed their pups less than other moms. Further, experienced parents contacted and aggressed their pups more frequently.

Third, I addressed whether pre-partum odor cues and breeding experience of parents affected boldness and hormonal physiology of pups (Chapter 4). Pup boldness was assessed using feeding and novel object tests with a predator stimulus (i.e. human observer) present for both tests. Cortisol and testosterone concentrations were analyzed using hair shaved from pups at 5, 10, and 15 weeks of age. Pups born to experienced parents re-emerged from their dens more during behavioral tests, and had lower hair testosterone at 5 weeks of age. Pre-partum fecal androgen metabolites of parents were negatively associated with how frequently pups ate during feeding tests, suggesting that decreased parental androgens are associated with increased pup boldness.

Finally, feeding and novel object tests were repeated on a subset of coyote pups during the yearling stage to determine if phenotypes were consistent over developmental time (Chapter 5). A set of 13 behaviors were analyzed using a principal components analysis to identify personality components. Further, I collected fecal samples over a 7-week period to quantify fecal glucocorticoid and androgen metabolites of coyote yearlings. Individuals that were more willing to eat when exposed to predator cues (i.e. humans) as pups were also more likely to be more active and exploratory. Yearling fecal androgen metabolites were also positively associated with pre-partum fecal androgen metabolites of their parents when those yearlings were developing neonates.

Taken together, these results suggest coyote parents were able to transduce their environmental (i.e. odor) and breeding experiences via androgens into meaningful phenotypic outcomes for pups. Specifically, decreased pre-partum androgens of parents were associated with increased boldness behaviors in their offspring. Boldness and other personality traits of pups were associated with the behaviors of those individuals as yearlings, highlighting the pervasive and long-term nature of parental effects in this system. In addition, fecal androgen metabolites of yearlings and their parents were positively associated, indicating that parental physiology over gestation affected the subsequent physiology of their offspring long-term. Parental effects in coyotes therefore may be implicated in affecting traits (i.e. boldness, behavioral plasticity) that have previously been considered integral in coyote colonization of nonnative habitats such as urbanized ecosystems.

CHAPTER 1: INTRODUCTION

Phenotypic variation across a population provides the raw material that selection can act upon (Darwin 1859; Reddon 2011; Sih et al. 2010). Individual differences in phenotype and the selection pressures (e.g. predation, environment, competition, climate change etc.) that act on those differences are readily observed across populations of various taxa (Hoffman & Sgró 2011; Mitchell-Olds et al. 2007). Meanwhile, an ongoing stem of research aims to determine the causes of phenotypic diversity to fully understand the mechanisms that generate the raw material integral to natural selection processes (Houle et al. 2010; Kussell & Leibler 2005; Mousseau & Fox 1998, McAdam et al. 2000; Mitchell-Olds et al. 2007; Reddon 2011; Sih et al. 2010). Previous work has either focused on target genetic markers (Benfey & Mitchell-Olds 2008; Hurst 2009) or isolated specific environmental variables (Kussell & Leibler 2005; Sgró & Hoffman 2004) to determine how genetic and environmental factors are associated with phenotypic variation. Studies on heritability and development in particular meticulously work to reduce environmental variance (Visscher et al. 2008), as it has previously been considered a source of error in assessing genotype-phenotype relationships (Falconer & Mackay 1996; Laland et al. 2014; Maestripieri & Mateo 2008). However, a surge of recent work has emphasized that certain sources of environmental variance are both resistant to experimental control (Falconer & Mackay 1996), as well as highly influential in shaping phenotypic traits and plasticity beyond the genetic contribution to phenotype (Ghalambor et al. 2007; Laland et al. 2014; Maestripieri & Mateo 2008; Mousseau & Fox 1998; Sgró & Hoffman 2004; Via & Lande 1985). Indeed, phenotypic plasticity produced via these non-genetic mechanisms in response to altered environmental conditions are hypothesized to drive latter genetic evolution and increase

colonization of novel habitats (Ghalambor et al. 2007; Laland et al. 2014; Price et al. 2003). Consequently, epigenetic mechanisms likely play a pivotal role in the evolutionary dynamics of a population (Bonduriansky & Day 2009; Mousseau & Fox 1998; Räsänen & Kruuk 2007; Wolf et al. 1998).

The non-genetic transmission of phenotypic traits from parents to offspring accounts for a substantial degree of the epigenetic mechanisms identified as integral to phenotypic variation in a population (Bonduriansky & Day 2009; Wolf et al. 1998). This form of non-genetic inheritance (i.e. maternal effects) demonstrates that the maternal phenotype (e.g. hormones during pregnancy, body size and condition, social status, etc.) provides an additional influence to offspring phenotype beyond inherited genes (Maestripieri & Mateo 2008; Mousseau & Fox 1998; Räsänen & Kruuk 2007; Reddon 2011). Similarities between maternal and offspring phenotypes can therefore significantly bias genetic correlations. For instance, positive correlations among maternal mass and offspring growth rates have been associated with both direct genetic effects and maternal foraging behaviors exhibited pre- and post-partum (Bowen et al. 2006; McAdam et al. 2002). Maternal habitat selection when rearing offspring is strongly associated with habitat preferences of dispersing offspring later in life (Höner et al. 2010; Mateo 2009; Sacks et al. 2008), as well as overall dispersal distances traveled by offspring (Bitume et al. 2014). In addition, physiological stress experienced over gestation (Love et al. 2013; Meylan et al. 2012; Uller 2008) and intrauterine position of offspring *in vitro* (Clark & Galef 1988; Clark et al. 1990; Reynolds et al. 2013; Vandenburgh 2008) are uniquely associated with subsequent hormonal and behavioral traits of developing offspring. These maternal effects occasionally interact with social stimuli or rank in which heightened status (Dloniak et al. 2006; Onyango et al. 2008) or experienced social challenges pre-partum (Dantzer et al. 2013; Kemme et al. 2007)

result in correlations among maternal social rank and offspring traits (Holekamp & Dloniak 2008; Onyango et al. 2008). Moreover, many of these maternal traits, such as size, habitat selection and social status, are often reinforced by intimate parenting and contact behaviors (e.g. licking and grooming, provisioning) performed by mothers post-partum that initiate offspring physiological and behavioral changes critical for development and survival (Champagne & Curley 2008; Meaney 2001). Thus, thorough research has demonstrated the innumerable influences mothers have to shape offspring traits through media other than genetic means.

Maternal effects mechanisms are not simply prolific at biasing parent-offspring genetic correlations, but are also effective at preparing offspring for success by transducing current environmental conditions into tangible phenotypic consequences for offspring (Räsänen & Kruuk 2007; Reddon 2011). Mothers are constantly challenged to mount an appropriate response toward predictable (e.g. seasonal variation, community structure), stochastic (e.g. severe weather, social challenges), or unidirectional (e.g. anthropogenic land conversion, global climate change) changes to environmental conditions that present credible threats to survival (Maestripieri & Mateo 2008). Adaptive responses mounted by mothers, especially during gestation, can alter physiological, morphological, and behavioral traits of the mother that affect developing neonates *in vitro* (Meylan et al. 2012; Reynolds et al. 2013; Uller 2008). These changes 'prepare' offspring for the environments they are predicted to face, affecting their fitness (Mousseau & Fox 1998; Schöpper et al. 2012; Schweitzer et al. 2014). This extraordinary facet of maternal effects has previously been deemed maternal (or parental) programming, in which changes to maternal phenotypic traits affect the developmental trajectories of offspring which are expected to be longstanding (Breuner 2008; Fish et al. 2004; Kemme et al. 2007; Sheriff et al. 2010; Stein & Bell 2014). Maternal programming is further broken down into two categories:

anticipatory, in which mothers induce offspring trait changes toward an optimum suitable for predictable environmental changes; and context-dependent, in which mothers hedge their bets by increasing the phenotypic variation of their offspring under unpredictable conditions to ensure survival of at least a few individuals (Badyaev and Uller 2009; Reddon 2011). The predictability of environmental change in both instances partially dictates maternal response, while individual differences among mothers remain prevalent even when conditions are identical across a population (Budaev et al. 1999; Meaney 2001; Reddon 2011; Stein & Bell 2015; Westneat et al. 2011; 2013). The interaction between individual maternal response and environmental change may result in varied phenotypic consequences for offspring, thus resulting in the phenotypic diversity essential to natural selection processes. Hence, by transducing environmental change into phenotypic variability of the next generation, maternal effects have the potential to impact evolutionary dynamics of a population.

OBJECTIVES OF STUDY

The objective of this dissertation was to investigate the extent to which parental effects impact personality traits and hormonal development in coyotes, a biparental canid. The majority of parental effects research has focused solely on maternal influences in single parent systems (Maestripieri & Mateo 2008; Mousseau & Fox 1998; Räsänen & Kruuk 2007; Uller 2008). Previous studies have observed paternal care in relation to mothers (Budaev et al. 1999; Creighton et al. 2014; Nakagawa et al. 2002; Schwagmeyer et al. 2002; Westneat et al. 2011); however, only a small number of studies have explicitly addressed paternal effects in biparental care systems (Harris et al. 2013; Lock 2012; Schweitzer et al. 2014; Stein & Bell 2014). Moreover, only a few studies directly address how parental effects influence personality

development in offspring (Hinde et al. 2014; Schuett et al. 2013). For this thesis, I directly address the impact of biparental effects on coyote offspring development, making this the first empirical study to examine non-genetic inheritance in a biparental mammal. In addition, I directly address how parental effects are associated with personality development in offspring, specifically focusing on the behavioral traits critical to coyote success in nonnative habitats (i.e. boldness, tolerance, aggression).

Coyotes qualify as an excellent species to further our understanding of parental effects for several reasons. First, long-term relationships among parents and pups that often extend into adulthood provide parents adequate time to bias offspring phenotypes beyond inherited genes (Asa & Valdespino 1998; Messier & Barette 1982; Sacks & Neale 2001). Second, rapid geographic expansion of the species into metropolitan and other novel environments suggest genetic change was not a driving factor affecting recent coyote colonization. Because rates of genetic mutation take thousands to millions of years (Wolf et al. 1998), non-genetic transmission is a more plausible mechanism affecting recent coyote colonization of nonnative habitats. Third, with the recent geographic expansion of the species, exposure of expectant coyote parents to multiple habitats conditions result in varying selection pressures. Adaptation to these varying pressures under short (i.e. decades) timescales likely results in physiological or behavioral changes of expectant parents that may later affect neonates (Reddon 2011; Uller 2008; Wolf et al. 1998). Examination of parental effects in coyotes thus not only expands the theory by direct assessment of the mechanism in a biparental care system, but also provides insight into how parental effects mechanisms may drive adaptation to rapid human-induced environmental change. Specifically, non-genetic inheritance in the species is a likely determinant of natural

variation in human-associated tolerance and boldness, behavioral traits hypothesized to facilitate coyote adaptation to cities and other nonnative habitats (Gehrt 2010).

Coyotes are typically clandestine and the chances of acquiring long-term repeated measures data from individuals in the wild are untenable. I therefore observed a captive population of coyotes at the National Wildlife Research Centers (NWRC) in Millville, UT to address parental effects in the coyote system. Following the general introduction, both Chapters 2 and 3 examine behavioral and hormonal traits of parents pre- (both) and post-partum (behavior only). I provide a comprehensive examination of fecal glucocorticoid (FGMs) and androgen (FAMs) metabolites in expectant coyote mothers and fathers from early to late gestation (i.e. February to April; Chapter 2). Pregnant mothers and accompanying fathers were repeatedly presented with odor cues mid-gestation (February to March) meant to be a proxy for high conspecific densities to induce increases in FGMs and FAMs. In addition, parents were observed as first-time and experienced breeders to determine how time affected hormones and behaviors over gestation. In Chapter 3, I observed coyote breeding pairs as parents over a critical stage of offspring development (i.e. 5 to 15 weeks of litter age). Previous studies have observed parental care behaviors in coyotes (Asa & Valdespino 1998; Fentress et al. 1987; Messier & Barette 1982; Way et al. 2001), though no study to date has quantified care differences among the sexes, or how individual variation in care behaviors change over breeding experiences and time. I therefore systematically characterize parenting behaviors for both mothers and fathers (Chapter 3).

Chapters 4 and 5 examine outcomes for pup phenotypes. In Chapter 4, I focused on the personality and hormonal traits of offspring over the same critical stage of development in Chapter 3. Both Chapters 4 and 5 focus on boldness and aggressiveness traits previously

characterized by Reddon (2011). Specifically, I used novel object and feeding tests previously used to measure boldness behaviors in other species (brown trout, *Salmo trutta*, Adriaenssens & Johnsson 2013; cichlid fish, *Oreochromis mossambicus*, Galhardo et al. 2012; ravens, *Corvus corax*, Stöwe & Kotrschal 2007), as well as coyotes (Darrow & Shivik 2009; Harris & Knowlton 2011; Mettler & Shivik 2007; Young et al. 2015). To characterize cortisol and testosterone concentrations of pups, I used shaved hair samples collected from individuals at 5, 10, and 15 weeks of age (Chapter 4). In Chapter 5, I then repeated novel object and feeding tests on a subset of coyote pups within the yearling stage to examine consistency of personality traits across life stages. Fecal samples were collected to quantify fecal glucocorticoid and androgen metabolites (Chapter 5). In both Chapters 4 and 5, the goal was to determine whether pre-partum environmental experience of parents and parity (i.e. first-time vs. experienced breeders) affected the behavioral and hormonal traits of offspring during infancy (Chapter 4) and the yearling stage (Chapter 5). If in fact parental experiences were associated with pup outcomes long-term, then these data would suggest that parental effects produce a tangible impact on coyote offspring fitness.

In the concluding chapter (Chapter 6) I synthesize the results and provide suggestions as to the evolutionary consequences of parental effects mechanisms in coyotes. The findings from this dissertation are assessed from the lens of hormonally mediated parental effects described in other species. Moreover, the role that parental effects play in affecting personality traits and behavioral plasticity of the species, as well as the overall importance of parental effects in affecting expansive coyote colonization of novel environments are discussed. I provide evidence to how the coyote system fits in the larger narrative centering on the role epigenetic inheritance plays in generating phenotypic plasticity (Reddon 2011; Wolf et al. 1998), and how phenotypic

plasticity facilitates adaptation (see Laland et al. 2014; Price et al. 2003). Finally, I give future directions of research that may provide further insight into rapid coyote adaptation, specifically elaborating on the genotypic markers that may co-vary with the hormonal and behavioral indices measured.

NATURAL HISTORY OF COYOTES

Geographic Expansion and Adaptation

Coyotes are mid-sized (11.4 to 15.9kg) social canids nearly ubiquitous across the North American continent (Bekoff & Wells 1982; Gehrt 2010). Historical geographical limits of the species had them situated within the Great Plains, and their increased geographic distribution (i.e. within the last 50 years) is likely due to a multitude of interacting factors. First, grey wolves (*Canis lupus*) were removed from North America in 1926 resulting in a competitive release for coyotes (Fox 2006; Gehrt 2010). Grey wolves actively exclude and aggress coyotes from habitats, and this interference competition restricts coyote ranges and resource acquisition (Atwood & Gese 2010; Merkle et al. 2009). Competitive conflict in many habitats vanished with the removal of wolves in the early 1900s, leaving niches that coyotes could exploit. Second, abundances of small prey species (e.g. eastern cottontail, *Sylvilagus floridanus*; prairie voles, *Microtus ochrogaster*) dramatically increased in both naturalized (e.g. forest preserves, grasslands, etc.) and developed habitats (e.g. agricultural fields, urban areas, etc.) within the last few decades (Buck & Kitts 2004; Fox 2006; Gehrt et al. 2009; Grinder & Krausman 2001; Morey et al. 2007; Tigas et al. 2002). Unlike related grey wolves and African wild dogs (*Lycaon pictus*) which need to hunt larger prey to survive (Courchamp & Macdonald 2001; Courchamp et al. 2002; Creel 1995), coyotes are flexible enough to either cooperatively hunt larger prey (e.g.

ungulates) or hunt small prey animals individually (Bekoff & Wells 1981). Thus, coyotes' tremendous dietary flexibility enabled individuals to capitalize on increased prey abundances in multiple nonnative habitats (Gehrt 2010). Last, coyotes are phenotypically flexible enough to modify their landscape use patterns (Gehrt et al. 2009; Grubbs & Krausman 2009), diet (Morey et al. 2007), and activity budgets (Grinder & Krausman 2001; Kitchen et al. 2000; Séquin et al. 2003) to avoid human detection. Despite an overall increase in anthropogenic landscape conversion over recent decades, inconspicuous yet bold individuals were able to exploit increased prey resources in urban and suburban environments (Ditchkoff et al. 2006; Lowry et al. 2013). Taken together, the combination of species' phenotypic flexibility (i.e. dietary, behavioral) and external environmental changes (i.e. extirpated wolves, landscape conversion) facilitated coyote colonization of novel environments.

Reproductive biology and socioecology

Coyotes are seasonally monestrous, mating once annually from December to February (Carlson & Gese 2008; 2009). Individuals are socially monogamous and mate with a single partner over successive years for an indeterminate amount of time (Carlson & Gese 2008; Bekoff & Wells 1982). Mating is characterized by a copulatory lock and tie in which males remain physically attached to the female over an extended period after copulation has completed (Carlson & Gese 2008). Females experience a general increase in progesterone and estradiol concentrations that proceed approximately 1-2 weeks post-ovulation, then declines until parturition (Carlson & Gese 2008). These hormonal changes occur within an estrus period that extends from early January to late March (Sacks 2005), and individuals that do not become pregnant exhibit a hormonal signature analogous to a viable pregnancy referred to as

pseudopregnancy (Carlson & Gese 2008). Meanwhile, male coyotes exhibit testosterone concentrations that peak in mid- to late- January and generally decline toward parturition in April (Minter & DeLiberto 2008). Older coyotes generally have greater reproductive success than yearlings, although yearling breeding success may increase for yearlings with exceptional nutritional condition (Sacks 2005). Mated pairs are increasingly territorial over the breeding season, as scent-marking, ground scratching, and physical aggression toward other conspecifics peak mid-January to late-February (Carlson & Gese 2010; Gese 2001; Gese & Ruff 1997; Messier & Barette 1982). Both males and females actively defend and maintain territorial boundaries, and dominant individuals among pack members perform territorial behaviors more frequently compared with subordinates (Gese 2001).

Litters are born late March to mid-May after a 62-65 day gestation period, and litter sizes range from 1 to 12 pups (Gehrt 2010; Sacks 2005). Parturition dates and litter sizes of older mothers tend to be earlier in the season and larger, respectively (Sacks 2005). Pups are generally born in dens constructed by their parents that are approximately 2 to 5 meters deep, and emerge 2 to 4 weeks post-birth (C.Schell pers. obs.; Way et al. 2001). Both mothers and fathers greatly interact with pups over development and provide complimentary care to the accompanying parent (Bekoff & Wells 1982). In fact, territorial defense from both parents is so critical to pup survival that the risk of mortality increases when either parent is removed (Messier & Barette 1982; Sacks & Neale 2001). During the first 8 to 10 weeks of life, parents attend dens relatively frequently and provision pups regularly (Bekoff & Wells 1982; Way et al. 2011). Male coyotes invest a considerable amount of time to pup guarding and contact behaviors (Asa & Valdespino 1998), in addition to provisioning females early in pup rearing (Sacks & Neale 2001). It is still unclear, however, how mothers and fathers differ in the rates at which they perform certain

parenting behaviors. When pups become approximately 15 to 20 weeks of age, they often disperse from their natal pack to establish a breeding pair with other conspecifics (Bekoff & Wells 1982). Some individuals occasionally remain with their parents as yearlings, and stay within their natal packs to assist in rearing offspring of the proceeding generation (Bekoff & Wells 1982). Consequently, parents and pups develop extended long-term relationships in which both parties can affect each other concurrently.

Social biology, resource availability, and conspecific densities

Coyote social systems are relatively flexible compared to related grey wolves and African wild dogs (Bekoff & Wells 1986). Individuals within a population may either be part of a pack containing a nuclear breeding pair (i.e. parents and pups), or as individual transients that are not directly associated with a pack group (Bekoff & Wells 1981; 1986). When individuals do form a pack group, social status within that pack dictates reproductive success and resource acquisition (Gese et al. 1996a; Gehrt 2010). This is similar to social group dynamics and reproduction in other social carnivores (spotted hyenas, *Crocuta crocuta*, Holekamp et al. 1996; meerkats, *Suricata suricata*, O'Riain et al. 2000; grey wolves; Peterson et al. 2002; African wild dogs, Vucetich & Creel 1999). Dominant individuals breed the majority of the time and tend to have greater offspring survival as beta individuals in the pack help in rearing offspring (Bekoff & Wells 1982; Sacks et al. 2008). Subordinate coyotes and transients (i.e. individuals without pack membership) occasionally reproduce, but the lack of help from other conspecifics or the absence of a parent (e.g. in the case of transients) increases the rate of offspring mortality (Sacks et al. 2008). Further, beta or transient mothers den in habitat patches between home ranges of alpha

females which usually have lower quality resources to rear young (Kamler & Gipson 2000; Sacks et al. 2008).

Resource availability (e.g. prey, source habitat, etc.) strongly influences coyote social organization, in which limited supply of source habitats and prey items frequently result in intra- and interspecies conflict (Geffen et al. 1996). Coyote groups and pairs maintain strict territorial boundaries that are patrolled and scent-marked frequently (Gese 2001; Gese & Ruff 1997; Neale et al. 2007). Residents of larger pack groups often outcompete neighboring packs for higher quality habitats with a greater percentage of contiguous forested or grassland ecospace (Atwood 2006). Coyote home ranges with more contiguous landscape contain a greater abundance of vegetation that prey species require (Bekoff & Wells 1986). Therefore, groups able to obtain prime territories increase their survival outcomes by securing habitat space essential to their prey base (Gese 2001).

Competition for resources within a group also indirectly influences social structure. Beta coyotes are partially restricted from ungulate carcasses and infrequently participate in social interactions with dominant individuals (Bekoff & Wells 1986; Gese et al. 1996a). As a result, individuals proficient in hunting small prey choose to disperse and establish a new pack (Bekoff & Wells 1986). However, the potential benefits of establishing a new group and home range need to outweigh the risk of mortality from conflict with adjacent coyote packs, or interspecies conflict (e.g. sympatric wolves). In many instances, beta individuals remain in their natal packs and wait to matriculate to higher social status once alpha individuals die (Gese et al. 1996a). Despite the rigidity of social hierarchies within the coyote social system, pack sizes are still flexible enough to decrease with decreasing large ungulate abundances (Bowen 1981) or increased abundance of lagomorphs and rodents (Mills & Knowlton 1991). These data highlight

how intraspecific competition for resources partially influence flexibility in coyote social systems.

Interference competition for resources with other canids and carnivores is also responsible for shaping coyote social flexibility. Species within Canidae share remarkable overlap in their prey resources (Atwood & Gese 2010; Merkle et al. 2009; Randa et al. 2009), and smaller canid species are often relegated to hunt smaller prey items as a consequence of their sympatry (Merkle et al. 2009; Randa et al. 2009). Moreover, a system of top-down aggression results in wolves actively killing intruding coyotes (Merkle et al. 2009; Palomares & Caro 1999), and coyotes killing red foxes (Gese et al. 1996b; Voigt & Edie 1983) in hostile habitat space. Consequently, sympatric canids regularly avoid each other by partitioning habitat space (Atwood & Gese 2010; Randa et al. 2009), and resource availability dictates home range parameters of wolves that ultimately affect the distribution of mesocarnivores across the landscape (Palomares & Caro 1999). Nevertheless, some coyotes may scavenge wolf-provisioned carcasses, actively augmenting their food intake when small prey are difficult to secure (Atwood & Gese 2010). Coyotes habituated to wolf presence become especially adept at navigating hostile habitat space to capitalize on scavenging opportunities by traveling individually and reducing their pack sizes (Switalski 2003). The avoidance behavior exhibited by these coyotes therefore suggests that individuals are able to perceive the degree of risk present with wolf-associated mortality (Atwood & Gese 2010).

Recent studies observing newly establish coyote populations in urban environments underscore the range of flexibility characteristic of coyote social organization. Several metropolitan areas within North America have witnessed recent increases in coyote populations that coincide with increased abundance of white-tailed deer (*Odocoileus virginianus*), eastern

cottontail rabbits, and other rodents (e.g. Norway rat, *Rattus norvegicus*, Gehrt 2010; Šálek et al. 2014). In addition, anthropogenic food sources such as garbage and pet food subsidize natural prey resources (Bateman & Fleming 2012; Fedriani et al. 2001; Morey et al. 2007). Thus, the combination of natural and anthropogenic foods in cities provide an ample amount of resources to sustain an increasing coyote population. Indeed, previous work has demonstrated that urban coyotes are at higher densities in urban versus rural or natural environments (Fedriani et al. 2001; Šálek et al. 2014). Coyotes additionally reduce their home range sizes (Atwood et al. 2004; Gehrt 2010; Šálek et al. 2014; although see Riley et al. 2003) and incorporate habitat patches that encompass park and residential areas (Grinder & Krausman 2001; Gehrt et al. 2009). Equally as important is the fact that the prey items made available are easily obtainable without coordinated pack hunting. As a result, coyotes and other carnivores in urban environments regularly persist as breeding pairs or transients rather than large packs (Bateman & Fleming 2012).

Several studies have demonstrated how interspecies conflict between humans and coyotes result in the expression of avoidance behaviors also observed with sympatric wolves (Gehrt 2010; Grubbs & Krausman 2009; Kitchen et al. 2000; Lowry et al. 2013; Tigas et al. 2002). Urban coyotes travel most frequently during crepuscular and evening hours when human activity is lowest (Gehrt et al. 2009; Grubbs & Krausman 2009; Tigas et al. 2002). Coyotes also utilize habitat patches with increased vegetation for cover during daylight hours to avoid detection (Gehrt et al. 2009; Grubbs & Krausman 2009; Tigas et al. 2002). Thus, coyotes temporally and spatially avoid human detection by modifying their activity budgets and movement patterns (Ditchkoff et al. 2006). Avoiding detection by people is similar to navigating hostile habitat space with wolves, as discovery by people often results in translocation or mortality (Bateman & Fleming 2012). Individuals and groups also become habituated to the predictable patterns of

people in urban areas, and become adept at navigating human-dominated landscapes (Lowry et al. 2013). The effect these behavioral modifications have had on the social organization of urban coyotes likely shift social dynamics among conspecifics. However, it is unknown what the proximate or ultimate consequences may be for coyotes in urbanized areas.

HORMONES AND MATERNAL EFFECTS

Hormones are critical catalysts that either initiate or accompany changes to behavioral and morphological traits (Creel et al. 2013; Groothuis et al. 2005; Möstl & Palme 2002). Glucocorticoids in particular are integral components of an organism's physiological response to environmental change, social challenges, or predatory pressures (Creel et al. 2013). Such external stressors initiate a biological stress response characterized by activity in the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is integral in the release and production of glucocorticoids (i.e., stress hormones; Herman et al. 2003; Jacobson & Sapolsky 1991; Ulrich-Lai & Herman 2009) and underlies the commonly referred fight or flight response. In short, innervation of hypothalamic nuclei in the paraventricular nucleus stimulates the release of corticotropin-releasing hormone (CRH), a primary neurotransmitter hormone responsible for initiating the HPA axis (Herman et al. 2003; Ulrich-Lai & Herman 2009). Corticotropin-releasing hormone then stimulates the pituitary gland, resulting in the release of the glucocorticoid adrenocorticotropin hormone (ACTH) and subsequent release of other glucocorticoids from the adrenal glands, most notably cortisol and corticosterone (Dedovic et al. 2009). These adrenal glucocorticoids ultimately migrate back to hypothalamic nuclei to inhibit further production of CRH, effectively down-regulating the release of glucocorticoids (Dedovic et al. 2009; Schulkin 2011). Short-term activation of this neuroendocrine mechanism is highly

adaptive, in which production of glucocorticoids mobilizes energy stores, suppresses secondary physiological functions (e.g., immune, reproductive, etc.), and attenuates memory retention (Schulkin 2011). Conversely, over-production of glucocorticoids in the long-term depletes available glucose, resulting in several physiological issues that can decrease overall health and fecundity (Love and Williams 2008; Schulkin 2011).

Androgens are also important hormonal factors that regulate behavioral and morphological responses to social challenges, reproduction, and assist in development and maturation from infancy into adulthood (Groothuis et al. 2005; Korte et al. 2005). The hypothalamic-pituitary-gonadal (HPG) axis regulates androgens and other reproductive hormones (Groothuis et al. 2005; Korte et al. 2005; Mastorakos et al. 2006; Viau 2002). Experienced external (e.g. conspecific confrontation, sexually-receptive conspecifics) or internal (e.g. puberty and maturation) stimuli innervate the paraventricular nucleus (PVN) within the hypothalamus to release gonadotropin-releasing hormone (GnRH). Increased concentrations of GnRH activate the release of lutenizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. The release of LH and FSH then proceed to innervate sex-specific end organs (e.g. ovaries, testes) to produce estrogen, progesterone, and testosterone (Mastorakos et al. 2006; Viau 2002). These sex steroids later migrate back to the hypothalamus to inhibit further production of GnRH to down-regulate the release of excess sex steroids. Factors such as seasonal variation or conspecific interactions over the breeding season often trigger HPG responses that greatly exaggerate concentrations of reproductive hormones (Korte et al. 2005). For instance, robust HPG-axis responses in males underlie phenotypic changes (e.g. badge size, beak color, muscle mass, etc.) that represent honest signals of quality and therefore increase their reproductive success (Gonzalez et al. 2001; Miles et al. 2007; Setchell et al. 2008).

The pervasive nature of glucocorticoids and androgens warrant considerable attention within a maternal effects context, as hormonal changes pre- and post-partum both affect offspring development directly through the placenta (Capellini et al. 2011; Reynolds et al. 2013), and indirectly through changes to care strategies employed by the mother (Rilling & Young 2014; Meylan et al. 2012). A few studies examining gestational maternal hormones in response to environmental or social cues have described the consequences experienced by offspring. For instance, red squirrel (*Tamiasciurus hudsonicus*) mothers exposed to heightened density cues (i.e. conspecific vocalization playbacks) over gestation demonstrate increased glucocorticoid concentrations that positively affect pup growth rates (Dantzer et al. 2013). Snowshoe hare mothers (*Lepus americanus*) consistently exposed to predation pressures over gestation exhibit increased fecal corticosteroid metabolites with increased predator densities, and their progeny exhibit more attenuated HPA axis responses (Sheriff et al. 2010). Further, dominant spotted hyena (*Crocuta crocuta*) mothers have higher androgens over gestation and produce cubs that exhibit increased rates of aggression and mounting post-partum (Dloniak et al. 2006).

Perhaps not surprisingly, the majority of hormonally mediated parental effects studies have focused on the role of the mother, particularly because the mother is the primary or sole individual rearing offspring (Maestripieri & Mateo 2008). Few studies have addressed parental effects mechanisms in which the father provides significant care (Charpentier et al. 2008; Schweitzer et al. 2014; Stein & Bell 2014). Fewer examine how paternal hormone concentrations pre-partum correspond with care strategies employed (Almond et al. 2008; Schradin et al. 2003; Ziegler & Snowdon 2000; Ziegler et al. 2009) or consequences for offspring (Stein & Bell 2014). Hormonal changes in mammalian fathers have been detailed sufficiently in the literature (Gordon et al. 2010; Reburn & Wynne-Edwards 1999; Rilling &

Young 2014; Wynne-Edwards 2001; Wynne-Edwards & Reburn 2000), yet it is unclear how hormonal changes of fathers coincide with changes in mothers, and if these changes in biparental systems produce significant consequences for offspring development.

PERSONALITY AND PARENTAL EFFECTS

Individuals often exhibit robust behavioral tendencies that persist across time and contexts (Biro & Stamps 2008; Dingemanse et al. 2010; Réale et al. 2007; Sih et al. 2004). These behavioral tendencies have commonly been referred to as animal personality (i.e. temperament, behavioral type; Dingemanse et al. 2010; Réale et al. 2007), which is traditionally demonstrated by estimating the repeatability of behavioral traits over time (Sih et al. 2004). For instance, eastern chipmunks (*Tamias striatus*) exhibit consistent differences in activity and exploration over repeated handling and hole-board tests traditionally used to quantify exploratory behaviors (Martin & Réale 2008). Female fallow deer (*Dama dama*) readily demonstrate consistent differences in boldness measures that correspond to the individual's willingness to ingest novel foods or forage in novel environments across time (Bergvall et al. 2011). In addition, zebra finches (*Taeniopygia guttata*) exhibit consistent individual differences in exploration, activity, and boldness when released into an unfamiliar cage setting to forage after an hour of fasting (David et al. 2011). Previous literature has also characterized animal personality as the covariance among separate personality traits (Dingemanse & Réale 2005; Sih et al. 2004). For instance, three-spined stickleback (*Gasterosteus aculeatus*) faced with predator pressures from brown trout exhibit consistent individual differences in boldness and aggression positively associated across the population (Bell & Sih 2007; Dingemanse et al. 2007). Moreover,

exploratory in Belding's ground squirrels (*Urocitellus beldingi*) is positively associated with activity as measured during hole-board testing (Dosmann et al. 2014).

Much of animal personality research has emphasized how behavioral traits remain consistent over time and across contexts. However, individuals do possess limited flexibility to modify their behavior according to current environmental context (Betini & Norris 2012; Briffa et al. 2008; Carter et al. 2012; Dosmann & Mateo 2014). Behavioral plasticity and personality are not mutually exclusive, but rather interact to form an individually specific reaction norm of behavior according to environmental conditions (Dingemanse et al. 2010). This allows the organism to mount an adaptive response toward environmental challenges or stressors that may compromise individual fitness (Biro & Stamps 2008). The interaction between personality type and individual plasticity have significant consequences for reproductive success (Bridger et al. 2015; Briffa et al. 2008; Pratt et al. 2005; Sih & Watters 2005) and survival (Adriaenssens & Johnsson 2013; Bell & Sih 2007; Bremner-Harrison et al. 2004; Dingemanse et al. 2007). Consequently, animal personalities and the varying degree of behavioral plasticity exhibited across organisms suggest evolutionary trade-offs that affect both individual fitness outcomes and population-level structure (Sih et al. 2004; Smith & Blumstein 2008). Indeed, many consider personality a critical driver to population structure and function (Bell & Stamps 2004; Cote et al. 2011; Lakowski et al. 2014; Réale et al. 2007; Sih et al. 2012), and behavioral plasticity as the currency by which population structure evolves (Biro & Stamps 2008; Dingemanse et al. 2004; Dingemanse & Réale 2005). Nevertheless, recent studies are taking a multivariate approach to address the underlying physiological and genetic factors contributing to personality and plasticity, as phenotypic correlations alone do not indicate the presence of evolutionary trade-offs (Duckworth 2015; Dochtermann et al. 2010).

The aggregate of individual personalities within a population results in a correlated suite of behaviors commonly referred to as a behavioral syndrome (Sih et al. 2004; Sih 2011), which summarizes the distribution of personality types across a population (Biro & Stamps 2008; Sih et al. 2012). Behavioral syndromes are exhibited in multiple taxa (Réale et al. 2007), and are unique because they emphasize that behavioral plasticity or flexibility is constrained by selection (Dochtermann et al. 2010; Sih et al. 2012). Individuals that exhibit excessively bold or aggressive behaviors, for instance, may increase their reproductive fitness but compromise their survival (Smith & Blumstein 2008). Increasingly exploratory individuals may capitalize on rarely exploited food resources but become more susceptible to predation (Sih et al. 2003). In addition, increasingly aggressive individuals may secure more reproductive opportunities and higher-quality resources through social status, yet jeopardize their physiological health (Sands & Creel 2004; Vucetich & Creel 1999). In all of these examples, organisms face trade-offs that impact their overall fitness (Smith & Blumstein 2008). Selection therefore imposes limits on behavioral plasticity (Sih 2011), implying that strong selection affects certain personality traits disproportionately (Dochtermann et al. 2010). Consequently, animal personalities reflect individuals repeatedly utilizing behavioral strategies to maximize fitness benefits in response to selection (Sih et al. 2012; Smith & Blumstein 2008).

Given the fundamental role personality plays in ecological and evolutionary processes, recent studies have suggested several mechanisms likely responsible for generating variation in personality (Duckworth 2015; Reddon 2011; Sih et al. 2012). Genetic heritability and genotypic correlations substantially contribute to the expression of animal personality (for reviews see Dochtermann et al. 2010; Turkheimer et al. 2014; Van Oers et al. 2005). However, parent-offspring heritability estimates for several personality traits in various taxa range from 0.04 to

0.66, suggesting that environmental variance plays a moderate if not critical role in affecting personality type (see Van Oers et al. 2005). Further, heritability estimates vary within populations and families, indicating that indirect genetic effects (e.g. epigenetic inheritance, Wolf et al. 1998) also provide significant input into personality development (Van Oers et al. 2005). Therefore, the additive influence from environmental and developmental experiences deserve consideration when interpreting the mechanisms responsible for variation in personality types. Parents have the ability to structure and modify the environment experienced by their offspring (Mousseau & Fox 1998), indicating that a substantial proportion of environmental variance can be attributed to parental influence beyond genetic inheritance (Wolf et al. 1998).

Epigenetic inheritance of personality traits is unique in that the personality traits of the parent inform care behaviors that subsequently affects offspring personality (Reddon 2011). This cascading influence may materialize in the way a parent provisions (Ghalambor et al. 2013; Schwagmeyer et al. 2003; Westneat et al. 2011; Wetzel & Westneat 2014), defends (Budaev et al. 1999; Mutzel et al. 2013; Stein & Bell 2015; Wetzel & Westneat 2014), and interacts with offspring (Francis et al. 1999; Stein & Bell 2015), stimulating offspring personality development. Parental care variation attributed to personality differences eventually affect the parenting strategies offspring employ as parents (Reddon 2011). Synthesizing the fields of animal personality and parental effects may therefore provide novel insights into the mechanisms that both generate wide variation in personality types but also impose limits on plasticity (Reddon 2011).

CONCLUSION

Parental effects research to date has examined how single-parent systems affect offspring morphological and physiological traits. In addition, previous studies have theorized that parental effects are paramount in shaping personality traits of subsequent generations (Duckworth 2015; Reddon 2011), but only a few have directly focused on the pervasive impacts these mechanisms on place on offspring personality (Hinde et al. 2014; Schuett et al. 2013). This dissertation is therefore novel in a multitude of approaches. This is the first approach at quantifying biparental effects in a non-traditional wildlife model, as well as the first empirically demonstrating the connections between parental effects and personality. Reddon (2011) previously emphasized the importance of considering parental effects mechanisms to generating individual variation in personality. Duckworth (2015) provides a compelling argument highlighting underlying hormonal mechanisms likely restrict and reinforce the range and extent of behavioral plasticity. Variation in personality types across a population may therefore correlate with individual differences in hormonal traits. This dissertation provides appropriate data meant to fuse the separate predictions by examining how hormonally mediated parental effects affect personality variation in offspring, and how individual variation in hormones are linked to personality traits critical for coyote survival in nonnative habitats.

Coyotes are an excellent fit to expand parental effects theory given their recent and rapid expansion beyond their indigenous range. Chapter 2 of this thesis is the first to characterize fecal glucocorticoid and androgen metabolites over gestation, as well as quantify consistent individual differences in both territorial behaviors and fecal hormone metabolites. Chapter 3 of this thesis is the first to systematically characterize parenting behaviors of both mothers and fathers over developmental time. Chapter 4 is the first to use coyote hair as a sample medium to quantify

long-term hormone concentrations. Finally, Chapter 5 is the first study to demonstrate consistency in personality traits across life stages. Stamps & Groothuis (2010a; 2010b) previously theorized that experiences over development may affect the expression of adult personality in individuals, but to date no data exist demonstrating how previous environmental experiences influence later behavioral traits. Chapter 5 therefore expands personality and temperament theory as well by Overall, this thesis is innovative and dynamic in that no other parental effects studies have observed individuals over successive breeding events and tracked developing offspring over a period of years. Previous restrictions made long-term assessment of wildlife development too difficult in the wild. Here, I provide empirical data that demonstrate the extent to which parental effects may operate in wildlife species.

This thesis emphasizes the far-reaching consequences parental effects mechanisms have on the evolutionary trajectories of a population by using the coyote system as a lens. The hope is that this research will spark conversation about the role epigenetic and non-genetic mechanisms play in rapid adaptation to an ever-changing global environment. With the rise of developed landscapes and the pervasive effects of anthropogenic disturbance in a multitude of environments, it will be critical to consider how and if organisms will be able to survive. This thesis work not only has future implications for how coyotes and similar carnivores may adapt to cities, but how other organisms may adapt to similar human-dominated landscapes under relatively short timescales. As a result, increasing work on parental effects mechanisms may affect conservation efforts as well as wildlife management efforts. I anticipate that the results from this thesis will generate such discourse.

CHAPTER 2: CONSPECIFIC ODOR CUES AND PARITY AFFECT PRENATAL ANDROGENS AND TERRITORIALITY OF COYOTE BREEDING PAIRS

INTRODUCTION

Organisms are constantly challenged by various external stimuli within their environment. Behavioral and morphological responses toward these environmental challenges are frequently initiated by underpinning neuroendocrine mechanisms and associated hormonal suites (Bijlsma & Loeschcke 2005; Boonstra 2013; Creel et al. 2013; Korte et al. 2005; McEwen & Wingfield 2003; Wingfield 2005). For instance, glucocorticoids increase gluconeogenesis to activate energy stores necessary to actively respond toward environmental stressors (Möstl & Palme 2002; Touma & Palme 2005). Glucocorticoids are also relevant physiological factors associated with individual social status (Creel 2001; 2005; Creel et al. 2013; Goymann & Wingfield 2004), mate preference and choice (Husak & Moore 2008; Miles et al. 2007), and individual behavioral differences (Atwell et al. 2012; Carere et al. 2010; Dosmann et al. 2014; 2015). Reproductive hormones such as androgens represent another pervasive suite of physiological factors that are intimately involved in reproduction and the social environment (Möstl & Palme 2002). For example, increased androgens are often associated with sexually-selected ornamentation that constitute an honest signal of both fitness and social rank (Buchanan et al. 2001; Drea et al. 2002; Gonzalez et al. 2001; Setchell et al. 2008). In many instances, increased androgens also augment territorial and aggressive behaviors that facilitate the acquisition and maintenance of heightened social status (Bales et al. 2006; Beehner et al. 2006; Creel et al. 1997; Goymann & Wingfield 2004; Koren et al. 2006). Further, individual differences in foraging (Chávez-Zichinelli et al. 2014), exploration (Van Oers et al. 2011), and territorial marking behaviors (Asa

et al. 1990; Fuxjager et al. 2014) have demonstrated positive associations with androgens. Therefore, endocrine responses to environmentally-induced changes have the potential to impact myriad factors linked to individual fitness.

Endocrine mechanisms have far-reaching consequences that not only affect the individual long-term, but also influence other conspecifics across generations. Specifically, changes to parental glucocorticoid and androgen concentrations directly interact with offspring *in vitro*, greatly dictating offspring development in the process (Meylan et al. 2012; Reynolds et al. 2013; Uller 2008). Environmental stressors experienced by pregnant individuals therefore have the potential to alter developmental trajectories of neonates that have long-term fitness consequences (Mousseau & Fox 1998; Marshall & Uller 2007). These hormone-associated parental effects are salient proximate mechanisms for inducing non-genetic phenotypic change across generations (Maestripieri & Mateo 2008; Räsänen & Kruuk 2007) and have been repeatedly demonstrated in the literature. For instance, red squirrel mothers (*Tamiasciurus hudsonicus*) exposed to heightened density cues (i.e. conspecific vocalization playbacks) over gestation demonstrate increased glucocorticoid concentrations that positively affect pup growth rates (Dantzer et al. 2013). Snowshoe hare mothers (*Lepus americanus*) consistently exposed to increased predation pressures over gestation exhibit increased fecal corticosteroid metabolites, and HPA axis responsiveness is more attenuated in their progeny (Sheriff et al. 2010). Further, dominant spotted hyena (*Crocuta crocuta*) mothers have higher androgens and produce cubs that exhibit increased rates of aggression and mounting (Dloniak et al. 2006).

Given the important role hormones play in the parental effects process, it is essential to assess the factors that generate hormonal variation over gestation. Previous work has demonstrated the role that social environment (Dantzer et al. 2013; Dloniak et al. 2006; Onyango

et al. 2006) and environmental change (Harris et al. 2013; Kemme et al. 2007) play in affect glucocorticoid and androgen production over gestation. However, few studies have addressed this in biparental systems, in which the father is present from conception through offspring development (Charpentier et al. 2008; Schweitzer et al. 2014). Males in socially monogamous systems frequently interact with their breeding partners over gestation (Kleiman & Malcolm 1981; Wynne-Edwards & Reburn 2000), and those interactions may influence the hormonal patterns of gestating females. Hormonal changes of expectant fathers also moderate individual behaviors in preparation for offspring rearing (Gubernick & Nelson 1989; Gubernick et al. 1995; Wynne-Edwards 2001; Ziegler & Snowdon 2000; Ziegler et al. 2004). In addition, few studies have repeatedly measured individuals over successive breeding events to assess how temporal variation impacts endocrine profiles and behavior (Almond et al. 2008; Sheriff et al. 2013; Ziegler & Snowdon 2000). Physiological and behavioral changes with prior breeding experience (Leuner et al. 2010) and aging may alter the degree or directionality of parental effects (Marshall & Uller 2007). Here, we investigated gestational hormones and behaviors of captive breeding coyote pairs, a biparental canid species.

Coyotes are an excellent system 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 (Carlson & Gese 2008; 2009; 2010; Minter & DeLiberto 2008). Second, mated individuals frequently demarcate the boundaries of their territories via continued scent-marking and enforce home range limits using aggression against intruding conspecifics when necessary (Gese 1998; 2001; Gese & Ruff 1997). Social territorial incursions may likely

represent a prominent stressor to breeding pairs. Third, previous work has detailed consistent individual differences in behavior of the species, especially in relation to novel stimuli (Darrow & Shivik 2009; Mettler & Shivik 2007; Young et al. 2015). Associations among consistent individual differences in behavior and hormones may suggest that behavioral profiles of the species are hormonally mediated. Finally, coyotes have previously exhibited individual variation in fecal glucocorticoid metabolites in response to human-associated stimuli (Schell et al. 2013), and it is therefore likely that similar stressors experienced during pregnancy may increase glucocorticoids that subsequently impact developing offspring. This is particularly relevant in the context of coyote adaptation to nonnative and urban habitats, as the intensity and duration of stressors experienced in such areas differ greatly compared with rural or forest preserve environments (Gehrt et al. 2010; Magle et al. 2014).

We observed coyote breeding pairs exposed to commercial scent lures mid-gestation (February to March, see Bekoff & Wells 1982) to determine whether conspecific odor cues influenced fecal glucocorticoid (FGM) and fecal androgen metabolites (FAM), as well as behavioral responses. Coyotes have previously demonstrated increased marking activity and investigation behaviors in response to provisioned commercial odors (Kimball et al. 2000; Shivik et al. 2011). Here, we predict that similar behavioral patterns will occur and correspond with FGM and FAM modifications. In addition, we examined breeding pairs over successive breeding events in 2011 and again in 2013 to quantify the impact of experience on hormonal and behavioral measures. Previous studies have detailed several hormonal changes that occur according to prior breeding experience (Almond et al. 2008; Ziegler & Snowdon 2000), though it is unclear how environmental and temporal variation simultaneously affect hormonal traits.

Finally, we collected repeated measures from all individuals to determine whether coyotes demonstrated consistent individual differences in hormonal and behavioral traits across time.

METHODS

Subjects

We observed 8 breeding 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 all 1 or 2 years of age (1.4 ± 0.1 years [*X*± SD]). Prior to breeding, animals at the facility were housed in multiple enclosure types ranging from large outdoor pens (1000–6000 m^2) to raised kennels (3.3m^2). In December 2011, we randomly paired study animals and moved them from single-housed pens to 1000 m^2 outdoor "clover" pens optimized for long-term behavioral observations (Gilbert-Norton et al. 2009a; Mettler & Shivik 2007). Pair relocation corresponded to the beginning of the breeding season (Bekoff & Wells 1982; Carlson & Gese 2008; 2009). To reduce the effects of potential stress of relocation on hormonal assays, each coyote pair was allowed 1 month to acclimate to their new pens. From late December to January, each breeding pair was fed 1300g of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, UT) daily and water was provided *ad libitum* (Brummer et al. 2010). 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 (*n*=43 pups).

Coyote parents were either hand-reared (5 females, 5 males) or 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*, Fox & Millam 2004; red foxes, *Vulpes vulpes*, Pedersen & Jeppesen 1990; gray wolves, *Canis lupus*, Gácsi et al. 2005). Therefore, we considered rearing condition as a main effect in subsequent analyses (see Statistical Analysis) to quantify the effect of early rearing experience on gestational hormones and scent-marking behaviors.

Odor cue testing

We administered odors to four pairs, and a control to the other four. In the experimental treatment, we provided four different commercial scent lures developed by Russ Carman® (Canine Call®, Pro's Choice®, and two versions of Magna Gland®, New Milford, PA) to assess physiological and behavioral responses from male-female pairs mid-gestation. The odors were a blend of fermented glandular materials, urines, and other volatile conspecific scents, known to elicit strong marking and territorial behaviors from coyotes (Kimball et al. 2000). We manually administered odor cues to a treatment breeding pair territory (n=4) every 5 days over a 20-day period (February 28th to March 15th) in 2011. We varied lure type order per pen to reduce potential presentation order effects. We provided control pairs with distilled water, and we provided all pairs with associated 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 (Gilbert-Norton et al. 2009a). The experimenter was in each pen for approximately 30 seconds and all pens received stimuli (either odor cues or water) within approximately 55 minutes (54.88 ± 3.08 minutes [X± SD]). We video-recorded pairs beginning immediately before stimuli were provided and continued recording for approximately 140 minutes (137.75 ± 4.03 minutes [X ± SD]) with 8

cameras operating remotely. Cameras were stationed inside a closed environment in the center compound of three clover pens (for schematic, see Mettler & Shivik 2007). We recorded at least 2 hours 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 experimental treatment: 2011 control pairs became 2013 treatment pairs and vice versa. In 2013 but not 2011, following Schell et al. (2013) we collected fecal samples in the afternoon directly following the foreign scent test period to determine if coyotes had an acute glucocorticoid response. 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 2012, pairs 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 and NWRC regulations. Our study animals were not on any other NWRC related projects in the interim between the 2011 and 2013 breeding seasons. In both years, we noted several marking and investigative behaviors following Gese & Ruff (1997) and Kimball et al. (2000); see Table 2.1. We began coding target behaviors at the moment of stimulus deposition using all-occurrence methods (Altmann 1974) for a 70-minute period.

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 (Guertin et al. 2010; Fuller et al. 2011; Young et al. 2008) to separate samples and determine

their freshness. Specifically, we mixed glitter with surplus mink food, partitioned that food into small biscuits, froze them at -20^{o}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: Loeding et al. 2011). Females have significantly higher progesterone concentrations compared with males over gestation (Carlson & Gese 2008). We thus hypothesized that we could confirm the sex of fecal samples via the progesterone assay (see Results).

We also assessed freshness by appearance, odor, and stiffness in response to freezing temperatures. We restricted sample collection to feces excreted between 0600 and 1000 hours MST, as FGMs content varies diurnally in coyotes (Schell et al. 2013). Samples were immediately stored at -20^{o}C to limit the amount of hormone metabolite degradation. 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, U.S.A.) for hormonal analyses.

Table 2.1 Behavioral ethogram used during foreign scent tests (behaviors adapted from Bekoff & Wells 1986; Gese & Ruff 1997; 1998)

Behavior	Description
Aggression	Teeth baring, growling, and/or physical confrontation directed toward pair-mate
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	No. of sniffs at the scent-affected area
Site visits	No. of instances individual gets within $\leq$1 meter from scent-affected area
Site time (sec)	Total time spent at the scent-affected area
Latency to visit (sec)	Length of time before individual gets $\leq$1 meter from scent-affected area

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, U.S.A.) for 3 days and crushed to a fine powder before extraction following Schell et al. (2013). Briefly, sample powder was weighed (0.2 ± SD 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 minutes at setting 60. The samples were then centrifuged for 20 minutes at 1500 rpm and 10^{o}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 30s, and re-centrifuged for 15 minutes 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^{o}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 minutes before analysis.

Enzyme Immunoassays

We used a previously validated cortisol enzyme immunoassay (Appendix A; Schell et al. 2013) to measure coyote fecal glucocorticoid metabolites. Polyclonal cortisol antiserum (R4866) and horseradish peroxidase were provided by C. Munro (University of California, Davis, CA, U.S.A.). Cortisol antiserum and cortisol horseradish peroxidase were used at dilutions of 1:8500 and 1:20,000, respectively (Santymire and Armstrong, 2010; Appendix A; Schell et al. 2013). 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 (Armstrong & Santymire 2013; Rafacz et al. 2011). 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; $\hat{y} = 0.8197x + 5.9562$; R^2 = 0.9960). Testosterone horseradish peroxidase and polyclonal antiserum were used at 1:30 000 and 1:10 000, respectively (Armstrong & Santymire 2013; Santymire & Armstrong 2010). 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 (see above) 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 (1:3,000; $\hat{y} = 0.9999x + 1.4882$; 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 (Loeding et al. 2011).

Statistical Analyses

To assess the impact of our odor cues on scent-marking and investigative behaviors, we used linear mixed models (LMMs). Here, we observed treatment group and breeding year as main effects in our model, as well as the interaction term between the two factors. To account for

repeated measures in our dataset, we also set coyote identity, breeding pair identity, and test order (i.e. the order a pair received their respective stimuli for a given test day in relation to the other 7 pairs) 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 also wanted to quantify whether there were any learning or habituation effects present in our dataset, as coyote pairs were provided stimuli 4 times over a 20-day period. We therefore used post-hoc Tukey contrasts in pair-wise comparisons to ask whether investigative measures such as latency to approach and time at the affected site varied with progressing trials. We arcsine square root transformed behavioral count for our analyses following Dosmann & Mateo (2014).

To quantify the influence of odors on FGMs and FAMs, we used LMMs once more to determine how odor treatment and breeding year were associated with hormonal outcomes. Prior to using LMMs, we partitioned hormonal values by weeks until birth as there were unknown differences in date of conception for each pair. Specific date of conception was uncertain primarily due to the difficulty of visually confirming copulatory events, often halted by the study animals when researchers were present, and the unreliability of visual confirmation as a means for determining when females conceived. Therefore, weeks until birth were projected according to each female's date of parturition and the typical length of coyote gestation (63 days; Bekoff & Wells 1982). 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 and to ask where significant effects of weeks until birth occurred. In subsequent LMMs, we set weeks until birth as a random factor to account for differences in conception that may have influenced FGM and FAM concentrations.

After general assessment of hormonal patterns according to our weeks until birth factor, we partitioned hormonal data into three descriptive categories: pre-test, testing, and post-test. The testing period specifically was the aforementioned 20-day period in which odor cues (treatment) and water (control) were provided. The pre-test period comprised the 4 weeks of fecal collection before odor cues were provided, while the post-test period 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, pair identity, and weeks until birth as random effect terms. We followed 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 with the corresponding random effects. It is worth mentioning that we anticipated that a significant effect of test period would correspond with a significant effect of weeks until birth, as both factors covaried with time and the progression of gestation. We tested hormonal data for normality using Shapiro-Wilk tests, and non-normally distributed data were log transformed following Schell et al. (2013).

To determine whether hormonal and behavioral measures demonstrated consistent individual differences, we used a likelihood ratio test previously used to effectively quantify trait consistency and repeatability (Betini & Norris 2012; Carter et al. 2012; Dosmann & Mateo 2014). Specifically, the likelihood ratio test compares a linear regression model with only fixed effects to a linear-mixed effects model (LMMs) that contains the same fixed terms but with subject identity as the random factor. Statistical significance between the models indicates that the variance observed in the dependent measure is repeatable and best approximated by the designated random term (Crainiceanu & Rupert 2004; Schielzeth & Forstmeier 2009). The likelihood ratio test computes the LRT test statistic using restricted maximum likelihood that

follows a chi-square distribution and produces a probability value based on 10,000 simulated iterations of the dataset (Crainiceanu & Rupert 2004; Schielzeth & Forstmeier 2009). Here, we examined the effect of treatment group (E_T), breeding year (E_Y), the individual (I), and the interaction among individual and environmental factors (I x E_T; I x E_Y) on hormones and behaviors. To test for a significant effect of coyote identity (i.e. effect of individual consistency), we compared a model without coyote identity (E_T or E_Y only) and one with identity (I) as a random effect term, keeping main effects consistent (Crainiceanu & Rupert 2004; Schielzeth & Forstmeier 2009). To determine if there were any interactions effects (i.e. individual plasticity) among treatment group, breeding year, and the individual, we compared models with and without a random slope (i.e. E_T or E_Y | coyote ID) for treatment group and year to investigate whether a significant I x E_T or I x E_Y interaction existed (Carter et al. 2012). If a significant I x E interactions existed, a secondary analysis was performed in which we examined the correlation between the intercept and slope estimates generated by the LMM, referred to as the best linear unbiased predictors (BLUPs; Betini & Norris 2012; Carter et al. 2012). Degrees of freedom were straightforward, as we had even samples sizes across treatment conditions. To later examine the relationships among hormonal BLUPs and behavioral responses to odor cues, we used Spearman rank correlations.

Linear mixed models were performed using the lmer function from 'lme4' (Bates et al. 2012) and 'lmerTest' (Kuznetsova et al. 2013) packages. We used restricted estimation maximum likelihood (REML) with a diagonal covariance structure for all of our models, with Satterthwaite approximation for degrees of freedom. Likelihood ratio tests were performed using the exactLRT function from the 'RLRsim' package (Crainiceanu & Rupert 2004). All Spearman correlations were performed using the corr function from the 'corrplot' package (Wei 2013). We

reported results from the best-fit models for all measures, determined using the lowest Akaike Information Criterion (AIC) values (Burnhamn et al. 2010). In all cases, alpha was set to $P<0.05$ and data were reported as mean ± S.E. None of our hormonal or behavioral measures demonstrated an effect of rearing condition (E_R), nor did we observe interaction effects among coyote identity and rearing condition. Rearing effects are therefore not addressed further.

RESULTS

Odor Cue Behaviors

We first observed overall differences (i.e. results across the four odor-water presentations) by treatment group and breeding year in each sex separately (Fig. 2.1). We found that both odor-treated females and males displayed aggression (females: $F_{1,44.9} = 21.80$, $P < 0.001$; males: $F_{1,43.3} = 6.14$, $P = 0.017$), urinated (females: $F_{1,45.1} = 77.07$, $P < 0.001$; males: $F_{1,36.3} = 55.8$, $P < 0.001$), rubbed (females: $F_{1,47.4} = 115.45$, $P < 0.001$; males: $F_{1,48.4} = 119.93$, $P < 0.001$), sniffed (females: $F_{1,48.6} = 128.90$, $P < 0.001$; males: $F_{1,51.9} = 72.59$, $P < 0.001$), and visited the affected site (females: $F_{1,44.7} = 28.25$, $P < 0.001$; males: $F_{1,45.6} = 6.64$, $P = 0.013$) more frequently than control individuals (Fig. 2.1). Odor-treated pairs also spent more time at the odor site (females: $F_{1,37.4} = 77.70$, $P < 0.001$; males: $F_{1,44.4} = 49.51$, $P < 0.001$). Only females differed by treatment group in ground scratching ($F_{1,47.5} = 28.50$, $P < 0.001$) and latency to visit the odor site ($F_{1,41.8} = 8.08$, $P = 0.007$).

We also found that pairs as experienced breeders scent-marked and investigated the affected areas more (Fig. 2.1). Specifically, both experienced males and females urinated (females: $F_{1,45.1} = 27.0$, $P < 0.001$; males: $F_{1,34.5} = 62.8$, $P < 0.001$), rubbed (females: $F_{1,47.4} = 115.45$, $P < 0.001$; males: $F_{1,45.7} = 19.04$, $P < 0.001$), sniffed (females: $F_{1,44.9} = 18.28$, $P < 0.001$; males: $F_{1,51.9} =$

14.43, $P < 0.001$), and visited the affected site (females: $F_{1,44.7} = 28.25$, $P < 0.001$; males: $F_{1,45.6} = 36.53$, $P < 0.001$) more frequently than first-time breeders despite the treatment group membership (Fig. 2.1). Experienced breeders also spent more time at the affected area (females: $F_{1,34.5} = 13.7$, $P < 0.001$; males: $F_{1,44.4} = 19.67$, $P < 0.001$). However, only females differed in their latency to visit the site ($F_{1,41.8} = 29.5$, $P < 0.001$), in which experienced breeders approached the affected site quicker than first-time breeders.

To assess whether individuals adjusted their test site time or latency to visit the test area, we additionally used Tukey contrasts to compare coyotes within treatment groups in each of the four odor provisioning events (Fig 2.2). Again, we found that odor-treated males and females spent more time at the affected site within each test date (Fig. 2.2). However, over each successive trial odor-treated females spent less time at the odor-affected site ($F_{3,27} = 4.237$, $P = 0.014$) compared with control females ($F_{3,27} = 0.646$, $P = 0.592$). We did not observe an overall 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. 2.2).

Finally, we aimed to determine whether territorial behaviors demonstrated consistent individual differences for males and females for the entire study population. We found that both sexes were individually consistent in ground scratching and site visit behaviors (Table 2.2), but demonstrated varying results for other observed behaviors. Specifically, females were individually consistent in aggression and urination behaviors, indicating that dominance behaviors were repeatable in females across successive breeding years (Table 2.2). Males demonstrated consistent differences in site time and latency to visit metrics, which suggests that investigative behaviors were repeatable in males across successive breeding years (Table 2.2).

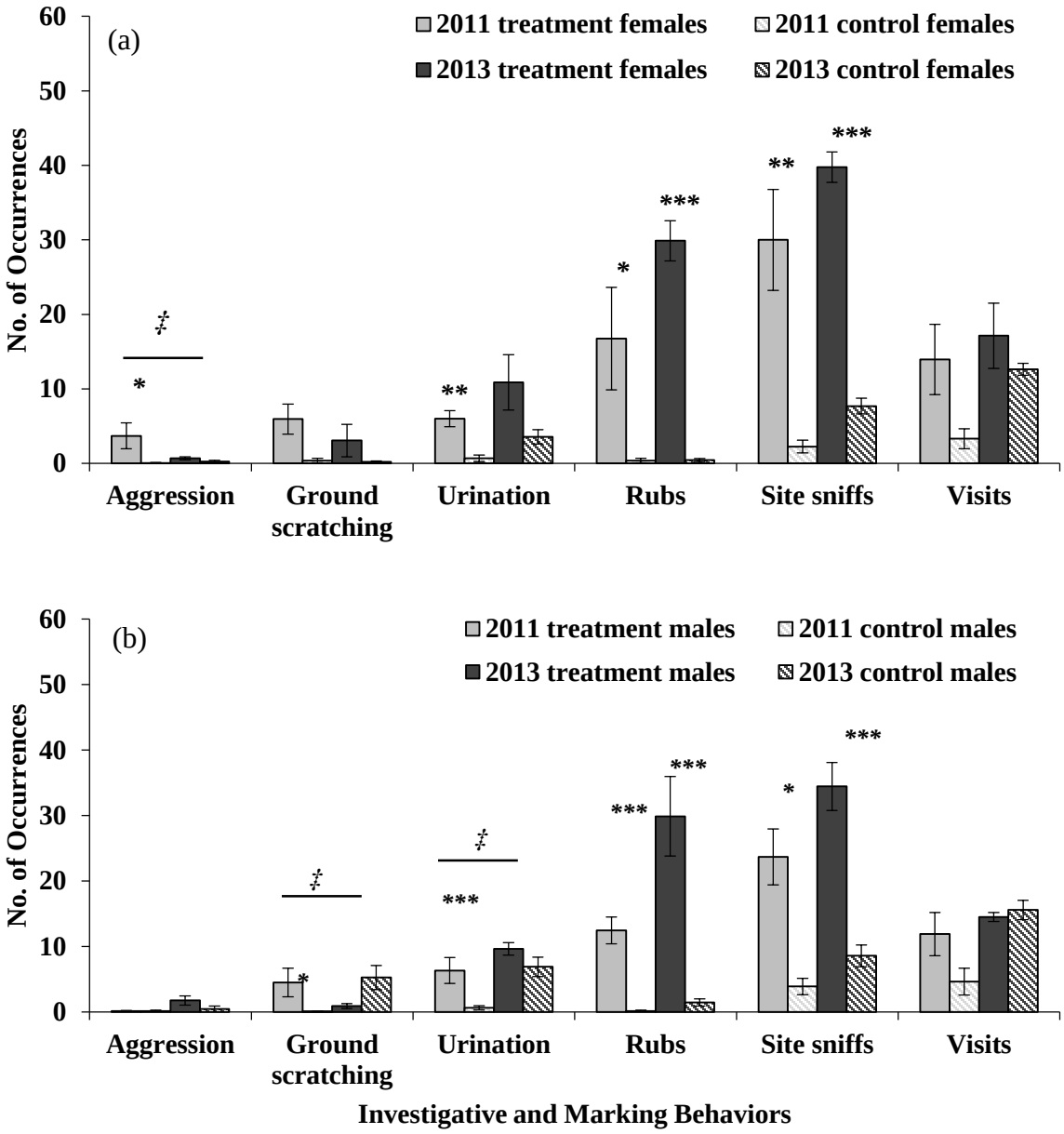

Fig. 2.1: Mean (± S.E.) instances of marking and investigative behaviors in response to commercial scent lures (treatment group) and water (control group) during gestation for females (a) and males (b). Asterisks indicate differences (**P*<0.05, ***P*<0.01, ****P*<0.001) between treatment conditions within each year. Crossbars indicate interactions (‡*P*<0.05) between main effects in the model.

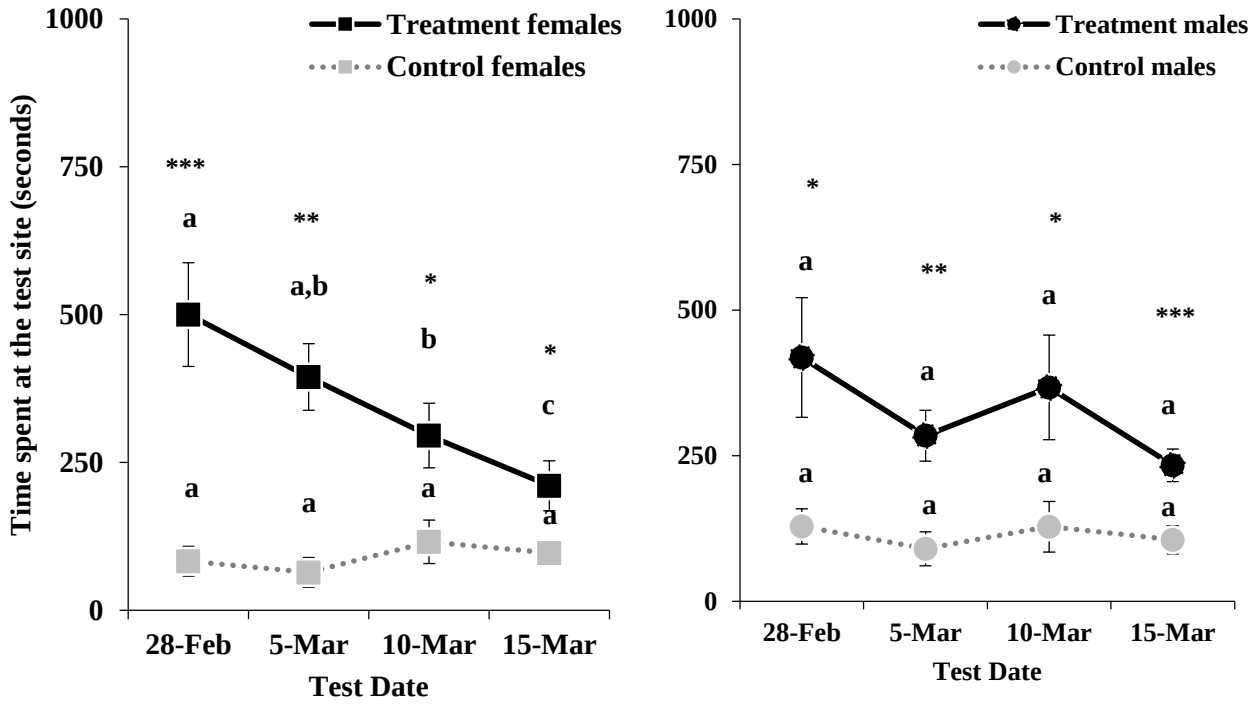

Fig. 2.2: Mean (± SE) time spent ≤1 meter within the odor- (treatment) or water-treated (control) test site. Asterisks indicate differences between treatment groups within each test date (**P*<0.05, ***P*<0.01, ****P*<0.001). Contrasts in subscript letters indicate statistical differences within treatment groups across test dates.

Table 2.2 Model comparisons to test for consistent individual differences in marking and investigative behaviors during the foreign scent test, including interactions among individual identity and environmental factors (E_T and E_Y)

Sex	Behavior	Treatment (E_T)		Year (E_Y)		Individual (I)		I x E_T		I x E_Y	
		t	***P***	***t***	***P***	***LRT***	***P***	***χ2***	***P***	***χ2***	***P***
Females	Aggression	13.34	**<0.001**	2.306	0.134	2.907	**0.024**	10.252	**0.006**	3.304	0.192
	Ground scratching	18.43	**<0.001**	0.569	0.453	5.572	**0.005**	20.16	**<0.001**	1.111	0.574
	Urination	39.31	**<0.001**	9.743	**0.003**	3.952	**0.012**	37.204	**<0.001**	19.033	**<0.001**
	Rubs	9.138	**<0.001**	1.449	0.152	1.484	0.063	55.116	**<0.001**	11.505	**0.003**
	Site sniffs	9.17	**<0.001**	2.33	0.023	0.295	0.187	45.136	**<0.001**	9.492	**0.009**
	Site visits	3.421	**0.001**	3.631	**<0.001**	7.36	**0.002**	20.018	**<0.001**	18.442	**<0.001**
	Site time (sec)	6.839	**<0.001**	2.209	**0.031**	1.035	0.098	30.747	**<0.001**	6.114	**0.047**
	Latency to visit	-2.098	**0.04**	-4.445	**<0.001**	0.51	0.147	4.432	0.109	19.547	**<0.001**
Males	Aggression	1.96	0.055	2.616	**0.011**	0.505	0.148	0.6524	0.722	4.886	0.087
	Ground scratching	0.209	0.835	1.155	0.252	26.718	**<0.001**	5.8159	0.055	7.316	**0.026**
	Urination	4.477	**<0.001**	4.967	**<0.001**	0.139	0.247	27.394	**<0.001**	32.089	**<0.001**
	Rubs	9.347	**<0.001**	2.496	**0.015**	0	0.365	49.635	**<0.001**	9.438	**0.009**
	Site sniffs	7.331	**<0.001**	2.511	**0.015**	2.59	0.061	30.327	**<0.001**	6.743	**0.034**
	Site visits	1.748	0.085	4.649	**<0.001**	2.878	**0.025**	5.6532	0.059	28.964	**<0.001**
	Site time (sec)	5.289	**<0.001**	2.956	**0.004**	3.677	**0.017**	12.569	**0.002**	2.559	0.278
	Latency to visit	-1.465	0.148	-4.506	**<0.001**	1.982	**0.045**	8.9464	**0.011**	31.65	**<0.001**

Tests for environmental effects (ET and EY) were done using linear regression models without coyote identity fit as a random effect term. *LRT:* the log likelihood ratio test followed a $\chi 2$ distribution to compare models for a significant effect of identity. Interactions among personality, year (EY) and treatment group (ET) were determined by comparing mixed models with coyote ID nested in study year or treatment status. Significant P values ($P<0.05$) are shown in bold

In addition, we found both within-individual consistency and context-specific plasticity (i.e. plasticity in response to odor treatment or breeding year) in many of the behaviors observed (Table 2.2). Urination, body rubs, site sniffs, and time spent at the affected site also demonstrated plasticity with both odor treatment and breeding year, indicating that individually-specific marking and investigative behaviors were sensitive to provisioned odor cues and overall breeding experience.

Odor cue 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. In 2013, fecal sample metabolites collected directly after (8 to 12 hours) the odor provisioning event did not differ between treatment groups, and did not differ according to specific odor type provided (i.e. Canine Call®, Pro's Choice®, and Magna Gland®) within treatment groups. Because our results were inconclusive, we do no report further acute stress results. Both FGMs and FAMs were significantly affected by weeks until 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 among gestational weeks demonstrated lower FGMs and FAMs in the latter half of gestation compared to early gestation (Fig. 2.3).

When hormonal data were partitioned by test period (i.e. pre-test, testing, and post-test), we found that odor-treated females – but not odor-treated males – had lower FGM concentrations

over 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. 2.4a). Odor-treated females also had lower FGM concentrations over the pre-test period ($F_{1,115.1} = 4.19$, $P = 0.043$), but did not differ from controls in the post-test period ($F_{1,57.3} = 1.61$, $P = 0.21$), indicating that differences in FGMs may have existed prior to odor cue provisioning. 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 cues did not influence male fecal glucocorticoids. Within both sexes, we generally did not observe any differences in FGMs between first-time and experienced breeders (females: $F_{1,259.1} = 0.20$, $P = 0.66$; males: $F_{1,256.8} = 1.08$, $P = 0.30$).

We did find higher FAM concentrations over 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. 2.4b). Fecal androgen 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. 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 over gestation compared with females ($F_{1,7} = 84.8$, $P < 0.001$).

Last, we found that both males and females demonstrated consistent individual differences (I) in FGMs across breeding events (Table 2.3). Fecal glucocorticoid profiles also demonstrated plasticity with odor treatment (I x E_T) and breeding year (I x E_Y), indicating that individual stress profiles are sensitive odor cues and parity. Only males demonstrated consistent differences in FAMs, and we did not observe plasticity with odor treatment or breeding year (Table 2.3).

Females, however, did demonstrate individual plasticity in fecal androgens in relation to breeding year, suggesting that androgen profiles of females are sensitive to breeding experience.

Correlations among Behaviors and Hormones

Because we found individual plasticity in FGMs and FAMs, we computed BLUPs to assess whether any relationships among individual-specific hormones and behaviors existed (Fig. 2.5). We did find that both female (r_s = 0.61, N = 16, P = 0.012) and male (r_s = 0.58, N = 16, P = 0.019) androgen BLUPs were positively correlated with latency to visit the affected site (Fig. 2.5), indicating that individuals with higher androgen BLUPs took longer to investigate the odor or water-affected site. Male androgen BLUPs in particular were also 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 (Fig. 2.5). Male androgen BLUPs were also negatively associated with the mean number of urinations (r_s = -0.67, N = 16, P = 0.005). Further correlational data among BLUPs for glucocorticoid metabolites, androgen metabolites, and behaviors, as well as behavior-behavior relationships are in Appendix B. Briefly, a suite of scent-marking and investigative behaviors were correlated for both sexes. Specifically, urination, body rubs, site sniffs, site visits, and time at the site all positively covaried (Table B1).

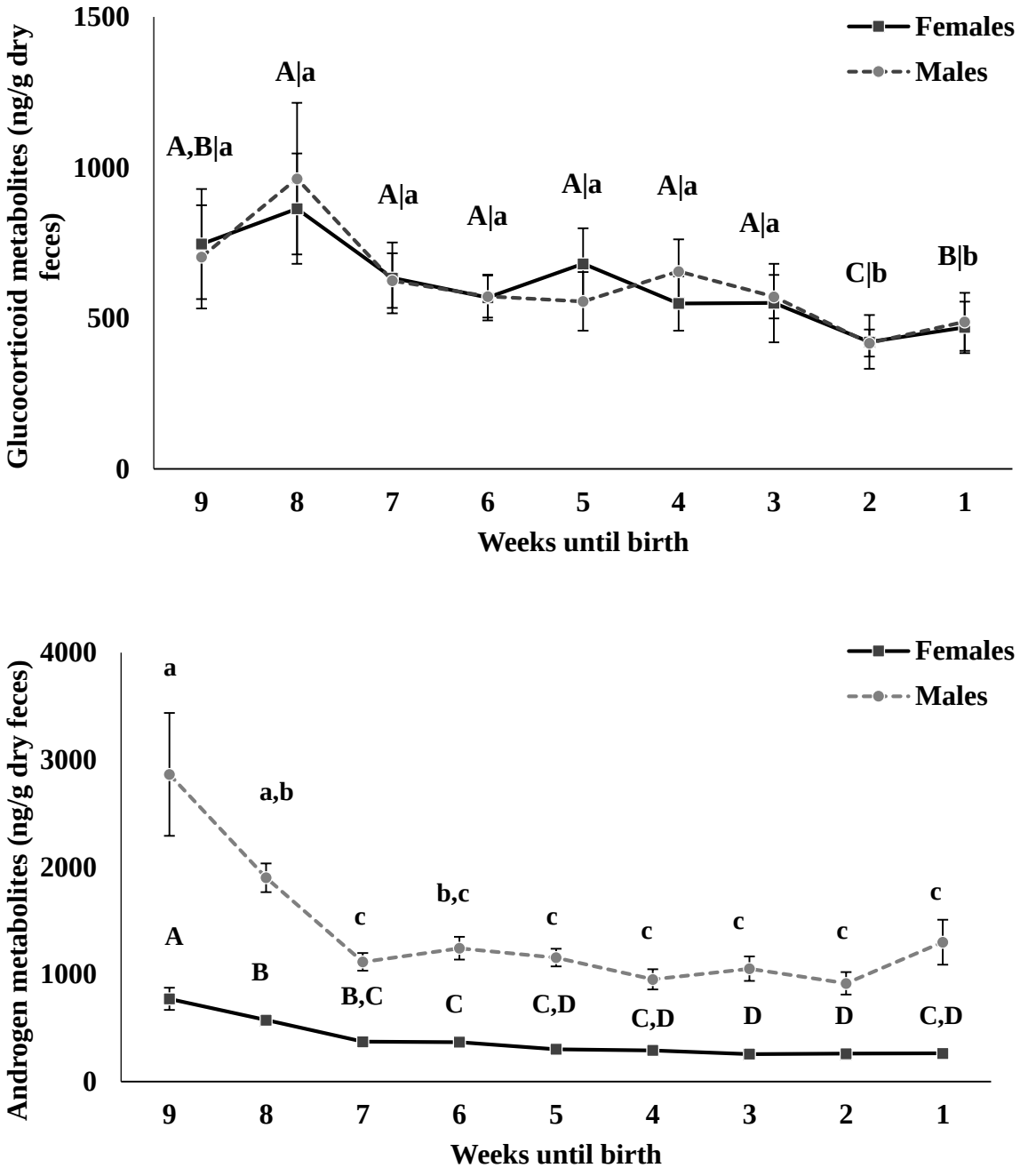

Fig. 2.3: Fecal glucocorticoid and androgen metabolite concentrations across gestation before parturition. Uppercase and lowercase superscripts correspond to maternal and paternal metabolites, respectively. Contrasts in letters above data points indicate a statistical difference within each sex.

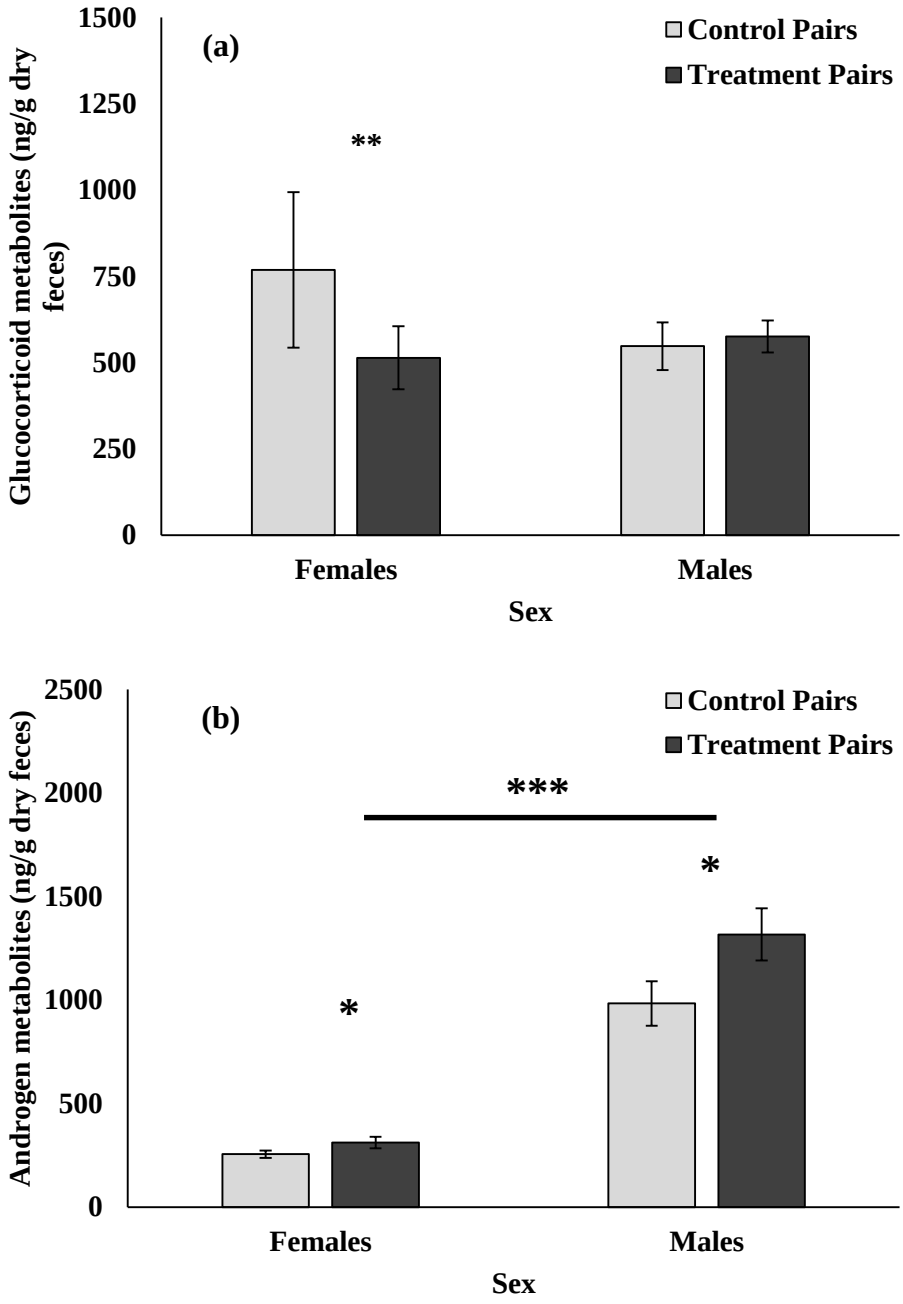

Fig. 2.4: Fecal glucocorticoid and androgen metabolite concentrations over the odor cue provisioning (i.e. testing) period. Data represent means ± S.E. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 2.3 Model comparisons to test for consistent individual differences in overall gestational hormones, including interactions among individual identity and environmental factors (E_T and E_Y)

Sex	Hormone	Treatment (E_T)		Year (E_Y)		Individual (I)		I x E_T		I x E_Y	
		t	***P***	***t***	***P***	***LRT***	***P***	***χ2***	***P***	***χ2***	***P***
Females	Glucocorticoids	7.593	**0.006**	0.324	0.57	72.108	**<0.001**	18.153	**<0.001**	10.786	**0.005**
	Androgens	0.925	0.337	9.828	**0.002**	0	0.351	1.247	0.536	6.568	**0.037**
Males	Glucocorticoids	1.567	0.118	0.893	0.372	12.948	**<0.001**	8.096	**0.017**	10.811	**0.004**
	Androgens	2.909	**0.004**	-2.642	**0.009**	2.436	**0.026**	2.054	0.358	0	1

Tests for environmental effects (ET and EY) were done using linear regression models without coyote identity fit as a random effect term. *LRT:* the log likelihood ratio test followed a *χ2* distribution to compare models for a significant effect of identity. Interactions among personality, year (EY) and treatment group (ET) were determined by comparing mixed models with coyote ID nested in study year or treatment status. Significant P values ($P<0.05$) are shown in bold.

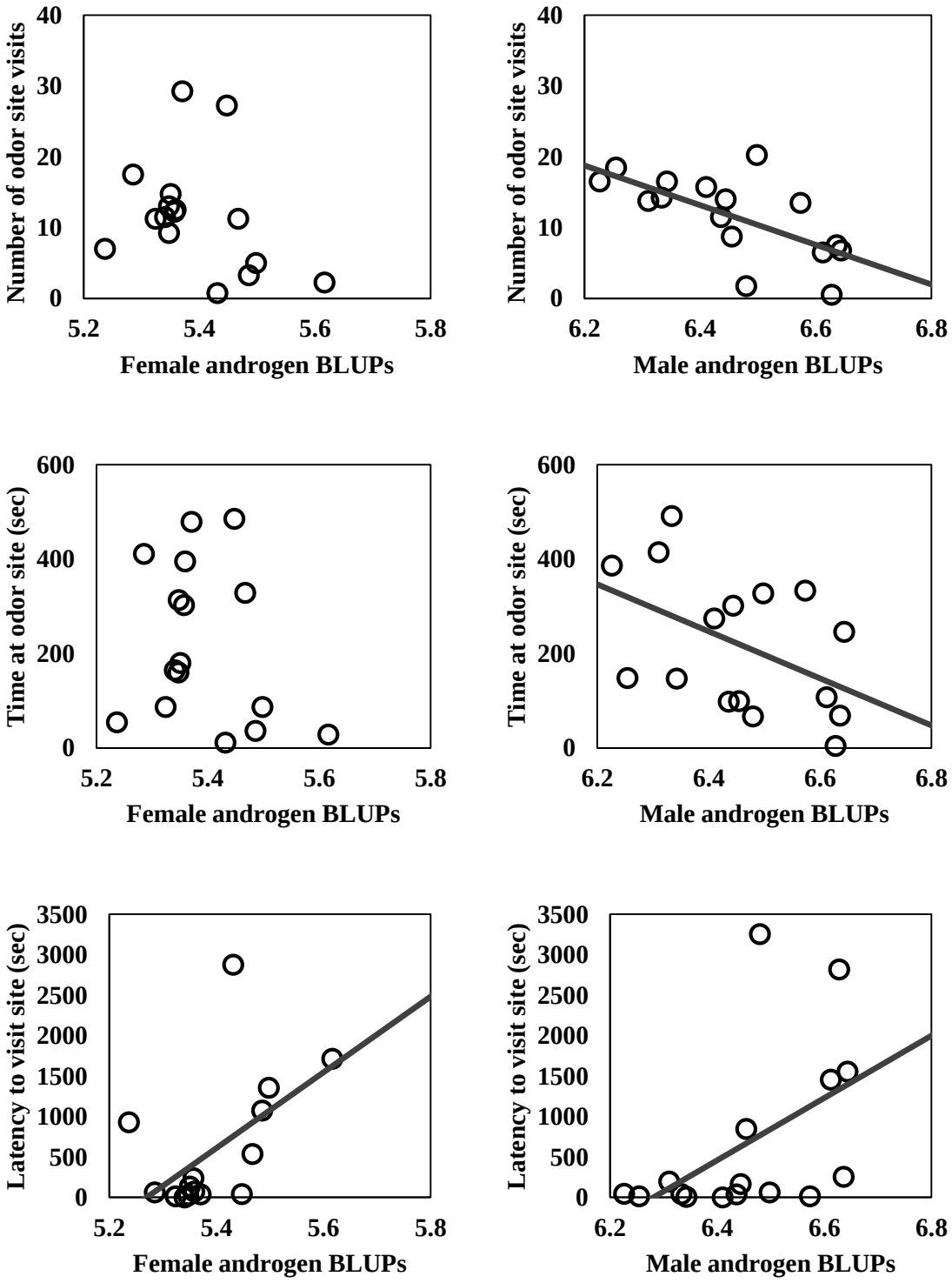

Fig. 2.5: Spearman rank relationships among best linear unbiased predictors (BLUPs) for fecal androgen metabolites and investigatory behaviors recorded over the odor cue provisioning tests. Trend lines denote significant relationships ($P < 0.05$), and behavioral data represent means ± S.E.

DISCUSSION

We have demonstrated here that odor cue provisioning was both effective at eliciting strong territorial responses, as well as increasing fecal androgens of both sexes. In addition, coyote pairs had increased FAMs as first-time versus experienced breeders, indicating that experience played some role in androgen metabolite concentrations. We observed steady declines in both FGMs and FAMs toward parturition for both sexes, elucidating the temporal component of these hormones over gestation. Many of our behavioral and hormonal measures demonstrated consistent individual differences that were also sensitive to odor cues and breeding experience, indicating plasticity in coyote personality and hormone profiles. Finally, androgen profiles (i.e. androgen BLUPs) were correlated with several behaviors, suggesting that behavioral responses to our odor cues were hormonally mediated.

Scent-marking and investigative behaviors greatly increased for individuals that received odor cues. This is consistent with other odor studies in coyotes (Kimball et al. 2000; Shivik et al. 2011), as well as with other studies in Canidae (African wild dogs, Rafacz & Santymire, 2013; Ethiopian wolves, *Canis simensis*, Sillero-Zubiri & Macdonald, 1998), highlighting the importance of olfactory cues in stimulating parallel territorial behaviors across the clade. What is unique to our study is that these behaviors were not only plastic toward odor cues, but demonstrated individual plasticity toward prior experience as well (see Table 2.2). 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 do mark more frequently than yearlings or early-aged adults (Gese & Ruff 1997). 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 previously did in 2011. These results suggest that coyotes remembered the mere process of application in 2011 and anticipated the overall event of stimulus provisioning when repeated in 2013. Coyotes have previously been documented to quickly detect and remember several patterns and sequences, though coyotes were only tested over a 16-day to 1-month period (Gilbert-Norton et al. 2009a). Here, we suggest that coyotes demonstrated habituation effects over a 2-year period.

In addition to strong environmental effects (i.e. odor cues), we observed within-individual consistency in scent-marking and investigative behaviors despite treatment group membership. However, the suite of behaviors that were repeatable between years differed by sex: females exhibited repeatability in territorial behaviors (e.g. aggression and urination) while males exhibited repeatability in investigative behaviors (e.g. site time and latency to visit). Repeatability differences between the sexes has previously been observed in biparental care in house sparrows (*Passer domesticus*), in which males exhibit high between-year repeatability in offspring provisioning rates, while females relatively do not (Nakagawa et al. 2007). Here, differences between the sexes may suggest that coyote males and females serve different roles in pair maintenance and territorial defense over gestation. Previous work has provided preliminary evidence to suggest that coyote parents undergo a division of labor during pup rearing (Asa & Valdespino 1998; Bekoff & Wells 1982; Sacks & Neale 2001). It is likely that territoriality and exploration are differentially expressed between the sexes during gestation as well, and the degree of individual differences among mated pairs may predict how breeding pairs raise offspring.

Multiple scent-marking and investigative behaviors were tightly correlated with one another irrespective of the odor treatment (Appendix B). For instance, ground scratching, urination, body rubs, and site sniffs all covaried. The multiple associations among these marking behaviors likely accentuate individual territoriality and social dynamics characteristic within this species (Gese & Ruff 1997; 1998). Specifically, only particular individuals ascend to alpha pair status, and those individuals demarcate territorial boundaries more frequently than betas or transients (Gese & Ruff 1997). Alpha individuals also maintain status via successful territorial defense from neighboring conspecifics and suppression of insurgency within a pack (Gese 1998). Because being an alpha coyote increases breeding opportunities for that individual (Gese 1998; Gehrt 2010), consistent 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 (Creel et al. 1997), Ethiopian wolves (Van Kesteren et al. 2012), and gray wolves (Asa et al. 1990). Future research should closely examine pack systems and how individual differences in behavior shape the development of pack dynamics in Canidae.

This study is the first to physiologically validate the measurement of gestational fecal glucocorticoids and androgens in coyotes of both sexes, as previous studies were restricted to plasma samples and did not measure both hormones in each sex (Amoss & Hodges, 1995; Carlson & Gese 2008; Minter & DeLiberto 2008). 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 (Carlson & Gese 2008) and male testosterone (Amoss & Hodges, 1995; Minter & DeLiberto 2008) 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 (Bekoff & Wells 1982; Carlson & Gese 2010; Messier & Barette 1982). It is likely that hormonal physiology accompanies the onset and regression of marking behaviors, similar to urine-marking and testosterone in gray wolves (*Canis lupus*; Asa et al. 1990; Asa & Valdespino 1998). 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 (Korte et al. 2005). 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*, Concannon, 2011; Ethiopian wolves, Van Kesteren et al. 2012). More distantly related mammals show the opposite trend (yellow baboons, *Papio cynocephalus*; Nguyen et al. 2008; pygmy rabbits, *Brachylagus idahoensis*, Scarlata et al. 2011), 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 fathers track glucocorticoid responses of paired pregnant partners (Ziegler et al. 2004). 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 fathers show similar responsiveness, as males exhibited increased

testosterone shortly after sniffing periovulatory scents of pregnant females (Zielger et al. 2009). 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 over gestation. Coincidentally, coyotes, cotton-top tamarins, and common marmosets are all socially and reproductively monogamous (Bekoff & Wells 1982; Gerht, 2010; Ziegler et al. 2004; Zielger et al. 2009), 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 over gestation. This study provides further evidence to suggest that hormonal patterns of expectant fathers are highly responsive to female stimuli.

Both sexes had increased FAM when provided odor cues, suggesting that novel odors were effective at soliciting a physiological response. Paired with increased scent-marking and investigatory behavior, it is likely that our odor cues were effective proxies for territorial incursion. These results support the challenge hypothesis previously categorized by Wingfield et al. (1990) and revisited by Goymann et al. (2007), 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 (Bales et al. 2006). Similarly, in male chacma baboons (*Papio hamadryas ursinus*) testosterone concentrations and

changes in rank are positively correlated, in which males rising in rank had higher testosterone than males falling in rank (Beehner et al. 2006). 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. odor cues) or actual challenges to social rank.

In addition to effects of our odor cues, we observed an effect of breeding experience on male and female androgens, in which pairs as first-time breeders had higher FAMs concentrations. This is in contrast to cotton-top tamarins, in which FAMs changes pre-partum are independent of breeding experience (Ziegler & Snowdon 2000). For coyotes, it is possible that as a young breeding pair, securing a territory and guarding against territorial intrusions may presents a greater challenge than maintaining a territory is for experienced breeders. Consequently, increased FAMs over 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.

Both sexes demonstrated within-individually consistency 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. (2005), where individuals employ different behavioral strategies that are facilitated by underlying physiology. Korte et al. (2005) 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 consistent individual differences in FAMs. Given that testosterone is generally higher in male mammals (Wynne-Edwards, 2001), it is likely that repeatability of androgen metabolites in coyote males reflect sex-linked traits important for reproduction.

Individually-consistent differences in hormones (i.e. BLUPs) were correlated with multiple scent-marking and investigative behaviors (Appendix B). This is similar to male great tits (*Parus major*), in which testosterone levels were both repeatable over time and correlated with exploratory behaviors (Van Oers et al. 2011). Likewise, individual white-eared hummingbirds (*Hylocharis leucotis*) show repeatability in testosterone over time and those with higher testosterone concentrations are more risk-prone foragers (quantified by frequent visits to variable flowers; Chávez-Zichinelli et al. 2014). For this study, increased androgen BLUPs are positively associated with latency to visit the affected site for both sexes (Table 3.4). For male coyotes specifically, androgen BLUPs were also correlated with the number of visits made and the time spent at the affected site, suggesting male coyote androgens are particularly salient for individually-specific investigative behaviors. Our results therefore suggest that individual differences in territoriality are hormonally mediated.

To conclude, we have demonstrated that both environmental cues (i.e. conspecific odors) and prior experience affect behavior and hormones of breeding coyotes. Moreover, several territorial

behaviors, glucocorticoid metabolites, and androgen metabolites are consistent within individuals across time. 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 long-term. If in fact experiences over gestation significantly affect coyote breeding pairs, then those experiences may alter the way a parent impacts their offspring. Therefore, future work should address how pre-partum experiences over time are connected with individual parenting styles.

CHAPTER 3: PRE-PARTUM ANDROGENS AND PARITY ARE PRINCIPAL DETERMINANTS OF COYOTE PARENTAL STYLES

INTRODUCTION

Parental care is an imperative yet costly component of reproduction, in which individuals invest in offspring at a level determined by their own future reproductive success (Clutton-Brock 1991; Klug & Bonsall 2010; Trivers 1974). Although fitness costs and benefits may resolve themselves to produce a single optimum, individuals consistently differ in their investment (Klug & Bonsall 2010; Trivers 1974). For instance, individual convict cichlid males (*Amatitlania nigrofasciata*) consistently differ in brood provisioning, which is negatively associated with parental activity (Budaev et al. 1999). Individual threespined stickleback fathers (*Gasterosteus aculeatus*) consistently differ in brood fanning and nest defense, and fathers that perform egg fanning more frequently also respond more rapidly to conspecific intruders or predators (Stein & Bell 2015). Female red-winged blackbirds (*Agelaius phoeniceus*) demonstrate individual consistency in food provisioning and foraging location preferences (Westneat et al. 2013). Moreover, house sparrows (*Passer domesticus)* are individually consistent in nestling provisioning, which covaries with nest defense from European starlings (*Sturnis vulgaris*), a common nest box competitor (Wetzel & Westneat 2014).

Individual variation in parental care may likely be explained by parental reactions to existing environmental conditions prior to birth, in which parents program offspring for success in future environments (Marshall & Uller 2007; Stein & Bell 2014). More specifically, this maternal matching hypothesis posits that environmental stressors at gestation are translated to offspring via pre-partum hormones that later program offspring physiology (Breuner 2008; Love &

Williams 2008; Love et al. 2013). Previous studies have shown associations between pre-partum hormones and post-partum parental behavior. For example, yellow baboon mothers (*Papio cynocephalus*) with high glucocorticoid concentrations late during pregnancy are more responsive to offspring (Nguyen et al. 2008). Savannah baboon mothers (*Papio hamadryas sp.*) show similar trends, in which higher pre-partum glucocorticoid levels are positively associated with infant-directed affiliative behaviors such as grooming, watching, and moderate contact (Bardi et al. 2004). Paternal hormones during pregnancy also affect care outcomes. For instance, male California mice (*Peromyscus californicus*) demonstrate increased prolactin 2 days post-partum, corresponding to periods of intensive paternal care (Gubernick & Nelson 1989). Decreased androgen concentrations of Siamang (*Symphalangus syndactylus*) fathers post-partum are associated with increased father-infant proximity (Rafacz et al. 2011). Moreover, human males demonstrate increased prolactin and decreased androgen concentrations post-partum correlated with emotional responsiveness to offspring (Wynne-Edwards & Reburn 2000).

Parental care variation in single-parent systems has been well documented (Meaney 2001; Stein & Bell 2015; Westneat et al. 2013; Wetzel & Westneat 2013). A recent swell of studies have quantified the cumulative impact of consistent individual differences in bi-parental systems (Budaev et al. 1999; Creighton et al. 2014; Nakagawa et al. 2007; Westneat et al. 2011; Wetzel & Westneat 2014), though none examine these differences in mammalian species. Each individual of a mated pair may have contrasting or complimentary responses to the same environmental cues. Quantifying the role individual differences play in biparental care helps elucidate mechanisms underlying cooperative care of mated pairs. Here, we investigated parental care of captive coyote pairs, a biparental canid species.

Coyotes are mid-sized (~11.5 to 16 kg) social canids that are nearly ubiquitous across North American (Bekoff & Wells 1982; Gehrt 2010). The species is seasonally monoestrous, with mated pairs breeding once annually from December to February (Carlson & Gese 2008; 2009; Sacks 2005). Scent-marking is an integral component of coyote territorial behavior and peaks between December and March (Carlson & Gese 2008; 2009; Gese 2001; Gese & Ruff 1997; Messier & Barette 1982; Sacks 2005). Mated pairs may also utilize physical (e.g., biting, chasing, etc.) and behavioral (e.g., teeth bearing, scent marking, etc.) aggression during territorial incursions from intruding conspecifics (Gese 1998; 2001). Gestation is 62 to 65 days long with litters (range: 1–12 pups) born between March and May (Gehrt 2010; Fentress et al. 1987; Sacks 2005). Pup survival positively correlates with parental territorial defense (Messier & Barette 1982) and significantly decreases when either parent is removed (Sacks & Neale 2001). Male coyotes invest a considerable amount of time to pup guarding and contact behaviors (Asa & Valdespino 1998), in addition to provisioning females early in pup rearing (Sacks & Neale 2001). Thus, males are highly responsive to the needs of both offspring and mothers.

We observed captive coyote pairs with accompanying pups to determine whether parents demonstrated consistent individual differences in care behaviors. In our previous study, we demonstrated that conspecific odor cues provided mid-gestation increased fecal androgen metabolites (FAMs), while we found decreased FAMs over successive breeding events for both sexes (Chapter 2). In addition, we found within-individual consistency in both fecal glucocorticoid metabolites (FGM) and FAMs, suggesting individuals possess hormone profiles. Here, we observed the same breeding pairs throughout a critical portion of the pup rearing stage (i.e. 5 to 15 weeks; see Bekoff & Wells 1982). We hypothesized that FGMs and FAMs, previously affected by parity and odor cues (Chapter 2), would also be associated with parental

care behaviors over pup rearing. To quantify repeatability in care behaviors, we observed male-female pairs as first-time versus experienced parents two years later. This enabled us to ask whether breeding experience affected plasticity in care, as observed in other species (cotton-top tamarins, *Saguinus oedipus*, Almond et al. 2008; Ziegler & Snowdon 2000; common marmosets, *Callithrix jacchus*, Ziegler et al. 2009). Finally, previous studies have documented the parenting behaviors exhibited by coyote breeding pairs (Bekoff & Wells 1982; Fentress et al. 1987; Way et al. 2001), but do not provide sufficient empirical data that effectively characterize differences in parenting between the sexes. Here, we measured differences in intensity of care behaviors among mothers and fathers to categorize the potentially varying influences provided by parents.

METHODS

Subjects

We observed 8 breeding pairs in 2011 as first-time parents and again in 2013 as experienced parents at the United States Department of Agriculture National Wildlife Research Center (NWRC) Predator Research Facility in Millville, UT. Before the onset of our study in 2011, no pairs had prior breeding experience and were all 1 or 2 years of age (1.4 ± 0.1 years [X± SD]). At the beginning of the breeding season (December), breeding pairs were each placed in 1000 m^2 outdoor "clover" pens optimized for long-term observations on coyotes (Mettler & Shivik 2007; Gilbert-Norton et al. 2009a; Young et al. 2008). Parent and pup family units were fed 2600g per unit of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, Utah) daily plus an additional 650g of food for every pup in the litter according to NWRC regulations. We provided water *ad libitum* (Brummer et al. 2010). We observed pups (n=29) with their parents until early August 2011. Pups were then relocated to outdoor enclosures separate from their

natal pens. Pup relocation corresponded to age of dispersal in the wild (Bekoff & Wells 1982). We observed the same eight breeding pairs again in 2013 as experienced parents giving birth to their second litters (*n*=43 pups). In 2011, 2 litters were slated for early removal from their natal pens for NWRC-specific projects. From the period between 11 and 14 weeks of age we therefore considered 6 litters in 2011 and the full 8 litters in 2013.

Parental Care and Activity

We observed parents twice weekly with accompanying litters when offspring were 5 to 15 weeks of age. Each adult had distinct individual differences in coat pattern, facial features, and tail color. These morphological features were used as a primary means of identification, with adult ear tags and previous shave marks as secondary markers. To reduce coyote wariness, we observed parent-pup units from a field vehicle familiar to the coyotes at the NWRC and specifically designated for long-term behavioral studies. The vehicle was parked at a vantage point 50 to 100 meters away from the breeding pair of interest. We combined live on-site observations were combined with video recordings. The five video-recorders who collected these observations were blind to the treatment group, rearing condition, and age of each animal. At any given observation, only 2 individuals were present: one individual who recorded video and the other coding behaviors. To eliminate interobserver variation, only a single individual coded behaviors.

We used a mixed-scan sampling design with 1-minute intervals (Altmann, 1974), specifically noting general state behaviors such as standing, resting, and walking, at each interval (Table 3.1). We coded pup-directed parenting behaviors previously described by Asa & Valdespino (1998), and also noted scent-marking or territorial behaviors previously described (Table 3.1), to

Table 3.1. Behavioral ethogram used during the gestation and weaning periods (behaviors adapted from Asa & Valdespino 1998; Bekoff & Wells 1986; Gese & Ruff 1997; 1998)

Behavior	**Description**
Ground scratching	Digs and kicks down and backward; often follows urination
Urination	Discharges urine
Locomotion	Individual is active and moving
Vigilant stares	Stares directly at a neighboring conspecific for $\geq$3 seconds
Howling	A single howling event marked by a series of howls separated by $\leq$1 minute
Grooming	Parent licks and cleans pup
Carrying	Parent grabs and lifts pup, transporting to a different location inside the pen
Provisioning	Provides mile and/or regurgitates food to the offspring
Pup play	Number of play bouts parent engages in with offspring
Aggresses pup	Teeth baring, growling, or shoving directed toward offspring
Den attendance	Parent looks directly into den
Pup checks	Parent sniffs and/or briefly contacts body of pup
Proximity	Proportion of time parent is <5 meters away from offspring

determine how they corresponded with pup-directed behaviors. Parental observations occurred over a 30-minute period at 0600-0900 and 1800-2130 hours Mountain Standard Time (MST), which corresponds to the time of peak activity in the wild (Gehrt 2010). We observed each once in the morning and again in the evening every week. We used a randomization without replacement design to assign pairs to particular observation days and times.

Statistical Analyses

We partitioned parenting behaviors into three biologically relevant periods of pup development: early weaning (5-7 weeks of age), late weaning (8-10 weeks of age), and independence (11-14 weeks of age). Developmental windows corresponded to critical periods previously described by Bekoff & Wells (1982). The foremost objective was to determine whether there were significant differences between the sexes in the parenting behaviors observed (Table 1). We used linear mixed models (LMMs) fit with a Poisson distribution for count data to ask whether mothers and fathers differed in the expression of specific care behaviors within each developmental period. Parent identity was set as a random effect in all Poisson distributed LMMs, as well as litter size particularly because litter sizes were greater in the 2013 season compared with the 2011 seasons ($F_{1,15.21} = 31.96$, $P < 0.001$). Because multiple parental care measures (Table 1) covaried, we subsequently used principal components analysis (PCA) to reduce the list of variables into uncorrelated components that described the majority of variance in parental behaviors. We used a PCA rather than a factor analysis because the variance structure was unknown (Abdi & Williams, 2010; Sussman et al. 2013). Previous studies have sufficiently used PCA to assess latent personality traits not evident from direct examination of singular behaviors (Bergvall et al. 2011; Cote et al. 2011; Dingemanse et al. 2007; Martin &

Reale 2008; Sih & Watters 2005; Sussman et al. 2013). Once again, principal component data were partitioned within the three designated developmental periods for assessment.

For principal component data specifically, we used LMMs to ask whether prior odor treatment during gestation (Chapter 2), breeding experience (i.e. first-time vs. experienced breeders), or sex were significant main effects accounting for variance in parenting components within each developmental stage. Once again we set coyote identity and litter size as random effect terms in our models. In addition, we analyzed sexes separately specifically to address how odor-treated mothers or first-time mothers differed from control and experienced mothers (and with fathers as well). To examine how parenting components were related to general activity and behaviors of parents, we used Spearman rank correlations. We also used Spearman rank correlations to assess the relationships among pre-partum hormones (Chapter 2), parenting components, general activity, and marking behaviors of parents over development. With Spearman correlations, we compared mean data for all behaviors over the 5 to 15 week developmental period. Again, we partitioned our dataset by sex to observe differences within sex across our main effects (i.e. treatment group and parity).

Finally, to determine whether parents demonstrated consistent individual differences in parenting components, we used likelihood ratio tests previously described (Betini & Norris 2012; Carter et al. 2012; Dosmann & Mateo 2014; Chapter 2). Specifically, likelihood ratio tests compare models with and without a random effect term and uses restricted log likelihood based on 10,000 simulated values to approximate whether the addition of the random term improves model estimation of the variance within the dataset (Betini & Norris 2012; Carter et al. 2012). To test for significance of coyote identity in parenting components and general activity, we compared a model without coyote identity (E_T or E_Y only) and one with identity (I) as the

random effect term, keeping main effects consistent (Crainiceanu & Rupert 2004; Schielzeth & Forstmeier 2009). We compared models with and without a random slope (i.e. E_T or E_Y | coyote ID) for treatment group and year to investigate whether a significant I x E_T or I x E_Y interaction existed (Carter et al. 2012). We also used likelihood ratio tests to determine whether parents demonstrated consistent individual differences in general activity and marking behaviors (Table 3.1).

We performed all statistical analyses using R version 3.1.3 (R Core Team, 2014). Linear mixed models were performed using the lmer and glmer function from 'lme4' (Bates et al. 2012) and 'lmerTest' (Kuznetsova et al. 2013) packages. Likelihood ratio tests were performed using the exactLRT function from the 'RLRsim' package (Crainiceanu & Rupert 2004). All Spearman correlations were performed using the corr function from the 'corrplot' package (Wei 2013). We used the Shapiro-Wilk test statistic to determine whether data were normally distributed. We reported results from the best-fit models for all measures, determined using the lowest Akaike Information Criterion (AIC) values (Burnham et al. 2010). Alpha was set to 0.05 for all cases and we report data as mean ± S.E.

RESULTS

Parental Care

Mothers and fathers primarily differed in the number of play bouts they participated in with pups, provisioning, and pup checks performed (Table 3.2). Specifically, over the early weaning period moms provisioned pups more frequently than dads (z = -2.436, P = 0.015), but did not differ in their rates of provisioning over the late weaning (z = -1.472, P = 0.141) and independence (z = -1.054, P = 0.292) periods. In addition, mothers engaged in play bouts more

frequently during the late weaning (z = -2.946, P = 0.003) and independence (z = -3.052, P = 0.002) periods, but not over the early weaning period (z = 0.874, P = 0.382). Finally, mothers pup checked more frequently over the independence period (z = -2.006, P = 0.045), and on average were in closer proximity to pups during the late weaning ($F_{1,178.0}$ = 5.48, P = 0.020) and independence periods ($F_{1,11.1}$ = 4.70, P = 0.053; Table 3.2). Mothers and fathers did not significantly differ in the expression of any other parenting behaviors for each developmental window (Table 3.2).

PCA revealed four components that explained approximately 68% of the variance (Table 3.3). Relationships among the original behavioral variables and the four components led to four categories of pup-directed behaviors: contact (pc1), sociability (pc2), aggression/provisioning (pc3), and attachment (pc4). We then partitioned these components by the three developmental periods previously mentioned to assess how parental components differ by our main effects. First, we found that maternal contact ($F_{2,290.5}$ = 4.80, P = 0.009), aggression/provisioning ($F_{1,291.6}$ = 5.71, P = 0.004), and attachment ($F_{1,291.4}$ = 10.64, P < 0.001) all decreased as pups aged (Fig. 3.1) despite treatment group or parity differences. In contrast, maternal sociability generally increased as pups aged ($F_{2,289.3}$ = 14.52, P < 0.001), indicating that mothers were engaged in play bouts with their pups more frequently as pups aged. Second, we found that first-time and experienced mothers differed in the degree of parental components expressed within each developmental stage (Fig. 3.1). Specifically, contact components of experienced mothers were higher compared to first-time mothers when pups were 11 to 14 weeks of age ($F_{1,108.1}$ = 4.59, P = 0.034), indicating that experienced mothers pup checked, groomed, and were in close proximity more frequently than when they were first-time parents (Table 3.4 and Fig. 3.1). Third, experienced mothers had higher aggression/proximity components at every phase (5-7 weeks:

$F_{1,94}$ = 6.26, P = 0.014; 8-10 weeks: $F_{1,84.1}$ = 5.91, P = 0.017; 11-14 weeks: $F_{1,110}$ = 9.12, P = 0.003), indicating that experienced mothers aggressed but also provisioned pups more frequently (Fig. 3.1). Last, maternal attachment components were greater for mothers over the early ($F_{1,88.2}$ = 5.53, P = 0.021) and late weaning phases ($F_{1,83.8}$ = 16.31, P < 0.001) as first-time parents versus experienced parents, suggesting that first-time mothers checked on pups within their den boxes more frequently early throughout development (Fig. 3.1).

Similar to mothers, paternal contact ($F_{2,289.0}$ = 8.45, P < 0.001) and attachment components ($F_{2,288.5}$ = 10.12, P < 0.001) decreased over developmental time, while paternal sociability components increased ($F_{2,290.6}$ = 8.00, P < 0.001). We also found that fathers had increasing aggression/provisioning components over time ($F_{2,289.6}$ = 3.25, P = 0.040), indicating that fathers increased their provisioning and pup-directed aggression when mothers finished weaning (Fig. 3.2). Also similar to mothers, males as experienced fathers aggressed and provisioned their pups more frequently ($F_{1,290.2}$ = 4.75, P = 0.030) than when they were first-time parents (Fig. 3.2). Moreover, first-time fathers had higher attachment component scores during the early ($F_{1,88.2}$ = 4.60, P = 0.035) and late weaning phases ($F_{1,83.2}$ = 10.85, P = 0.001), suggesting that fathers also attended dens more frequently early in development (Fig. 3.2). When we examined sex as a main effect in our mixed models, we did not observe any differences in our parenting components between mothers and fathers (contact: $F_{1,13.7}$ = 2.54, P = 0.133; sociability: $F_{1,13.6}$ = 2.18, P = 0.16; aggression/provisioning: $F_{1,14.0}$ = 1.89, P = 0.19; attachment: $F_{1,13.3}$ = 1.32, P = 0.27). In addition, we did not observe any odor treatment differences for parental components of either sex.

We also found that mothers and fathers were individually consistent in their parenting components (Table 3.4). Mothers demonstrated consistent individual differences in sociability

and aggression/provisioning component scores between years, showing that social behaviors directed towards pups were consistent within moms. Fathers also demonstrated consistent individual differences in all parenting components except sociability, indicating that the paternal involvement in parental was consistent within individual dads (Table 3.4). None of the parenting components for either mothers or fathers demonstrated individual plasticity with prior odor treatment mid-gestation. In contrast, both mothers and fathers demonstrated individual plasticity in attachment component scores, suggesting that parents modified the intensity of den attendances they performed as first-time versus experienced parents (see Figs. 3.1 and 3.2). Within mothers, we found that aggression/provisioning component scores were individually plastic with parity, indicating that moms varied their pup-directed aggression and provisioning behaviors as first-time versus experienced moms. Further, within fathers we found that contact components scores were individually plastic with parity, signifying that dads varied their general proximity with and pup checks to offspring as first-time versus experienced dads (Table 3.4).

General Activity and Parenting

We found that general activity and marking behaviors were individually consistent for coyote parents (Table 3.5) and these behaviors were associated with several parenting components (Table 3.6). Specifically, both mothers and fathers demonstrated repeatability between years in locomotion, urination, ground scratching, howling, and vigilant stares direct toward neighboring coyotes (Table 3.5). In addition, individual maternal responses in urination, ground scratching, and vigilant stares were sensitive to both receiving odor cues and prior breeding experience, demonstrating that mothers exhibited individual plasticity with our environmental factors. Individual paternal activity and marking behaviors were not individually plastic with odor

treatment. Paternal locomotion and urination did demonstrate individual plasticity with parity, implying that fathers individually augmented their activity and the frequency of urinations according to whether they were first-time or experienced dads.

General activity and marking behaviors were differentially associated with parenting components of mothers and fathers (Table 3.6). Maternal sociability component scores were negatively associated with general locomotion and the mean frequency of urinations, indicating that mothers that play more with their pups move and scent-mark less (Table 3.6). In addition, maternal aggression/provisioning component scores were positively associated with the mean frequency of urination and howling events, demonstrating that moms that aggress pups more frequently also scent-mark and vocalize more. Only paternal contact component scores demonstrated a significant correlation with our marking behaviors; specifically, dads that had higher contact component scores also urinated more frequently (Table 3.6).

Behavior-Hormone Correlations

We used Spearman rank correlation to determine whether previously observed (Chapter 2) pre-partum hormones (i.e. fecal glucocorticoid and androgen metabolites) were associated with our parenting components and general behaviors. We specifically focused on fecal androgen metabolites (FAMs) over the odor cue provisioning period, as odor cues increased FAMs within the testing phase (Fig. 3.3). We also focused specifically on fecal glucocorticoid metabolites (FGMs) over the entire gestation as mothers demonstrated higher FGMs irrespective of odor treatment or parity (Fig. 3.4).

We found that litter size negatively corresponded with maternal FAMs (r_s = -0.76, $P < 0.001$, Fig. 3.3a), suggesting that moms with higher FAMs produced smaller litters. In addition, we

found that maternal contact component scores were negatively associated with maternal FAMs (r_s = -0.54, P = 0.031, Fig. 3.3c), indicating that mothers with higher FAMs during odor cue provisioning pup checked less and were in close proximity less frequently than other moms. Similarly, maternal aggression/provisioning scores were negatively associated with maternal FAMs (r_s = -0.59, P = 0.016, Fig. 3.3e), signifying that mothers with higher FAMs during odor cue provisioning aggressed pups less but also provisioned their pups less over development. Maternal FAMs were also negatively associated with the mean frequency of ground scratching over development (r_s = -0.59, P = 0.016), indicating that mothers with higher FAMs ground scratched less. Mean fecal glucocorticoids in moms were not associated with any parenting components, but were positively associated with the frequency of urinations (r_s = 0.56, P = 0.027, Fig. 3.4a) and ground scratching (r_s = 0.52, P = 0.039, Fig. 3.4c), indicating that mothers with higher FGMs pre-partum performed scent-marking behaviors more frequently.

Paternal FAMs meanwhile did not demonstrate any associations among litter size (r_s = -0.42, P = 0.11, Fig. 3.3b), contact component scores (r_s = -0.11, P = 0.69, Fig. 3.3d), or aggression/provisioning components scores (r_s = -0.43, P = 0.10, Fig. 3.3f). Paternal FAMs were also not associated with any of the general activity or scent-marking behaviors. Comparatively, paternal FGMs were positively associated with the frequency of urinations (r_s = 0.59, P = 0.10, Fig. 3.4b) and howling (r_s = 0.50, P = 0.049, Fig. 3.4f), indicating that fathers with higher FGMs pre-partum tended to scent-mark and vocalize more frequently. Paternal FGMs were also positively associated with the frequency of ground scratching (r_s = 0.49, P = 0.054, Fig. 3f), although not significant.

Table 3.2 Mean (± S.E.) differences in parenting behaviors between mothers and fathers corresponding to litter age. Bold values and asterisks indicate significant differences between mothers and fathers within each age period (*P < 0.05; **P<0.01; ***P<0.001). Arrows denote significant (P<0.05) trends in behaviors over time; lines indicate no relationship over time.

	Behavior	Litter age			Direction
		5-7 weeks	8-10 weeks	11-14 weeks	
Mothers	Grooming	4.38 ± 0.68	1.81 ± 0.45	1.21 ± 0.39	↓
	Carrying	1.00 ± 0.57	0.38 ± 0.22	0.00 ± 0.00	---
	Provisioning	**2.06 ± 0.62***	0.31 ± 0.15	0.64 ± 0.27	↓
	Pup play	8.13 ± 2.24	**9.19 ± 1.99****	**15.14 ± 3.29***	↑
	Aggresses pup	3.44 ± 1.37	6.00 ± 3.26	6.21 ± 2.07	↑
	Den Attendance	6.69 ± 1.35	2.06 ± 0.49	1.21 ± 0.43	↓
	Pup Check	8.63 ± 2.03	7.69 ± 1.44	**6.43 ± 1.19***	↓
	Proximity	28.22 ± 0.03%	**34.19 ± 0.04%***	32.82 ± 0.03%	---
Fathers	Grooming	3.56 ± 0.80	0.88 ± 0.38	0.79 ± 0.26	↓
	Carrying	2.31 ± 1.36	0.00 ± 0.00	0.00 ± 0.00	↓
	Provisioning	**0.19 ± 0.14***	0.06 ± 0.06	0.36 ± 0.17	---
	Pup play	9.88 ± 1.83	**3.88 ± 0.71****	**7.29 ± 1.67***	---
	Aggresses pup	2.31 ± 1.10	4.13 ± 1.71	4.86 ± 1.50	↑
	Den Attendance	5.31 ± 1.09	1.13 ± 0.42	0.86 ± 0.39	↓
	Pup Check	9.75 ± 3.36	9.63 ± 1.99	**3.64 ± 0.90***	↓
	Proximity	25.75 ± 0.03%	**24.56 ± 0.03%***	24.16 ± 0.04%	---

Table 3.3 Results of principal components analysis for parental care behaviors. Variables that contribute with a loading of >|0.40| are shown in bold (Sussman et al. 2013). Care behavior definitions are further elucidated in Table 1. PC=Principal Component

Parental care behavior	PC1: contact	PC2: sociability	PC3: aggression/provisioning	PC4: attachment
Grooming	**0.462**	-0.220	-0.128	-0.116
Carrying	0.286	**-0.526**	-0.283	-0.290
Provisioning	0.197	-0.054	**0.757**	0.065
Pup play	0.344	**0.532**	-0.283	0.270
Aggresses pup	0.295	0.268	**0.446**	**-0.435**
Den attendance	0.217	-0.288	0.161	**0.795**
Pup checks	**0.448**	-0.272	-0.013	-0.060
Proximity	**0.462**	**0.401**	-0.157	0.028
Eigenvalue	2.251	1.136	1.061	0.977
Variance explained	0.281	0.142	0.133	0.1221
Total variance explained	0.281	0.423	0.556	0.6782

Table 3.4 Model comparisons to test for consistent individual differences in parental care principal components over pup development (i.e., 5 to 15 weeks of age), including interactions among individual identity and environmental factors (E_T and E_Y). Bold values indicate statistical significance

	Behavior	Treatment (E_T)		Year (E_Y)		Individual (I)		I x E_T		I x E_Y	
		t	***P***	***t***	***P***	***LRT***	***P***	***χ2***	***P***	***χ2***	***P***
Mothers	Contact	-2.147	**0.033**	1.937	0.054	0.506	0.128	1.156	0.561	1.590	0.452
	Sociability	-0.774	0.440	-0.938	0.349	5.603	**0.003**	0.074	0.964	0.000	1.000
	Aggression/Provisioning	-1.861	0.064	3.982	**0.000**	3.004	**0.019**	2.686	0.261	9.259	**0.010**
	Attachment	-0.213	0.831	-4.057	**0.000**	0.360	0.163	0.630	0.730	9.312	**0.009**
Fathers	Contact	-0.472	0.637	1.636	0.103	7.331	**0.001**	1.229	0.541	7.191	**0.027**
	Sociability	-0.688	0.492	-1.613	0.108	0.858	0.090	0.815	0.665	1.666	0.435
	Aggression/Provisioning	-1.512	0.132	2.386	**0.018**	2.569	**0.026**	0.000	1.000	3.517	0.172
	Attachment	-0.465	0.642	-3.453	**0.001**	9.044	**0.001**	0.655	0.721	7.975	**0.019**

LRT: $\chi 2$ value for the log likelihood ratio test. Interactions among odor-treatment, year, and individual identity were determined by comparing mixed models with animal ID nested in each respective environmental variable.

Table 3.5 Model comparisons to test for consistent individual differences in general marking, social, and vigilance behaviors over pup development (i.e. 5 to 15 weeks of litter age), including interactions among individual identity and environmental factors (ET and EY)

	Behavior	Treatment (E_T)		Year (E_Y)		Individual (I)		I x E_T		I x E_Y	
		t	*P*	*t*	*P*	*LRT*	*P*	$\chi 2$	*P*	$\chi 2$	*P*
Mothers	Locomotion	1.542	0.124	-0.910	0.363	17.579	**<0.001**	1.586	0.453	1.945	0.378
	Urination	1.411	0.159	4.214	**<0.001**	79.768	**<0.001**	7.109	**0.029**	20.499	**<0.001**
	Ground scratching	1.697	0.098	2.425	0.016	97.929	**<0.001**	12.793	**0.002**	15.059	**0.001**
	Howling	0.949	0.343	0.013	0.990	14.327	**<0.001**	1.401	0.496	3.605	0.165
	Vigilant stares	1.055	0.292	5.076	**<0.001**	18.987	**<0.001**	16.452	**<0.001**	42.669	**<0.001**
Fathers	Locomotion	-0.858	0.391	-3.564	**<0.001**	28.232	**<0.001**	2.166	0.339	11.707	**0.003**
	Urination	-0.535	0.593	3.179	**0.002**	22.335	**<0.001**	0.556	0.757	10.446	**0.005**
	Ground scratching	-1.223	0.222	1.556	0.121	25.680	**<0.001**	3.213	0.201	4.204	0.122
	Howling	0.383	0.702	-1.547	0.123	8.602	**0.001**	0.000	1.000	4.372	0.112
	Vigilant stares	-2.365	**0.019**	2.627	**0.009**	9.860	**<0.001**	0.000	1.000	4.287	0.117

LRT: $\chi 2$ value for the log likelihood ratio test. Interactions among odor-treatment, year, and individual identity were determined by comparing mixed models with animal ID nested in each respective environmental variable.

Table 3.6 Spearman rank relationships among mean (± S.E.) parenting components and general behaviors of parents over pup development (5 to 15 weeks of age). Bold values and asterisks indicate statistically significant relationships ($N = 16$, $P < 0.05$)

		Parenting components			
	General behaviors	Contact	Sociability	Aggression/Provisioning	Attachment
Mothers	Locomotion	-0.24	**-0.51***	0.14	0.37
	Urination	-0.11	**-0.53***	**0.56***	-0.20
	Ground scratching	0.07	-0.23	0.32	0.11
	Howling	-0.28	-0.35	**0.53***	0.00
	Vigilant stares	0.00	-0.49	0.45	-0.40
Fathers	Locomotion	0.13	-0.19	-0.41	0.36
	Urination	**0.54***	-0.45	0.23	0.09
	Ground scratching	0.21	-0.10	0.15	-0.30
	Howling	0.34	0.23	-0.17	0.41
	Vigilant stares	0.22	0.27	-0.30	-0.27

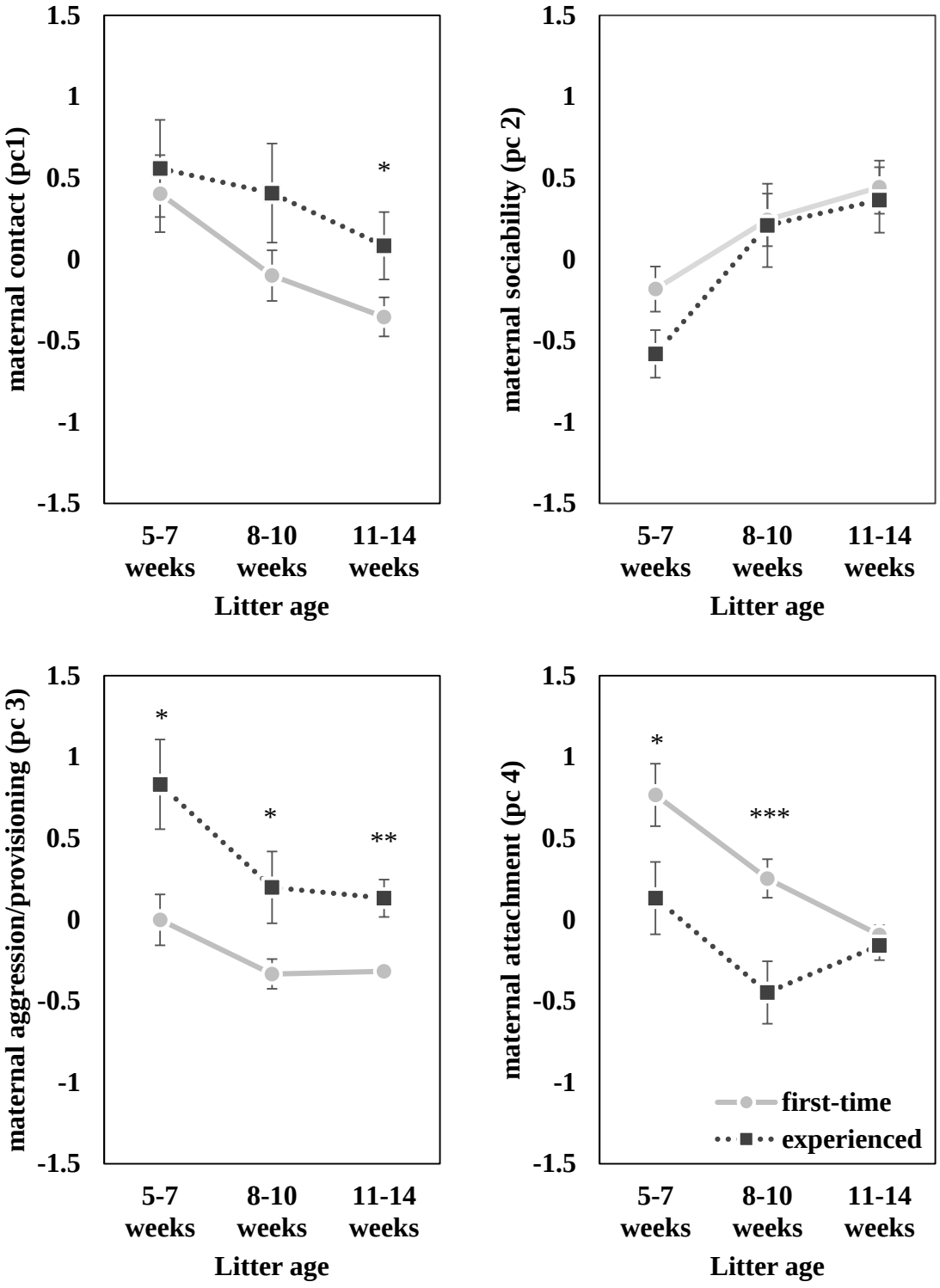

Fig. 3.1: Mean (± S.E.) parental care principal component scores of first-time and experienced mothers over pup development. Asterisks indicate differences (*P<0.05, **P<0.01, ***P<0.001) between first-time and experienced mothers within each period

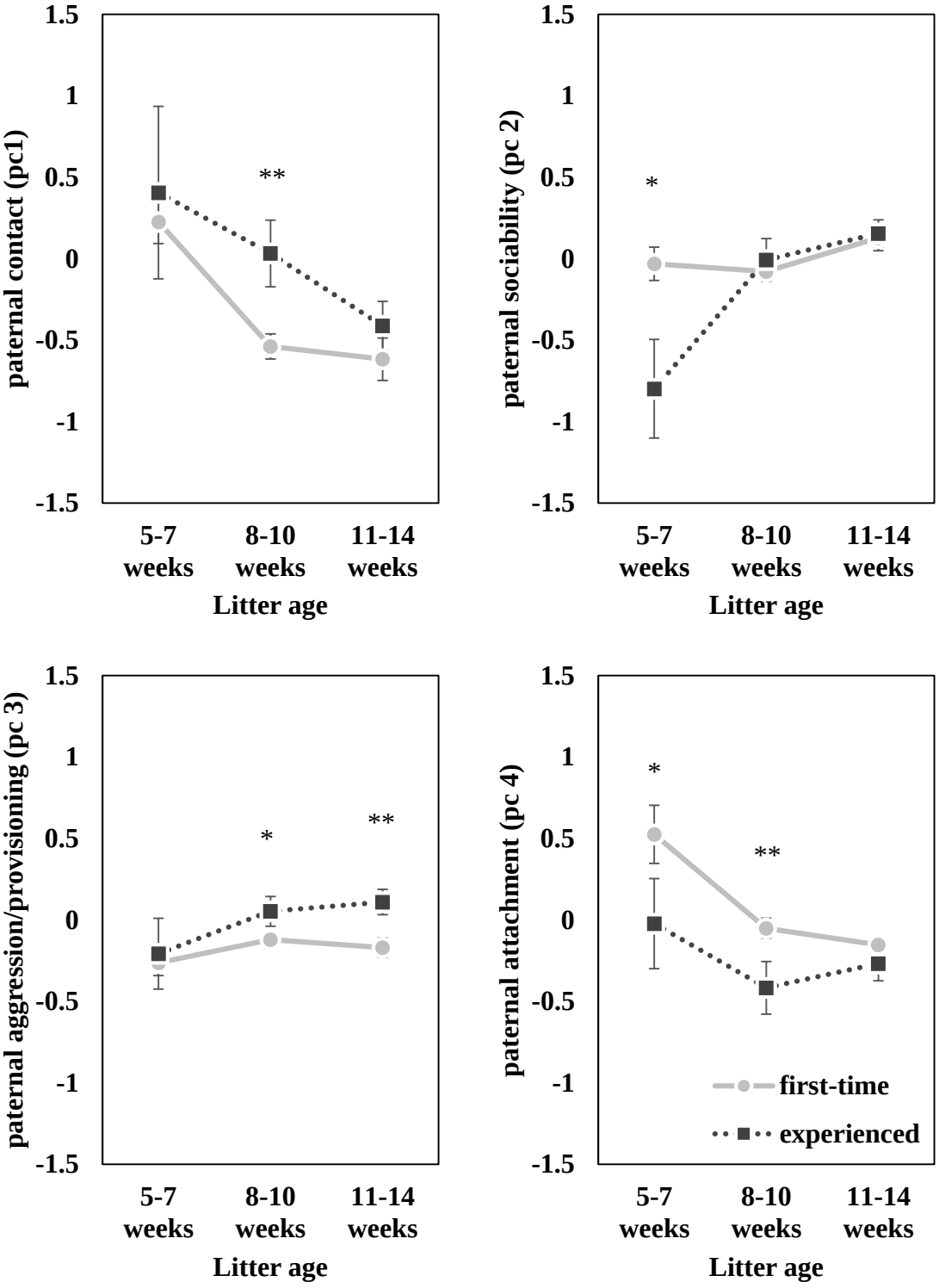

Fig. 3.2: Mean (± S.E.) parental care principal component scores of first-time and experienced fathers over pup development. Asterisks indicate differences (**P*<0.05, ***P*<0.01, ****P*<0.001) between first-time and experienced fathers within each period

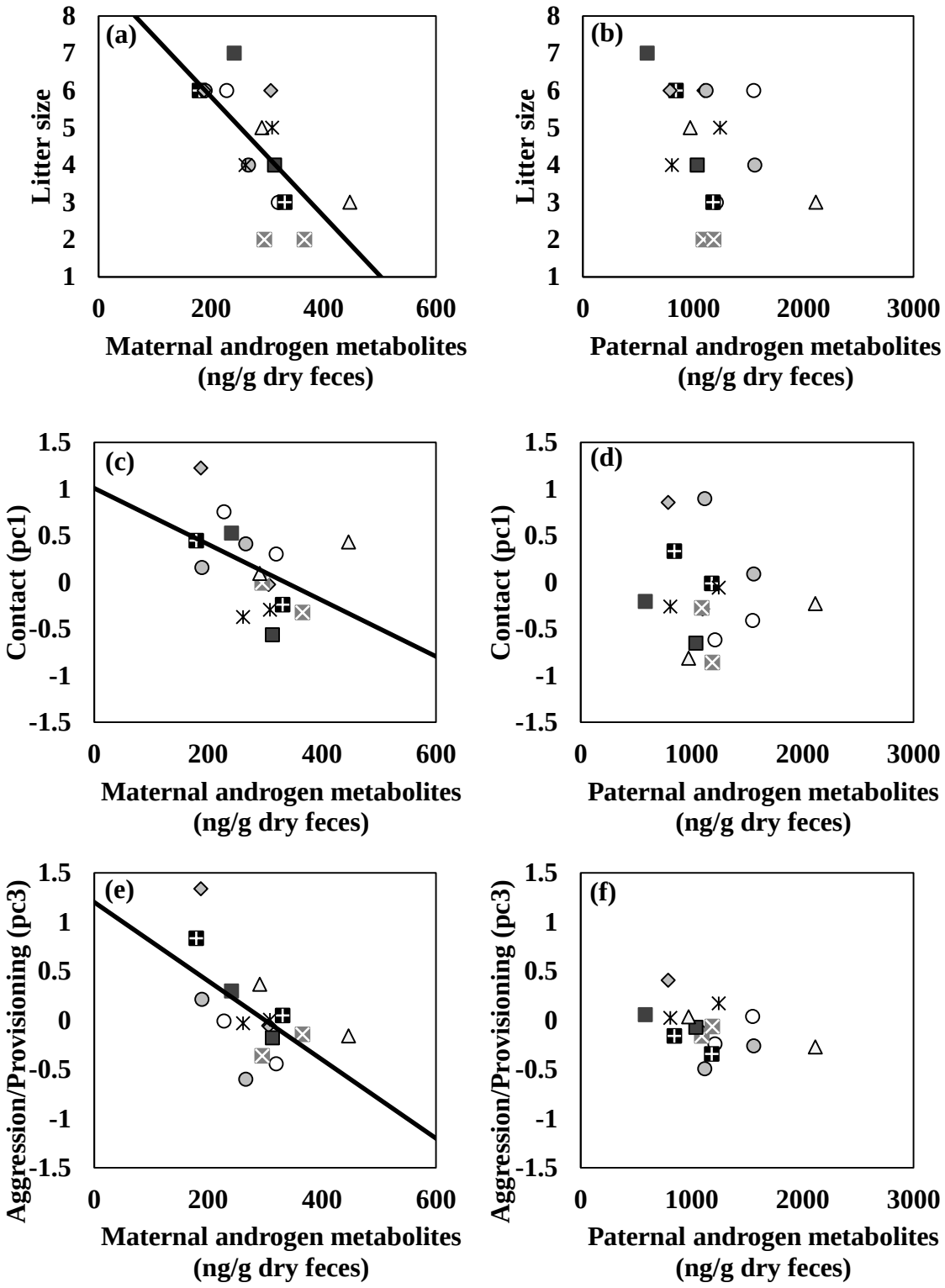

Fig. 3.3: Litter size (a-b), mean (± S.E.) contact (c-d), and mean aggression/provisioning components (e-f) over pup development in relation to androgen metabolites (Chapter 2) over the odor cue test. Symbols represent individual mothers and fathers over both seasons (N = 16), and pairs share the same symbols. Solid lines represent significant relationships

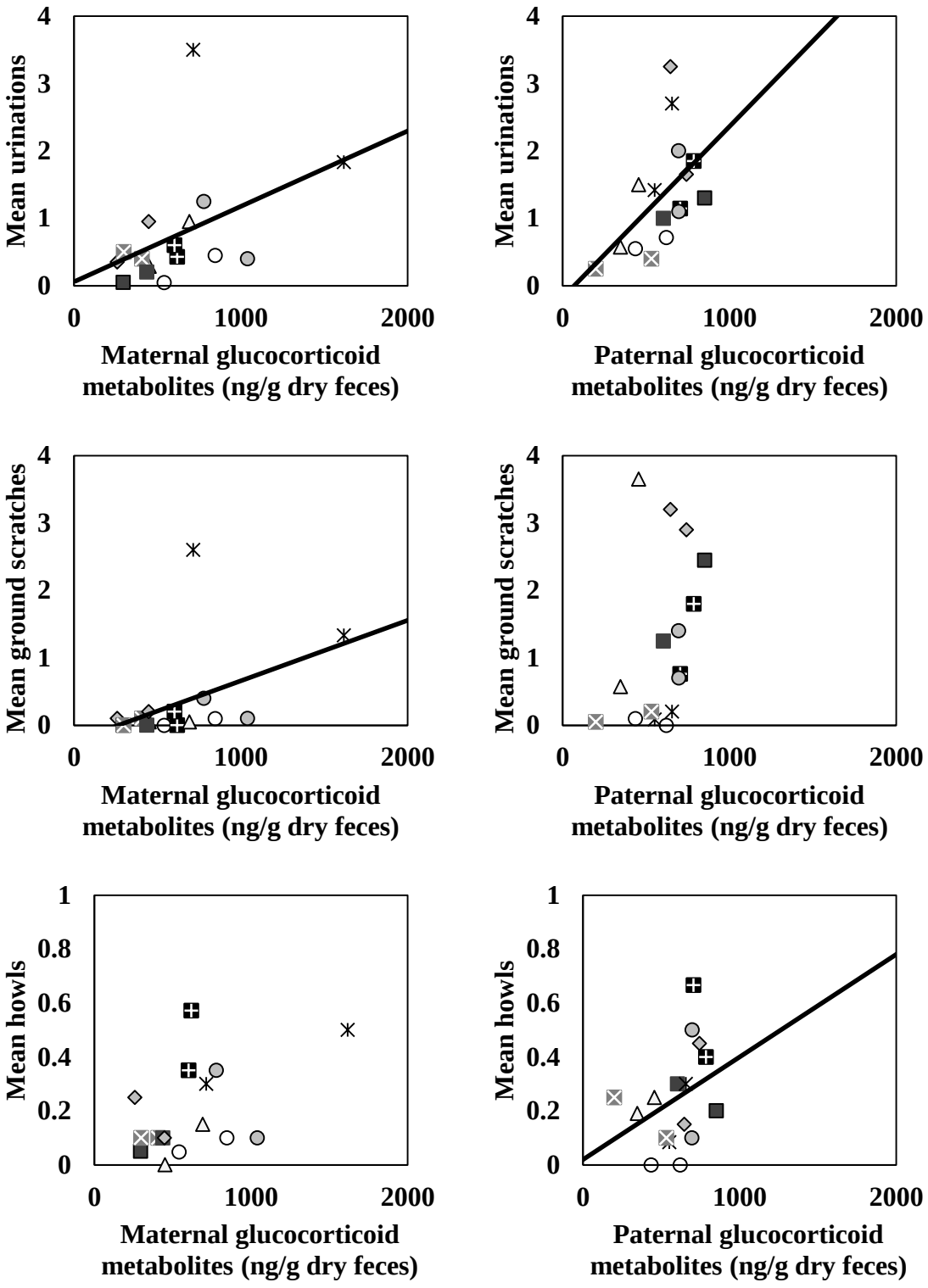

Fig. 3.4: Mean (± S.E.) urinations (a-b), ground scratches (c-d), and howls (e-f) over pup development in relation to fecal glucocorticoid metabolites (Chapter 2) over gestation. Symbols represent individual mothers and fathers over both seasons (N = 16), and pairs share the same symbols. Solid lines represent significant relationships

DISCUSSION

Coyote parents demonstrated consistent individual differences in parenting components, as well as general activity and marking behaviors. Parenting component scores changed over development for both parents, and though we observed differences in components over time, we did not find component differences between mothers and fathers. Parents demonstrated individual plasticity in their parenting components as a function of prior parenting experience. In addition, maternal FAMs previously influenced by odor cue provisioning were correlated with litter size and parenting components over pup development. Thus, our results suggest that consistent individual differences (i.e. parenting styles) are not only hormonally mediated, but that prior environmental experiences and parity have the potential to alter androgen metabolites connected with parenting styles. Previous studies have demonstrated behavioral types and syndromes in coyotes (Darrow & Shivik 2009; Gilbert-Norton et al. 2009a; Mettler & Shivik 2007; Young et al. 2015), as well as consistent individual differences in parenting of other taxa (Budaev et al. 1999; Stein & Bell 2015; Wetzel & Westneat 2013). However, to our knowledge this is one of the first studies to demonstrate the interplay between consistent behavioral and hormonal differences in a biparental system.

Parental components from mothers and fathers both changed within a rearing. For instance, we observed general decreases in parental contact and attachment component scores, in which parents pup checked and den attended less, and were in close proximity of offspring less frequently. This is likely due to the maturation of pups and the developmental milestones they experience, which have been previously described (Bekoff & Wells 1982; Fentress et al. 1987; Messier & Barette 1982; Way et al. 2001). Offspring become more mobile and independent from their parents, and are able to eat for themselves as early as 5 weeks of age (Fentress et al.

1987; Way et al. 2001). Pups 11 to 14 weeks of age have a different set of requirements than younger individuals, so pup maturation likely imposes changes to parental components and thus changes to parenting strategies. Temporal trade-offs in parenting behaviors have previously been observed in house sparrows, in which both mothers and fathers reduced their provisioning rates with increasing age of their offspring (Schwagmeyer & Mock, 2003). In contrast, both mother and father house sparrows increase their feeding rate (per hour) and nest visitation rate as chicks age (Westneat et al. 2011). Similar to our results, both Schwagmeyer & Mock (2003) and Westneat et al. (2011) demonstrate the degree of flexibility inherent in parental care, particularly in response to offspring growth.

Similarly, we observed temporal variation in aggression/provisioning components of both parents; specifically, mothers decreased their pup-directed aggression and provisioning while fathers conversely increased their pup-directed aggression and provisioning. Both parental aggression and provisioning were influential variables explaining variance in the third principal component, which is biologically relevant to the weaning process. Pups often solicit food from their parents using muzzle licks and tail wags, signifying their desire to eat (Fentress et al. 1987). As mothers begin the weaning process, pups attempt to solicit food and parents either capitulate by regurgitating food to pups or aggress pups to halt further begging (Schell pers. obs). Consequently, the number of pups within a litter may affect the expression of our aggression/provisioning components scores. It is worth reiterating that litter size was significantly larger with experienced parents, and aggression/provisioning scores of experienced parents were as well (Figs. 3.1 and 3.2). It is therefore likely that hungry pups partially explain the between year differences were observed in the aggression/provisioning components of both mothers and fathers. This is similar to great tits (*Parus major*), in which food allocation is

dictated by the number of hungry nestlings (Tanner et al. 2008). Litter size does not fully explain variance in our aggression/provisioning components though, as both mothers and fathers demonstrated consistent individual differences between rearing years. Moreover, mothers (but not fathers) demonstrated individual plasticity in the aggression/provisioning component with parity, indicating that mothers differentially augment how they provision and aggress weaning offspring over time. It is possible that mothers are differentially responsive to their pups, and those pups accentuate individual differences in maternal behavior. Meanwhile, fathers have individually-specific responses to begging offspring regardless of how many pups he has or prior paternal experience. The differences observed among maternal and paternal individual differences here is likely to impact variance in offspring traits, and future work should examine the cumulative impact of both parents on pup phenotype.

There were no sex differences in our parenting components, suggesting that fathers are providing care that compliments the mothers. Our results further support previous findings in coyote biparental care (Asa & Valdespino 1998; Bekoff & Wells 1982), as well as other species within Canidae (Malcolm 1985). We did, however, observe sex-linked differences in parenting component repeatability between years: mothers demonstrated individual consistency in socially-oriented parenting components (i.e. sociability and aggression/provisioning), while fathers demonstrated individual consistency in all components except sociability. These differences may underscore investment differences that mothers and fathers exhibit in biparental systems (Brown 1985; Creighton et al. 2014). For instance, biparental burying beetles (*Nicrophorus orbicollis*) overcompensate for a partner's lack of productivity in nest building by contributing more time to preparing vertebrates carcasses for larval development (Creighton et al. 2014). It is therefore likely that sex-specific differences in component repeatability highlights

overcompensation from either the mother or father to affect the cumulative parental effort of a breeding pair.

Our results may also underscore the interaction between sex-specific investment differences and individual variability. For example, male house sparrows demonstrate high repeatability in provisioning rates between breeding years, while females demonstrate low to moderate repeatability (Nakagawa et al. 2007). These are similar to our findings in that parenting components such as contact and attachment were consistent within fathers, but not within mothers. Nakagawa et al. (2007) posited that differences in investment among male house sparrows followed a "sealed-bid" model for biparental systems (Schwagmeyer et al. 2002) in which paternal investment in offspring proceeds despite deviation in parental care behaviors of their partner. This differs from the negotiation model in biparental systems (McNamara et al. 1999), in which either parent increases investment in offspring when the other parent decreases their parental care. Similar to the biparental house sparrows observed by Nakagawa et al. (2007), our coyote parents seem to employ both strategies according to sex, in which fathers follow the "sealed-bid" model and mothers the "negotiation" model. This may indicate that differential selective forces operate on mothers and fathers during parenting. Individually consistent parental investment from coyote fathers indicate that internal state drives variation in paternal care, while low to moderate individual differences in mothers suggest maternal care is more flexible. The interplay between consistency in males and flexibility in females, as well as the intensity of paternal care provided by fathers, provide a multidimensional set of influences that may impact the development of offspring traits.

Parenting components were correlated with a suite of general behaviors. Specifically, maternal sociability was negatively associated with locomotion and urination, while maternal

aggression/provisioning was positively associated with urination and howling events (Table 3.6). Both sociability and aggression provisioning components were individually consistent in mothers, as well as all scent-marking and general behaviors. For fathers, we also observed associations between contact component scores and urination, suggesting that more territorial males are also more involved in pup checking and proximity behaviors. Our results therefore provide evidence that parenting styles in coyotes are also associated with behavioral syndromes for territoriality. Previous work has demonstrated behavioral syndromes exist in coyotes in regards to ambiguous threat (Dawson & Jaeger 2009), handling and predator (i.e. human) response (Young et al. 2015), and human-associated items (Darrow & Shivik 2009; Mettler & Shivik 2007). However, this is the first study to demonstrate that coyotes have a parental syndrome (i.e. parental style), which corresponds with a behavioral syndrome for territoriality. If in fact personality influences the parenting strategies of coyotes, then personality variation among breeding mothers represents a critical source of non-genetic influence that could shape phenotypic variation for coyote populations. Reddon (2011) previously hypothesized that non-genetic transmission of traits via personality differences of parents could drive change and adaptation. Further, Meaney (2001) hypothesized that individual differences in maternal care provide a foundation for the transmission of phenotypic differences across generations. We have provided preliminary evidence to support that claim, as personality differences and parenting styles are associated in coyotes.

Remarkably, parenting components observed over pup development were negatively associated with prenatal androgen metabolites suggesting that hormonal physiology during gestation mediates resultant parenting behaviors during the rearing stage. This is similar to California mice (*Peromyscus californicus*), in which fathers exposed to seven different stressors

over a 7-day period exhibited higher corticosterone concentrations and consequently spent less time with their pairmate and pups over development, compared to control fathers (Harris et al. 2013). Swiss-Webster mice demonstrate similar trends, in which moms prenatally stressed over gestation groomed and nursed their pups significantly less than control dams (Meek et al. 2001). Here, pre-partum androgen metabolites underlie behaviors that are individually consistent in mothers, suggesting that androgens are key initiators of parenting styles in coyotes. Pre-partum glucocorticoid metabolites were also associated with scent-marking and howling behaviors, which in turn were individually consistent for both mothers and fathers. Thus, hormonal metabolites pre-partum predicted future parenting styles and personality. Previous work has suggested that individual differences in behavior correspond to hormonal physiology (Atwell et al. 2012; Carere et al. 2010; Chávez-Zichinelli et al. 2014; Van Oers et al. 2011). However, this is the first study to demonstrate that consistent individual differences in hormones, parenting, and other behaviors are all interconnected.

To conclude, coyote parents exhibited individually-distinct parental styles that were both flexible to within-year experiences and prior breeding experiences. These parental styles were associated with personality traits unrelated to pup-directed behaviors in the species, suggesting that personality traits before pup rearing predicted subsequent parenting behaviors. Furthermore, parental styles and personality traits were mediated by hormonal physiology over gestation. As we previously demonstrated, odor cues provided mid-gestation increased FAMs (Chapter 2). If external stimuli such as conspecific odor cues have the potential to influence hormonal physiology of coyotes, this provides credible support to the theory of hormones operating as mediators of non-genetic inheritance (i.e. parental effects; Meylan et al. 2012; Reynolds et al. 2013; Uller 2008). For coyotes, experienced stimuli over gestation may be transmitted to

offspring both *in vitro* and through realized changes in parental care strategies. Consequently, experiences of parents over gestation are directly and indirectly transmitted to offspring and likely affect their developmental trajectories. Further empirical study of parental effects in the coyote system may suggest that coyote parents are the linchpin to phenotypic and behavioral flexibility in the species, factors that greatly influence the adaptive capacity of coyotes to urban habitats (Gehrt 2010).

CHAPTER 4: COYOTE PARENTING EXPERIENCE AND PRENATAL ANDROGENS IMPACT BOLDNESS AND HORMONES IN PUPS

INTRODUCTION

Parents can impact offspring phenotypic traits beyond their genetic contribution (Maestripieri & Mateo 2009; Mousseau & Fox 1998; Räsänen & Kruuk 2007). These parental effects have received considerable attention in recent decades given the potential for this mechanism to influence both the direction and strength of evolutionary change in a population (Bonduriansky & Day 2009; Marshall & Uller 2007). Previous research has attempted to address the contributing factors, such as environment, social group, and aging, that generate or sustain parental trait variation to elucidate the proximate processes connected to non-genetic inheritance. For example, social stressors (red squirrels, *Tamiasciurus hudsonicus*, Dantzer et al. 2013; California mouse, *Peromyscus californicus*, Harris et al. 2013; guinea pigs, *Cavia aperea f. porcellus*, Kemme et al. 2007), hierarchical status within a social group (spotted hyenas, *Crocuta crocuta*, Dloniak et al. 2006; Höner et al. 2010; yellow baboons, *Papio cynocephalus*, Onyango et al. 2008), or predatory stimuli (snowshoe hares, *Lepus americanus*, Sheriff et al. 2010; three-spined stickleback, *Gasterosteus aculeatus*, Stein & Bell 2014) affect pre- and post-partum parental hormones associated with offspring social behaviors, dispersal patterns, and hormones. Parental effects often span more than one generation as grand-offspring traits, including physiological stress (Norway rat, *Rattus norvegicus*, Francis et al. 1999; guinea pigs, Schöpper et al. 2012), dispersal (two-spotted spider mite, *Tetranychus urticae*, Bitume et al. 2014), mass gain (burying beetle, *Nicrophorus vespilloides*, Lock 2012), and maternal behavior (Francis et al. 1999) are correlated with grandparental traits. In fact, Kirkpatrick & Lande (1989) previously

hypothesized that parental effects reflect generations going back an infinite distance into the past, suggesting that non-genetic influences span throughout evolutionary time. Parental effects are therefore inherently complex and malleable, allowing parents' flexible responses to environmental fluctuations be passed on to offspring in adaptive ways (Marshall & Uller 2007; Scordato et al. 2012).

Hormonal expression often represents a primary catalyst underlying the phenotypic changes parents may experience that later correlate with offspring traits (Brown 1985; Meylan et al. 2012; Mateo 2008). For instance, parents with increased pre-partum glucocorticoids produce and sire offspring with faster growth rates (red squirrels, Dantzer et al. 2013) or increased body condition (zebra finches, *Taeniopygia guttatta*, Crino et al. 2014). Glucocorticoids post-partum may also be transferred to offspring via colostrum, increasing infant nervousness and confidence while positively impacting infant weight gain (field voles, *Microtus agrestis*, Helle et al. 2013). Androgens represent another key suite of hormones underlying parental effects. Early exposure to testosterone *in vitro* augments offspring sexual and aggressive behaviors (Groothuis et al. 2005; guinea pigs, Kemme et al. 2007), as well as territoriality and social status (spotted hyenas, Dloniak et al. 2006). It is therefore essential to consider underlying endocrine mechanisms of parental effects when exploring non-genetic inheritance in previously unexplored systems.

Prior studies have thoroughly addressed parental effects from the maternal perspective because primary care is exhibited solely by mothers, with fathers either absent or providing very little care (Maestripieri & Mateo 2008). Comparatively fewer studies have examined the impact of paternal effects on offspring (Charpentier et al. 2008; Harris et al. 2013; Stein & Bell 2014), or influences from both parents (Donelson et al. 2009; Lock 2012). Fathers in many species readily exhibit extensive nest building, provisioning, guarding, and other care behaviors

(Canidae species, Asa & Valdespino 1998; California mice, Gubernick & Nelson 1989; titi monkeys, *Callicebus cupreus*; common marmosets, *Callithrix jacchus*; Goeldi's monkeys, *Callimico goeldii*, Schradin et al. 2013). For some species, the intensity and duration of paternal care is directly associated with offspring traits and survival (Stein & Bell 2014; Sacks & Neale 2001; Woodruff et al. 1994; Wynne-Edwards & Lisk 1989). Subsequently, paternal contributions to offspring traits may produce a complimentary effect to maternal effects.

The majority of research on maternal effects is also focused on single reproductive efforts rather than observations of parents over successive rearing events (Bowen 2008; Wilson & Festa-Bianchet 2008). Parental experience is a key source of variation for parental effects. For instance, prolactin and behavioral sensitivity to infant cues increase with prior infant experience (cotton-top tamarins, *Saguinus oedipus*, Almond et al. 2008; humans, *Homo sapiens*, Delahunty et al. 2007). Individuals with prior parenting also experience changes in neural circuitry of both the hippocampus and prefrontal cortex, which are associated with hormonal correlates of caregiving behaviors (Leuner et al. 2010). Parity also affects measures such as birth mass (Pinnipeds, Bowen et al. 2006; Ungulates, Wilson & Festa-Bianchet 2008), in which increased experience positively correlated with offspring mass. Experience is therefore one facet of plasticity in parental effects. Consideration of temporal variation and experience in parental effects may thus prove valuable to understanding how changes over reproductive episodes affect offspring. Here, we investigated a biparental canid species – the coyote – to address both the cumulative contribution of maternal and paternal effects on offspring behavior and hormones.

Coyotes are a compelling system in which to address how biparental mechanisms affect pup traits. First, pups have long-term relationships with their parents from infancy to the yearling stages (Bekoff & Wells 1982; Patterson & Messier 2001; Way et al. 2001), allowing for multiple

parent-offspring interactions. Second, both mothers and fathers exhibit care (Bekoff & Wells 1982; Messier & Barette 1982; Way et al. 2001) and observational studies posit that the lack of paternal care reduces maternal and offspring fitness (Sacks & Neale 2001). Third, individual differences in neophobia, wariness, and aggression have been detailed (Darrow & Shivik 2009; Dawson & Jaeger 2009; Gilbert-Norton et al. 2009a; Gilbert-Norton et al. 2009b, Mettler & Shivik 2007; Young et al. 2015); these are key personality traits likely sensitive to parental influences. Last, multiple populations have successfully colonized urban areas despite increased landscape conversion and human populations (Gehrt 2010). Coyotes modify their landscape use patterns (Gehrt et al. 2009; Grubbs & Krausman 2009), diet (Morey et al. 2007), and activity budgets (Grinder & Krausman 2001; Kitchen et al. 2000; Séquin et al. 2003) to avoid human detection, and such adjustments by first-time colonizers, i.e. parents, may have facilitated coyote adaptation to cities. Successful experiences of both mothers and fathers in urban areas may have a cumulative impact on offspring survival.

For this study, we observed captive breeding pairs with pups (5 to 15 weeks of age) in two separate breeding episodes, separated by two years. In our previous studies, we provided these same breeding pairs with foreign conspecific odor cues (i.e. Russ Carman© coyote scent lures) predicted to increase prenatal fecal glucocorticoid (FGM) and androgen (FAM) metabolites (Chapter2), that were late associated with parental behaviors (Chapter 3). Odor cues were effective at increasing FAMs of expectant mothers and fathers (Chapter 2), and maternal FAMs were strongly associated with contact and aggressive care behaviors (Chapter 3). Likewise, coyotes demonstrated individual differences in FGMs, FAMs, and parenting styles that were plastic in response to parity (Chapter 3). We hypothesized that exposing parents to odor cues prenatally, parenting experience, and prenatal parental hormones would influence pup behavior

and hormones. To determine the influence of parental experience, we observed litters born to first-time and experienced parents. Pups were behaviorally assessed via a combination of feeding and novel object tests. We used hair samples collected at 5, 10, and 15 weeks of pup age to quantify hair cortisol and testosterone concentrations of pups. We then examined the relationships between our behavioral, hormonal, and physiological measures with pre-partum parental hormones.

MATERIALS AND METHODS

Study Animals and Housing

We observed captive breeding pairs (8 males and 8 females) and their offspring in 2011 and again in 2013 at the United States Department of Agriculture National Wildlife Research Center (NWRC) Predator Research Facility in Millville, UT. Parents were all 1 or 2 years of age (1.4 ± 0.1 years [X± SD]) and had no prior parenting experience at the start of the 2011 season. Pairs gave birth to their first litters (n=29 total pups) in 2011, and second litters (n=43 total pups) in 2013. Parent-pup family units were housed in 1000 m^2 outdoor pens from gestation (early January) until dispersal age in the wild (i.e. 15 weeks of age; Bekoff & Wells 1982). Pups were then relocated from their natal pens to outdoor enclosures separate from their parents to reduce parent-juvenile conflict. From December through January, animals were fed 1300g of commercial mink food per pen (Fur Breeders Agricultural Cooperative, Logan, Utah) daily and water was provided *ad libitum* (Brummer et al. 2010). We increased food rations two-fold in February to ensure that pregnant females received adequate nutrition. Food rations increased cumulatively for each pup in a litter according to NWRC standards.

Table 4.1 The five different novel objects provided and presentation order for parent-pup family units over development. The frightening devices were previously used by Darrow & Shivik 2009.

Litter age	2011 objects	2013 objects
6 weeks	Dogzilla® braided rope toy	Toyshoppe® plush frisbee
8 weeks	Boomer Ball coated in food	Dogzilla® chewer bone with peanut butter
10 weeks	Stationary human observer	Flambeau® coyote decoy
12 weeks	Frightening device w/o lights*	Portable box fan
14 weeks	Frightening device with lights	Amber police light beacon

Presentation of prenatal odors

In 2011 and 2013, we provided odor cues for coyote breeding pairs mid-gestation as a proxy for territorial intrusion and increased conspecific density. Detailed methods, behavioral, and hormonal outcomes are detailed in Chapter 2. Briefly, pairs in the odor group (*n*=4) received four different commercial odors developed by Russ Carman (Canine Call®, Pro's Choice®, and two versions of Magna Gland®, New Milford, PA) administered to the interior of their pen. Odor presentation was done every 5 days over a 20-day period (February 28th to March 15th). We provided control pairs with distilled water (n=4). All pairs either received odor cues or water simultaneously. Pairs that were part of the odor group in 2011 became controls in 2013 and vice versa. For this study, we specifically addressed offspring traits in relation to the treatment status of their parents. In addition, we used FGMs and FAMs reported in Chapter 2 to examine the relationships between prenatal hormones and pup behavior.

Novel object tests

Previous work has used novel object tests to quantify boldness and exploration traits in multiple taxa (brown trout, Adriaenssens & Johnsson 2013; cichlid fish, Galhardo et al. 2012; ravens, Stöwe & Kotrschal 2007), including coyotes (Darrow & Shivik 2009; Harris & Knowlton 2011; Mettler & Shivik 2007; Young et al. 2015). We therefore provided each parent-pup family unit with five different novel objects over a 30-minute period from 6 to 14 weeks of age to quantify pup boldness and exploration traits (Table 4.1). Each object test was presented twice weekly, with all objects provided in the same order for all families. We did not choose to randomize the object presentation order because of the potential for increased response variance produced by differences in developmental stage. To ensure that novelty was maintained and

responses were not biased by previous object encounters by parents two years prior, we changed objects used in 2011 to objects with similar qualities in 2013. To reduce coyote wariness, we observed family units from a familiar, neutral NWRC field vehicle that was specifically designated for long-term behavioral studies. We parked the vehicle at a vantage point 50 to 100 meters away from the observed pair. Live on-site observations were coupled with video recordings. The five observers recording behavior were blind to the treatment group of observed family units and were trained to consistently recognize individuals from a distance. At any given time, only two observers (one coding behaviors, one recording behaviors) were present. To reduce inter-observer variation, the same observer (Schell CJ) coded target behaviors for all observations.

For the first two tests, objects were placed directly in the center of the pen. For the last three tests, the observer placed objects against the outer fencing of the pen. In all cases, we presented objects at a location only visible by the tested family. In some cases (e.g. the frightening device, box fan) objects were covered by a cardboard box and pressed against the fencing to ensure that only the observed pen could see the object. We presented objects at 1800-2130 hours MST. Once objects were placed inside or adjacent to the tested family pen, we immediately began the 30-minute test period using all-occurrence methods (Altmann 1974). At test completion, we removed objects from the pen. To quantify boldness we measured: (1) pup emergence or re-emergence from their den hole, (2) whether a pup approached the object (i.e. came within 5 meters), and (3) whether the parents approached the object. Time to re-emergence has traditionally been considered a measure of boldness (Pratt et al. 2005; Seaman & Briffa 2015; Wilson et al. 2010). Measures were considered as both a binary response (i.e. Yes/No), and latencies (in seconds).

Feeding tests

Feeding paradigms have previously been used to assess boldness and aggression traits in several taxa (three-spined stickleback, Bell & Stamps 2004; pumpkinseed sunfish, *Lepomis gibbosus*, Coleman & Wilson 1998), including coyotes (Dawson & Jaeger 2009; Gilbert-Norton et al. 2009a; Gilbert-Norton et al. 2009b). Coyotes in particular are highly cautious and wary of humans (Gehrt 2010; Kitchen et al. 2000; Séquin et al. 2013); therefore individuals willing to feed in front of an observer can be considered increasingly bold or tolerant of human presence. We measured willingness of pups and parents to eat in the presence of an observer from 2 to 14 weeks of litter age to assess boldness and aggression traits in coyotes. Generally, daily food rations are haphazardly scattered throughout their outdoor pens. We modified these feeding events and provided rations in 3 to 6 food piles concentrated at the front half of the pen. To transport food to each pen, we used an all-terrain vehicle used regularly by NWRC care staff to provide food to all animals throughout the facility. A single observer (Schell CJ) conducted feeding tests and was the only individual in sight during testing. After approaching the focal pen, the observer turned off the food vehicle, walked into the pen, provided food rations on the floor inside the pen interior, then walked back out of the pen to sit and monitor feeding behaviors of the focal individuals 2 meters from the pen entrance.

Coyotes have previously demonstrated stereotypic behaviors towards specific individuals and created associations with NWRC care staff (Schell CJ, pers. obs.). Therefore, we had only one observer (Schell CJ) perform feeding observations to eliminate the potential for coyote neophobic responses caused by novel feeders. We used all-occurrence methods over a 7-minute period and gained similar measures compared with novel object testing: (1) whether a pup

emerged or re-emerged from their den holes; (2) whether a pup ate the food provided; and (3) whether the parents ate. We chose a 7-minute observation period because in preliminary feeding observations 7 minutes was the maximum amount of time for coyotes to consume all food provided, regardless of whether all animals ate or a few individuals monopolized food rations. We observed each pen three times over the course of one week. We randomized the order in which pens were observed during each feeding test using a random number generator. Once we began feeding tests at the first focal pen, we continued our observations until all family units were fed. Feeding observations for all pens took approximately 72 minutes (71.9 ± 5.05 minutes [X± SD]). Each family group had 3 feeding tests per week for a total of 13 weeks.

Pup hormones and validation

Extraction and enzyme immunoassay methods had not been previously biochemically validated for the species. In addition, previous studies have demonstrated that hair cortisol concentrations differ as a function of body region (MacBeth et al. 2010). We therefore quantified pup hair cortisol and testosterone using hair samples from captive coyote pups (N=12 pups from 3 families) unrelated to our study subjects. To assess the influence of body location on hair cortisol and testosterone concentrations, we shaved our 12 captive coyote pups in six distinct locations: abdomen, above tail, shoulders, hips, mid-back, and neck. We particularly used pups to quantify body location hormone differences because it was less hazardous for research assistants and staff to handle pups for the extended time necessary to shave all six locations. For each location, we shaved a 4cm area from and stored samples in a plastic bag. Shaving was done with commercially available pet grooming clippers, which were brushed and wiped with 70% alcohol before each shave to avoid cross-contamination from previous samples

(Stalder & Kirschbaum 2012). Experimenters that handled pups also wore gloves to reduce further potential for cross-contamination. Bags were then stored and maintained at room temperature until extraction.

To extract hormones from our hair samples, we washed the hair by combining it with 5.0 ml of 90% methanol (methanol:distilled water), and agitated on a mixer (Glas-col, Terre Haute, Indiana) for 1 minute (setting 50). The methanol was poured off and an additional 5.0 ml were added to the hair. This process was repeated for a total of three times, and then hair samples were placed to individual plastic trays to dry for 3-5 days. Once dry, we cut hair into 2 to 3 mm sections using scissors and removed the follicle before pulverizing the strands to a fine powder (Omni Bead Ruptor 24, settings: 6.8 m/s, four 50 second intervals; Omni International, Kennesaw, GA). We then weighed out 0.2 ± 0.005g of pulverized hair into pre-labeled 16x125mm plastic tubes. Pulverized hair was then combined with 2 ml of 90% methanol, vortexed briefly, and agitated on the Glas-col mixer for 4 hours (setting 50). Tubes were later centrifuged for 15 minutes at 1500 rpm at 10^{o}C, the supernatant was poured into clean plastic tubes, and then dried down under forced air and a hot-water bath (60^{o}C). Once all samples were dried, we reconstituted samples with 500 µl of phosphate-buffered saline (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, NaCl) to produce a 4x concentrated extract. These samples were briefly vortexed, then sonicated for 20 minutes before analysis.

We analyzed hair cortisol using a previously described cortisol enzyme immunoassay (Schell et al. 2013; Santymire et al. 2012). Polyclonal cortisol antiserum (R4866) and horseradish peroxidase were provided by C. Munro (University of California, Davis, California). Cortisol antiserum and horseradish peroxidase were used at dilutions of 1:8500 and 1:20,000, respectively. We also analyzed hair testosterone using a previously described testosterone

enzyme immunoassay (Armstrong & Santymire 2013; Rafacz et al. 2011). Testosterone horseradish peroxidase and polyclonal antiserum were used at 1:30,000 and 1:10,000, respectively. We biochemically validated the enzyme immunoassays by demonstrating parallelism between binding inhibition curves of fecal extract dilutions (8 times concentrated-1:4), the cortisol standard (R^2 = 0.986), and the testosterone standard (R^2 = 0.983). In addition, we found a significant percent recovery (> 90% - Armstrong & Santymire, 2013) of exogenous cortisol (1:4; y = 1.413x – 9.511, R^2 = 0.995) and exogenous testosterone (1:4; y = 1.108x – 7.469, R^2 = 0.962) added to pooled fecal extracts. Assay sensitivity for cortisol and testosterone enzyme immunoassays were 1.95 pg/well and 2.3 pg/well, respectively, and intra- and interassay coefficient of variation was <10% for all enzyme immunoassays.

For our study pups born to known coyote parents, we repeated hair collection and extraction methods from our validation work. We captured pups at 5, 10, and 15 weeks old and collected shaved hair samples to examine pup hair cortisol and testosterone. We shaved pups in one of six individually-specific locations: abdomen, shoulders, hips, mid-back, above tail, or neck. We later used these shave marks as temporary identification markers for feeding and novel object tests. One litter had 7 pups, and we therefore shaved the seventh pup on only one side of its abdomen. We placed shaved hair in plastic bags and kept them at room temperature for subsequent hormonal analyses.

Statistical analyses

We analyzed re-emergence and object approach data primarily as a binary response (i.e. Yes or No) for all individuals, because several individual pups either did not emerge or approach objects. We first calculated the overall proportion of re-emergence and object approach for each

individual pup and parent across the five novel object tests (Table 4.1). We then calculated the average proportion of re-emergence and object approaches for each litter for direct comparison with maternal or paternal object approach proportions. Wilcoxon signed-rank tests were used to compare litters within breeding pairs across prenatal odor treatment groups (i.e. odors versus control) and breeding years (i.e. first-time versus experienced). In addition, we used Wilcoxon signed-rank tests to compare approach proportions of mothers and fathers across prenatal odor treatment groups and breeding years. We subsequently used linear-mixed models (LMMs) to determine how parental approach proportions affected litter variables, particularly because we had repeated measures and were able to set parental identity as a random effect. Litter size was also set as a random effect to control for litter size effects, as we previously observed greater litter sizes with experienced parents (mean = 3.6 ± 1.2 pups) versus first-time parents (mean = 5.4 ± 1.5 pups; $F_{1,15.21} = 31.96$, $P < 0.001$; Chapter 2 & 3), a trend observed in the wild (Sacks 2005).

For feeding tests, re-emergence and eating data were partitioned into four different critical developmental stages (Bekoff & Wells 1982): suckling (2-4 weeks); early weaning (5-7 weeks); late weaning (8-10 weeks); and independence (11-14 weeks). For each developmental period, we measured the number of times that each litter re-emerged and divided that value by the total number of tests performed to get a proportion. We also calculated the proportion of time the individual ate. Proportions were then averaged by each developmental period. The proportion a pup ate did not include when the parents regurgitated food to their offspring. We compared proportions within each developmental period using LMMs setting parental treatment and experience as fixed effects, and parental identity and litter size as random effects. We compared proportion a litter ate food rations with parental proportions to determine whether parental choice

to eat impacted offspring. Similar to the pups, proportion of eating for each parent was calculated as the number of instances the focal parent ate divided by the total number of feeding tests performed.

For pup hair cortisol and testosterone, we used LMMs to observe the impact of prenatal odor cues and parental experience. We have shown that hair cortisol and testosterone in coyotes do not differ according to shave location across the body (Appendix B). However, 5-week old male pups had significantly higher hair cortisol than female pups (Appendix B). We therefore tested for an effect of sex in our models. In addition, we used Spearman rank correlations to examine the associations among hair hormones and recorded behaviors such as object approach (novel object tests) and eating proportions (feeding tests). We also used spearman correlations to examine the associations among maternal and paternal FGMs and FAMs.

All statistical analyses were performed using R version 3.1.3 (R Core Team, 2015). Linear mixed models were performed using the lmer function from the 'lme4' (Bates et al. 2012) and 'lmerTest' (Kuznetsova et al. 2013) packages. We reported results from the best-fit models for all measures, determined using the lowest Akaike Information Criterion (AIC) values (Burnham et al. 2010). Spearman correlations were performed using the corr function from the corrplot package (Wei, 2013). Wilcoxon signed rank tests were performed using the wilcox.test function from the 'MASS' package (Venables & Ripley; 2002). Alpha was set to 0.05 for all cases, and we report data as mean ± S.E. In 2011, two of the four control litters were removed from the study at 10 weeks of age for NWRC-related research work. Four additional pups between 2011 and 2013 died of unknown causes at 6 to 7 weeks of age. Thus, we observed n=72 pups up to 5 weeks of age, n=68 pups up to 10 weeks, and n=60 pups up to 15 weeks.

RESULTS

Novel object tests

We first used LMMs to determine whether parents differed in their re-emergence and approach behaviors, as parental behaviors may have affected offspring behaviors. Both moms ($F_{1,13}$ = 12.22, P = 0.004) and dads ($F_{1,6}$ = 294, P < 0.001) approached more objects when an experienced (mean = 1.2 ± 0.4 objects) versus a first-time (mean = 4.3 ± 0.2 objects) parent. The proportion of objects approached by moms and dads did not differ as a function of prenatal odor treatment (moms: $F_{1,13}$ = 0.69, P = 0.42; dads: $F_{1,6}$ = 0.00, P = 1.00). We did find that object approach proportions of litters were positively associated with maternal ($F_{1,7.9}$ = 18.34, P = 0.003) and paternal ($F_{1,13.4}$ = 29.49, P < 0.001) proportions, indicating that parents that approached more objects had litters that approached more objects.

We then sought to determine whether litters differed in their re-emergence and approach behaviors as a function of parental odor treatment or prior breeding experience. Litters born to experienced parents re-emerged from their dens more often than those born to first-time parents (Wilcoxon: z = 2.366, P = 0.018; Fig. 4.1a). Litters from experienced parents also approached more objects compared with those of first-time parents (Wilcoxon: z = 2.366, P = 0.018; Fig. 4.1c), further indicating that pups approached objects more frequently when parents approached objects more frequently. Odor-treated and control litters did not differ in the proportion of re-emergence (Wilcoxon: z = -0.169, P = 0.866; Fig. 4.1b), nor did they differ in the proportion of objects approached (Wilcoxon: z = 0.676, P = 0.499; Fig. 4.1d).

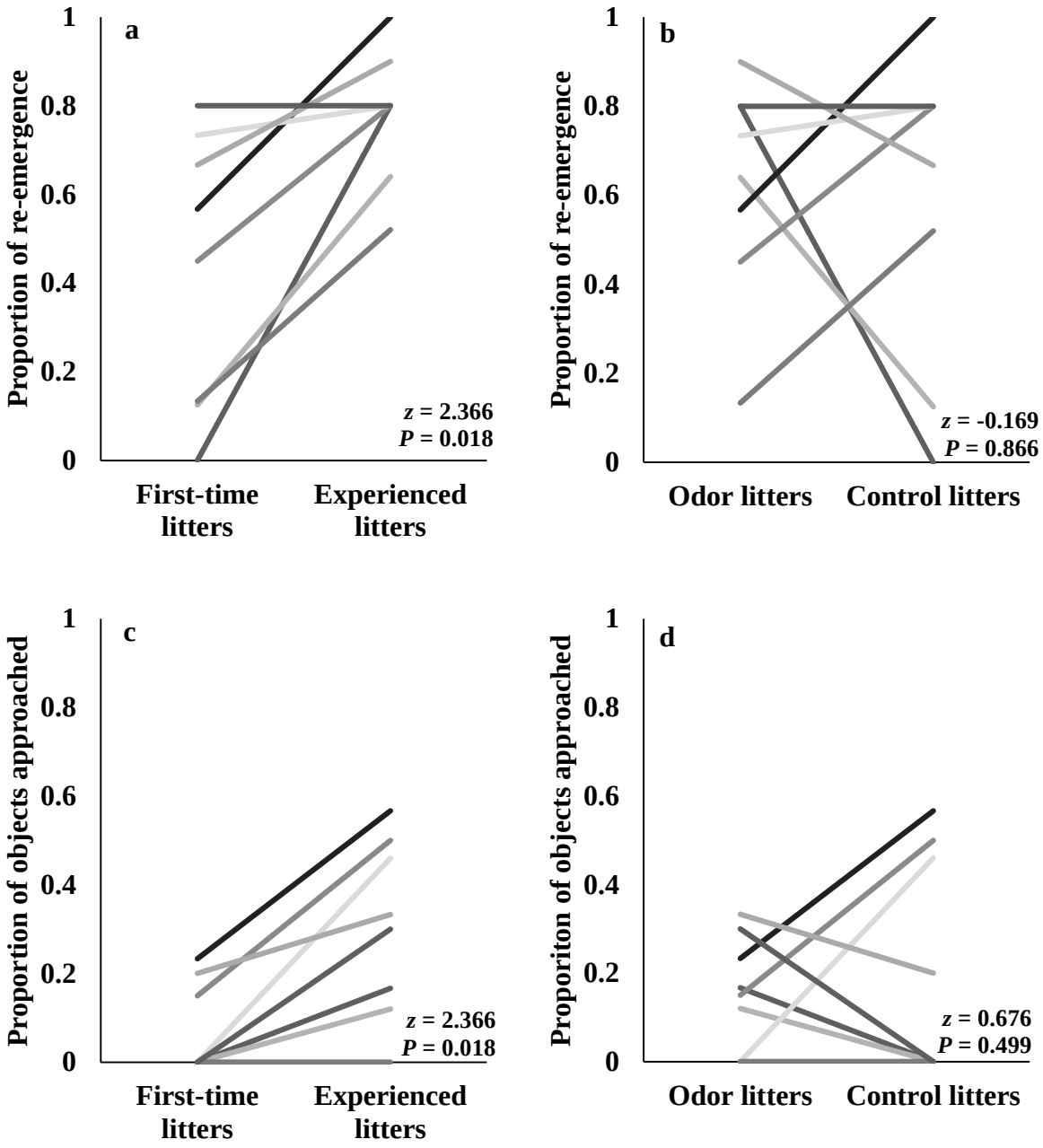

Fig. 4.1: Mean proportion that litters re-emerged from their dens during novel objects tests (a-b); and approached novel objects (c-d). Litter differences are displayed by parental breeding experience (a,c) and prenatal odor treatment group (b,d). Lines represent litters from each individual mother-father pair (N = 8). Wilcoxon sign tests were used to assess statistical significance within pairs, between litters

Feeding tests

Similar to novel object tests, we first used LMMs to determine how parental feeding behaviors were associated with litter feeding behaviors. Mothers that ate more frequently tended to have litters that also ate food rations more frequently during both the early weaning ($F_{1,14}$ = 13.08, P = 0.003) and late weaning ($F_{1,11}$ = 20.95, P < 0.001) periods. Likewise, fathers that ate more frequently tended to have litters that also ate more frequently during the early weaning ($F_{1,8.2}$ = 19.7, P = 0.002) and late weaning ($F_{1,8.6}$ = 8.69, P = 0.017) periods. Both moms and dads generally ate more frequently as experienced (93.8 ± 2.9% of feeding tests) versus first-time (50.5 ± 8.2% of feeding tests) parents (moms: $F_{1,6}$ = 13.65, P = 0.010; dads: $F_{1,6}$ = 42.84, P < 0.001). Dads in particular ate more frequently with a human observer present when a member of the odor-treated group (78.9 ± 8.0% of feeding tests) versus the control group (60.3 ± 14.4% of feeding tests; $F_{1,6}$ = 7.37, P = 0.035). Odor treatment did not affect the proportion of instances mothers ate food rations ($F_{1,6}$ = 0.10, P = 0.77).

Litter re-emergence increased over time for all family units, though there were differences within developmental periods and across time (Fig. 4.2a). Re-emergence and eating were not significantly different by odor treatment ($F_{1,4.6}$ = 4.13, P = 0.10) or experience ($F_{1,7}$ = 1.83, P = 0.22) during the suckling period (i.e. 2 to 4 weeks; Fig. 4.2a). Over the early weaning period, we found that experienced litters ($F_{1,12}$ = 29.80, P < 0.001) and odor-treated litters ($F_{1,12}$ = 10.19, P = 0.008, Fig. 4.2a) emerged more frequently. In addition, there was a significant interaction between the two factors ($F_{1,12}$ = 7.73, P = 0.017), in which odor-treated litters from first-time parents emerged from their dens more frequently than control litters from first-time parents (Fig. 2a). Litters born to experienced parents generally emerged more frequently than those born to first-time parents, regardless of parental odor treatment (Fig. 4.2a).

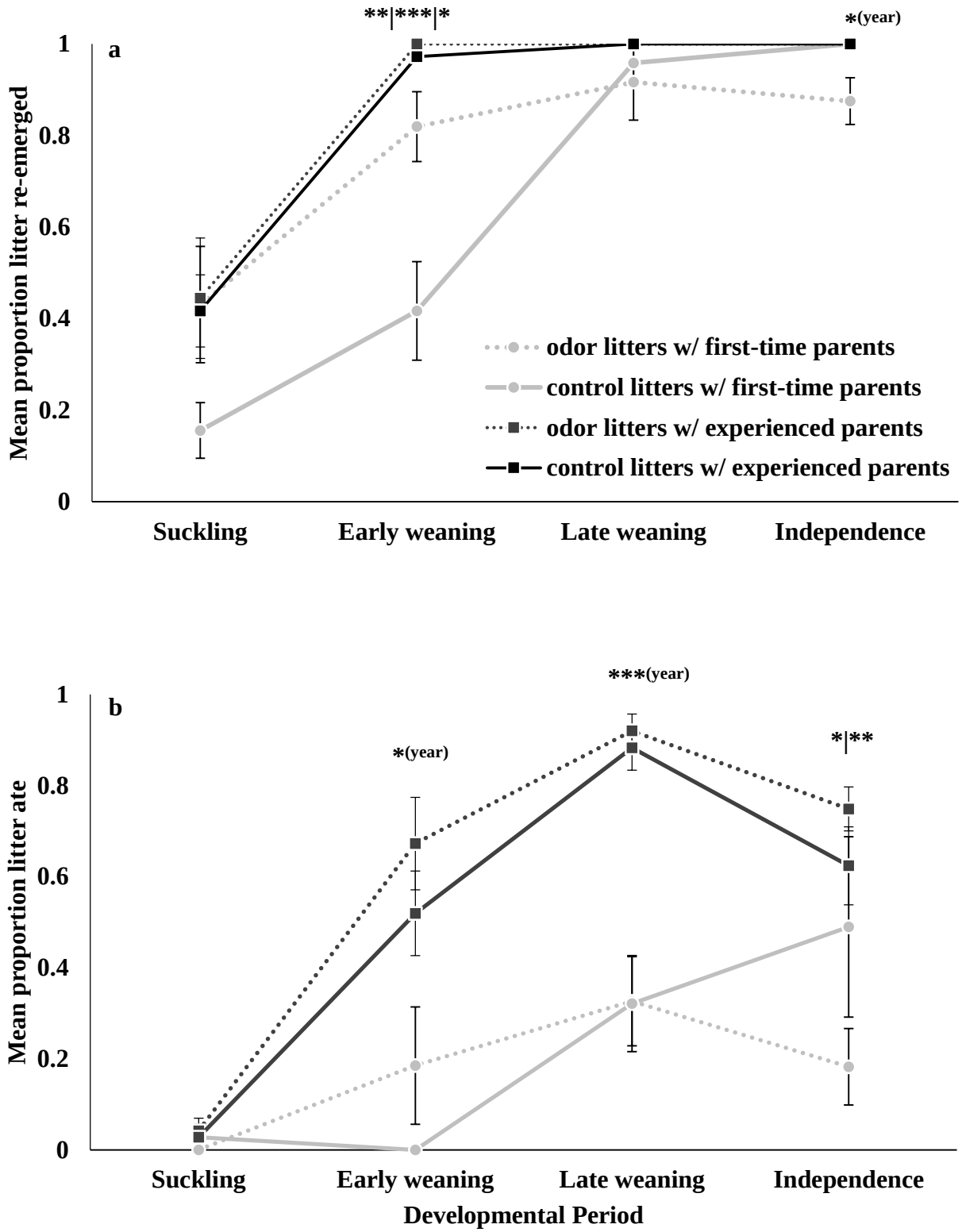

Fig. 4.2: Mean proportion that (a) litters re-emerged from their dens during feeding tests; and (b) ate independently from their parents at food piles with a human observer present. Asterisks indicate significance (P<0.05) for parental treatment | experience | interaction unless noted otherwise in parenthesis. *P<0.05, **P<0.01, ***P<0.001

We found that the mean proportion a litter ate over feeding tests exhibited similar trends to the re-emergence data (Fig. 4.2b). In the early weaning period, litters born to experienced parents ate more frequently than litters of first-time parents ($F_{1,14}$ = 26.2, P < 0.001; Fig. 4.2b). This continued into the late weaning ($F_{1,14}$ = 63.0, P < 0.001) and independence periods ($F_{1,4.4}$ = 8.27, P = 0.04, Fig. 4.2b). Only in the independence period did odor-treated litters eat less than their control counterparts ($F_{1,6.3}$ = 4.94, P = 0.07), though this was not significant (Fig. 4.2b). There were no treatment effects on the proportion a litter ate for any developmental period.

Pup hormones

Because we shaved our study pups in varying locations for identification, we first assessed whether body region influenced our subsequent hormonal measures. We first assessed hair cortisol and testosterone in the unrelated coyote pups previously designated to validate hair hormone differences according to body region. We used a linear mixed model with shave location and sex as fixed effects, and pup identity as the random effect. We found that hair cortisol concentrations did not differ by shave location on the body ($F_{5,50}$ = 0.45, P = 0.81; Appendix C). We also did not observe any differences by shave location for androgen metabolites ($F_{5,50}$ = 1.15, P = 0.35, Appendix C). We did, however, find that male pups had higher hair cortisol compared to females ($F_{1,10}$ = 6.45, P = 0.029; Appendix C). We did not observe sex differences in hair testosterone concentrations of coyote pups ($F_{1,10}$ = 0.94, P = 0.36; Appendix C). There were no interaction effects between shave location and sex for hair cortisol ($F_{5,50}$ = 0.76, P = 0.58) or testosterone ($F_{5,50}$ = 1.87, P = 0.12) concentrations. We therefore proceeded with comparing different shave locations across our original sample population.

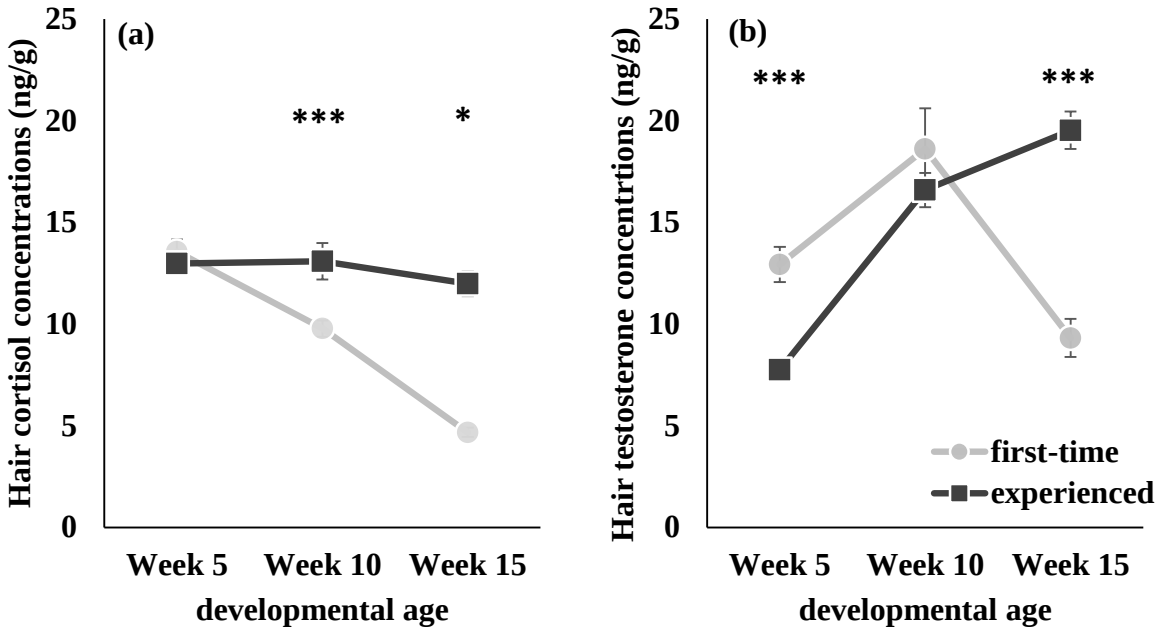

Fig. 4.3: Hair cortisol and testosterone concentrations over developmental time of litters born to first-time and experienced parents. Asterisks indicate significant difference within each developmental period (*P<0.05, **P<0.01, ***P<0.001)

At 5 weeks of age, we found that odor-treated pups had higher hair cortisol compared to control pups ($F_{1,63.9} = 4.83$, $P = 0.032$). By 15 weeks of age control pups had higher hair cortisol ($F_{1,68.0} = 11.15$, $P = 0.001$). We also found breeding year differences, in which litters born to experienced parents had higher hair cortisol at 10 ($F_{1,63.1} = 18.56$, $P < 0.001$) and 15 ($F_{1,68.0} = 5.67$, $P = 0.02$) weeks of age compared with control litters (Fig. 4.3a), indicating that parental parity affected pup hair cortisol. We did not find an interaction effect among odor treatment and breeding year at 5 ($F_{1,7.0} = 0.79$, $P = 0.24$), 10 ($F_{1,6.5} = 0.07$, $P = 0.81$), or 15 ($F_{1,68.0} = 0.59$, $P = 0.45$) weeks of age. We also did not find any differences between male and female hair cortisol concentrations at 5 ($F_{1,67.3} = 3.80$, $P = 0.06$), 10 ($F_{1,67.3} = 0.20$, $P = 0.66$), or 15 ($F_{1,68.0} = 1.00$, $P = 0.32$) weeks of age.

We found that pups from experienced parents had lower hair testosterone compared with pups from first-time parents ($F_{1,47.2} = 15.00$, $P < 0.001$; Fig. 4.3b) at 5 weeks of age. This trend reversed over developmental time, and experienced litters had higher hair testosterone at 15 weeks of age compared with first-time litters ($F_{1,63} = 40.4$, $P < 0.001$; Fig. 4.3b). Litter hair testosterone did not differ as a function of parental odor treatment at any developmental age (5 weeks: $F_{1,61.1} = 1.57$, $P = 0.22$; 10 weeks: $F_{1,60.5} = 0.01$, $P = 0.94$; 15 weeks: $F_{1,63} = 0.23$, $P = 0.64$). We also did not find any differences between male and female pup hair testosterone at 5 weeks ($F_{1,63} = 0.06$, $P = 0.80$), 10 weeks ($F_{1,62.8} = 2.82$, $P = 0.098$), or 15 weeks of age ($F_{1,63} = 1.31$, $P = 0.26$).

Relationships among behaviors and hormones

Several of our measures of pup boldness were correlated across tests, with physiological traits, and with parental hormones at gestation. First, the mean proportion each pup ate food

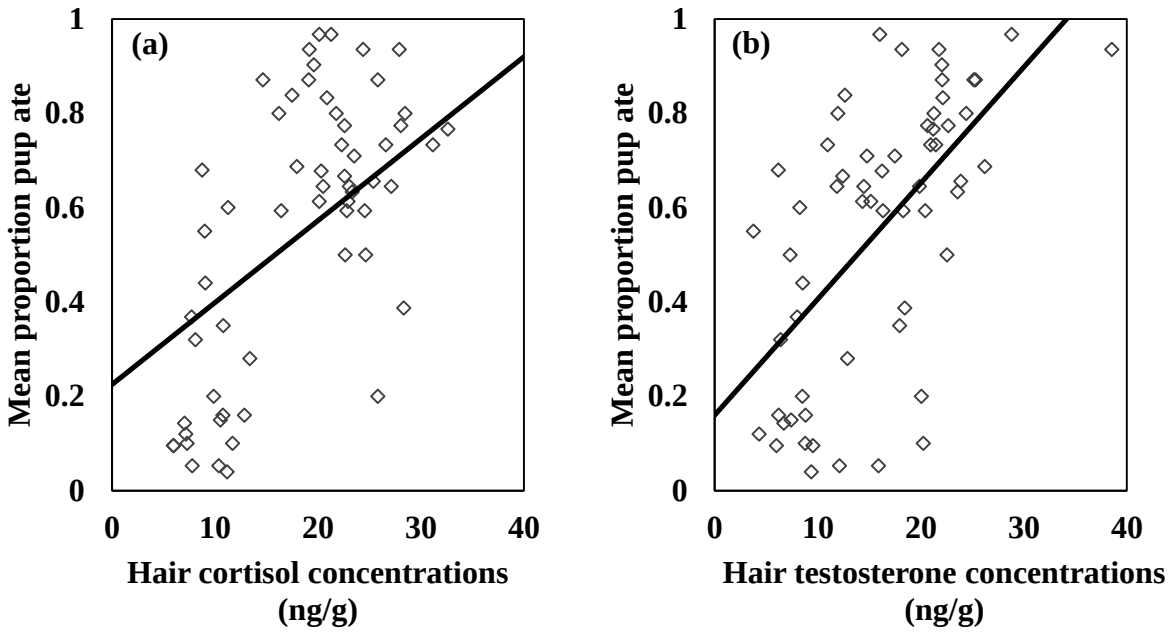

Fig. 4.4: Relationships among mean proportion each pup ate during feeding tests, their hair cortisol concentrations (a) and hair testosterone concentrations (b) at 15 weeks of age. Trend lines indicate significant ($P<0.05$) Spearman rank correlations among variables

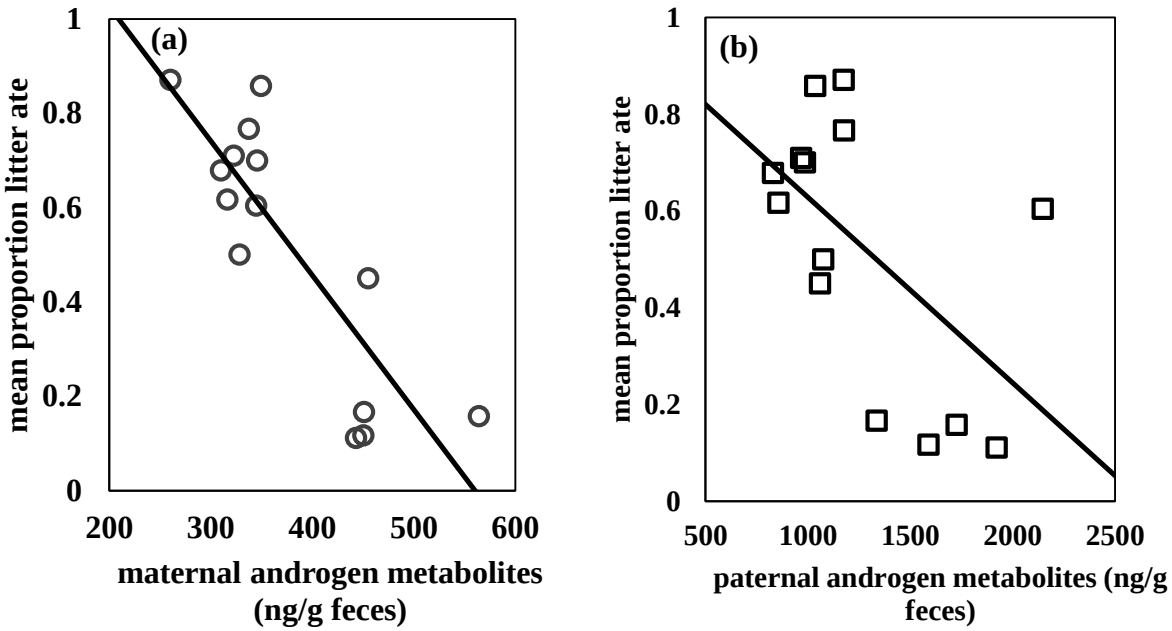

Fig. 4.5: Relationships among mean proportion each litter ate during feeding tests with mean prenatal maternal (a) and paternal (b) androgen metabolites over gestation. Trend lines indicate significance ($P<0.05$).

rations was positively associated with the mean proportion that pup re-emerged during novel object testing (r_s = 0.40, N = 60, P = 0.002). Second, the mean proportion each pup ate was also positively correlated with the proportion of objects approached (r_s = 0.31, N = 60, P = 0.016), indicating that approach behaviors were consistent across test contexts. Third, the mean proportion each pup ate was positively correlated with both hair cortisol (r_s = 0.52, N = 60, P < 0.001; Fig. 4.4a) and hair testosterone (r_s = 0.63, N = 60, P < 0.001, Fig. 4.4b) at 15 weeks of age. Fourth, both hair cortisol and testosterone at 15 weeks of age were positively associated with total emergence (cortisol: r_s = 0.39, N = 60, P = 0.002; testosterone: r_s = 0.48, N = 60, P < 0.001) and object approaches (cortisol: r_s = 0.38, N = 60, P = 0.003; testosterone: r_s = 0.38, N = 60, P = 0.003) during novel object testing, indicating that pups with greater hair cortisol and testosterone were more likely to re-emerge and approach objects. Finally, prenatal fecal androgen metabolites of both parents were negatively correlated with the mean proportion their litter ate during feeding tests (moms: r_s = -0.67, N = 14, P = 0.009, Fig. 5a; dads: r_s = -0.54, N = 14, P = 0.045, Fig. 4.5b). There were no relationships between mean litter proportions and maternal (r_s = -0.32, N = 14, P = 0.261) or paternal (r_s = 0.09, N = 14, P = 0.747) glucocorticoid metabolites.

DISCUSSION

Parents can act as conduits of change by non-genetically transmitting ambient environmental conditions to offspring, effectively contributing to their future fitness (Bonduriansky & Day 2009; Marshall & Uller, 2007; Mousseau & Fox 1998; Räsänen & Kruuk 2007). In this study, we demonstrated that parity and odor cues experienced during gestation influenced boldness, hair cortisol, and hair testosterone of coyote pups over development. Pups born to experienced

parents emerged more frequently from their dens earlier during development. Experienced parents and pups alike ate more frequently while being monitored by a human observer, and approached more human-associated objects. Further, parenting experience played a role in pup hair cortisol and testosterone concentrations, as second litter pups had lower hair testosterone levels early in development, and higher hair cortisol and testosterone levels late in development. Finally, pups demonstrated within-individual consistency in boldness behaviors across feeding and novel object contexts, and parental prenatal androgens coincided with these individual differences. To our knowledge this is the first study to demonstrate that prenatal androgens of both mothers and fathers covary with offspring boldness (Fig. 4.4).

Our results implicate several potential pathways in which coyote parents non-genetically influence their pups. Environmental cues before parturition represent one pathway, and work in a variety of other taxa have elucidated the impact of prenatal exposure to environmental, social, or predatory cues on offspring outcomes (North American red squirrels, Dantzer et al. 2013; spiny damselfish, *Acanthochromis polyacanthus*, Donelson et al. 2009; three-spined stickleback, *Gasterosteus aculeatus*, Stein & Bell 2014). Our current study attempted to assess whether conspecific odor cues would operate as high-density cues for coyotes, in a manner analogous to the presence of extra territorial vocalizations in the red squirrel (Dantzer et al. 2013). The high-density context is relevant for coyotes in both nonnative and natural habitats, as coyote population densities both increase on a natural to urban habitat gradient (Šálek et al. 2014) and with decreased intraspecific competition from wolf populations across the North American continent (Peterson 1996). However, population structure for coyotes are not solely mediated by socio-spatial dynamics of conspecifics at the individual level (Atwood & Gese 2010; Atwood & Weeks, 2003; Sacks et al. 2004; 2005), but by prey availability and dietary resources as well

(Magle et al. 2014; Poessel et al. 2014). Our coyote parents exhibited increased fecal androgen metabolites and territorial behavior in response to the odor cues (Chapter 2), and litters born to odor-treated parents had higher hair cortisol concentrations early during development. All breeding pairs were provided the same amount of food over gestation, indicating that high-density cues alone were effective environmental cues to augment parental fecal androgens associated with offspring physiological traits. Nevertheless, future work could experimentally manipulate prey availability to assess impact of dietary resources on pre-partum parental hormones.

Parental experience also appeared to be a significant factor affecting parental affects in coyotes. Second-litter pups approached more novel objects (Fig. 4.1c), ate more frequently in front of an observer (Fig. 4.2b), had increased hair cortisol throughout development (Fig. 4.3a), and decreased hair testosterone at 5 weeks of age (Fig. 4.3b). Note that experienced parents also had lower pre-partum fecal androgen metabolites and were bolder over development: mothers and fathers approached more novel objects and ate more frequently as experienced parents. Being a first-time parent both presents challenges related to the novelty of raising offspring while simultaneously responding to any potential environmental changes. When environmental conditions are stable and predictable over time, parental response likely confers benefits to offspring programming their future success (Mousseau & Fox 1998). Our coyote parents had a maximum of 1.5 years at the captive facility during the first breeding event, with an additional 2 years by the time they were second-time parents. Consequently, that increased time at the facility allowed them multiple opportunities to observe people and adjust their behavior accordingly. Previous work has demonstrated that coyotes learn from human patterns of behavior and modify

their activity in response (Gehrt 2010; Séquin et al. 2003). Increased parental familiarity with human patterns and associated contexts may have likely been transferred to second-litter pups.

The transmission of boldness and human tolerance was likely further mediated by both indirect and direct learning experiences. Increased frequency of eating by parents during feeding tests positively correlated with the frequency that each pup ate for both the early and late weaning periods. Likewise, parental object approach corresponded with increased pup re-emergence and object approach. In these examples, parents may have indirectly increased pup boldness by providing visual confirmation of safety, i.e. the lack of negative consequences for interactions with human-associated items. Experiential learning may have subsequently occurred when pups ate or interacted with objects personally. Personal experiences of parents may have therefore informed their future behaviors, which in turn facilitated increased boldness of both pups and parents. Our results generally support the theory of context-dependent parental effects previously suggested by Reddon (2011). More specifically, our coyotes appear to demonstrate what Reddon (2011) referred to as anticipatory parental effects in which parents act to bias their offspring toward phenotypes best suited to environments they are likely to face. This type of context-dependent parental effect is observed most often when environmental conditions are predictable or stable over time. Anticipatory effects have been demonstrated in several other taxa (rhesus macaques, *Macaca mulatta*, Hinde et al. 2014; yellow baboons, Onyango et al. 2008; snowshoe hares, Sheriff et al. 2010; three-spined sticklebacks, Stein & Bell 2014). For this study, other than weather conditions and occasional relocation of animals, the clover pens that housed coyote family units were relatively stable and predictable for coyote parents. Thus, increased experience within the captive setting may have allowed coyote parents to bias boldness in their offspring to capitalize on predictable environmental cues. Indeed,

experienced parents produced pups that ate more frequently in front of an observer (Fig. 4.2) and approached human-associated objects more often (Fig. 4.1), an indication that anticipatory parental effects are present.

Remarkably, we found that prenatal testosterone of both parents were closely associated with several pup outcomes. Pup object approach, re-emergence, and feeding behaviors were all associated with parental pre-partum androgens, suggesting that variation in personality traits such as boldness are affected by maternal androgen concentrations *in vitro*. Androgen-mediated parental effects on offspring behaviors and hormones have also notably been documented in a related carnivore, the spotted hyena (Dloniak et al. 2006; Höner et al. 2010). Specifically, elevated prenatal androgens of spotted hyena mothers are linked to high social rank, which positively corresponds with cub aggression and mounts during development (Dloniak et al. 2006). Aggression and mounts in cubs can impact the social standing of that individual, ultimately affecting the resources and potential mates available to them as adults. Further, it has been demonstrated that sons of high-ranked mothers both had faster growth rates and were more likely to disperse to neighboring hyena clans and sire offspring (Höner et al. 2010). The female dominance structure of hyena clans makes successful breeding opportunities difficult to obtain for males (Holekamp & Dloniak 2008). Thus, increased growth rates, aggression, and reproductive success imparted by high-rank mothers to male cubs have direct consequences for the fitness of those developing offspring (Holekamp & Dloniak 2008). There are several similarities between the coyote and hyena social and parental systems (e.g. structured hierarchies among packs or clans, extended care of young) that suggest prenatal testosterone in coyotes may also represent an integral mechanism underlying phenotypic variation in the species. We could not directly assess whether feeding and risk-prone behaviors of coyote pups had long-term

fitness consequences, because our study subjects were captive. The behaviors observed, however, have previously been hypothesized to directly impact the adaptation and expansion of coyotes into nonnative such as urban areas (Gehrt 2010). We may therefore consider that androgen-mediated parental effects facilitate coyote population adaptation, and pre-partum or environmental factors can affect those androgen-mediated parental effects.

Our study was unique is that we found parental prenatal androgens of both mothers and fathers were negatively correlated with feeding behaviors of litters. Previous work has demonstrated the cumulative or compensatory impacts of biparental effects, the effects of external stressors on breeding pairs, and resultant offspring outcomes (Harris et al. 2013; Lock, 2012; Schuett et al. 2013; Schweitzer et al. 2014). Our previous work demonstrated how prenatal glucocorticoids and androgens covary among expectant parents within a breeding pair (Chapter 2), similar to previous work cotton-top tamarins (*Saguinus oedipus,* Almond et al. 2008; Ziegler et al. 2004). Paternal androgen metabolites of coyotes, however, could not directly impact offspring *in vitro*, which suggests that hormone profiles of fathers may have been sensitive to cues provided by expectant mothers. Thus, even if paternal traits were not directly transmitted to offspring *in vitro,* hormonal synchrony of mothers and fathers may have resulted in a feedback mechanism that further shaped maternal traits and subsequently influenced developing pups. Indeed, our previous work demonstrated that pre-partum hormones of coyote parents are strongly associated with their parenting styles (Chapter 3). Both the hormonal and behavioral synchrony between coyote parents therefore suggests that they produced a comprehensive influence for developing offspring.

Pup feeding and emergence behaviors were positively correlated with both hair cortisol and testosterone concentrations, suggesting that boldness traits of pups are mediated by their

physiology. Previous work on female vervet monkeys (*Chlorocebus aethiops sabaeus*), also demonstrated an association between hair cortisol concentrations and boldness, although vervets with lower concentrations tended to score higher in novelty-seeking (Laudenslager et al. 2011). We also found that hair cortisol of pups generally decreased over time, a trend previously reported in vervet monkeys (Laudenslager et al. 2012). Most notably, we found that pups born to experienced parents had higher hair cortisol and testosterone at 15 weeks of age (Fig. 4.3), implicating parental effects and parity in shaping hormonal traits of offspring that are connected to their behavioral traits. Studies on other taxa have demonstrated similar findings, in which environmental stressors experienced by parents pre-partum affect hormonal (vervet monkeys, Fairbanks et al. 2011; guinea pigs, Kemme et al. 2007; yellow baboons, Onyango et al. 2008) and behavioral traits (spotted hyenas, Dloniak et al. 2006; rhesus macaques, Hinde et al. 2004) of offspring. These widespread effects implicate the importance of endocrine mechanisms as potential regulators of both personality traits and parental effects. In an extensive review across an array of taxa, Duckworth (2015) previously suggested that hormonal mechanisms are not only integral factors underlying behavioral flexibility and personality, but also shape parental programming across generations and subsequently dictate evolutionary trajectories of populations. Consequently, it is likely that hormonal mechanisms are at the forefront of coyote adaptation to nonnative habitats, as both pups and parents demonstrate myriad associations among behaviors and hormones.

Boldness measures in pups were both positively correlated across contexts (i.e. feeding and novel object tests). Previous work in the species has consistently highlighted how individuals readily demonstrate differences in risk-aversion and exploration (Darrow & Shivik 2009; Gilbert-Norton et al. 2009a; 2009b; Harris & Knowlton 2001; Mettler & Shivik 2007). Select

studies have also provided evidence to suggest that coyotes have behavioral syndromes for boldness and exploration (Dawson & Jaeger 2009; Young et al. 2015), as well as territoriality and parenting behaviors (Chapter 2 & 3). Our current study is novel because we demonstrated that coyotes demonstrate behavioral syndromes for boldness as early as infancy. Still to be determined, however, is whether the behavioral traits we observed here are stable over the lifetime of an individual coyote. This is critical, as it would provide further evidence to suggest that parental effects have long-term fitness consequences for developing offspring. Both Stamps & Groothuis (2010) and Stamps (2015) have highlighted the importance of considering ontogeny on personality trait expression, particularly because external stimuli experienced over development can significantly change the physiological or behavioral traits of an individual into adulthood. If in fact traits of coyote offspring are consistent into adulthood or affect adult trait expression, then the gravity of pre- and post-partum parental effects we have observed here becomes increasingly relevant.

Parental effects in coyotes operate both at the pre- and post-partum stages to affect boldness and hormonal physiology of pups. The coyote system demonstrated cumulative parental effects in which androgens of both parents influenced pup traits in the same direction. Androgens appear to be key underlying hormonal mediators of non-genetic trait transmission. In addition, parenting experience and behavior indirectly influenced pup boldness over development. It is therefore reasonable to suggest that changes in care strategies across time also mediate trait transmission to offspring. Pups demonstrated behavioral syndromes for boldness across test contexts. This is one of the first non-human studies to demonstrate that infants exhibit personality early during development. If in fact personality traits observed during infancy reflect adult behavioral outcomes in coyotes, this creates a sizeable potential for multigenerational

transmission of boldness. Further, behavioral consistency of pups over time would indicate that coyote parents affect primary behavioral traits (i.e. boldness) previously hypothesized to influence coyote adaptation to nonnative habitats such as urban areas (Gehrt 2010). Duckworth et al. (2015) recently provided empirical evidence to support this hypothesis, as dispersal and successive colonization into post-fire habitats by mountain bluebirds (*Sialia currucoides*) then western bluebirds (*Sialia currucoides*) is an indirect consequence of aggressive behavioral types within each species. These behavioral types were primarily explained by parental effects, indicating that cycles of colonization and community structure are a product of parental effects mechanisms (Duckworth et al. 2015). It is possible that coyote populations follow similar biological constructs, because the species' geographic range has continually expanded beyond historical limits. Coyote expansion and adaptation to human-associated landscapes may very well be facilitated by parental effects.

CHAPTER 5: DEVELOPMENTAL EXPERIENCES PREDICT YEARLING PERSONALITY TRAITS AND ANDROGENS IN COYOTES

INTRODUCTION

Individual differences in behavior (i.e. animal personality, temperament, behavioral types, etc.) intimately affect individual fitness, group-level dynamics, and ecological patterns (Biro & Stamps 2008; Dingemanse & Réale 2005; Dingemanse et al. 2010; Réale et al. 2007; Sih et al. 2004; Sih et al. 2012). Previous work has demonstrated how increased individual aggression and boldness may affect group-level reproductive success and dispersal (Bridger et al. 2015; Sih & Watters 2005). For instance, heightened conspecific aggression of single male water striders (*Aquarius remiges*) alters social group composition and affects the reproductive success of neighboring individuals (Sih & Watters 2005). For male hermit crabs (*Pagurus bernhardus*), increased boldness (i.e. latency to respond toward a startling stimulus) negatively correlates with spermataphore size implying that boldness reduces fecundity (Bridger et al. 2015). Further, mosquitofish (*Gambusia affinis*) populations with more asocial or bolder individuals have higher dispersal rates by group members irrespective of the personality type of the dispersing individual (Cote et al. 2011). Personality also affects individual survivorship. For example, less active juvenile brown trout suffer greater mortality (Adriaenssens & Johnsson 2013), and increasingly risk-averse captive-bred swift foxes (*Vulpes velox*) suffer greater mortality six months following reintroduction into the wild (Bremner-Harrison et al. 2004). Lastly, personality traits may have trans-generational effects that influence offspring development (Reddon 2011). For example, more aggressive and defensive tree swallow males (*Tachycineta bicolor*) fledge more young (Betini & Norris 2012).

It is important to consider the proximate mechanisms that influence personality traits given the myriad fitness consequences such traits have for the individual. External factors such as weather or predation pressures are a few factors previously documented. For example, small within-day increases in temperature increase individual boldness, activity, and aggression for two species of juvenile coral reef fish (damselfish, *Pomacentrus moluccensis* and *Pomacentrus bankanensis*; Biro et al. 2010). Exposure to predation induces strong correlations between aggressiveness, activity, and boldness in three-spined stickleback (*Gasterosteus aculeatus;* Bell & Sih 2007), and the absence of predation relaxes those behavioral relationships (Dingemanse et al. 2007). Increases in conspecific densities and female movement result in increased courting by male sand fiddler crabs (*Uca pugilator*), which correspond with consistent individual differences in boldness (Pratt et al. 2005). Further, drastic seasonal changes from the dry to wet seasons decrease risk-taking behaviors in Namibian rock agamas (*Agama planiceps*), all of which readily demonstrate consistent individual differences in boldness (Carter et al. 2012).

Though it is evident that personality has consequences for the individual and beyond, few existing studies address the developmental mechanisms shaping personality and the stability of behavioral traits across different life stages (Hoeve et al. 2013; Weintraub et al. 2010). Moreover, studies that have addressed the impact of early life experience on behavioral traits primarily focus on laboratory rodents with limited detail on juvenile behavioral traits and corresponding traits at the adult stage (Stamps 2015). Current personality traits have been theorized to be intimately affected by past experiences (Stamps 2015; Stamps & Groothuis 2010a, 2010b). Stamps (2015) describes the influence of past experiences on observed personality traits as ontogenetic plasticity which recognizes that early life experiences may predict the expression of adult personality traits. If in fact developmental experiences affect

behaviors that remain relatively stable into adulthood, then those experiences represent a catalyst for personality. Here, we investigated coyotes over multiple years to address whether behaviors and hormonal traits observed in pups predicted congruent traits at the yearling stage.

Coyotes are an intriguing study organism to address consistency of personality traits across developmental time. Studies have previously demonstrated individual differences in foraging behaviors in response to unpredictable changes in food location (Gilbert-Norton et al. 2009a; Gilbert-Norton et al. 2009b) or ambiguous anthropogenic threat (Dawson & Jaeger 2009). Consistent individual differences in risk-aversion to startling light and sound stimuli have also been documented (Darrow & Shivik 2009). Because behavioral flexibility has previously been hypothesized to facilitate coyote adaptation to nonnative habitats (Gehrt 2010), individual differences in behavior may be implicated in adaptation. Several studies have observed flexibility in landscape use patterns (Gehrt et al. 2009; Grubbs & Krausman 2009), diet (Morey et al. 2007), and activity budgets (Grinder & Krausman 2001; Kitchen et al. 2000; Séquin et al. 2003) that corroborate the crucial role that behavioral plasticity plays in coyote adaptation to urban and nonnative environments. Further, we have demonstrated that parental experience and prenatal androgens increase boldness in developing offspring (Chapter 4), implicating parents as key components that shape personality traits across generations. Examining the role of early life experiences and the stability of traits over developmental time thus provides an adequate measure of long-term, non-genetic inheritance for target traits that may impact coyote survival.

For this study, we observed coyotes at the pup stage (5 to 15 weeks of age) and again at the yearling stage (1 year old) to examine the consistency of behavioral and hormonal traits across time. Our previous work demonstrated that boldness, hair cortisol and testosterone concentrations were differentially associated with pre-partum androgen metabolites of both

parents in addition to prior parenting experience (Chapter 4). Here, we repeated both feeding and novel object tests previously experienced by yearling coyotes as pups to quantify consistency of behavioral traits. We asked if behavioral traits observed during the pup stage correlated with yearling stage behaviors and if correlated behavioral suites were individually consistent. We also tested whether hair cortisol and testosterone concentrations at the pup stage positively correlated with fecal glucocorticoid (FGMs) and fecal androgen (FAMs) metabolites at the yearling stage. Coyote yearlings were born to parents that were previously exposed to foreign conspecific odor cues (i.e. Russ Carman scent lures) in an effort to augment prenatal hormones that would potentially have a cascading effect on offspring development (Chapter 2). Parents of study yearlings also demonstrated individual parenting styles that varied with prior breeding experience (Chapter 3). We therefore considered parental odor exposure, prenatal hormones, and breeding experience as potential parental factors associated with observed yearling traits.

METHODS

Subjects

We observed a subset of coyote pups born in 2011 and 2013 (Chapter 4) again as yearlings in 2012 and 2014 (13 males and 14 females). Yearlings were housed at the United States Department of Agriculture National Wildlife Research Center (NWRC) Predator Research Facility in Millville, UT in 1000 m^2 outdoor "clover" pens previously used for extended behavioral observations (Gilbert-Norton et al. 2009a; 2009 b; Darrow & Shivik 2009; Chapters 2-4). Pups were either born to first-time parents in 2011 (6 males and 7 females, from 8 breeding pairs) or experienced parents in 2013 (7 males and 7 females, from 8 breeding pairs), and all

individuals were 1 year old at the onset of the study. Individual coyotes at the NWRC are customarily paired with another unrelated coyote of the opposite sex at 20 weeks of age in accordance with NWRC regulations for long-term studies. We therefore randomly paired our yearlings amongst each other when subjects were 20-weeks old. Yearling pairs were maintained until the onset of the breeding season (mid-December) and were temporarily separated until early April to eliminate the possibility for study individuals to breed (breeding season extends from late December to February; Bekoff & Wells 1982; Carlson & Gese 2008). Yearling pairs were reunited mid-April and observed over a 7-week period from April to June in both 2012 and 2014. To control for biased behavioral responses of individuals as a function of familiar environmental settings, all pairs were reunited in pens they had not previously inhabited. Prior experience with a mate may likely have reduced potential conflict that occasionally occurs when unfamiliar conspecifics are forming new bonds (Bekoff & Wells 1982). Nevertheless, we controlled for mate identity in our statistical analyses as personality differences from the partner yearling may have influenced behavior of the focal individual (see Statistical Analyses). A single female yearling (ID: 1172) born in 2011 did not have a male counterpart yearling and was therefore paired with a 2-year old unrelated male. Study animals were fed 1300g per pen of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, Utah) daily and water was provided *ad libitum* according to NWRC regulations.

Feeding tests

Similar to Chapter 4, we measured willingness of each yearling to eat in the presence of an observer to quantify boldness and other personality traits. We provided daily food rations in 3-6 food piles concentrated at the front half of the pen. We used an all-terrain vehicle customarily

used by NWRC care staff to provide food to all captive animals at the facility. A single observer (Schell CJ) conducted feeding tests and was the only individual on animal grounds during testing. For each focal pen, the observer parked the food vehicle approximately 3 meters in front of the pen entrance and walked into the pen to deposit food rations along the floor. The observer then walked back out of the focal pen to sit and monitor feeding behaviors from the parked (and powered-off) vehicle. Subjects willing to feed in front of an observer were previously categorized as increasingly bold or tolerant of human presence.

We used a mixed-scan sampling design over a 5-minute period with 15-second intervals (Altmann 1974) for 6 days per week. At each interval we recorded state behaviors (e.g. eating, walking, running, sitting, etc.) that were later used to assess activity and other personality traits. We also coded target behaviors opportunistically (Table 5.1). Similar to Chapter 4, we recorded whether an individual chose to eat or not as a binary response (Y/N) as well as the latency to eat (Table 5.1). Feeding tests began the moment that the observer left the pen and all occurrence behaviors were coded immediately after exiting the pen. Each yearling had distinct coat patterns, facial features, and tail type that we used as identifying markers during feeding tests. We frequently changed the order in which pens were tested, and once we began feeding tests at the first focal pen, observations continued until all yearling pairs were fed. The onset of feeding tests began at varying times each day. Feeding tests for all pens took approximately 42 minutes (41.4 ± 3.14 minutes [X±SD]. Only one observer performed feeding tests to eliminate the potential for coyote neophobic responses caused by novel observers (Chapter 4). We began feeding tests the day we paired yearlings, and tests continued for the entire 7 weeks of observation in 2012 and 2014.

Table 5.1 Behavioral ethogram used during the feeding and novel object tests (behaviors adapted from Bekoff & Wells 1986; Gese & Ruff 1997; 1998).

Behavior	**Description**	**Test Context**
Latency to eat	Length of time (in seconds) before individual ingests food	Feeding test
Proximity	Proportion of time individual is <5 meters from food pile or object	Both
Eating	Proportion of time individual ingests food at provisioned food pile	Feeding
Approaches	Number of instances individual gets within ≤5 meter from object	Novel object
Stationary	Proportion of time individual sits or stands	Both
Locomotion	Proportion of time individual is active and moving	Both
Resting	Proportion of time individual lies down	Both
Sniffing	Individual directs nose to the ground and investigates for ≥1 second	Both
Urination	Discharges urine	Both
Ground scratching	Digs and kicks down and backward; often follows urination	Both
Aggression	Teeth baring, growling, and/or physical confrontation directed toward pair-mate	Both
Vigilant stares	Stares directly at a novel object OR human observer for ≥3 seconds	Both
Play	Individual is engaged in a play bout (>1 minute, see Bauer & Smuts 2007) with pair-mate	Novel object

Table 5.2 The four objects presented to yearlings that were previously provided to them as pups (Chapter 4).

Presentation Order	2012 yearling objects	2014 yearling objects
1st	Dogzilla® braided rope toy	Toyshoppe® plush frisbee
2nd	Boomer Ball coated in food	Dogzilla® chewer bone with peanut butter
3rd	Frightening device w/o lights*	Portable box fan
4th	Frightening device with lights	Amber police light beacon

Repeated object tests

We presented yearlings with objects they had previously experienced as pups (Table 5.2) to further quantify behavioral traits. In addition, we presented yearling pairs with the objects in the same order as they originally experienced them during the pup stage. We did not choose to vary object presentation order because we were specifically interested in repeatability of measures from the pup stage into the yearling stage. The object order was the same for all trials. To reduce coyote wariness and ensure responses directed toward presented objects, we observed yearling pairs from a familiar and neutral field vehicle at the NWRC specifically designated for long-term behavioral studies. We parked the vehicle at a vantage point 50-100 meters away from the focal yearling pair. Live on-site observations were supplemented by with secondary video recordings. Two observers were on-site during tests at any given time, with one individual coding behaviors and the other video recording the tests. Five total observers recorded behavior and were blind to the past experiences of individuals, their parentage, and related conspecifics. All observers were also trained and proficient in recognizing individuals from a distance using the coat differences previously mentioned.

We presented objects to yearlings once per week. The first two objects (e.g. plush dog toys and food-associated toys) were placed directly in the center of the pen, whereas the last two objects (e.g. large boxed items and flashing lights) were placed adjacent to the outer fencing of the pen. In all cases, we presented objects at a location only visible to the tested pair. To ensure this, we covered the last two objects with a cardboard box and pressed the box against the fencing to ensure that only the focal pair could see the presented object. Yearling pairs were presented with each object over a 30-minute period, and we used a mixed-scan sampling design with 30-second intervals (Altmann 1974) to record state behaviors as in feeding tests. The

behaviors of interest are detailed in Table 5.1. We immediately began the 30-minute observation period the moment that the object was placed inside the pen or adjacent to it. We measured whether each yearling approached the object (i.e. when an individual came within 5 meters of an object) as a binary response (i.e. Yes/No) and the latency to approach within the 30-minute period (Table 5.1). A lack of object approach was therefore coded as '1800 seconds'. We presented all objects to yearlings at 1800-2130 hours MST, which corresponds to the time of peak activity (Gehrt 2010). Once each test was completed, we removed objects from the pen. We presented objects once per week over the final 4 weeks of study in 2012 and 2014.

Fecal sample collection, processing, and analysis

We collected fresh fecal samples twice weekly from yearling pairs to quantify glucocorticoids and androgens. Sampling methodology closely followed that previously used (Chapter 2). Specifically, we mixed multi-colored glitter particles into surplus mink food and partitioned the food into small biscuits that were later frozen at -20^{o}C. We then provided these mink food biscuits to each individual in a yearling pair simultaneously the evening prior to sample collection. Individuals opportunistically ate food biscuits and the color of the biscuit ingested was recorded. Glitter-marked feces retained their color once excreted the following morning allowing us to identify samples by individual and whether those samples were fresh. We also assessed freshness by appearance, odor, and stiffness in response to desiccation. We restricted our sample collection between 0600 and 1000 hours MST because fecal cortisol metabolite output varies diurnally in coyotes (Schell et al. 2013). We immediately stored samples at -20^{o}C to reduce hormone metabolite degradation associated with differential bacterial breakdown of metabolites across fecal samples (Goymann 2012). Further, we collected multiple

samples for each sampling period (n=4 per day per pair) to ensure we collected suitable fresh samples for each individual yearling. For each object test specifically, we collected samples before and after object presentation to examine whether yearlings exhibited acute increases in FGMs in response to the object provided. Our previous work demonstrated that coyotes have pronounced FGM responses to human-associated disturbances (Appendix A; Schell et al. 2013). We therefore considered our human-associated objects as potentially relevant biological stressors. All samples were later shipped overnight on dry ice to the Lincoln Park Zoo Endocrinology Laboratory (Chicago, Illinois) for hormonal analyses.

We freeze-dried the samples on a lyophilzer (Thermo Modulyo Freeze Dryer; Thermo Scientific, Waltham, Massachusetts) for 3 days then crushed the samples to a fine powder. Hormone metabolite extraction followed previously described methods (Appendix A; Schell et al. 2013). Specifically, we weighed sample powder (0.2 ± SD 0.02 g) then combined the weighed out sample with 5.0 ml of 90% ethanol (ethanol:distilled water). The samples were agitated on a mixer for 30 minutes (Glas-col, Terre Haute, Indiana, setting: 60) and subsequently centrifuged for 15 minutes at 1500 rpm and 10^{o}C. We poured the sample liquid into a second set of corresponding clean glass tubes. Fecal pellets in the original test tubes were then resuspended in 5.0 ml of 90% ethanol, vortexed for 30 seconds, and re-centrifuged for 15 minutes at 1500 rpm. The supernatant was once again poured into the second set of glass tubes and dried down under air and a hot-water bath (60^{o}C). We then reconstituted dry samples 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 minutes before analysis.

We used a previously validated cortisol enzyme immunoassay (EIA, Appendix A; Schell et al. 2013) to quantify yearling coyote FGMs. Polyclonal cortisol antiserum (R4866) and

horseradish peroxidase (HRP) were provided by C. Munro (University of California, Davis, California). Cortisol antiserum and cortisol HRP were used at dilutions of 1:8500 and 1:20,000, respectively (Santymire and Armstrong 2010; Schell et al. 2013). Assay sensitivity was 1.95 pg/well and intra- and interassay coefficient of variation was <10%. We also used a previously validated testosterone enzyme immunoassay (Chapter 2) to measure coyote FAMs. Testosterone HRP and polyclonal antiserum were used at 1:30,000 and 1:10,000, respectively (Armstrong & Santymire 2013; Chapter 2). Assay sensitivity was 2.3 pg/well and intra- and interassay coefficient of variation was <10% for the testosterone EIA.

Statistical analyses

We used a likelihood ratio test (LRT) to determine whether yearlings were individually consistent across developmental stages (i.e. pup to yearling stage) in the proportion of food rations they ate with a human observer present. The likelihood ratio test compares a linear regression model with only fixed effects to a linear-mixed effects model (LMMs) that contains the same fixed terms but with subject identity as the random factor. Statistical significance between the models indicates that the variance observed in the dependent measure is repeatable and best approximated by the designated random term (Crainiceanu & Rupert 2004; Schielzeth & Forstmeier 2009). The likelihood ratio test computes the LRT test statistic using restricted maximum likelihood that follows a chi-square distribution and produces a probability value based on 10,000 simulated iterations of the dataset (Crainiceanu & Rupert 2004; Schielzeth & Forstmeier 2009). The likelihood ratio test has previously been used to effectively quantify trait consistency and repeatability for several other taxa (tree swallows, Betini & Norris 2012; Namibian rock agama, Carter et al. 2012; Belding's ground squirrels, *Urocitellus beldingi*,

Dosmann & Mateo 2014). Similar to previous studies, we arcsine square root transformed proportional data (Betini & Norris 2012; Carter et al. 2012; Dosmann & Mateo 2014). We specifically compared the mean proportion an individual pup ate (i.e. the number of instances an individual ate/the total number of tests) from the pup stage with the mean proportion that same individual ate as a yearling at two descriptive time periods: the acclimation and testing periods. The acclimation period encompassed the first 2 weeks when yearlings were reunited with their respective mates. The testing period encompassed the remaining 5 weeks of observation when yearlings were presented with repeated objects. For LMMs testing consistency of eating proportions across developmental time, we considered developmental stage (i.e. pup, yearling) and parental breeding experience (i.e. year) as the main fixed effects. We anticipated that coyote mate identity may influence behavioral measures recorded during the yearling stage. We therefore used additional likelihood ratio tests to assess whether significance in the proportion an individual coyote ate was attributed to either coyote identity or mate identity.

Many of the variables coded during yearling feeding tests (Table 5.1) were highly correlated. We therefore used principal components analysis (PCA) on yearling test data to reduce the list of variables into uncorrelated components describing the majority of variance across yearlings (Abdi & Williams 2010). Further, previous studies have used PCA to efficiently summarize suites of correlated behavioral traits to assess latent personality traits unobservable from direct examination of singular behaviors (Bergvall et al. 2011; Cote et al. 2011; Martin & Réale 2008; Dingemanse et al. 2007; Sih & Watters 2005; Sussman et al. 2013). We did not use a PCA for any pup behavioral data. We then used likelihood ratio tests to assess the consistency of feeding principal components within the yearling stage across the acclimation and testing periods. To assess relationships among feeding components, yearling FGMs, FAMs, and previously

categorized pup stage boldness (assessed via feeding and repeated object tests), mean hair cortisol concentrations (i.e. from 5 to 15 weeks of age), and mean hair testosterone concentrations we used Spearman rank correlations. We further used LMMs to determine if parental odor treatment group, parental breeding experience, sex, or period (i.e. acclimation and testing) were associated with differences in feeding components. We nested coyote identity in mate identity as random factors in all LMMs.

Similar to feeding tests, we used likelihood ratio tests for repeated object tests to examine whether individuals demonstrated consistency in the proportion of objects they approached from the pup stage to the yearling stage. Likewise, proportions for object approach for each developmental stage were arcsine square root transformed. For LMMs testing consistency across developmental time, we again considered developmental stage and year as the main fixed effects in the model. Multiple variables recorded during repeated object tests (Table 5.1) were also highly correlated, and we therefore performed a separate PCA for repeated object testing. Again, we used likelihood ratio tests to assess the consistency of object components within the yearling stage across the four different objects presented to yearlings. For LMMs testing consistency within the yearling stage across weeks, parental treatment group, year, sex, and object type were all considered fixed effects, with coyote identity and mate identity as random effects. We used Spearman rank correlations to determine if there were any relationships among yearling FGMs, FAMs, and pup variables. Spearman rank correlations also helped us determine if there were any associations among feeding and repeated object test components.

Likelihood ratio tests were used once more to assess consistency of glucocorticoids and androgens across developmental time. We compared the average hair cortisol and testosterone concentrations of individuals at the pup stage with their FGMs and FAMs at the yearling stage.

Here, we restricted our comparison of hormone concentrations across developmental stages to yearling FGMs and FAMs within the acclimation period. This restriction was done specifically because our previous work demonstrated that human-associated items (e.g. box fan and fireworks) elicited a pronounced physiological stress response (Appendix A; Schell et al. 2013). There was minimal contact or perturbation by NWRC staff or other researchers during the acclimation period, thus we were able to consider hormonal measures during the acclimation period as baseline values for our yearling coyotes. We later quantified whether individuals exhibited acute hormonal responses to each object presented using similar methodology to Schell et al. (2013). Specifically, we used paired t-tests to determine whether individuals had significantly higher or lower hormone metabolites post-object presentation compared to FGMs and FAMs observed during the acclimation period. We then used LMMs to determine whether period, parental treatment, year, or sex were associated with FGMs and FAMs in yearlings. Finally, we used Spearman rank correlations to assess the relationships between feeding components, repeated object components, and pup variables. We also examined the correlations among yearling and parental FGMs and FAMs.

All statistical analyses were performed using the R version 3.1.3 (R Core Team, 2015). Linear mixed models were performed using the lmer function from 'lme4' (Bates et al. 2012) and 'lmerTest' (Kuznetsova et al. 2013) packages. Likelihood ratio tests were performed using the exactLRT function from the 'RLRsim' package (Crainiceanu & Rupert, 2004). All Spearman correlations were performed using the corr function from the 'corrplot' package (Wei 2013). Fisher's exact tests were performed using the exact.test function from the 'Exact' package (Calhoun 2015). Nonparametric hormonal data were natural log transformed similar to previous studies (Schell et al. 2013). We reported results from the best-fit models for all

measures, determined using the lowest Akaike Information Criterion (AIC) values (Burnham et al. 2010). Alpha was set to 0.05 for all cases and we report data as mean ± S.E.

RESULTS

Feeding tests

PCA revealed five components that explained approximately 73% of total variance (Table 5.2). Relationships among the original behavioral measures and principal components led to five broad interpretations: boldness (pc1), activity (pc2), habituation (pc3), territoriality (pc4), and aggressiveness (pc5). We then compared our components with pup feeding behaviors. Individuals that ate more frequently as pups were bolder and more active as yearlings (Fig. 5.1a-b), particularly in the first few weeks of testing (i.e. the acclimation period). However, the frequency an individual ate food rations within the pup stage was not correlated with boldness (r_s = 0.09, P = 0.66) or activity (r_s = -0.16, P = 0.43) components within the testing period. In addition, we found that the frequency each pup ate first within their natal litter positively correlated with their habituation (Fig. 5.1c) and aggression (Fig. 5.1d) component scores over the testing period, indicating that pups which regularly ate first among siblings were more aggressive and habituated to feeding tests quicker when yearlings. The relationships among pup feeding behaviors, yearling habituation, and yearling aggression components were not significant over the acclimation period (habituation: r_s = -0.01, P = 0.96; aggression: r_s = 0.34, P = 0.083).

We then assessed whether yearling principal components were individually consistent within the yearling stage and across test periods (i.e. acclimation versus testing period). We did find that yearling boldness components were individually consistent from the acclimation period into the testing period (LRT = 11.28, P = 0.002; Fig. 5.2a). Additionally, we found that mate identity

significantly affected individual yearling boldness (LRT = 25.65, P < 0.001), while the boldness components for all individuals increased over time ($F_{1,20}$ = 34.7, P < 0.001; Fig. 5.2a). Parental odor treatment ($F_{1,20}$ = 0.49, P = 0.49), year ($F_{1,20}$ = 0.57, P = 0.46), and sex ($F_{1,20}$ = 0.29, P = 0.60) were not significant factors in the model. The other components are displayed in Appendix D (Fig. D1). Briefly, the habituation (LRT = 14.81, P < 0.001) and territoriality components (LRT = 4.55, P = 0.036) were individually consistent from the acclimation to the testing period. Further, yearling habituation components increased from the acclimation to the testing period ($F_{1,20}$ = 46.91, P < 0.001), and males generally had lower activity component scores ($F_{1,20}$ = 7.77, P = 0.011).

The frequency an individual ate over feeding tests was not individually consistent from the pup to the yearling stage (restricted log likelihood ratio test, LRT = 1.53, P = 0.11; 54 observations from 27 individuals). We also did not find consistent mate identity differences explaining variance in the frequency an individual ate (LRT = 0.61, P = 0.14). Individuals generally ate the provided food rations considerably more frequently as yearlings (93.8 ± 0.03% of tests) than as pups (54.4 ± 0.06% of tests, $F_{1,50}$ = 51.12, P < 0.001). Yearlings born to experienced parents generally ate more frequently (98.4 ± 0.01% of tests) than yearlings born to first-time parents (89.2 ± 0.05% of tests; $F_{1,50}$ = 31.86, P < 0.001). Parental treatment group (i.e. prenatal odor cues), however, was not a significant factor affecting feeding behavior of yearlings ($F_{1,48}$ = 0.03, P = 0.87).

Repeated object tests

PCA revealed five components that explained approximately 71% of the variance in the data after rotation (Table 5.3). Relationships among the original behavioral variables and the five

components led to five broad interpretations: boldness (pc1), activity (pc2), vigilance (pc3), territoriality (pc4), and wariness (pc5). We then compared our components with pup object approach. We found that the proportion of objects approached as a pup negatively correlated with wariness (Fig. 5.3c), indicating that bolder pups were less wary of objects as yearlings (Table 5.4c). We did not observe any other correlations among our object principal components and pup object approach (Fig. 5.3). Individuals generally approached more objects as yearlings (2.6 ± 0.2 objects) than as pups (1.3 ± 0.2 objects, $F_{1,50} = 21.27$, $P < 0.001$). There was also an interaction between parental experience and developmental stage ($F_{1,50} = 10.22$, $P = 0.002$), indicating that individuals from experienced parents generally approached more objects at the pup stage. We did not find any differences in proportion of objects approached by parental odor treatment ($F_{1,48} = 0.29$, $P = 0.59$) or sex ($F_{1,48} = 2.60$, $P = 0.11$).

We then assessed all components for individual consistency within the yearling stage across the different object types. Yearlings demonstrated consistent individual differences in their boldness component scores across different objects ($LRT = 5.86$, $P < 0.001$; Fig. 5.2b). Mate identity was also a significant factor explaining variance in boldness component scores ($LRT = 44.93$, $P < 0.001$; Fig. 5.2b), indicating that boldness of a mate affected the latency to eat, proximity, and sniffing behaviors of focal individuals (Table 5.3). Boldness component scores generally decreased with each successive object presentation ($F_{1,83} = 59.5$, $P < 0.001$; Fig. 5.2b). The other components are displayed in Appendix D (Fig. D2). Briefly, we found that vigilance was the only other principal component that was individually consistent across the four different object presentations ($LRT = 3.21$, $P = 0.004$). Mate identity was a significant factor affecting vigilance ($LRT = 1.53$, $P = 0.032$), territoriality ($LRT = 1.00$, $P = 0.05$), and wariness ($LRT = 2.73$, $P = 0.012$) components suggesting that pair members consistently scent-marked territories

Table 5.3 Results of principal components analysis (PCA) for feeding test measures, specifying five components rotated with varimax rotation. Variables that contributed with a loading of > |0.40| are shown in bold (Abdi & Williams 2010; Sussman et al. 2013).

Variable	pc1: boldness	pc2: activity	pc3: habituation	pc4: territoriality	pc5: aggressiveness
Latency to eat	**-0.460**	0.222	-0.031	0.153	0.157
Proximity	**0.493**	0.083	0.219	0.011	0.199
Eating	0.060	**-0.653**	-0.204	-0.228	-0.143
Stationary	-0.018	-0.108	**0.691**	**0.443**	-0.081
Locomotion	0.125	**0.653**	-0.243	-0.155	-0.170
Resting	-0.328	0.007	-0.143	0.081	**0.716**
Sniffing	**0.456**	0.188	0.203	-0.067	0.221
Urination	0.246	0.038	-0.332	**0.512**	-0.049
Ground scratching	0.088	-0.071	-0.344	**0.644**	-0.134
Aggression	0.147	-0.119	0.031	0.090	**0.482**
Vigilant stares toward person	-0.347	0.151	0.285	0.093	-0.258
Eigenvalue	**3.243**	**1.957**	**1.376**	**1.299**	**0.997**
Variance explained	**23.5%**	**16.5%**	**12.5%**	**11.7%**	**9.0%**
Total variance explained	**23.5%**	**39.9%**	**52.4%**	**64.1%**	**73.1%**

Table 5.4 Results of principal components analysis (PCA) for novel object test measures, specifying five components rotated with varimax rotation. Variables that contributed with a loading of > |0.40| are shown in bold (Abdi & Williams 2010; Sussman et al. 2013).

Variable	pc1: boldness	pc2: activity	pc3: vigilance	pc4: territoriality	pc5: wariness
Latency to approach	-0.321	-0.061	0.364	-0.188	0.183
Proximity	**0.526**	-0.130	-0.042	-0.027	0.154
Number of approaches	0.298	0.295	-0.015	0.150	**0.529**
Stationary	0.231	0.053	**0.625**	-0.003	**-0.450**
Locomotion	-0.213	**0.489**	-0.264	-0.351	0.231
Resting	-0.254	-0.354	-0.117	**0.567**	0.186
Sniffing	0.200	**0.463**	0.215	0.103	-0.009
Urination	0.118	0.360	-0.371	0.130	**-0.406**
Ground scratching	0.096	0.198	-0.020	**0.637**	-0.050
Aggression	0.380	-0.134	0.205	-0.056	0.396
Vigilant stares toward object	-0.231	0.192	0.262	0.098	0.201
Play bouts	0.327	-0.294	-0.310	-0.225	-0.091
Eigenvalue	**2.818**	**2.079**	**1.519**	**1.154**	**1.003**
Variance explained	**23.5%**	**17.3%**	**12.7%**	**9.6%**	**8.4%**
Total variance explained	**23.5%**	**40.8%**	**53.5%**	**63.1%**	**71.4%**

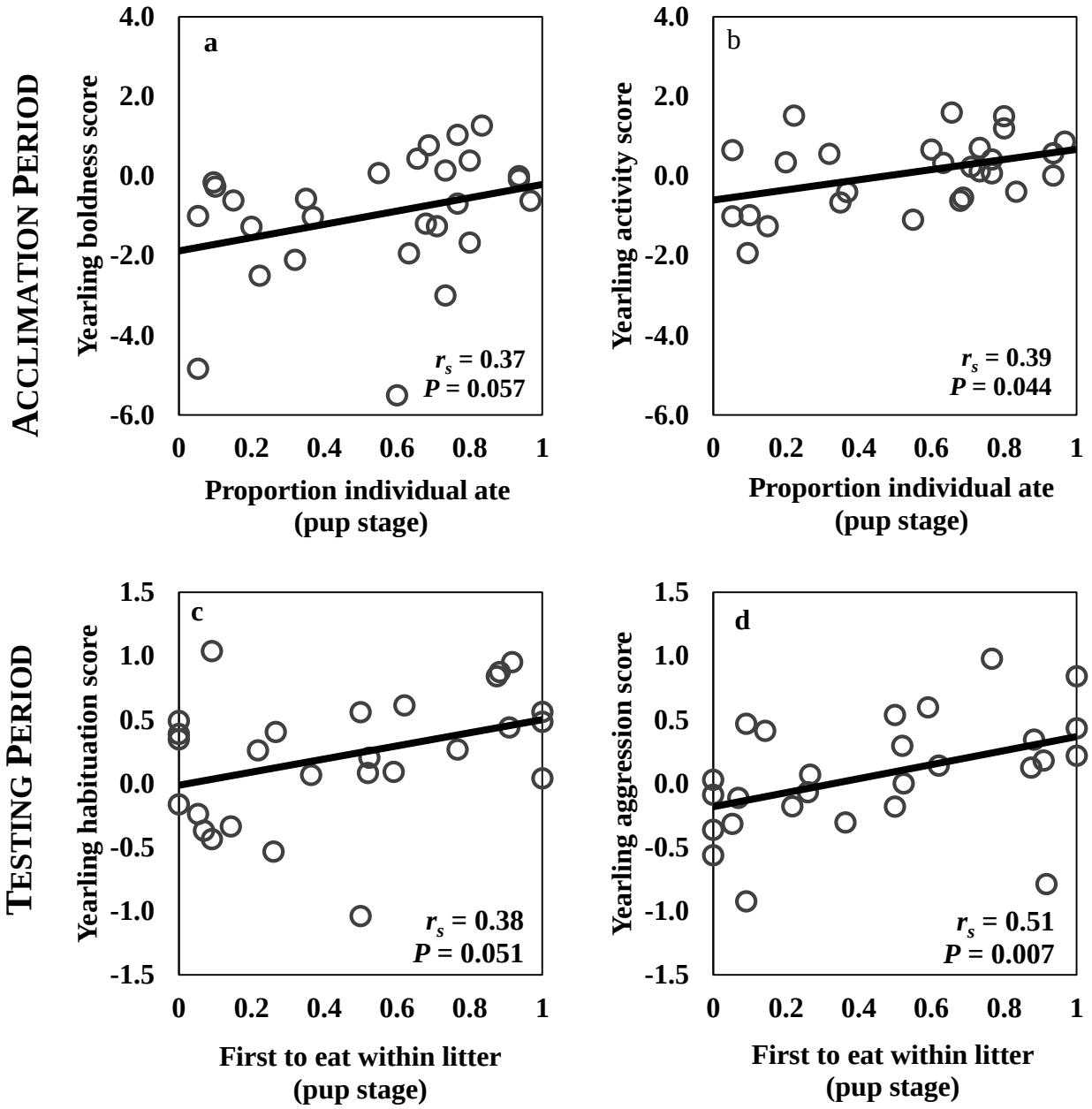

Fig. 5.1: Spearman rank correlations among the proportion an individual pup ate food in front of a human observer (a,b), the propensity of each pup to eat first within their litter (c,d), and select yearling feeding principal component scores during the acclimation (a,b) and testing periods (c,d) over feeding tests (*N*=27 yearlings).

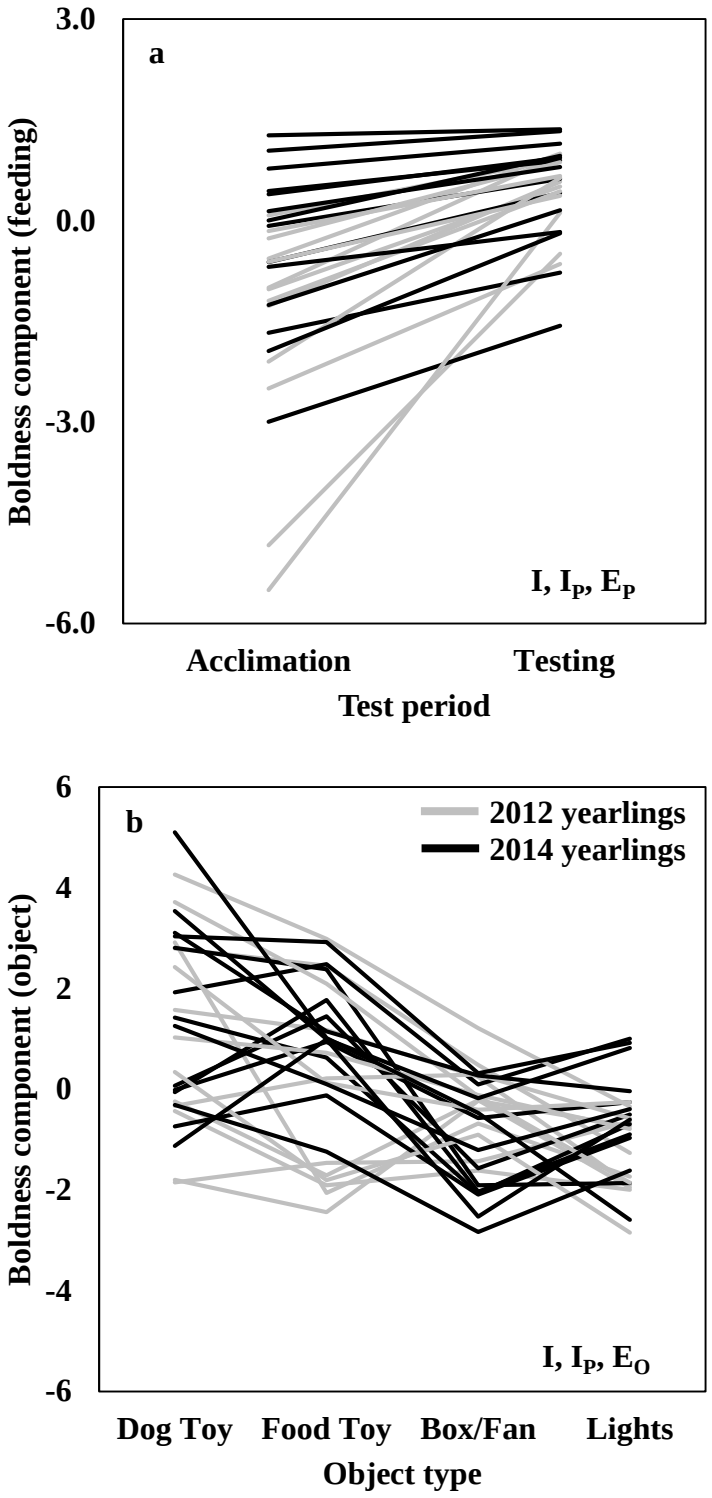

Fig. 5.2: Boldness principal components for yearling feeding and repeated object tests across the (a) acclimation and testing periods and (b) different presented objects. ***I*** denotes a significant effect (P<0.05) of coyote identity, $\boldsymbol{I_P}$ an effect of pair identity, $\boldsymbol{E_P}$ an effect of period, $\boldsymbol{E_Y}$ an effect of year, and $\boldsymbol{E_O}$ an effect of object (specifically for repeated object testing).

and vigilantly stared at presented objects.

The number of objects approached was not individually consistent from the pup to the yearling stage ($LRT = 0.18$, $P = 0.35$; 54 observations from 27 individuals). In addition, mate identity was not a significant factor explaining the variance in proportion of objects approached ($LRT = 0$, $P = 0.38$; Fig. 5.1b).

Hormones

Pup hair cortisol was not associated with fecal glucocorticoid metabolites at the yearling stage ($LRT = 0.15$, $P = 0.32$). In addition, pup hair testosterone was not associated with fecal androgen metabolites at the yearling stage ($LRT = 0.037$, $P = 0.43$). Within the yearling stage, however, individuals demonstrated consistency from the acclimation to the test periods in both FGMs ($LRT = 12.07$, $P < 0.001$) and FAMs ($LRT = 2.13$, $P = 0.047$). Both FGMs and FAMs generally decreased from the acclimation to the test periods (FGMs: $F_{1,26} = 14.48$, $P < 0.001$; FAMs: $F_{1,26} = 35.67$, $P < 0.001$). Fecal androgen metabolites were higher for male yearlings compared with females ($F_{1,23} = 219.82$, $P < 0.001$), and yearlings born to first-time parents had higher FAMs for both sexes ($F_{1,26} = 10.36$, $P = 0.004$; Fig. 5.4b). Additional information on the fold change in FGMs and FAMs in response to presented objects are provided in Appendix D (Tables D1-D3, Fig. D3). Briefly, only three individuals exhibited acute increases in FGM response (ID: 1130, 1100, 1347) to the first object provided (i.e. dog toy; Table D2). In fact, for all other objects and individuals, FGMs post-object presentation were 1 to 12-fold lower than FGM values during the acclimation period.

Correlations across contexts

We used Spearman rank correlations to examine the relationships of our aforementioned measures across contexts. Individuals that ate more frequently as pups also tended to be more exploratory and less wary during yearling repeated object testing (Fig. 5.3). Pups with higher mean hair cortisol concentrations were more active as yearlings in feeding tests (r_s = 0.43, P = 0.025), whereas pups with higher mean hair testosterone concentrations were more territorial (r_s = 0.41, P = 0.034) and aggressive (r_s = 0.42, P = 0.029) as yearlings in feeding tests. Those relationships, however, were not consistent over time: pup hair testosterone concentrations, yearling territoriality (r_s = 0.05, P = 0.80), and yearling aggression (r_s = 0.02, P = 0.92) over the test period of feeding observations did not demonstrate significant correlations. Moreover, pups with higher hair cortisol concentrations had lower territoriality (r_s = -0.41, P = 0.034) and wariness (r_s = -0.41, P = 0.034) component scores over repeated object testing, indicating pups with higher cortisol were scent-marked less but approached objects more frequently.

Within the yearling stage, we found that boldness and territoriality components of both the feeding and repeated object tests were positively correlated (Fig. 5.5). Individuals that were bolder in the feeding context were bolder in the object test context (r_s = 0.58, P = 0.001; Fig. 5.5a). Further, individuals that scent-marked more during feeding tests also scent-marked more during repeated objects tests (r_s = 0.51, P = 0.007, Fig. 5.5b). Individual boldness scores for repeated object tests were also positively associated with aggressiveness during feeding tests (r_s = 0.56, P = 0.002), suggesting that bolder individuals were more aggressive toward conspecifics. Finally, yearlings with greater FAMs during the test period were more territorial (r_s = 0.43, P = 0.025) during feeding tests, indicating that individuals with higher androgens scent-marked more frequently.

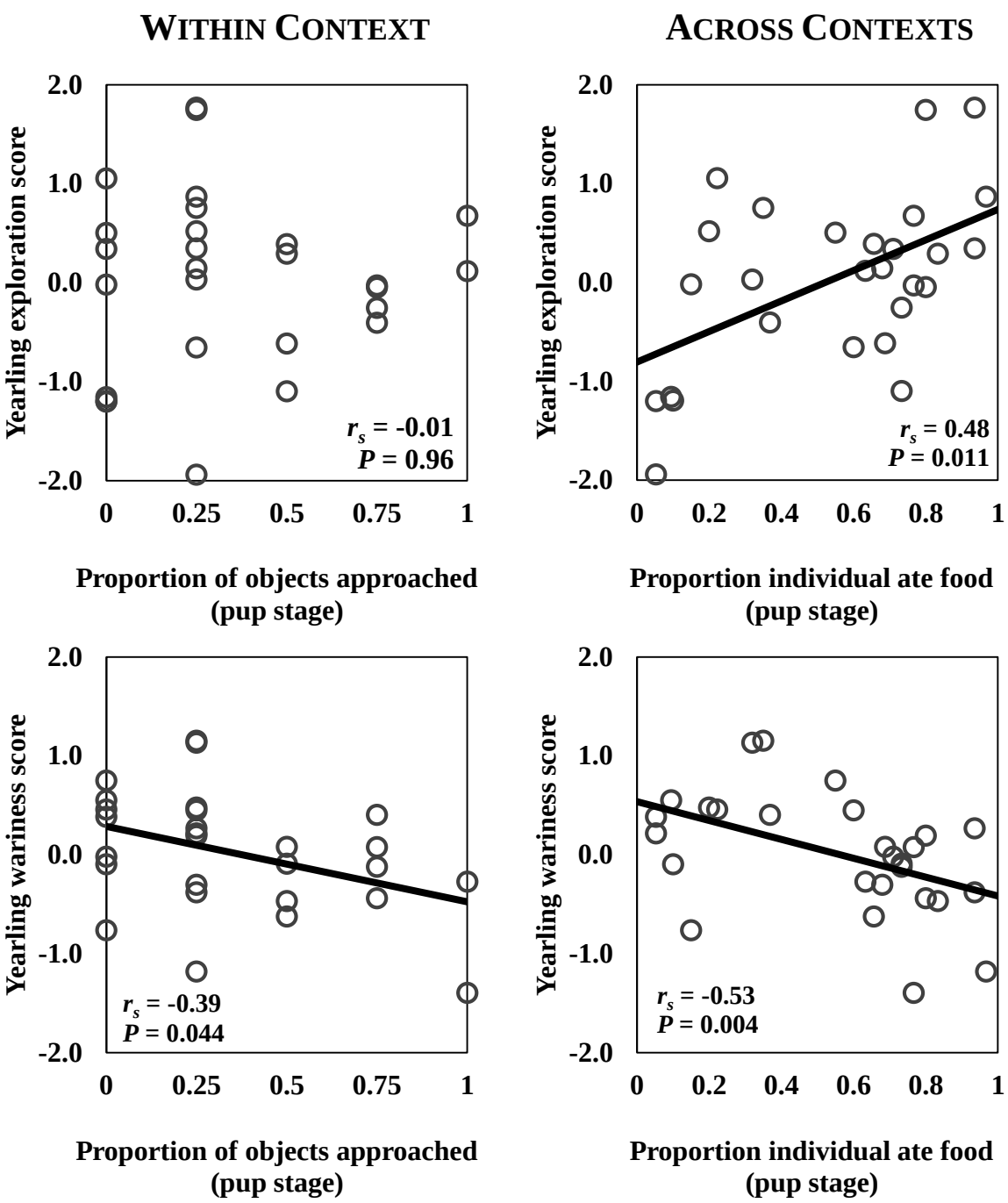

Fig. 5.3: Spearman rank correlations among pup variables and repeated object principal component scores. Relationships are between the proportion of objects approached as a pup and their mean (± S.E.) yearling wariness and exploration principal component scores (i.e. within context); as well as the proportion an individual ate during feeding tests as a pup and their mean yearling wariness and exploration component scores (i.e. across contexts). *N*=27 yearlings.

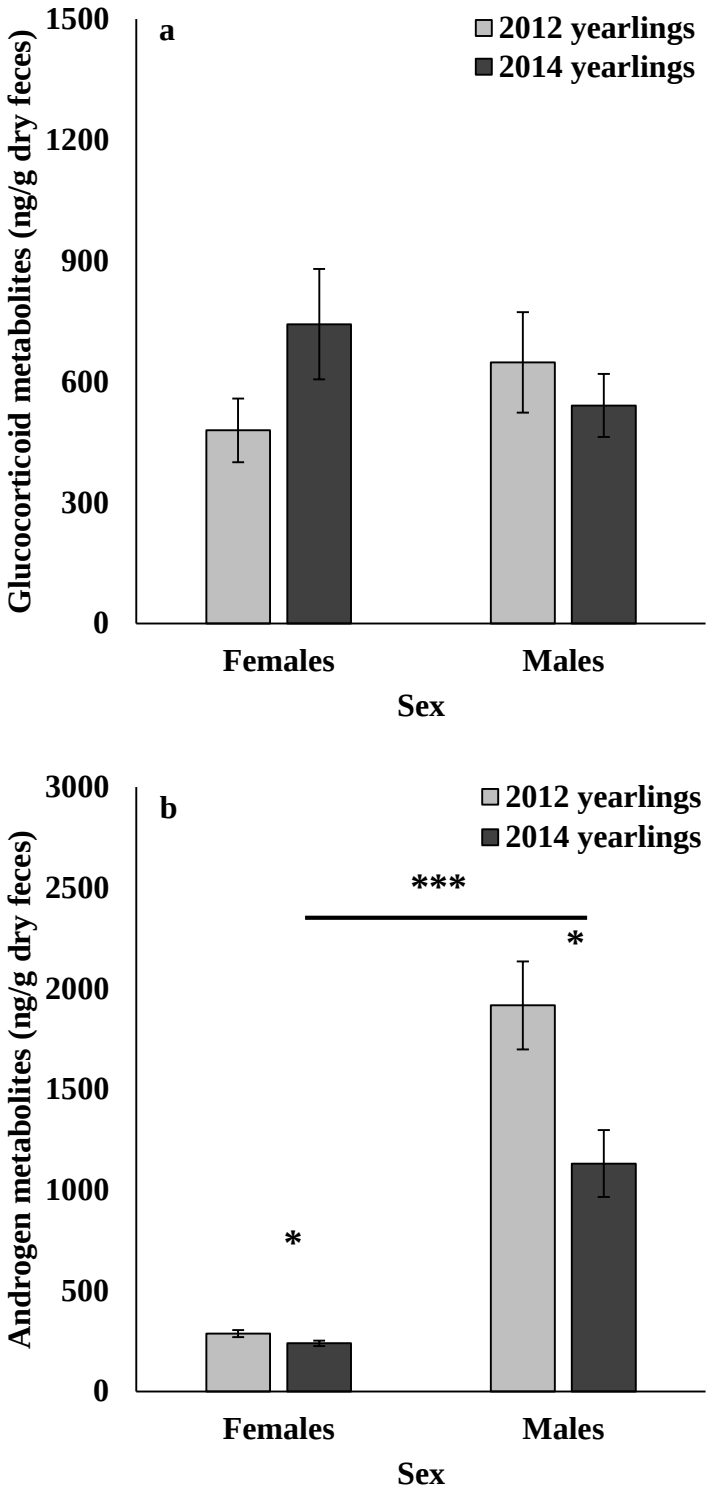

Fig. 5.4: Mean (± S.E.) overall fecal glucocorticoid (a) and androgen (b) metabolites of male and female yearlings from first-time (i.e. 2012 yearlings) and experienced parents (i.e. 2014 yearlings). *P<0.05, **P<0.01, ***P<0.001

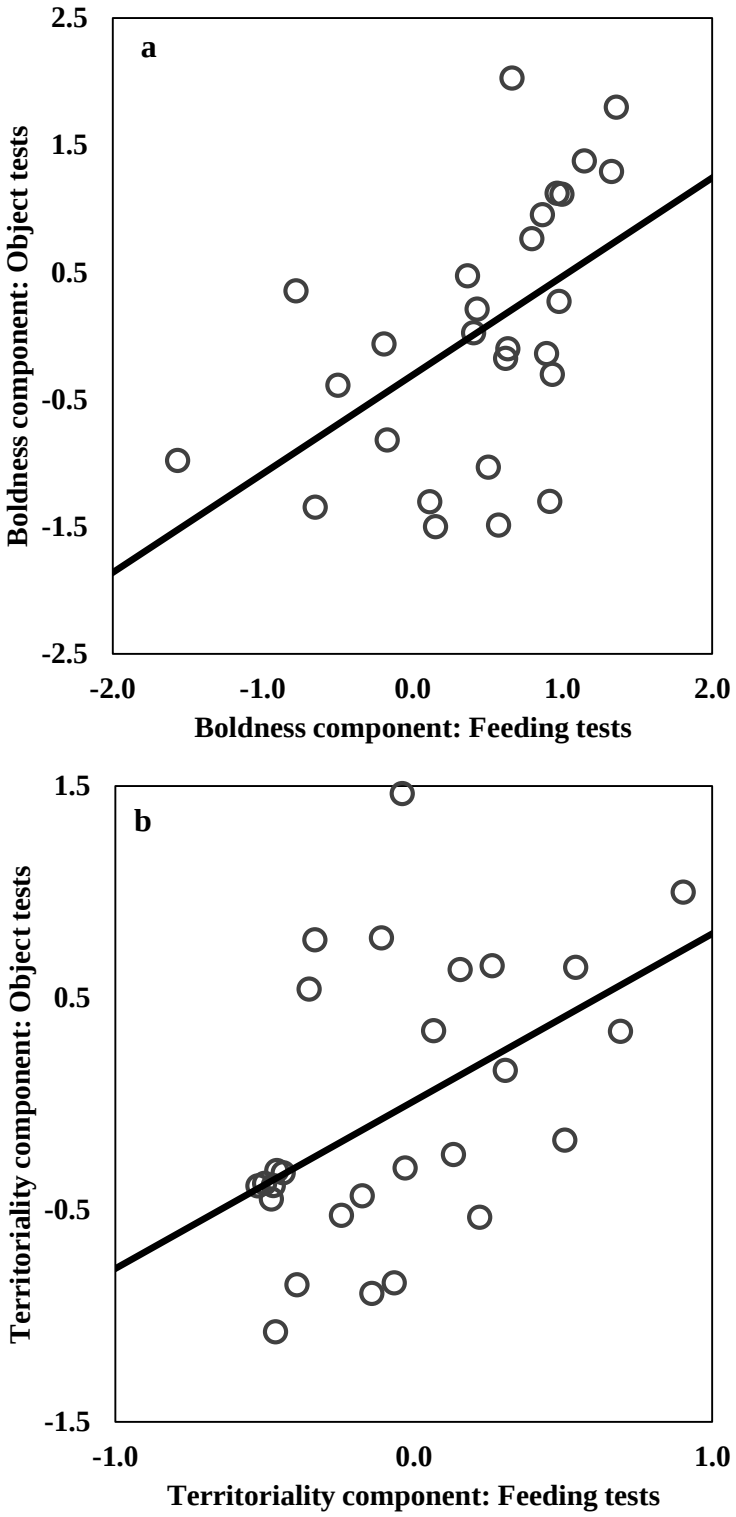

Fig. 5.5: Spearman rank relationships between mean (± S.E.) boldness and territoriality principal component scores across feeding and repeated object test contexts during the yearling stage. Trend lines denote statistical significance at $P<0.05$ level.

DISCUSSION

Early life experiences can have a profound impact on the maturation of phenotypic traits into adulthood (Stamps 2015; Stamps & Groothuis 2010a; 2010b). Here, we have demonstrated that behavioral and hormonal traits exhibited by individual coyotes as pups were intimately connected with subsequent behavioral and hormonal traits expressed as yearlings. Pups that ate more frequently with a human observer had higher boldness and activity component scores as yearlings during feeding tests. In addition, individuals that frequently ate first within their natal litter as pups were more aggressive as yearlings. As yearlings, coyotes were individually consistent in their boldness, habituation, and territoriality components for feeding tests across time. In many instances, there were consistent within-pair differences in feeding and repeated object test components, suggesting that both personality and social influences interact to shape behavior. Yearlings demonstrated consistent individual differences in fecal glucocorticoid and androgen metabolites within the yearling stage, but were not individually consistent from the pup to the yearling stage. Further, fecal androgen metabolites were lower in yearlings born to experienced parents (Fig. 5.4) for both sexes, a trend previously observed from these individuals in the pup stage (Chapter 4). Fecal glucocorticoids and androgens also decreased over time, suggesting the potential for coyote habituation to our test regimes. Last, we observed between-individual consistency in boldness and territoriality components across test contexts, providing further evidence behavioral syndromes exist for these traits in coyotes (Young et al. 2015).

Likelihood ratio tests did not demonstrate individual consistency of identical measures (i.e. objects approached, frequency of eating) recorded across developmental stages. This may be due to physiological or developmental processes that occur throughout maturation and aging of the individual. Stamps (2015) categorized potential age-related changes to behavior as

developmental trajectories, in which parallel behaviors observed over development are differentially expressed due either to prior learning or changes to the internal state (i.e. physiology, morphology, etc.) of an organism. Neophobia and caution are hallmark coyote behaviors, especially in relation to human activity and associated stimuli (Darrow & Shivik 2009; Kitchen et al. 2000; Mettler & Shivik 2007; Séquin et al. 2003). However, our captive coyotes were fed by people daily from birth, and our feeding tests were performed regularly over a 2-month period. With multiple experiences of being fed by people, risk assessment may have been reduced. Indeed, this behavioral habituation to human activity is not restricted to coyotes, as activity budgets of eastern grey squirrels do not deviate when a human pedestrian is nearby on a designated footpath in urban settings (Bateman & Fleming 2014). It is only when pedestrians diverged from the footpath that squirrels fled from humans in close proximity, indicating that risk assessment can be relaxed over successive and predictable experiences with humans. Our results support this interpretation, as boldness and habituation component scores increased over time. Experience gained with increasing age may have therefore played a pivotal role in feeding behaviors over development.

Our results also demonstrated that mate personality significantly impacted eating proportions, suggesting that the proportion an individual ate was strongly informed by the behavior of their mate. Mates that were particularly food-aggressive or ingested the majority of rations may have inhibited the focal individual's ability to secure adequate food. Because of the potential consequence of a missed foraging opportunity, focal individuals may have modified their activity to acquire as much food as possible before their mate obtained all provisions. This hypothesis is supported by the fact that boldness feeding component scores during the acclimation period were positively correlated among mates ($r_s = 0.71$, $N = 14$, $P = 0.004$),

suggesting that mates mimicked each other to maximize food intake. Local enhancement of foraging behavior in response to social cues has previously been observed for other species. For instance, zebra finches (*Taeniopygia guttata*) in flocks eat much faster, are more exploratory with novel stimuli than solitary individuals (Coleman & Mellgren 1994), and preferentially eat from feeders that a familiar conspecific previously ate from (Benskin et al. 2002). Red-winged blackbirds (*Agelaius phoeniceus*) increase their food consumption in the presence of conspecifics and preferentially eat novel foods that they previously witnessed other conspecifics eating (Mason & Reidinger 1981). Further, feeding rates and foraging efficiency of downy woodpeckers (*Picoides pubescens*) steadily increase with increasing flock size (Sullivan, 1984). Previous work on in coyotes has demonstrated that prior information and experience can improve foraging success, but social status greatly affects individual coyote foraging efficiency (Gilbert-Norton et al. 2013). Our results support previous work, and suggest that the additive influences of social facilitation, social learning, and experiential learning increase foraging intensity and override any potential individual consistency in eating frequencies.

As with feeding tests, we did not observe repeatability in the proportion of objects approached across developmental stages. In general, yearlings approached objects more frequently than pups. At the onset of our study, we moved yearlings to novel pen environments with new neighboring conspecifics in adjacent pens. The influence of social familiarity on exploratory behaviors has previously been demonstrated in zebra finches, in which individual response to novel objects is dependent on the familiarity shared among conspecifics (Benskin et al. 2002). Male cichlid fish demonstrate similar trends in which individuals are more exploratory and less neophobic when in the presence of a familiar conspecific (Galhardo et al. 2012). Moreover, exploratory ravens delay their approach to novel objects in the presence of more risk-

averse conspecifics (Stöwe & Kotrschal 2007). Social facilitation still played a role in shaping individual boldness and exploratory behaviors for yearling coyotes, as evidenced by significant within-pair differences. The degree of social facilitation experienced by related individuals versus a single conspecific merely presented a varying set of social conditions that may have influenced overall object approach. Familiarity with pen settings may have also contributed to the differences observed in overall object approach. This is similar to Harris & Knowlton (2001), who previously demonstrated that novel stimuli provided in familiar settings increase risk aversion in coyotes. Although we provided a 2-week period for individuals to adjust to their new surroundings, the amount of time provided may have been insufficient to produce a familiar setting. However, differences in weather conditions at the time objects were presented may have also influenced object approach, as coyotes occasionally avoided novel stimuli in rainy or storm-associated conditions (C. Schell pers. obs.).

Despite the absence of repeatability in identical behaviors measured across developmental time, within-individual consistency was readily demonstrated. Individuals that ate more frequently as pups were bolder and more active in feeding tests at the yearling stage. Likewise, individuals that ate first within their natal litters more frequently were more aggressive as yearlings when food was present. Our results therefore imply that experiences during development shaped personality traits expressed during adulthood. These correlations exemplify the concept of ontogenetic plasticity proposed by Stamps (2015), in which traits at designated life stages influence the expression of traits into the next life stage of an organism. Thus, bolder and more food-motivated coyotes demonstrated consistency across time, suggesting that personality is stable over development in this species. We also observed relationships between pup and yearling behaviors differed relative to the observational period (i.e. acclimation versus

testing). Thus, temporal variation within the yearling stage played some role in behavioral change. Further, we found that pup behaviors in one context corresponded with yearling behaviors in another context, suggesting that coyotes have distinct behavioral syndromes that not only persist through time but across contexts as well.

Stability of syndromes across time and context imply that underlying physiological mechanisms may have played some role in observed coyote personality traits. Glucocorticoids and androgens specifically represent key physiological components that are often associated with variation in consistent individual differences in behaviors (Biro & Stamps 2008). Exploration in Belding's ground squirrels, for example, positively correlates with fecal glucocorticoid metabolites (Dosmann et al. 2015). Rainbow trout (*Oncorhynchus mykiss*) that exhibit decreased cortisol responsiveness when exposed to a stressor tend to be more aggressive and dominant toward conspecifics (Pottinger & Carrick 2001). Further, male great tits (*Parus major*) with higher levels of baseline testosterone were generally less exploratory (Van Oers et al. 2011). The unique feature of this study is that we observed hormone-behavior relationships across developmental time. Specifically, individuals with higher mean hair cortisol concentrations as pups were more active during yearling feeding tests, suggesting that individuals with increased glucocorticoids early were active more frequently and foraged more intensely. We also found that individuals with higher hair cortisol concentrations as pups were also less territorial (i.e. scent-marked less) and risk-averse as yearlings during repeated object tests. Yearlings with greater FAMs tended to be more territorial, implicating testosterone as an underlying physiological mechanism impacting dominance-related behaviors in coyotes. However, we did not specifically find repeatability of hormones from the pup to yearling stage. Excretion window of hormone metabolites differs greatly between hair and fecal mediums (Stalder & Kirschbaum

2012), so incongruences may be due to differences in sample type. It is also possible that other hormones are associated with our behavioral indices, as glucocorticoids and androgens occasionally do not underlie aggression or exploratory behavior (see Mutzel et al. 2011). The most likely explanation is that we compared hormone concentrations of pups with hormone metabolites of yearlings. Hormones in the hair are stored concentrations deposited via passive diffusion from blood vessels (Stadler & Kirschbaum 2012), while hormones metabolites in feces are byproducts of the actual hormone of interest (Goymann 2012). Repeated collection of hair samples may allow better assessment of repeatability of hormone metabolites over developmental time.

Within the yearling stage, coyote boldness and territoriality components were positively correlated across test contexts, indicating strong behavioral syndromes for boldness and dominance in the species. Previous work in wild brown trout (Adriaenssens & Johnson 2013), fallow deer (*Dama dama*; Bergvall et al. 2011), Namibian rock agama (Carter et al. 2012), hermit crabs (Briffa et al. 2008), and Belding's ground squirrels (Dosmann & Mateo 2014) are only some of the species that also demonstrate behavioral consistency across environmental contexts. Consistent individual differences across contexts are a clear indication of personality, yet that does not mean that individuals remain static (Dingemanse et al. 2010). For instance, coyotes in our study demonstrated consistent individual differences in boldness toward objects but varied their responses according to object type. Coyotes therefore exhibited context-dependent plasticity, in which the rate of change in boldness and other component traits (i.e. the slope) was individually consistent (Stamps 2015).

Finally, we observed androgen metabolite differences for yearlings according to whether they were born to first-time (2012 yearlings) or experienced (2014 yearlings) parents. Specifically,

2012 yearlings had higher FAMs compared to 2014 yearlings. This result is consistent with our previous findings, in which individuals born to first-time parents had higher hair testosterone concentrations at 5 weeks of age (Chapter 4). Our current results are also consistent with prenatal FAMs exhibited by parents, in which first-time mothers and fathers consistently had higher FAMs compared with experienced parents (Chapter 2). Our cumulative results suggest that the non-genetic inheritance of androgen profiles begins over gestation and persists well into adulthood of offspring. Given that yearling territoriality and boldness are tangentially related to androgen metabolites, epigenetic processes likely play a critical role in shaping traits paramount to coyote adaptation. Reddon (2011) previously hypothesized that parental effects were integral to generating animal personalities and behavioral variation that would ultimately lead to fitness consequences and evolutionary change within a population. Previous literature in zebra finches demonstrated that offspring exploratory behavior is best predicted by exploratory behavior of foster parents (Schuett et al. 2013). Moreover, rhesus macaques (*Macaca mulatta*) reared by mothers or socially reared by other conspecifics differed in their activity and aggression (Gottlieb & Capitanio 2013). Our study is unique in that we provide supporting evidence showing epigenetic mechanisms affect behavior early in development, and those influences have long-term consequences on yearling traits. Therefore, non-genetic influences of parents shape personality traits and subsequent experiences of offspring that persist into adulthood, likely to affect fitness outcomes for those individuals.

To conclude, previous work has demonstrated repeatability of behavioral traits over relatively short (days to months) timescales and varying contexts (Bergvall et al. 2011; Biro et al. 2010; Carter et al. 2012), in addition to epigenetic influences and non-genetic transmission of personality traits across generations (Carere et al. 2005; Gottlieb & Capitanio 2013; Laviola &

Terranova 1998; Stein & Bell 2014; Reddon 2011). To our knowledge this is the first study using longitudinal behavioral and hormonal indices recorded from individuals over years to demonstrate developmental stability of personality traits. Coupled with our previous findings (Chapters 2-4), it appears that parental effects are an integral and comprehensive mechanism impacting coyote adaptation to changing environments, potentially affecting the directionality of evolutionary change in a population. Environmental experience and learning were critical factors affecting coyote reactions, and repeated experiences with human-associated stimuli reduced neophobic responses in individuals. This is relevant to management and wildlife studies focused on understanding human-wildlife conflict, as habituation and boldness to human-associated stimuli appear to increase indefinitely when negative consequences to the individual coyote are absent. Social factors were also relevant to shaping personality and should be considered when quantifying consistent individual differences in other social taxa. Finally, future work should consider genetic or genomic perspectives to determine the degree to which coyote behavioral syndromes are genetically associated (see Van Oers et al. 2005). Determining how non-genetic and genetic parental effects operate to influence offspring traits could prove invaluable to parental effects theory and an overall understanding of how populations adapt to human-induced environmental change.

CHAPTER 6: CONCLUSION

Evidence from a growing number of animal taxa are documenting the pervasive and enduring impacts parental effects have on the ecological and evolutionary processes of populations (Duckworth et al. 2015; Maestripieri & Mateo 2008; Mousseau & Fox 1998; Reddon 2011; Wolf et al. 1998). Here, the overall objective of my thesis was to determine if parental effects mechanisms were operating in the coyote system, and to what extent those effects influenced traits relevant to coyote adaptation. To achieve this goal, I attempted to induce a hormonal response in gestating breeding pairs, specifically predicting that expectant coyote parents were able to transduce environmental experiences toward offspring phenotype via hormonal physiology. Density-dependent cues (i.e. novel conspecific odors) were effective at eliciting an androgen response from both mothers and fathers (Chapter 2). In addition, these hormonal measures were strongly associated with the parenting behaviors exhibited post-partum (Chapter 3), the cortisol and testosterone concentrations of pups, and pup boldness behaviors (Chapter 4). Moreover, behaviors exhibited by individuals as pups were stable into the yearling stage, indicating that developmental experiences significantly biased the personality traits of coyote offspring. However, more importantly, this constancy of behavioral and hormonal traits across time suggest that parental effects in coyotes impart a persistent and indelible influence on phenotypic traits essential for coyote success and survival.

Amazingly, provisioned external stimuli (i.e. odor cues) were not the only influential factors mediating parental effects. Prior breeding experiences also play a significant role in shaping parental and pup traits. Experienced parents generally lower fecal androgen metabolites over gestation (Chapter 2), which were negatively correlated with increased aggression and

provisioning over pup development (Chapter 3). Offspring born to experienced parents were more likely to approach novel objects, eat independent of their parents, and emerge from their dens with predator cues (i.e. a human observer present; Chapter 4). These results indicate that parental experience was influential in shaping hormone physiology over gestation, parenting strategies post-partum, and offspring boldness behaviors. Further, yearlings born to experienced parents were bolder and more aggressive as well (Chapter 5), again implicating the longstanding influence produced by parents. In sum, both the provisioned odor cues and experiences over time interacted to produce multivariate effects on developing offspring. A comprehensive and distilled synopsis of the findings from this thesis are further provided in Table 6.1.

This thesis provides a series of novel methods and findings that attempt to expand the fields of parental effects, personality, and evolutionary biology. First, this thesis provides cohesive empirical data on the integral role fathers play in biparental care systems. Coyote fathers both witnessed increases in fecal androgen metabolites (Chapter 2) and were instrumental in caring for developing offspring (Chapter 3). Second, this research is the first instance in which both fecal androgen and glucocorticoid metabolites were assessed over gestation. Previous work eagerly characterized other facets of coyote reproductive biology (Carlson & Gese 2008; 2009; 2010; Minter & DeLiberto 2008), but here is the first instance that the stress and androgenic physiology of both sexes was characterized. Third, I both provide categorical and longitudinal data on the rate parenting behaviors are performed by both mothers and fathers (Chapter 3). Previous work has indicated that coyotes care for pups nearly-equally over the first 3-months of development (Bekoff & Wells 1982; Fentress et al. 1987; Way et al. 2001), but a substantial lack of data and detail was evident. Fourth, parents remarkably demonstrated consistent individual differences in hormonal and behavioral measures, as well as plasticity in response to both odor

cues and prior breeding experience. This thesis thus provides palpable evidence to support the claim that personality and plasticity are not mutually exclusive, but rather work in tandem for organisms to respond adaptively toward environmental change **(**Dingemanse et al. 2010). Fifth, this thesis was the first to utilize coyote hair to quantify cortisol and testosterone concentrations in coyotes. Several other studies document the feasibility of measuring hormone concentrations in hair (Bryan et al. 2013; Davenport et al. 2006; Laudenslager et al. 2011; 2012; Macbeth et al. 2010; Siniscalchi et al. 2013; Stalder & Kirschbaum 2012), with one specifically documenting epigenetic inheritance of hair hormonal concentrations (Fairbanks et al. 2011). This research adds to the currently limited sample size of studies utilizing hair as a hormonal media. Finally, this is the first study in a wildlife species (i.e. other than nonhuman primates and rodents) to demonstrate stability of personality traits across time. Stamps & Groothuis (2010a; 2010b) theorized that animal personalities likely demonstrate some developmental bias, in which prior experiences inform future behavioral phenotypes. This thesis provides empirical evidence to support the claims previously proposed by Stamps & Groothuis (2010a; 2010b).

Provided below are several additional implications this study has for coyote adaptation and colonization of novel environments. Further, the hope is that this work will inform future directions of research that combine epigenetic and genetic measures to gain a complete picture of how species may cope with current and future environmental changes.

THE PERVASIVE ROLE OF HORMONES

Androgens versus glucocorticoids

Androgens were fundamental to coyote parental effects at nearly every life stage observed (Fig. 6.1). Pre-partum fecal androgen metabolites were associated with individual parenting

strategies, as well as pup boldness and hormones (Fig. 6.1). Moreover, the observed phenotypic traits of developing pups corresponded with behaviors and hormones exhibited later in life, indicating that pre-partum hormonal outcomes of parents have long-term consequences for offspring. These data also suggest that parental effects mechanisms are sufficient to transduce environmental (i.e. odor cues) and internal experiences (i.e. parity) into meaningful components relevant to offspring phenotype. Other studies have provided complimentary evidence to highlight the extensive role of endocrine function in parental effects mechanisms (Dantzer et al. 2013; Fairbanks et al. 2011; Love et al. 2013; Reynolds et al. 2013; Schöpper et al. 2012; Sheriff et al. 2010; Yehuda et al. 2005). However, the majority of previous studies have demonstrated relationships among maternal glucocorticoids and offspring phenotype, rather than androgens (however, see Dloniak et al. 2006). Examining glucocorticoids as the primary responder is certainly justified when attempting to decipher which suites of hormones are most likely responsible for transmitting environmental experiences toward offspring (Meylan et al. 2012). Glucocorticoids are essential components of the HPA-axis and operate as primary factors regulating behavioral responses toward environmental or social challenges (Creel et al. 2013; Groothuis et al. 2005; Möstl & Palme 2002). Moreover, glucocorticoids permeate multiple biological systems and greatly accelerate or impede the process of such systems (Love and Williams 2008; Schulkin 2011). Consequently, glucocorticoids are frequently expected to be the leading hormones responsible for programming offspring phenotype (Meylan et al. 2012).

The primacy of glucocorticoids in prior studies begs the question: why were coyote androgens the primary hormonal suite sensitive to conspecific odor cues and parity, rather than glucocorticoids? To determine the overall importance of androgens to the coyote system, it may first be beneficial to establish with what functions these steroids are associated. Androgens such

as testosterone generally stimulate aggressive and territorial behaviors, particularly under conditions of sexual competition or conflict (Groothuis et al. 2005; While et al. 2010). These steroids are integral to sexual maturation and differentiation in several taxa, occasionally coinciding with sexually-selected characters of males and thus providing an honest signal of fitness (Gonzalez et al. 2001; Setchell et al. 2011). In addition, androgens can variably impact factors that contribute to individual fitness outcomes such as growth rates (Helle et al. 2013; Von Englehardt et al. 2006), sexual behaviors (Kemme et al. 2007; Koren et al. 2006; Wingfield et al. 1990), or social group status (Beehner et al. 2006; Dloniak et al. 2006; Van Kesteren et al. 2012). Moreover, comprised immune health (Van Oers et al. 2011) and decreased basal metabolite rates (Buchanan et al. 2001) reflect palpable fitness costs attributed to increased androgens. The myriad impacts of androgens on fitness have motivated recent studies demonstrating associations among androgens and personality (Chávez-Zichinelli et al. 2014; While et al. 2010), as well as the significance of androgens in parental effects (Clark et al. 1990; Kemme et al. 2007; Helle et al. 2013).

Species comparisons examining hormonally mediated maternal effects may best elucidate the significance of androgens in coyotes. Few studies have examined hormonally enabled parental effects in carnivores (Bowen 2008; Holekamp & Dloniak 2008), making phylogenetic comparisons of parental effects mechanisms scarce. However, several studies on spotted hyenas have demonstrated pronounced androgenic responses of mothers in relation to social challenges and rank status (Dloniak et al. 2006; Holekamp & Dloniak 2008). More specifically, spotted hyena mothers with increased androgens over gestation produce aggressive offspring that have higher reproductive success and survival, highlighting the significance of androgens in facilitating parental effects mechanisms for hyenas (Dloniak et al. 2006; Höner et al. 2010).

Table 6.1 Summary of findings from Chapters 2 through 5

Year(s)	Months	Focal group	Effect(s) of pre-partum odor cues	Response Type	Effect(s) of breeding experience	Response Type	Chapter
2011, 2013	Feb. – Apr.	Parents	Increased fecal androgen metabolites (FAMs); no changes in fecal glucocorticoid metabolites	Hormonal	Experienced parents had lower fecal androgen metabolites over gestation	Hormonal	2
2011, 2013	Feb. – Apr.	Parents	Females (not males) had lower fecal glucocorticoid metabolties (FGMs)	Hormonal	No relationship between fecal glucocorticoid metabolites (FGMs) and breeding experience	None	2
2011, 2013	Feb. – Apr.	Parents	Increased scent-marking and investigative behaviors	Behavioral	Experienced pairs scent-marked and investigated more frequently	Behavioral	2
2011, 2013	Apr. – Aug.	Parents	No association with parental care behaviors	None	Both mothers and fathers were move aggressive; provisioned pups more.	Behavioral	3
2011, 2013	Apr. – Aug.	Parents	Pre-partum fecal androgen metabolites negatively correlated with parental care behaviors	Both* (Correlation)	Pre-partum fecal androgen metabolites negatively correlated with parental care behaviors	Both* (Correlation)	3
2011, 2013	Apr. – Aug.	Pups	Pups born to odor-treated parents had higher cortisol at 5 weeks	Hormonal	Litters born to experienced parents had higher cortisol at 10 and 15 weeks	Hormonal	4
2011, 2013	Apr. – Aug.	Pups	No association with pup hair testosterone at any age	Hormonal	Litters born to experienced parents had lower testosterone at 5 weeks	Hormonal	4
2011, 2013	Apr. – Aug.	Pups	Pups emerged more frequently from dens at an earlier age (2011 only)	Behavioral	Litters born to experienced parents were bolder (emerged earlier, ate more frequently)	Behavioral	4
2011, 2013	Apr. – Aug.	Pups	Bold behaviors from litters negatively associated with pre-partum fecal androgen metabolites	Both* (Correlation)	Bold behaviors from litters negatively associated with pre-partum fecal androgen metabolites	Both* (Correlation)	4
2012, 2014	Apr. – Jul.	Yearlings	Yearling fecal androgen metabolites positively associated with parental pre-partum FAMs	Hormonal (Correlation)	Yearlings born to experienced parents had lower fecal androgen metabolites	Hormonal	5
2012, 2014	Apr. – Jul.	Yearlings	No association among yearling behaviors and parental odor treatment	None	Yearling boldness behaviors positively correlated with pup emergence; aggressiveness positively correlated with pup feeding behaviors	Behavioral (Correlation)	5

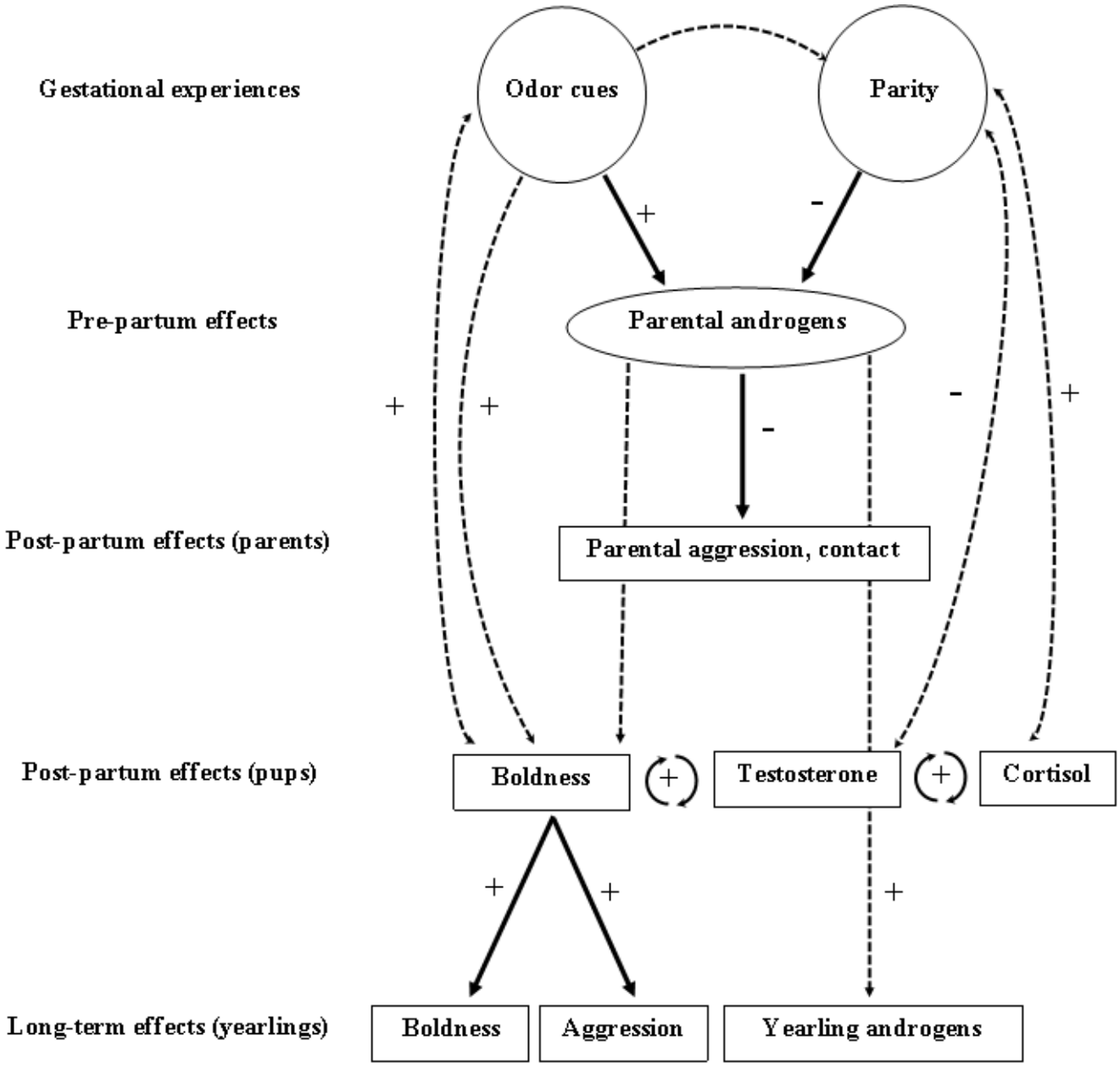

Fig. 6.1: Schematic depicting the cascading effects of pre-partum odor cues and breeding experience (i.e. parity) on parental and pup phenotypic traits. Dashed and solid lines indicate direct and indirect relationships among factors, respectively. Symbols (+) and (-) signify the direction of each relationship.

The evolutionary and social ecology of coyotes and spotted hyenas share remarkable similarity. Both are mesocarnivores that coexist with other larger predators across a landscape (Atwood & Gese 2010; Holekamp & Dloniak 2008). In addition, the two species occasionally scavenge kills and subsequently partition space with other sympatric carnivores to avoid conflict (Atwood & Gese 2010; Holekamp & Dloniak 2008). Further, coyotes and spotted hyenas are highly social and frequently use aggression as social currency in establishing and maintaining social bonds (Bekoff & Wells 1986; Holekamp & Dloniak 2008; Holekamp et al. 1996).

These similarities among species imply that selection pressures experienced by spotted hyenas are partially analogous to those experienced by coyotes. This prediction is strengthened by the fact that phenotypic consequences for both coyote and hyena offspring include associations among personality and hormonal traits essential to survival (i.e. boldness and aggression, respectively). The corresponding phenotypic outcomes for coyote offspring therefore infer pre-partum parental androgens have an adaptive function. The pairing among parental androgens and offspring phenotype is referred to as the maternal/fetal match hypothesis (Breuner 2008). Specifically, offspring phenotype is expected to reflect maternal hormones over gestation because such endocrine factors facilitate success under current conditions (Breuner 2008; Love & Williams 2008). This thesis provides evidence to support the maternal/fetal match hypothesis in coyotes, as parents with lower FAMs produced 5-week old pups with lower testosterone, whom subsequently had lower FAMs when they reached the yearling stage. It was beyond the scope of this thesis to assess the fitness outcomes (i.e. survival and reproduction) of captive coyote offspring, and future research will be necessary to establish the true adaptive function of androgen-facilitated parental effects in coyotes. Nevertheless, the adaptive

significance of these data can be extrapolated from the comprehensive network of interactions among parental androgens, parenting strategies, and offspring personality (i.e. boldness).

Hormones reinforced parenting styles and personality

Duckworth (2015) proposed that neuroendocrine mechanisms were inextricably linked to personality and subsequently imposed constraints on the range and depth of behavioral plasticity exhibited. Moreover, consistent individual differences in behavior often reflect underlying physiological function, suggesting that hormonal profiles are an integral component to understanding individual variation in behavior (Duckworth 2015). Similarly, Carere et al. (2010) hypothesized that covariance between personalities and individual hormonal differences implied a functional significance to consistent differences in behavior. In addition, changes to underlying physiological function enable adaptive modification of behaviors or other traits subject to selection pressures that may themselves change over time (Carere et al. 2010). Meanwhile, Reddon (2011) expressed the relevance of parental effects in generating the variation behavioral types observed in populations. Parental effects sufficiently bias offspring phenotype and generate variation in populations sensitive to selection, so it is logical to assume these mechanisms affect behavioral traits as well (Reddon 2011). Indeed, the coyote system provides evidence to support claims from Duckworth (2015), Carere et al. (2010), and Reddon (2011).

First, gestational FAMs were negatively associated with parental aggression and contact, parenting behaviors that were individually consistent within coyote parents across successive breeding events (Chapter 3). In addition, coyotes demonstrated consistent individual differences in glucocorticoids and androgens; and to a lesser extent, glucocorticoids underpinned individual

variation in activity and marking behaviors of coyote parents (Chapter 3). These results suggest individual differences in parental hormones and behavior reinforce each other to continually bias offspring phenotype. Consequently, coyote parents had the opportunity to directly (i.e. *in vitro*) and indirectly (i.e. through parenting) program offspring phenotype nongenetically. Further, individual differences in parenting strategies suggested coyotes possessed parental syndromes salient to generating phenotypic variation as posited by Reddon (2011). Individual differences in parenting strategies have previously been documented in other taxa (Budaev et al. 1999; Liu et al. 1997; Stein & Bell 2015; Westneat et al. 2011; 2013; Wetzel & Westneat 2014). The novelty here is that parental styles corresponded with hormone profiles, indicating that these individual differences are robust.

Second, both cortisol and testosterone concentrations of pups were positively associated with their boldness behaviors (Chapter 4). These results imply that personality traits of pups are also reinforced by underlying physiology. Further, consistency of boldness across contexts (i.e. feeding and novel object tests) at both the pup and yearling stages suggest behavioral syndromes for boldness (Chapters 4 and 5). Taken together, these data suggest behavioral syndromes may also be hormonally mediated, a prediction previously made by Carere et al. (2010) and Duckworth (2015). Perhaps most importantly, pup personality traits were directly associated with the pre-partum hormone profiles of their parents, corroborating predictions from Reddon (2011). Relatively new data provide complimentary findings to this thesis that further support parental effects as a mechanism shaping offspring personality. For instance, increased secretion of glucocorticoid concentrations in the colostrum of rhesus macaque mothers increase nervousness and cautious behavior in offspring (Hinde et al. 2014). Moreover, mothers with less breeding experience had higher concentrations of glucocorticoids in their colostrum. As a result,

less experienced mothers produced offspring that exhibited more cautious and nervous offspring personalities (Hinde et al. 2014). In zebra finches, a cross-fostering study demonstrated that foster chicks exploratory behaviors were positively associated with their foster rather than their genetic parents, whereas offspring body size was attributed to the genetic parents (Schuett et al. 2013). The results therefore indicated non-genetic inheritance as a key factor affecting zebra finch personalities (Schuett et al. 2013). Future research will be necessary to elucidate the breadth of personality traits potentially affected by parental effects mechanisms. Data from this thesis should appropriately be added to that catalogue of empirical examples.

Finally, temporal variation in gestational androgens of breeding pairs matched variation in parental styles and personality traits (Chapters 3 and 4). Parity and prior experience decreased androgens that affected both parental behavior and litter size, factors that continually interacted to result in the behaviors observed. These results therefore highlighted how plasticity in behavioral traits (e.g. parenting behaviors) are tightly correlated with hormonal profiles, as predicted by Duckworth (2015). Previous work has documented plasticity in care under varying environmental conditions (Ghalambor et al. 2013; Westneat et al. 2011; 2013), as well as plasticity in behavioral traits (Betini & Norris 2012; Briffa et al. 2008; Carter et al. 2012). Several studies have also documented plasticity of hormones and care as a function of successive breeding experience (Almond et al. 2008; Ziegler & Snowdon 2000). However, this thesis is one of the first studies to marry these previously distinct yet interconnected themes of plasticity in hormones and behavior, and demonstrate how covariance among these factors facilitate parental effects mechanisms

In sum, parenting styles, offspring personality, and inherent plasticity in parenting and personality were closely associated with androgens and glucocorticoids. The behaviors observed

were relevant to survival for wild populations of coyotes, indicating that hormonal profiles may have functional significance to how individuals behaviorally navigate environments. As a result, this thesis was able to provide information relevant to the complex proximate mechanisms affecting the adaptive capacity of the species, especially in the context of colonizing novel environments.

THE ROLE OF TEMPORAL VARIATION IN PARENTAL EFFECTS

Revisiting context-dependent and anticipatory programming

A primary goal of this dissertation was to determine how parental effects operated in the coyote system, as well as how such mechanisms may change as a function of time. With the data from this thesis I attempted to address the theoretical basis set forth by Reddon (2011), in which parental programming was predicted to either be context-dependent (i.e. increasing variance in offspring phenotypes under uncertain circumstances) or anticipatory (i.e. biasing offspring toward an optimum of predictable future conditions). Using the captive coyote system as a model to explore the theoretical constructs of parental programming, a critical element became apparent in addressing the issue: temporal variation. More specifically, increasing breeding experience of coyote parents was a principal determinant of pre-partum hormones (Chapter 2), parenting behaviors (Chapter 3), pup boldness and hormones (Chapter 4), and subsequent yearling boldness and hormones (Chapter 5; Fig. 6.1). Though parents demonstrated robust and distinct parenting styles and personalities, there was still a degree of plasticity attributed to overall experience with the breeding process, and even perhaps the environmental conditions established at the captive NWRC facility. Thus, it is paramount to explain the overarching

influence of time and parental experience to determine what parental programming mechanisms (i.e. context-dependent or anticipatory) affected coyote offspring.

First-time parents provided odor cues over gestation produced offspring that scored bolder on feeding tests (i.e. emerged from dens more frequently) early in development (Chapter 4; Fig. 6.1). The differences among litters from odor-provisioned and non-provisioned parents eventually waned as those pups aged, and the effects of novel conspecific odors pre-partum became less apparent. Results from the first breeding event provides evidence to support context-dependent programming because parents biased offspring phenotypes under relatively stochastic conditions. Specifically, the provisioned odors as well as the process of application (i.e. a human observer manually applying odor cues) were wholly novel experiences to coyote breeding pairs, who were also experiencing the breeding process for the first time. The combination of novel events presented an artificial context of unpredictability that likely elicited context-dependent programming. Comparisons across breeding events, however, indicate potential changes in the programming mechanisms exhibited by breeding pairs.

Coyote pairs as experienced parents produced offspring that scored bolder on feeding and novel object tests regardless of prior odor condition (Chapter 4). Both parents and pups were bolder and more willing to forage with a predator cue (i.e. human observer) present. Moreover, experienced pairs and 5-week old pups had lower fecal androgens and hair testosterone concentrations. These findings implicate anticipatory programming as the most appropriate mechanism attributing to the observed phenotypic outcomes. It is reasonable to suggest that an additional breeding opportunity, as well as 2 years of additional exposure to the captive facility, produced a relatively predictable environment. All testing regimes were also identical to the first year of observation, providing an additional layer of predictability. Thus, if coyote pairs

remembered test procedures and made associations among people, then they would have reliable information to predict future conditions and tailor offspring phenotypes accordingly. Indeed, amplified boldness of parents and pups toward human observers is likely a result of parental habituation and tolerance of people. Anticipatory parental effects therefore appeared to be the prevailing mechanism for coyotes as experienced breeders.

Consolidating the results from both breeding events suggests that both context-dependent and anticipatory programming played an active role in shaping coyote offspring phenotype. Context-dependent programming was prevalent when parents were first-time breeders, whereas parents appeared to transition to anticipatory programming with increasing breeding experience and environmental predictability. The inherent temporal variation in coyote parental programming highlights how critical it is to consider environmental predictability and stochasticity, particularly over contemporary timescales. Alterations to habitat conditions or biological communities produce complementary changes to selection pressures (Bonduriansky & Day 2009; Räsänen & Kruuk 2007). As a result, parents are likely to experience variable pressures over time that differentially affect their phenotypes, and in turn can differentially affect successive litters or broods (Marshall & Uller 2007; Uller 2008). The immigration of novel predators, habitat degradation and modification, or shifts in conspecific social dynamics may all contribute to the phenotypic changes parents experience over their lifetimes (Bonduriansky & Day 2009; Wolf et al. 1998). Therefore, how quickly and sufficiently parents mount an adaptive response toward environmental changes dictates the success of future offspring (Marshall & Uller 2007). This empowers parents with the ability to augment the evolutionary trajectories of populations and contribute to the rapid divergence of those populations (Sheldon 2002; Wolf et al. 1998).

Determining the rate of phenotypic response to rapid environmental change has become increasingly important for many species, particularly in the context of human-induced environmental change (Hansen et al. 2005). The common thread that persists across other wild coyote studies and this thesis is how individuals habituate to people over time. Here, offspring boldness in relation to human observers reflected increased parental experience with humans (Chapter 4). These findings implicate parental effects as the primary mechanism affecting offspring boldness and risk assessment of people and associated stimuli. Such factors are critical for population persistence of multiple species in human-dominated landscapes (Atwell et al. 2012; Bateman & Fleming 2014; Lowry et al. 2013). Perhaps it is also important to consider the role such factors play in the initial colonization process of urban environments. Previous work by Duckworth et al. (2015) adeptly demonstrated how maternal effects drive cycles of habitat colonization by augmenting personality variation of individuals from two species of bluebirds (*Sialia currucoides* and *S. mexicana*). Similarly, I argue that parental influences on boldness behaviors in coyotes enabled the species to infiltrate previously unexploited urban and suburban environments across the North American continent. Thus, epigenetic inheritance provides a lens to appreciate the rapid historical colonization of metropolitan areas by coyotes, while simultaneously predicting future colonization events and long-term persistence.

Stability of personality over time

If parental influences on pup personality traits were momentary, then it would be difficult to suggest parental effects drive coyote adaptation and colonization of nonnative habitats. This is particularly because we could not infer that developmental processes have any bearing on adult traits, and therefore not affect survival outcomes or reproductive success. On the contrary, this

thesis established a clear association between pup and yearling phenotype (Chapter 5). Moreover, yearling androgen profiles paralleled parental androgens pre-partum, when yearlings were developing *in vitro* (Chapter 5). The persistence of personalities and hormonal profiles strongly suggest that parental influences are dynamic and enduring. Stamps & Groothuis (2010a; 2010b) predicted that developmental experiences should bias adult behavioral phenotypes, emphasizing that experiences early in life have the potential to affect developmental trajectories relevant to phenotypic maturation. Indeed, bolder pups were bolder and more aggressive yearlings (Chapter 5), indicating that experiences during feeding and novel object tests were salient.

Perhaps most importantly, the stability of personality traits across life stages further support the central role of parental effects in coyote colonization and persistence in nonnative habitats. Parents were able to transduce environmental experiences via androgens, which in turn affected pup development. By extrapolating these data, we can conclude that parental experiences may ultimately impact the parental behaviors of coyote offspring from the F1 generation. Consequently, offspring of the F2 generation and beyond may continually be affected by grandparental phenotype because parents from the F1 generation were heavily biased their parents. This multigenerational, epigenetic transmission of traits have previously been addressed (Lock 2012; Love et al. 2013), though few provide empirical support for this prediction. The evolutionary significance of parental effects may lie in the duration to which these nongenetic biases persist, and how strongly they permeate throughout generations. Longstanding effects that proceed over successive generations are likely to have stark consequences for evolutionary lineages, ultimately leading to population divergence (Bonduriansky & Day 2009; Mousseau & Fox 1998; Sheldon 2002). It is therefore critical for future research to delve into the persistence

of parental effects over multiple generations, trace the impact of such processes on traits relevant to individual fitness, and determine how time modifies traits facilitating epigenetic inheritance.

FUTURE DIRECTIONS

To fully understand the gravity of parental effects in the coyote system, it will be essential to examine both genetic and epigenetic influences on phenotypic traits. Phenotype alone is insufficient to determine evolutionary trade-offs or population adaptation (Duckworth 2015; Dochtermann et al. 2010). It will therefore be necessary to utilize a behavioral genetics approach to assess whether observed personality traits also have a genetic basis, or if the results observed here are relatively fleeting. Determining the genes that regulate hormonal secretion, for instance, helps to establish a link between genotype and behavioral phenotype (Dochtermann et al. 2010; van Oers et al. 2005). This thesis has thoroughly demonstrated the robust associations among androgens and personality traits. Consequently, future research on the genetic factors regulating androgen receptors or the HPG-axis may prove a fruitful endeavor to decoding rapid coyote adaptation.

Another productive avenue of research may include previously documented genetic associations with boldness. Specifically, polymorphisms at the exon 3 region of the dopamine receptor D_4 (DRD4) gene have been linked to activity, impulsivity, and novelty-seeking behaviors in multiple taxa. In vervet monkeys (*Chlorocebus pygerythrus)*, individuals heterozygous for the 5-repeat (i.e. "short") DRD4 allele are more socially-impulsive and explore novel objects more readily compared to homozygous individuals for the 6-repeat (i.e. "long") allele (Bailey et al. 2007; Fairbanks et al. 2012). Siberian huskies (*Canis familiaris*) with the short DRD4 alleles are more active and less attentive to handlers versus individuals with the long

alleles (Wan et al. 2013). Moreover, DRD4 gene polymorphisms are associated with personality variation in exploratory behavior of great tits (*Parus major*; Fidler et al. 2007) and neophobia of yellow-crowned bishops (*Euplectes afer*; Mueller et al. 2014). Thus, DRD4 associations with personality traits extend across various mammalian and avian clades. Tolerance and novelty-seeking have previously been hypothesized to greatly impact colonization of human-dominated landscapes by coyotes (Ditchkoff et al. 2006; Gehrt 2010; Lowry et al. 2013; Šálek et al. 2014; Young et al. 2015). Thus, evidence of genotype-personality associations would suggest that behaviors relevant to coyote adaptation have a genetic basis.

The combination of genetic and epigenetic data, however, have broader implications that extend beyond coyotes. Collective work from leaders in evolutionary theory are beginning to validate the ultimate consequences of epigenetic inheritance and the ever-increasing significance of interactions among genetic and non-genetic factors (Houle et al. 2010; Kussell & Leibler 2005; Laland et al. 2014; Price et al. 2003). Recently, a group of prominent evolutionary biologists came together to assess the state of evolutionary biology as a theory and in practice (Laland et al. 2014). Laland et al. (2014) addressed the pervasiveness of epigenetic effects, gene expression, development, and other disciplines that both drive and bias evolutionary trajectories of populations. Consequently, the authors established an extended evolutionary synthesis (ESS) that encompassed fields previously not considered part of mainstream evolutionary theory (Laland et al. 2014).

Albeit a single species and perhaps a special circumstance, the coyote system embodies the theoretical constructs established by Laland et al. (2014). Parental effects permeated throughout this system, affecting phenotypic traits relevant to selection. It is likely that the observed trait correlations among behaviors and hormones in this thesis represent latent phenotypic

correlations that were not only selected in other habitat settings (i.e. naturalized areas), but also genetically associated. If in fact gene frequencies correspond with personality differences in the species, then the ESS framework is far more suitable to understanding evolutionary processes than exclusive focus on genetic inheritance. Further, future work could infer patterns of rapid adaptation in other species inhabiting urban ecosystems (Atwell et al. 2012; Bateman & Fleming 2014) to evaluate the factors integral to species persistence amid vast ecological change. Therefore, narrowing the focus to either genetic or epigenetic influences on phenotype present only an abridged version of the evolutionary story. My hope here is that this thesis sufficiently provided the first half of a coyote adaptation narrative that continues to expand and evolve.

APPENDIX A: FECAL HORMONE VALIDATIONS

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Anthropogenic and physiologically induced stress responses in captive coyotes

CHRISTOPHER J. SCHELL,* JULIE K. YOUNG, ELIZABETH V. LONSDORF, AND RACHEL M. SANTYMIRE

Committee on Evolutionary Biology, University of Chicago, 900 E 57th St. CULV 402, Chicago, IL 60637, USA (CJS)
United States Department of Agriculture–Wildlife Service–National Wildlife Research Center, Predator Research Facility, and Department of Wildland Resources, Utah State University, USDA-WS-NWRC Predator Research Facility, Department of Wildland Resources, USU - BNR 163, Logan, UT 84322-5292, USA (JKY)
Department of Psychology, Franklin and Marshall College, Lancaster, PA 17603, USA (EVL)
Conservation and Science, Lincoln Park Zoo, 2001 N. Clark St., Chicago, IL 60614, USA (RMS)

* *Correspondent: cschell@uchicago.edu*

Repeated stressful events can negatively impact overall health by continuous stimulation of the hypothalamic–pituitary–adrenal axis, which leads to depletion of glucose stores and suppression of immune and reproductive function. The influence of stressors on survivability is particularly salient for coyote (*Canis latrans*) populations, because understanding how coyotes cope with stressors may provide relevant context on coyote adaptation to urbanized ecosystems. Our objectives were to physiologically validate fecal glucocorticoid metabolite (FGM) analysis in coyotes by performing an adrenocorticotropic hormone (ACTH) challenge in 12 captive individuals (6 treatment and 6 control) housed at the United States Department of Agriculture National Wildlife Research Center Predator Research Facility in Millville, Utah; to quantify potential changes in FGM output due to diurnal variation and sex; and to determine the effects of 2 anthropogenic events (placement of a novel cooling fan and state holiday celebrations) on the coyotes' stress responses (via FGM production). Results demonstrated that in response to the ACTH injection, treatment animals (3 males and 3 females) displayed FGM concentration peaks $\geq$ 5-fold (range: 5- to 30-fold) above their preinjection means approximately 8 h after injection, which was a greater ($P = 0.037$) response than control animals. FGM output was lowest for morning fecal samples compared with midday ($P = 0.001$) and evening ($P < 0.001$) samples. Within the evening period, FGM output for male samples tended to be higher ($P = 0.056$) than for female samples, although not significant. The anthropogenic events elicited FGM concentration peaks $\geq$ 5-fold above pre-event means for several of the study animals occurring approximately 12 and 9 h later, respectively. This study is the 1st to physiologically validate the measurement of stress physiology using FGM analysis in coyotes and demonstrate the impact of anthropogenic events on their stress response. Furthermore, this work provides a foundation for future studies of FGMs, stress, and anthropogenic effects in wild and captive systems.

Key words: adrenocorticotropic hormone (ACTH) challenge, anthropogenic stressors, *Canis latrans*, diurnal variation, enzyme immunoassay, fecal glucocorticoid metabolite analysis, stress response

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Coyotes (*Canis latrans*) provide an interesting system to examine stress physiology because of the species' ability to successfully persist in nonnative environments. Recent ecological literature has suggested that increased fragmentation, development, and reduction of tertiary predators such as gray wolves (*Canis lupus*) in multiple niches facilitated coyote geographic expansion (Grubbs and Krausman 2001a; Fox 2006). Coyotes are relatively flexible in their landscape use, enabling them to survive despite environmental change (Bekoff and Wells 1980, 1986; Séquin et al. 2003; Gehrt 2007). Changes to coyote home-range size and movement patterns (Way et al. 2001; Tigas et al. 2002; Riley et al. 2003; Gehrt et al. 2009), habitat use and diet (Grinder and Krausman 2001a; Randa and Yunger 2006; Morey et al. 2007; Grubbs and Krausman 2009), health and disease (Grinder and Krausman 2001b; Liccioli et al. 2012), and social interactions (Atwood et

al. 2004) have been documented in multiple urban and suburban landscapes. However, no study has evaluated potential changes to stress physiology as a result of increased urban living or validated methods to analyze glucocorticoids in this species. The instances of stochastic events, novel stimuli, and chronic stressors are arguably greater in metropolitan areas compared with native environments, and increased chronic stress negatively influences overall health and survival (Love and Williams 2008; Schulkin 2011). Despite these factors, coyotes in metropolitan areas do not have greater disease prevalence compared with other populations, and population densities are increasing (Grinder and Krausman 2001b). Therefore, measuring glucocorticoids may be an informative tool in understanding how coyotes cope with nonnative environments.

Monitoring adrenocortical activity also may prove valuable in determining the physiological influence social group dynamics have on coyotes. Unlike related Canidae social systems (e.g., African wild dogs [*Lycaon pictus*] and gray wolves, coyote populations may persist in an array of resident and transient individuals across a landscape (Atwood et al. 2004; Fox 2006; Gehrt 2007). Territorial incursions by transients or neighboring residents into home ranges of resident coyotes can result in conflict (Gese et al. 1996; Gese 1998, 2001). Additionally, competition for and reinforcement of dominance status within a group can result in instances of physical (e.g., biting and chasing) and behavioral (e.g., teeth baring and scent marking—Gese et al. 1996; Gese 1998, 2001) aggression. These instances of conflict may differentially increase and affect adrenocortical activity of an individual as a function of social status, subsequently influencing overall health. Consequently, glucocorticoid analysis may prove beneficial in examining the effects of social stimuli on coyote adrenocortical activity.

The biological stress response is characterized by the activation of the hypothalamic–pituitary–adrenal axis, which is a negative feedback system that regulates the production of glucocorticoids (Jacobson and Sapolsky 1991; Herman et al. 2003; Ulrich-Lai and Herman 2009). At the onset of hypothalamic–pituitary–adrenal—axis activation, corticotropin-releasing hormone is released by the hypothalamus and stimulates the anterior pituitary gland, resulting in the production of adrenocorticotropic hormone (ACTH). The ACTH then stimulates the adrenal cortex to release glucocorticoids, such as cortisol and corticosterone (Dedovic et al. 2009). These glucocorticoids circulate (via blood) back to the hypothalamus as part of the negative feedback loop to cease hypothalamic–pituitary–adrenal—axis stimulation (Dedovic et al. 2009; Schulkin 2011). Short-term activation of the hypothalamic–pituitary–adrenal axis is highly adaptive, because the production of glucocorticoids mobilizes energy stores via increased gluconeogenesis, suppresses secondary physiological functions (e.g., immune, reproductive, etc.), and attenuates memory retention. Conversely, overproduction of glucocorticoids in the long term depletes available glucose, resulting in several physiological issues that can decrease overall health and fecundity (Love and Williams 2008; Schulkin 2011). As a result, chronic stress can be a significant threat to the fitness of an organism by disrupting proper hypothalamic–pituitary–adrenal—axis function.

Noninvasive fecal glucocorticoid metabolite (FGM) monitoring is ideal for wildlife species for several reasons. First, repeated capture events and blood draws of wild individuals are impractical and can result in rapid increases in serum glucocorticoid concentrations due to handling stress that ultimately influence subsequent analysis of adrenocortical activity (Möstl and Palme 2002; Touma and Palme 2005; Santymire et al. 2012). Second, plasma glucocorticoids only sample a short time period and are more susceptible to fluctuations due to handling or blood draws (Harper and Austad 2000; Möstl and Palme 2002; Santymire and Armstrong 2010; Santymire et al. 2012). In contrast, fecal glucocorticoid metabolites provide an inclusive view of adrenocortical activity over a 12- to 48-h period and are less vulnerable to rapid fluctuations in glucocorticoid concentrations (Wielebnowski et al. 2002; Loeding et al. 2011; Santymire et al. 2012). The validation and analysis of FGMs has been used on several canid species including domestic dogs (*Canis lupus familiaris*—Schatz and Palme 2001), gray wolves (Sands and Creel 2004), red wolves (*C. lupus rufus*—Young et al. 2004), African wild dogs (Monfort et al. 1998), and maned wolves (*Chrysocyon brachyurus*—Vasconcellos et al. 2011).

Our goal was to develop noninvasive methods to study the influence of stressors on overall health, which is particularly salient for coyote populations, because understanding how coyotes cope with stressors may provide relevant context on coyote adaptation to urbanized ecosystems. Our objectives were to physiologically validate FGM analysis in coyotes by performing an ACTH challenge in 12 captive individuals (6 treatment and 6 control) housed at the United States Department of Agriculture National Wildlife Research Center (NWRC) Predator Research Facility in Millville, Utah; to quantify potential changes in FGM output related to diurnal variation and sex; and to determine the effects of anthropogenic events (placement of a novel cooling fan and state holiday celebrations) on the coyotes' stress responses (via FGM production). We hypothesized that individuals receiving the ACTH injection would demonstrate peak FGM concentrations greater than those receiving saline injections and that the FGM concentrations would be greater for females compared with males. Previous literature on Carnivora has demonstrated higher glucocorticoids in females compared with males (African wild dogs [Creel et al. 1997], domestic dogs [Schatz and Palme 2001], and cheetahs [*Acinonyx jubatus*—Wielebnowski et al. 2002]), although this trend is not demonstrated consistently in the clade (African wild dogs [Monfort et al. 1998], and gray wolves [Sands and Creel 2004]). Additionally, we predicted that FGM output would gradually increase from morning to evening samples because coyotes are mainly crepuscular (Way et al. 2001; Gehrt 2007); thus, we anticipated higher FGMs in the evening and midday due to the lag time between circulating plasma hormones and excreted fecal

Table 1.—Descriptive values detailing the treatment condition, proximity to the facility entrance, total injection dose, fold-increase values for the adrenocorticotropic hormone (ACTH) injection and anthropogenic stressors (cooling fan introduction and Pioneer Day festivities), and excretion lag times for the ACTH injection and anthropogenic stressors for each study animal. Excretion lag times are reported as total hours and minutes elapsed between experience of a stressor and excretion of fecal glucocorticoid metabolite (FGM) peak values. FGMs were observed from 17 July 2011 to 1 August 2011 at the National Wildlife Research Center (NWRC) Predator Research Facility in Millville, Utah. F = female, M = male, NA = not available.

					ACTH		Cooling fan		Pioneer Day	
Identification	Sex	Treatment group	Proximity to facility entrance (m)	Total ACTH given (IU)	Fold increase	Excretion lag time	Fold increase	Excretion lag time	Fold increase	Excretion lag time
06102	F	ACTH[a]	4	40.4	19	7 h 33 min	1	24 h 01 min	3	9 h 46 min
1032	F	ACTH[a]	8	42.2	5	7 h 33 min	4	9 h 57 min	2	9 h 29 min
1052	F	ACTH[a]	12	41	30	7 h 29 min	2	24 h 00 min	1	23 h 40 min
0422	F	Control	2	46.6	17	7 h 37 min	16	9 h 58 min	6	9 h 40 min
06052	F	Control	6	39.6	7	7 h 36 min	3	24 h 00 min	0	9 h 40 min
1050	F	Control	10	41.8	2	17 h 35 min	0	24 h 00 min	5	9 h 33 min
06063	M	ACTH[a]	3	47	13	7 h 32 min	9	9 h 58 min	9	9 h 24 min
1051	M	ACTH[a]	7	46.2	12	10 h 53 min	2	9 h 53 min	10	9 h 30 min
1071	M	ACTH[a]	11	46.2	13	7 h 28 min	2	9 h 48 min	1	9 h 40 min
08171	M	Control	1	45.6	4	7 h 24 min	7	9 h 58 min	18	9 h 40 min
06133	M	Control	5	42.2	4	7 h 45 min	5	9 h 55 min	1	23 h 41 min
1041	M	Control	9	46.2	3	7 h 31 min	0	24 h 00 min	NA	NA

[a] The ACTH dosage was 4 IU/kg.

hormonal metabolites. Finally, we predicted that salient anthropogenic stressors would reliably correlate with FGM peaks witnessed post–anthropogenic event. We conducted this study in captivity specifically to be able to monitor and account for prominent environmental or anthropogenic stressors, in addition to obtaining repeated fecal samples from known individuals.

Materials and Methods

Animals.—Twelve captive-born individuals (6 males and 6 females) ranging in age from 1.0 to 7.0 years (3.0 ± 0.7 years [$\bar{X} \pm SEM$]) were housed at the NWRC. Coyotes at the facility were housed in multiple enclosure types ranging from large outdoor pens (0.1–6.0 ha) to kennel environments (3.3 m^2). Study animals were moved to outdoor raised kennels (3.3 m^2) on 5 July 2011 and given 12 days to acclimate to their environment prior to fecal sample collection. To reduce the stress response to researcher presence during collection events, we approached animals daily at 0800, 1300, and 1800 h starting on 13 July 2011, which corresponded to the collection schedule. Animals were fed 650 g of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, Utah) daily and water was provided ad libitum (Brummer et al. 2010). Additionally, kennels were cleaned daily in accordance with standard operating procedures for the Millville site (SOP: AC/UT 001.00 Daily coyote check and care for Millville Predator Research Facility).

Adrenocorticotropic hormone challenge.—We randomly selected coyotes for 1 of 2 treatment groups: control (saline) and treatment (ACTH). Both groups had an equal number of males and females ($n = 6$; 3 males and 3 females). The ACTH (Sigma-Aldrich, St. Louis, Missouri) dosage was 4 IU/kg given intravenously, determined by a prior challenge experiment conducted in 2011 (S. French, Utah State University, pers. comm.). The ACTH (range: 40.4–47 IU total) and saline (range: 39.6–46.6 IU total) injections were administered from 0757 to 0831 h on 27 July 2011 (Table 1). All animals were weighed a week before injection to determine proper doses. Immediately before all injections, doses were drawn up in saline solution and animals were moved into standard NWRC den boxes to physically restrain them during injection. This capture procedure is standard at the NWRC facility and is used to reduce injury to the animals and staff.

The time of injection was alternated by sex and treatment, with the exception of 2 anxious individuals (1 control female and 1 control male), which were injected 1st to reduce the potential for injury. Average amount of time to restrain, inject, and release each animal was 0 h 2 min ± *SD* 1 min (range: 1–4 min). After the injection period, animals were kept in the den boxes and all kennels were cleaned to ensure fresh fecal samples were collected. Individuals were then released back into their kennels, as staff and researchers withdrew from the area to reduce potentially stressful activity. Total time to inject all animals was 38 min, and total time of human presence on site was 127 min from initial arrival to the end of kennel cleaning.

We collected fecal samples from 17 July to 1 August 2011, 2 or 3 times daily at 0800–0900 h (AM period), 1300–1400 h (midday: MD period), and 1800–1900 h (PM period). Additionally, all samples defecated were collected 5 days post–ACTH injection to ensure that samples containing the ACTH-induced peaks would be observed in our analyses. Time of collection was recorded and multiple samples collected within the same time frame were ordered by freshness. We determined sample freshness by location of the defecated sample compared with samples collected previously during the

day or week, in addition to stiffness and overall appearance. During collection events, several samples were found in pools of urine. These samples were considered contaminated and were not collected for analysis. All samples were immediately stored at –20°C to limit the amount of hormone metabolite degradation. Samples were then shipped overnight on dry ice to the Lincoln Park Zoo Endocrinology Laboratory (Chicago, Illinois) for FGM analysis. All research conforms to guidelines of the American Society of Mammalogists for research on live animals (Sikes et al. 2011), and was approved through the University of Chicago Institutional Animal Care and Use Committee (ACUP 72185), Lincoln Park Zoo Research Committee, and the NWRC Institutional Animal Care and Use Committee (QA-1809).

Diurnal variation and sex differences in FGM output.—Samples collected during the study period also were used to examine potential diurnal variation and sex differences in FGM output. Briefly, samples were collected from 17 July to 1 August 2011, 2 or 3 times daily. Additionally, samples were collected at different periods during the day (AM, MD, and PM) to compare FGMs across collection periods. All animals were sampled during the same time period each day. Samples that were contaminated by urine were not collected.

Previous literature has demonstrated that degradation of glucocorticoid hormones due to bacterial decomposition occurs near linearly over a 12-h period, which could influence diurnal variation in FGM output (Möstl and Palme 2002; Shutt et al. 2012). This study does not specifically examine differences in FGM output due to increasing ambient environmental exposure. However, to account for this issue we cleaned and monitored kennels daily to obtain fecal samples as close to defecation as possible. Further, captive coyotes at the NWRC facility defecate 3 or 4 times daily, with the majority of fecal samples defecated from 0500 to 0900 h (staff, NWRC, pers. obs.).

Anthropogenic stressors.—An anthropogenic stress event was characterized as a human-associated event resulting in a loud audible noise or visual stimuli that occurred within or near the kennel environment. Several minor events were documented during the study period; however, 2 major events also occurred. On the morning of 21 July 2011, a large cooling fan was introduced into the kennel area near the facility entrance. A few days later marked the beginning of Pioneer Day, which is a Utah state holiday celebrated with a combination of fireworks and parades. During the 2011 year, approximately 25,000 people participated in the day's activities from the morning of 23 July and continued into 24 July 2011. Neighboring residents near the NWRC facility used fireworks on 22 July 2011, and continued use throughout the weekend. One control male did not defecate from the morning of 23 July to 26 July 2011, and was therefore excluded from Pioneer Day analyses. The date and diurnal period (AM, MD, or PM) were noted when the events occurred.

Fecal sample processing.—All samples were freeze-dried on a lyophilizer (Thermo Modulyo Freeze Dryer; Thermo Scientific, Waltham, Massachusetts) for 3 days and crushed to a fine powder before extraction using previously described methods (Brown et al. 1994; Santymire et al. 2012). Briefly, samples were then weighed (0.2 ± *SD* 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 (setting 60). The samples were then centrifuged for 20 min at 1,500 rpm at 10°C, and the supernatant was poured into clean glass tubes. The fecal pellet was resuspended in 5.0 ml of 90% ethanol, vortexed for 30 s, and recentrifuged for 15 min at 1,500 rpm. The supernatant was poured into the corresponding glass tubes and the combined supernatants were dried down under air and a hot-water bath (60°C). Once dry, all samples were 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.

Enzyme immunoassay.—We examined the effectiveness of 2 previously described corticosterone and cortisol enzyme immunoassays (Young et al. 2001; Santymire and Armstrong 2010; Heintz et al. 2011; Santymire et al. 2012) to observe coyote FGMs. Polyclonal cortisol antiserum (R4866), corticosterone antiserum (CJM006), and horseradish peroxidase were provided by C. Munro (University of California, Davis, California). Cortisol antiserum, corticosterone antiserum, cortisol horseradish peroxidase, and corticosterone horseradish peroxidase were used at dilutions of 1:8,500, 1:15,000, 1:20,000, and 1:15,000, respectively (Santymire and Armstrong 2010; Heintz et al. 2011). The enzyme immunoassays were biochemically validated by demonstrating parallelism between binding inhibition curves of fecal extract dilutions (1:2–1:1,024), the cortisol standard ($R^2 = 0.980$), and the corticosterone standard ($R^2 = 0.976$); and significant percent recovery (> 90%—Santymire and Armstrong 2010; Santymire et al. 2012) of exogenous cortisol (1:1,500; $\hat{y} = 0.8702x + 8.2525$, $R^2 = 0.9955$) and corticosterone (1:600; $\hat{y} = 0.848x + 22.642$, $R^2 = 0.9385$) added to pooled fecal extracts. Assay sensitivity was 1.95 pg/well and intra- and interassay coefficients of variation were < 10% for both enzyme immunoassays. Further, we used Pearson product moment correlation to compare our cortisol and corticosterone standards with serially diluted fecal extracts (Santymire et al. 2012). Our cortisol enzyme immunoassay had a higher correlation ($r = 0.997$) compared with our corticosterone enzyme immunoassay ($r = 0.968$). As a result, we used cortisol as our primary enzyme immunoassay for this study.

Data analyses.—We tested FGM data for normality using the Shapiro–Wilk test statistic. Data that were not normally distributed were natural log–transformed. For physiological validation, samples collected 72 h preinjection ($n = 3$ or 4) for each individual were averaged and compared to elevated values post–ACTH injection for each individual. We used fold increase to quantify the FGM response to the ACTH injection (Monfort et al. 1998; Brown et al. 1999). Specifically, fold increase was determined by computing the quotient between the pre–ACTH injection mean and the FGM peaks (Table 1). To determine if fold-increase values differed

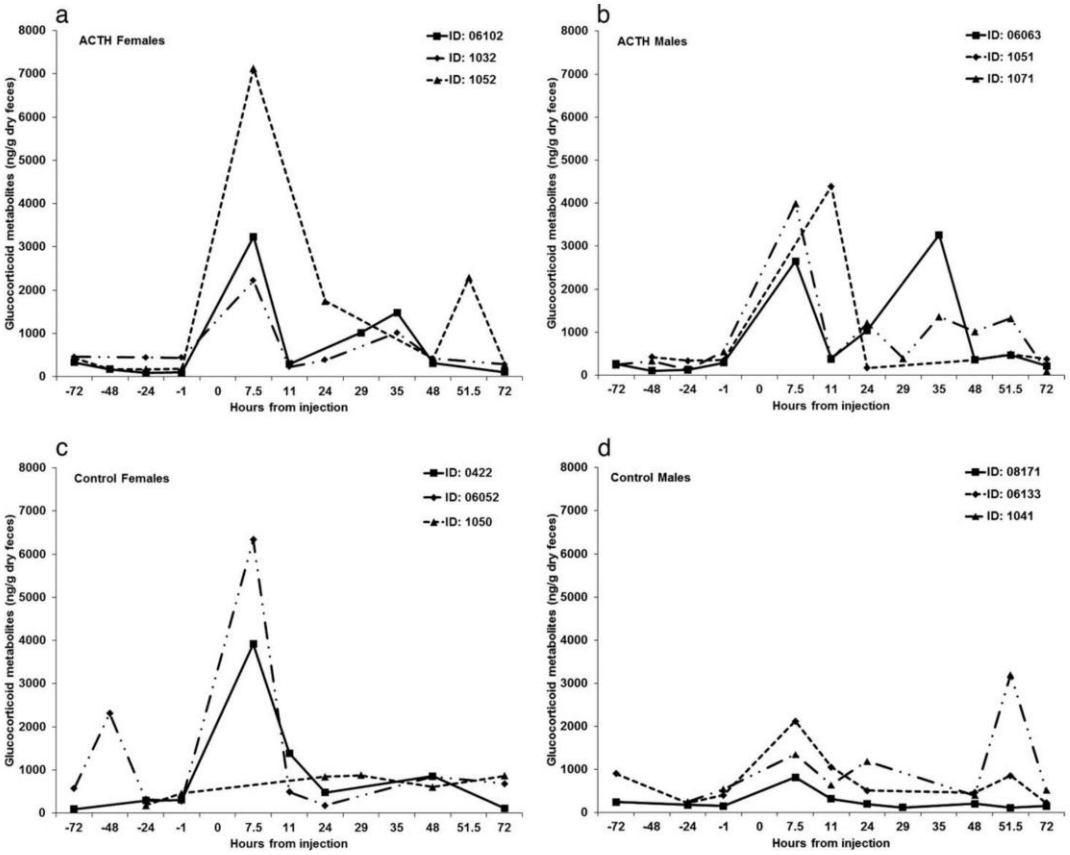

Fig. 1.—Fecal glucocorticoid metabolite (FGM) concentrations for a) females given adrenocorticotropic hormone (ACTH; $n = 3$), b) control females ($n = 3$), c) males given ACTH ($n = 3$), and d) control males ($n = 3$) 72 h before and after injections. The ACTH (range: 40.4–47.0 IU total) and saline (range: 39.6–46.6 IU total) injections were administered from 0757 to 0831 h on 27 July 2011 (hour 0) at the National Wildlife Research Center (NWRC) Predator Research Facility in Millville, Utah.

as a function of treatment condition or sex, we utilized Mann–Whitney *U*-tests for post–ACTH injection data. Additionally, we used Spearman rank correlation analyses to determine if order of injection, mass, or proximity to the facility entrance was related to ACTH fold-increase values. Time of ACTH injection was compared to time of collections postinjection to determine the approximate excretion lag time for FGMs.

To determine the potential for diurnal variation (comparing AM, MD, and PM samples) we used mixed regression models to measure the influence of time on FGMs. We specified collection period as the main fixed effect and animal identification as a random effect to account for repeated measures from the same individual. Sex, treatment condition, and animal mass also were specified as fixed effects in the model. The model was fitted with a random intercept and slope to examine whether individual differences in FGMs were consistent within subjects. Additionally, paired *t*-tests were performed to further examine diurnal variation of FGM output within subjects. Results were Bonferroni adjusted to account for multiple comparisons between collection periods (AM, MD, and PM). We excluded peak FGM values 7.5 h (11 h for coyote 1051) post–ACTH injection from these analyses.

Fold increase in response to anthropogenic stressors was quantified similarly to our ACTH results. Briefly, fold increase in response to anthropogenic events was quantified by computing the quotient between the pre-event means and the FGM peaks; then Mann–Whitney *U*-tests were used to determine differences as a function of a priori treatment condition and sex. Spearman rank correlation analyses were used to determine if animal mass or proximity to the facility entrance had any relationship with fold increase in FGMs post–anthropogenic event. Time of the anthropogenic stressor was

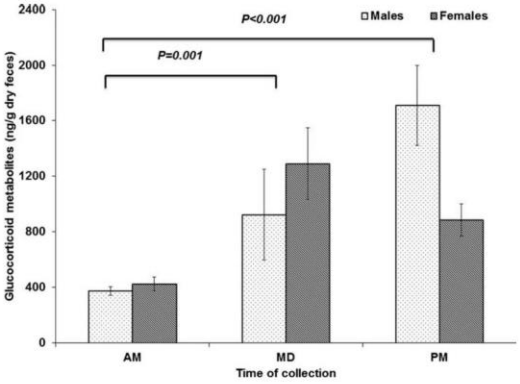

FIG. 2.—Mean (± *SE*) fecal glucocorticoid metabolites (FGMs) over time (0800–0900 h [AM], 1300–1400 h [midday: MD], and 1800–1900 h [PM] for all study coyotes. Peak FGM values 7.5 h (11 h for coyote 1051) post–adrenocorticotropic hormone injection were excluded from these analyses.

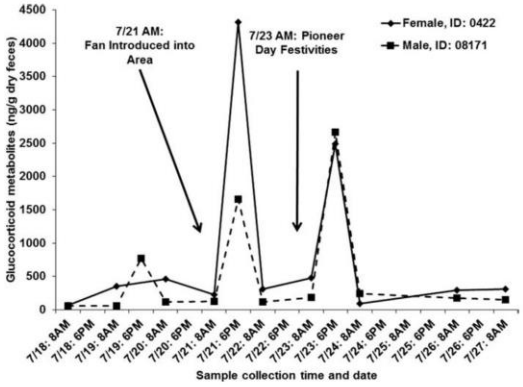

FIG. 3.—Fecal glucocorticoid metabolite (FGM) concentrations of 2 study coyotes (1 male and 1 female) representative of the sample population that experienced pronounced FGM peaks to the observed environmental events.

compared to time of collection postevent to determine the approximate excretion lag time. These anthropogenic excretion lag-time data were then compared to our ACTH challenge for further biological validation. All statistical analyses were performed using SYSTAT 11 (Systat Software, Inc. 2008). Results are presented as mean ± *SE*, where *P*-values < 0.05 were considered significant and *P*-values between 0.051 and 0.1 were considered a trend. Cortisol metabolite data were used for all analyses.

RESULTS

Adrenocorticotropic hormone challenge.—Pre–ACTH injection averages did not differ by treatment ($n = 12$, $t_{10} = -1.155$, $P = 0.275$), sex ($n = 12$, $t_{10} = 0.440$, $P = 0.670$), or mass ($b = -0.449$, $t_{10} = -1.587$, $P = 0.144$). Fold-increase values above the pre–ACTH injection mean were greater (Mann–Whitney $U_{11} = 31.00$, $P = 0.037$) in the treatment group (5- to 30-fold) compared with the control group (1- to 17-fold). Peak values postinjection for all ACTH animals were at least 5-fold higher than the preinjection average; 3 control individuals (2 females and 1 male) had comparable fold-increase values (Table 1). There was no relationship between fold-increase values and order of injection ($\rho = -0.014$, $P = 0.964$), or fold-increase values and kennel proximity to the facility entrance ($\rho = 0.0$, $P = 1.0$). Approximate excretion lag time for all individuals was 8 h 39 min ± 0 h 10 min (range: 0724–1735 h; 0 h 10 min, Fig. 1; Table 1).

Diurnal variation in FGM output.—Overall FGM output did exhibit diurnal variation ($z = -9.055$, $SE = 0.078$, $P < 0.001$). Post hoc paired *t*-tests demonstrated significant differences between AM and MD FGMs ($t_{10} = -4.416$, $P = 0.001$), and AM and evening FGMs ($t_{11} = -6.481$, $P < 0.001$). MD and PM FGM values were not statistically different ($t_{10} = -0.994$, $P = 0.343$; Fig. 2). There was a trend for greater FGMs in males ($t_{10} = -2.163$, $P = 0.056$; Fig. 2) compared with females for the PM samples. Additionally, individual variation in FGM increase from AM to PM samples was consistent within individuals (random effect intercept: $z = 3.441$, $SE = 2.292$, $P = 0.001$). Sex ($z = -0.440$, $SE = 0.139$, $P = 0.660$), treatment condition ($z = -0.322$, $SE = 0.102$, $P = 0.748$), and mass ($z = -0.657$, $SE = 0.209$, $P = 0.511$), however, did not influence the diurnal variation observed.

Anthropogenic effects on FGM concentrations.—In response to the cooling fan introduced to the kennels, 4 coyotes (3 males and 1 female) produced ≥ 5-fold increases in FGM output, similar to the ACTH challenge. The approximate excretion lag time for the cooling fan introduction (11 h 58 min ± 5 h 53 min; range: 9 h 55 min–24 h; Table 1; Fig. 3) also was similar to the ACTH challenge excretion lag time. There was a significant negative relationship ($\rho = -0.720$, $P = 0.006$) between fold-increase values and proximity to the facility entrance, with closer individuals more likely to witness fold increases similar to the ACTH challenge (Table 1). There were no differences a priori between assigned ACTH and control animals (Mann–Whitney $U_{11} = 16.00$, $P = 0.749$) or between males and females (Mann–Whitney $U_{11} = 15.00$, $P = 0.631$; Table 1).

In response to Pioneer Day festivities, 5 coyotes (3 males and 2 females) produced ≥ 5-fold increases in FGM output with an approximate excretion rate of 9 h 21 min ± 5 min (range: 9 h 24 min–9 h 46 min; Table 1; Fig. 3). Similar to the fan introduction, there was a significant negative relationship ($\rho = -0.609$, $P = 0.027$) between fold-increase values postevent and proximity to the facility entrance, with closer individuals more likely to witness fold increases similar to the ACTH challenge (Table 1). Again, there were no a priori differences between assigned treatment groups (Mann–Whit-

ney $U_{10} = 14.00$, $P = 0.855$) or between males and females (Mann–Whitney $U_{10} = 8.00$, $P = 0.201$; Table 1).

Discussion

This study was the 1st to physiologically validate the measurement of FGM via an ACTH challenge to noninvasively assess adrenocortical activity in coyotes. All treatment individuals had an ACTH-induced peak approximately 8 h after the injection. This excretion lag time is rapid compared to results from related Canidae. Lag time between ACTH injection and peak glucocorticoid values for African wild dogs (Monfort et al. 1998), gray wolves (Sands and Creel 2004), and maned wolves (Vasconcellos et al. 2011) are ~24, 16–20, and ~20 h, respectively. This difference may partly be due to various sizes within the Canidae: the coyote is currently the smallest species of the family for which adrenocortical activity has been physiologically validated. However, this trend is not extended to all of Carnivora, because smaller species in the order have longer lag times between ACTH injection and peak glucocorticoid concentrations (20–44 h in black-footed ferrets [*Mustela nigripes*—Young et al. 2001]). Other carnivores demonstrate longer excretion lag times (15 h for grizzly bears [*Ursus arctos horribilis*—Hunt and Wasser 2003], 25 h for brown hyenas [*Hyaena brunnea*—Hulsman et al. 2011], and 16 h for spotted hyenas [*Crocuta crocuta*—Benhaiem et al. 2012]). It is more likely that the method of injection influenced the rapid excretion rate, because all other previous ACTH studies within Canidae administered the injection intramuscularly rather than intravenously (Monfort et al. 1998; Sands and Creel 2004; Young et al. 2004; Vasconcellos et al. 2011). Our intravenous injection would have resulted in the direct transfer of ACTH to the bloodstream (Möstl and Palme 2002; Touma and Palme 2005). It is important to note that the reduced excretion lag time also may be partially explained by the frequent (3 or 4 times daily) defecation events of the captive coyotes.

Fold increases above the preinjection mean were significantly greater for treatment group individuals compared to control individuals. The ACTH males and females experienced increased FGM output 12- to 13-fold and 5- to 30-fold above their preinjection means, respectively. Monfort et al. (1998) demonstrated similar pronounced responses to ACTH administration in African wild dogs. Fecal corticosteroid metabolites in male and female African wild dogs increased approximately 9- and 19-fold above pre-ACTH concentrations (Monfort et al. 1998). The similar fold increase in African wild dogs is surprising given that the study animals from Monfort et al. (1998) were given 400 IU compared with a maximum injection of 47 IU for this study. Results from Monfort et al. (1998) are similar to those of Young et al. (2004), who utilized a 140 IU dose for red wolves and observed only a 4- to 11-fold increase in FGMs above pre-ACTH concentrations. In contrast, Sands and Creel (2004) administered a lower dosage of 0.5 IU/kg of ACTH to gray wolves and the 2 treatment gray wolves demonstrated a 5-fold increase above the pre-ACTH concentrations.

This variation in FGM fold increase among Canidae may suggest that coyotes are more sensitive to hypothalamic–pituitary–adrenal—axis stimulation. More broadly, an increased attenuation to glucocorticoids could influence physiological adaptation of coyotes to repeated stressors. However, differences in methodologies across studies may partially explain the variation in fold-increase values. First, contrary to our study, Monfort et al. (1998) and Sands and Creel (2004) used a corticosterone and cortisol radioimmunoassay, respectively. Young et al. (2004) used both a corticosterone radioimmunoassay and a cortisol enzyme immunoassay for ACTH analyses. Second, the other authors used varying methodologies to extract glucocorticoids from the feces; and the specific ACTH compound used differed across studies. Third, potential differences in species' metabolism may account for the variance in fold-increase values. As a result, it is difficult to directly compare fold-increase values across these studies.

It is important to note that FGM concentrations for 3 control individuals did emulate fold increases of the treatment group, with FGM peaks $\geq$ 4-fold above the preinjection means. These results are likely due to the actual stress experienced during handling and injection. Previous literature on multiple wildlife species has demonstrated FGM increases (Harper and Austad 2000) and plasma glucocorticoid increases (Morton et al. 1995; Kenagy and Place 2000) due to handling and capture. Examination of data from Harper and Austad (2000) showed increased FGM responses due to brief handling during captive cage transfer in 3 species of Rodentia (house mice [*Mus musculus*], deer mice [*Peromyscus maniculatus*], and red-back voles [*Myodes gapperi*]). Similarly, results from Kenagy and Place (2000) on wild-caught female yellow-pine chipmunks (*Tamias amoenus*) demonstrated plasma glucocorticoid increases in response to trap capture for 1–3 h. Our results further suggest that capture, restraint, and handling stress can augment FGM concentrations. Previous ACTH challenge papers on related Canidae (domestic dog [Schatz and Palme 2001], gray wolf [Sands and Creel 2004], red wolf [Young et al. 2004], African wild dog [Monfort et al. 1998], and maned wolf [Vasconcellos et al. 2011]) did not have established control groups to compare with ACTH animals, making it difficult to know whether those study animals also responded to capture and restraint similar to our control individuals.

Interestingly, examination of our data demonstrates diurnal variation in FGM concentrations. Specifically, FGM output was greatest in MD and PM samples compared with AM samples. This pattern may be occurring for several reasons. First, this trend may reflect the circadian pattern of a crepuscular species due to the 8- to 12-h delay of metabolism (from blood to feces). According to Touma and Palme (2005) it might be almost impossible to detect circadian patterns in carnivores primarily because of an infrequent diet or longer digestive tract. However, it is possible that the constant diet of our captive coyotes may have inadvertently augmented their

diurnal fluctuations in glucocorticoids. Second, staff activity around the kennel area is greatest in the morning and early afternoon (0730–1600 h), and dissipates in the evening hours (1600–2400 h). It is likely that increased staff presence influences circulating glucocorticoids early in the day, and those influences are reflected in the feces at MD and PM collection periods. Third, the diurnal variation observed may be an artifact of reduced FGM concentrations from increased bacterial enzymatic activity in morning samples (Möstl and Palme 2002; Shutt et al. 2012). Morning samples potentially had longer exposure time to ambient temperatures; the elapsed time between PM and AM collection periods (13 h) is greater than between AM and MD (4 h) or MD and PM (4 h) periods. Shutt et al. (2012) previously described a near-linear decrease in FGMs over a 12-h period when feces had prolonged exposure to ambient conditions. However, given that our captive coyotes often defecated from 0500 to 0900 h, the potential for increased environmental exposure of AM samples is low. Further research that controls for degradation potential is needed to properly address the effects of feeding and human activity on circadian and seasonal patterns in FGMs.

Contrary to our initial prediction, our study did not demonstrate sex differences between preinjection means or FGM peaks. However, sex differences were observed for PM samples, with males demonstrating higher FGM concentrations than females. Sex differences in adrenocortical activity have been observed previously in Carnivora (Monfort et al. 1998; Terio et al. 1999; Wielebnowski et al. 2002), although the differences vary across studies. Results from Monfort et al. (1998) suggested that female African wild dogs had smaller fold increases (~9-fold above basal FGM levels) compared with males (13- and 25-fold increase above basal FGM levels) in response to an ACTH challenge. African wild dog males also had lower pre-ACTH means compared with females (Monfort et al. 1998). Results from Terio et al. (1999) demonstrated higher baseline fecal corticoid concentrations for 1 of 2 male cheetahs compared to the 2 study females. The small sample size used by Terio et al. (1999), however, limits the conclusions that can be drawn about sex differences in cheetahs. In contrast, Wielebnowski et al. (2002) observed greater baseline fecal corticoid concentrations in female North American clouded leopards (*Neofelis nebulosa*) compared to males within a large sample size ($n = 72$). Yet, fold increase of fecal corticosteroid concentrations in response to an ACTH challenge did not differ among sexes. Wielebnowski et al. (2002) suggest that the differences observed may be a result of increased vigilance in females to protect young against infanticide and avoid aggressive encounters with markedly larger males. The potential for reproductive influences on FGMs in our study is unlikely because we observed the study animals during the summer of 2011, which is well outside the coyote breeding season (Carlson and Gese 2008). However, it is possible that sex-related differences in hormone metabolism (Touma and Palme 2005) resulted in the quantitative differences observed by Monfort et al. (1998), Terio et al. (1999), and Wielebnowski et al. (2002). The lack of differences observed in our study may indicate that sex-related differences in coyote metabolism do not exist. Future research looking at metabolic differences among sexes within different seasonal periods (breeding versus nonbreeding) may provide further insight into the patterns observed.

Anthropogenic events documented during this study period allowed us to biologically validate FGM analysis. In both events mentioned above, 4 or 5 of the study animals witnessed a peak promptly following the event (11 h 58 min ± 5 h 53 min and 9 h 21 min ± 5 h 0 min, respectively). However, only 3 individuals (2 males and 1 female) consistently demonstrated increases > 5-fold above the pre-event means for the fan introduction and Pioneer Day. Coincidentally, those 3 individuals were closest to the facility entrance. Examination of our data suggested that proximity to the facility entrance accounts for some of the variance in peak fold values. The most likely explanation for this trend is that coyotes in kennels closer to the entrance have greater visibility of the facility. This result of increased visibility may be 2-fold: the coyote can more readily detect a visitor to the kennel area and also may perceive a decreased sense of cover themselves. Coyotes often use vegetation or brush as cover when frightened (Gehrt 2007), and when unrestricted will move to vantage points to assess threat (Séquin et al. 2003). The perceived lack of adequate cover paired with restricted movement in a kennel environment may provoke stronger responses to human-induced stressors. It is important to note that not all events documented produced pronounced FGM peaks above pre-event means. On 20 July 2011 new coyotes were introduced into the kennel area, presumably a proxy for territorial incursion by novel conspecifics (Gese et al. 1998, 2001) and an acute stressor. However, none of the study animals had fold increases similar in magnitude (> 5-fold) to the other anthropogenic events.

Differences in our FGM results also may reflect individual differences in perception of the event. For instance, FGM concentrations for 1 control female and 1 treatment male were consistently > 5-fold above the pre-event means during the ACTH period, the fan introduction to the kennel, and the Pioneer Day fireworks. Repeated behavioral measures were not noted for this study. However, it is likely that temperament, social status, or both are correlated with the adrenocortical response, as observed in other species. Specifically, results from de Villiers et al. (1997) demonstrated that dominant African wild dogs had higher plasma glucocorticoid levels compared with others. Sands and Creel (2004) provided similar results in gray wolves, because aggressive and dominant individuals had greater basal FGM concentrations. Further, Séquin et al. (2003) demonstrated that coyote response to novelty is closely correlated with social status and temperament, although this study did not directly look at adrenocortical relationships with temperament. Future longitudinal research on temperament, social status, and their relationship to FGMs may provide a unique framework in understanding the variation in responses we have observed here.

To conclude, our results demonstrated robust FGM responses to both the ACTH challenge and 2 anthropogenic events.

The consistency between approximate adrenocortical responses to the ACTH challenge and anthropogenic stimulants suggests that rapid FGM excretion rates in coyotes are species-specific. Heightened metabolism can be an adaptive coping mechanism for persistence in nonnative environments, particularly given that examination of our data suggests that coyotes are responsive to human-associated events. This may have larger implications for coyote management practices in urban and suburban areas. Potential anthropogenic stressors occur relatively frequently in large metropolitan areas. A rapid adrenocortical response to these stressors from resident coyotes may partially enable their success in urban areas. Additionally, these adaptive hormonal responses may be transferred in vitro to developing pups, resulting in a multigenerational mechanism for tolerance to stressors in urban settings. Thus, FGM analysis in the species could prove useful in understanding the proximate mechanisms influencing their overall success as a species. Future work on FGMs could be useful in determining the long-term health of coyote populations and the potential adaptive physiological mechanisms.

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APPENDIX B: SPEARMAN RANK CORRELATIONS AMONG TERRITORIAL BEHAVIORS AND HORMONES OF EXPECTANT PARENTS

Table B1 Spearman rank correlations among scent-marking and investigative behaviors observed over odor cue provisioning for females (a) and males (b). $*P<0.05$, $**P<0.01$, $***P<0.001$; $N = 16$).

	Aggression	Ground scratching	Urination	Body Rubs	Site sniffs	Visits	Site Time	Latency to visit
(a) Females								
Aggression	1							
Ground scratching	**0.66****	1						
Urination	0.47	**0.61***	1					
Body rubs	**0.63****	**0.63****	**0.74****	1				
Site sniffs	**0.69****	**0.68****	**0.81*****	**0.83*****	1			
Visits	0.41	**0.56***	**0.64****	**0.51***	**0.75*****	1		
Site Time	**0.63****	**0.69****	**0.81*****	**0.83*****	**0.95*****	**0.85*****	1	
Latency to visit	-0.17	-0.01	-0.43	-0.25	-0.45	**-0.63****	**0.52***	1
(a) Males								
Aggression	1							
Ground scratching	0.13	1						
Urination	**0.51***	**0.68****	1					
Body rubs	**0.66****	0.39	**0.75*****	1				
Site sniffs	**0.60***	0.37	**0.77*****	**0.95*****	1			
Visits	0.49	**0.52***	**0.70****	0.37	0.44	1		
Site Time	**0.67****	0.34	**0.78*****	**0.87*****	**0.93*****	**0.64****	1	
Latency to visit	-0.39	-0.31	**-0.58***	-0.30	-0.31	**-0.77*****	-0.46	1

Table B2 Spearman rank correlations among fecal glucocorticoid (FGMs) and androgen metabolites (FAMs) over the testing period, as well as best linear unbiased predictors (BLUPs) for both hormonal metabolites, with scent marking and investigative behaviors exhibited over odor cue provisioning. Asterisk indicate significant relationships (†P<0.10, *P<0.05, **P<0.01, N = 16).

Variable	Aggression	Ground scratching	Urination	Body rubs	Site sniffs	Visits	Site Time	Latency to visit
Females								
Glucocorticoid metabolites	-0.29	-0.12	0.06	-0.12	-0.14	-0.18	-0.26	0
Androgen metabolites	**0.49†**	**0.47†**	0.06	0.28	0.19	-0.07	0.15	**0.57***
Glucocorticoid BLUPs	0	0.15	0.14	0	-0.16	-0.29	-0.19	**0.43†**
Androgen BLUPs	0.16	0.07	-0.26	-0.07	-0.06	-0.34	-0.14	**0.61***
Males								
Glucocorticoid metabolites	0.13	0.33	**0.47†**	0.13	0.2	0.23	0.16	0.01
Androgen metabolites	0.01	-0.27	-0.13	0.3	0.27	**-0.43†**	0.13	**0.46†**
Glucocorticoid BLUPs	0.13	0.25	**0.52***	0.12	0.15	0.31	0.22	-0.24
Androgen BLUPs	**-0.49†**	-0.42	**-0.67***	-0.3	-0.29	**-0.69****	**-0.51***	**0.58***

APPENDIX C: DIFFERENCES IN HAIR HORMONES BY BODY REGION

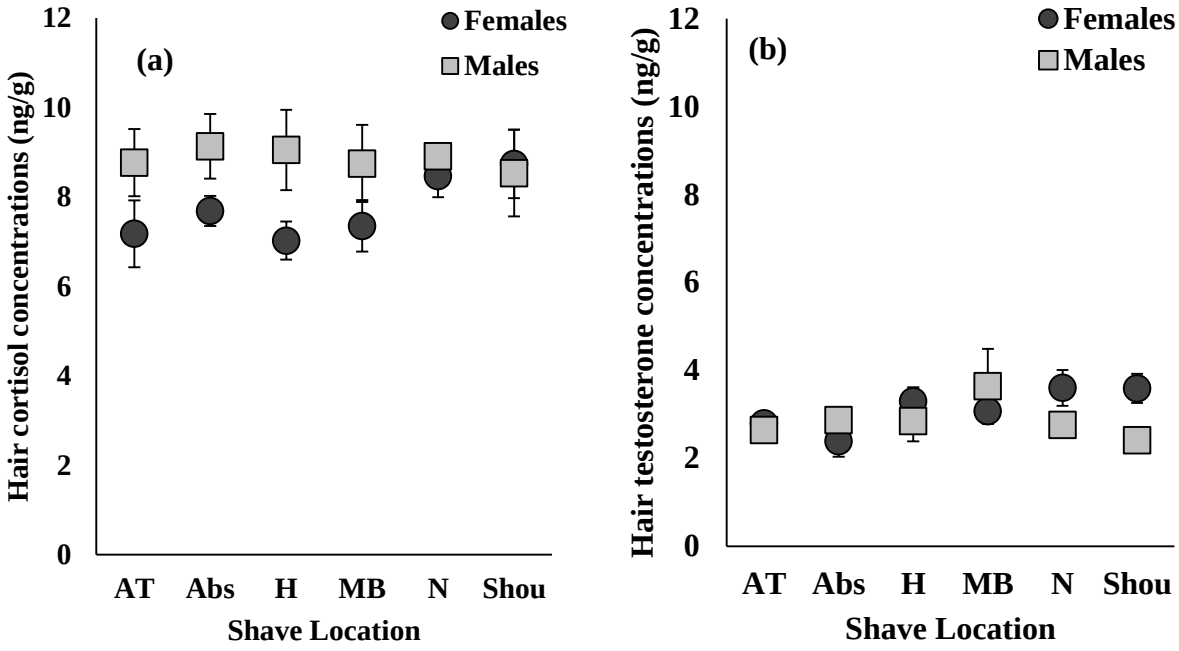

Fig. C1: Mean (± S.E.) hair cortisol (a) and hair testosterone (b) concentrations of 5 week old pups (N = 12) from six different shaved body regions. AT = above tail; Abs = abdomen; H = Hips; MB = mid-back; N = Neck; Shou = Shoulders.

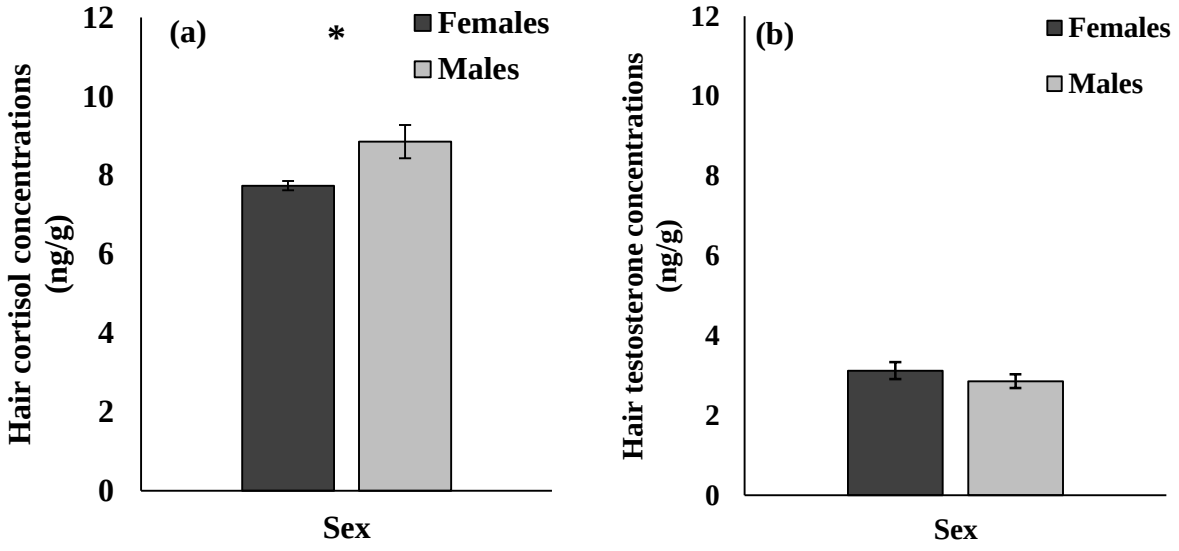

Fig. C2: Sex differences of mean (± S.E.) hair cortisol (a) and testosterone concentrations (b) for 5-week old pups (6 males, 6 females). Asterisk indicate significant differences in hair concentrations between males and females ($P < 0.05$).

APPENDIX D: YEARLING COMPONENTS AND HORMONAL RESPONSES TO HUMAN-ASSOCIATED OBJECTS

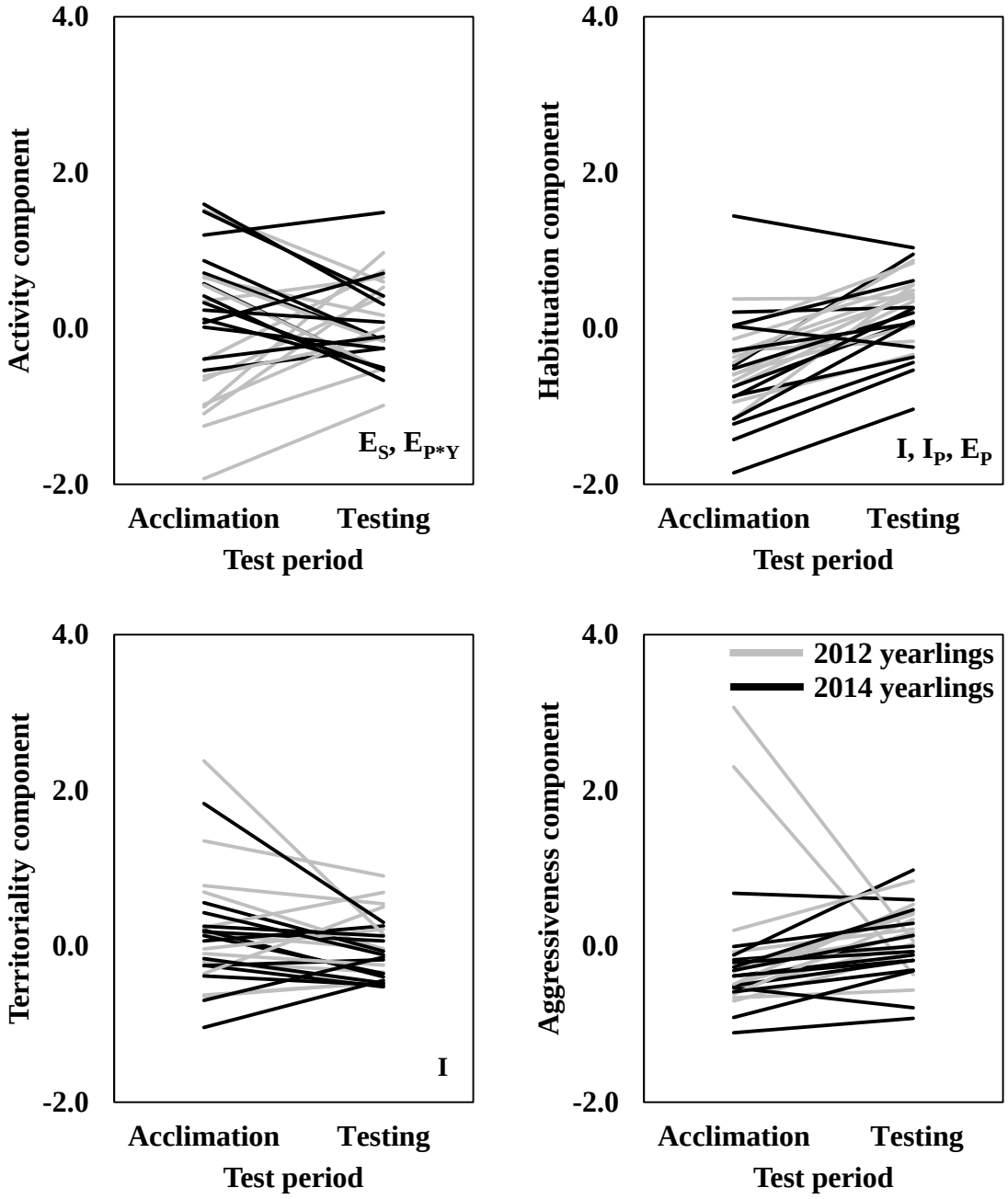

Fig. D1: Reaction norms of feeding test principal components for yearling across the acclimation and testing periods. ***I*** denotes a significant effect (P<0.05) of coyote identity, $\boldsymbol{I_P}$ an effect of pair identity, $\boldsymbol{E_P}$ an effect of period, $\boldsymbol{E_Y}$ an effect of year the individual was born, and $\boldsymbol{E_{P*Y}}$ an interaction effect between test period and year.

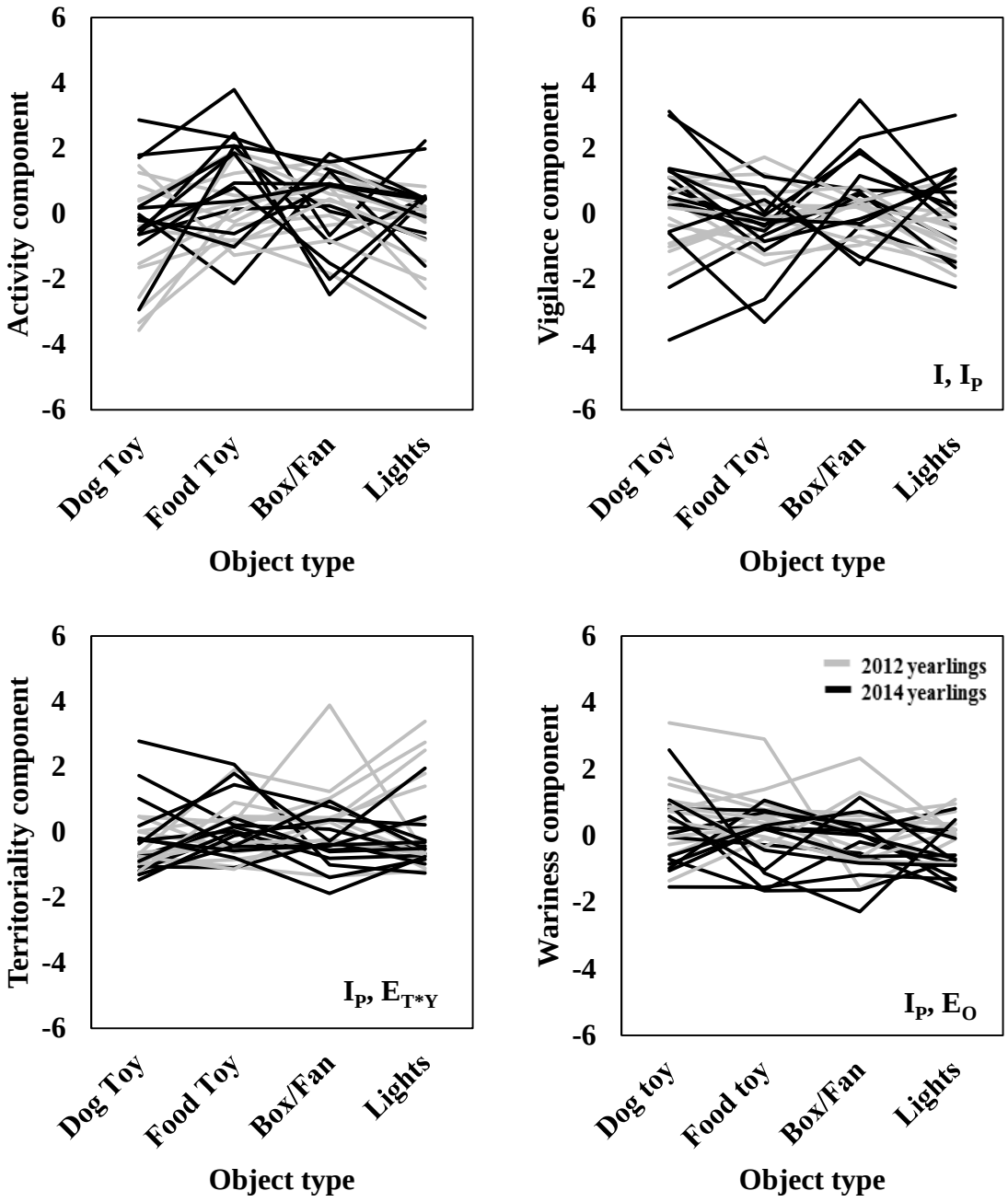

Fig. D2: Reaction norms of repeated object test principal components for yearlings across the acclimation and testing periods. ***I*** denotes a significant effect ($P<0.05$) of coyote identity, $\boldsymbol{I_P}$ an effect of pair identity, $\boldsymbol{E_T}$ an effect of parental odor treatment, $\boldsymbol{E_Y}$ an effect of year, and $\boldsymbol{E_{T*Y}}$ an interaction effect between parental treatment group and year.

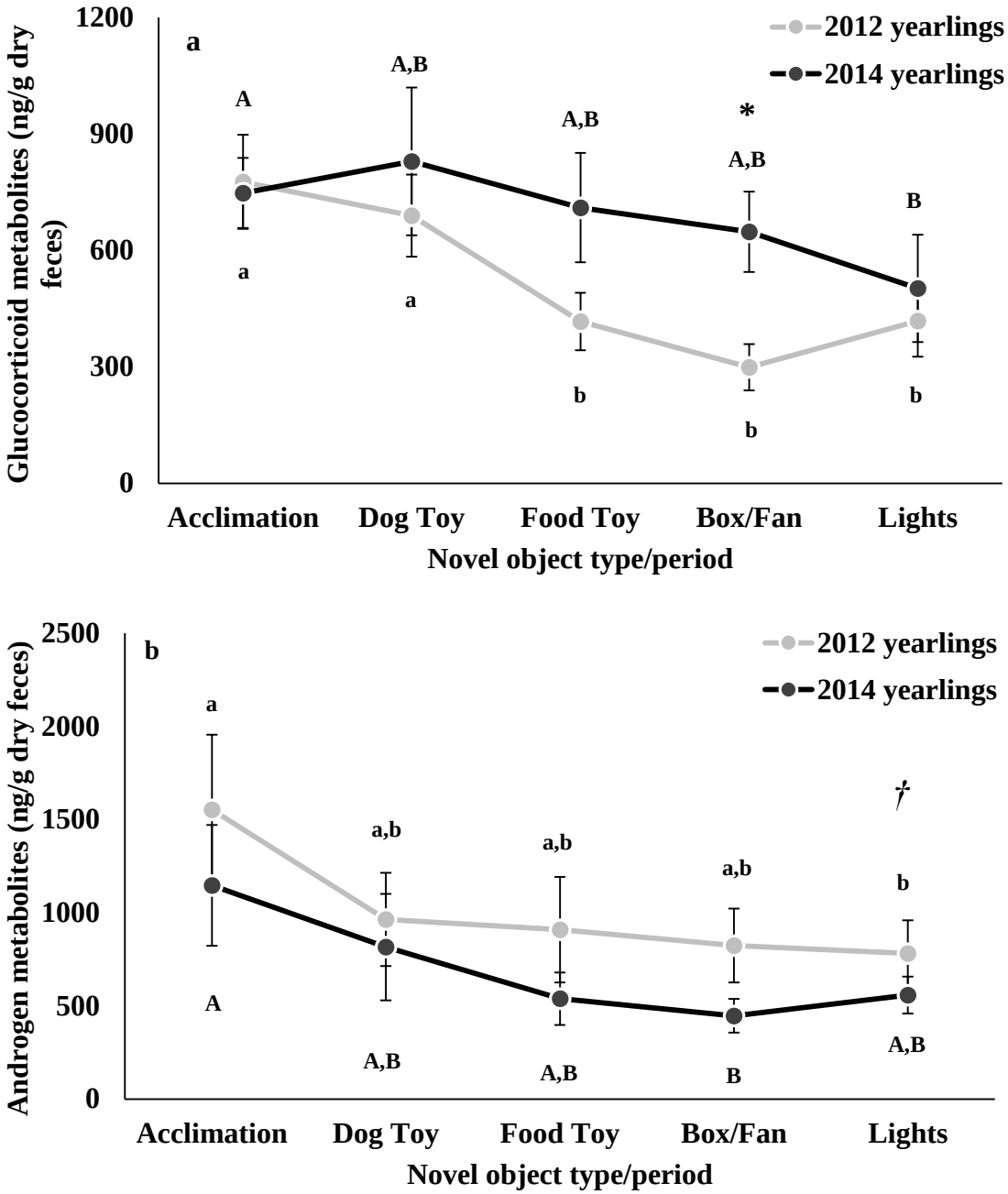

Fig. D3: Fecal glucocorticoid (a) and androgen (b) metabolite differences among novel object type. Lowercase subscripts indicate significant differences ($P < 0.05$) among object types within the 2012 yearling group (N=13). Uppercase subscripts indicate significant differences among object types within the 2014 yearling group (N=14). Asterisks indicate significance between yearling groups within an object type, whereas crossbars indicate an interaction between year and sex.

Table D1 Descriptive values and paired t-tests examining the degree of change in fecal glucocorticoid and androgen metabolites from the acclimation period, post-object presentation for all novel object tests. Metabolites are represented as means ± S.E. for all study individuals (N=27). *P < 0.05, **P < 0.01, ***P < 0.001

	Acclimation period	**Dog Toy(s)**	**Food Toy(s)**	**Box/Fan**	**Light object**
FGM means	761.73±73.58	761.81±109.93	568.78±84.78	479.62±68.90	461.56±83.09
t-value		-0.43	1.85	2.55	6.38
df		26	26	24	26
P-value		0.671	0.076	**0.017***	**<0.001****
FAM mean	1342.04±254.48	887.49±187.72	716.87±155.65	628.77±110.61	665.90±100.44
t-value		3.28	3.43	3.89	3.7
df		26	26	24	26
P-value		**0.003****	**0.002****	**0.001****	**0.001****

Table D2 Descriptive values detailing fecal glucocorticoid (FGMs) during the acclimation period (i.e. Pre-test), post-object presentation for each repeated object type (i.e. Post-test), and the degree of change (Δ; also known as fold change, see Schell et al. 2013) from the pre- to post-test FGM values for all yearling coyotes. Year denotes both when the yearlings were observed and the litter to which they were born (2012 yearlings born to first-time parents; 2014 yearlings born to experienced parents). F = female, M = male, NA = not available.

					1st Object		2nd Object		3rd Object		4th Object	
ID	**Sex**	**Pair ID**	**Year**	**Pre-test**	**Post-test**	**Δ**	**Post-test**	**Δ**	**Post-test**	**Δ**	**Post-test**	**Δ**
1130	F	1	2012	299.59	1580.47	5.28	90.01	-3.33	100.92	-2.97	269.52	-1.1
1160	F	2	2012	743.96	326.15	-2.28	636.66	-1.17	239.67	-3.1	291.09	-2.6
1132	F	3	2012	1470.67	197.22	-7.46	238.29	-6.17	420.4	-3.5	1541.9	1.05
1134	F	4	2012	273.5	345.13	1.26	271.74	-1.01	60.27	-4.54	212.56	-1.3
1172	F	5	2012	534.49	965.68	1.81	233.02	-2.29	221.17	-2.42	140.67	-3.8
1162	F	6	2012	589.98	387.23	-1.52	166.93	-3.53	287.14	-2.05	666.42	1.13
1100	F	7	2012	405.57	1579.72	3.9	856.46	2.11	369.19	1.1	385.66	-1.1
1320	F	8	2014	414.06	156.67	-2.64	377.07	-1.1	198.35	-2.09	616.8	1.49
1360	F	9	2014	1313.4	818.77	-1.6	751.36	-1.75	335.31	-3.92	564.36	-2.3
1350	F	10	2014	889.27	1876.27	2.11	425.45	-2.09	1159.19	1.3	589.7	-1.5
1340	F	11	2014	675.74	659.4	-1.02	371.45	-1.82	964.31	1.43	505.86	-1.3
1370	F	12	2014	1023.13	784.37	-1.3	2905.36	2.84	1085.99	1.06	720.5	-1.4
1372	F	13	2014	1440.56	117.99	-12.2	910.21	-1.58	243.3	-5.92	759.96	-1.9
1310	F	14	2014	311.07	220.78	-1.41	247.83	-5.81	987.8	3.18	105.47	-3
1151	M	1	2012	482.26	401.4	-1.2	85.57	-5.64	76.9	-6.27	210.62	-2.3
1101	M	2	2012	1488.13	473.68	-3.14	899.14	-1.66	1017.46	-1.46	1090.73	-1.4
1111	M	3	2012	764.87	421.45	-1.81	327.94	-2.33	343.2	-2.23	93.22	-8.2
1113	M	4	2012	557.54	417.94	-1.33	882.31	1.58	484.02	-1.15	141.84	-3.9
1071	M*	5	2012	349.2	NA	NA	243.66	-1.43	161.03	-2.17	415.53	1.19
1143	M	6	2012	1377.81	1422.16	1.03	315.92	-4.36	134.03	-10.3	674.87	-2
1141	M	7	2012	1107.98	604.75	-1.83	420.61	-2.63	120.26	-9.21	249.96	-4.4
1331	M	8	2014	770.61	343.88	-2.24	1243.25	1.61	1393.5	1.81	161.87	-4.8
1333	M	9	2014	826.2	1924.61	2.33	529.18	-2.48	710.58	-1.16	573.39	-1.4
1347	M	10	2014	658.34	4895.24	7.44	506.28	-1.76	1132.58	1.72	99.4	-6.6
1301	M	11	2014	659.16	214.78	-3.07	176.82	-3.82	305.35	-2.16	69.32	-9.5
1351	M	12	2014	662.13	240.02	-2.76	120.91	-10.9	836.88	1.26	159.41	-4.2
1311	M	13	2014	540.58	993.31	1.84	235	-6.13	215.06	-2.51	320.03	-1.7
1361	M	14	2014	286.15	574.01	2.01	725.34	-1.99	NA	NA	78.68	-3.6

Table D3 Descriptive values detailing fecal androgen (FAMs) during the acclimation period (i.e. Pre-test), post-object presentation for each repeated object type (i.e. Post-test), and the degree of change (Δ; also known as fold change, see Schell et al. 2013) from the pre- to post-test FGM values for all yearling coyotes. Year denotes both when the yearlings were observed and the litter to which they were born (2012 yearlings born to first-time parents; 2014 yearlings born to experienced parents). F = female, M = male, NA = not available.

					1st Object		**2nd Object**		**3rd Object**		**4th Object**	
ID	**Sex**	**Pair ID**	**Year**	**Pre-test**	**Post-test**	Δ	**Post-test**	Δ	**Post-test**	Δ	**Post-test**	Δ
1130	F	1	2012	365.01	414.91	1.14	190.64	-1.91	141.89	-2.57	321.9	-1.1
1160	F	2	2012	417.67	266.44	-1.57	339.19	-1.23	226.41	-1.84	229.65	-1.8
1132	F	3	2012	435.83	170.86	-2.55	114.5	-3.81	122.89	-3.55	315.25	-1.4
1134	F	4	2012	524.22	272.17	-1.93	433.12	-1.21	199.8	-2.62	253.02	-2.1
1172	F	5	2012	375.61	461.43	1.23	184.27	-2.04	706.36	1.88	119.89	-3.1
1162	F	6	2012	213.64	467.33	2.19	227.17	1.06	163.99	-1.3	175.07	-1.2
1100	F	7	2012	329.43	339.99	1.03	157.78	-2.09	94.59	-3.48	219.99	-1.5
1320	F	8	2014	213.98	194.14	-1.1	84.4	-2.54	101.27	-2.11	252.61	1.18
1360	F	9	2014	237.24	195.83	-1.21	159.71	-1.49	186.02	-1.28	392.09	1.65
1350	F	10	2014	256.54	440.2	1.72	172.63	-1.49	217.92	-1.18	328.48	1.28
1340	F	11	2014	418.15	176.55	-2.37	146.19	-2.86	215.84	-1.94	240.58	-1.7
1370	F	12	2014	314.92	142.01	-2.22	206.05	-1.53	144.42	-2.18	162.74	-1.9
1372	F	13	2014	535.78	131.13	-4.09	152.64	-3.51	132.7	-4.04	395.99	-1.4
1310	F	14	2014	280.81	202.94	-1.38	295.45	1.05	210.46	-1.33	206.31	-1.4
1151	M	1	2012	4027.52	4014.58	-1	1894.61	-2.13	596.33	-6.75	1305.85	-3.1
1101	M	2	2012	3594.82	1521.83	-2.36	816.44	-4.4	1527.72	-2.35	734.27	-4.9
1111	M	3	2012	2038.69	1639.84	-1.24	1402.85	-1.45	1504.51	-1.36	1459.44	-1.4
1113	M	4	2012	1909.49	1129.03	-1.69	850.64	-2.24	1820.33	-1.05	867.2	-2.2
1071	M*	5	2012	1080.07	NA	NA	1040.21	-1.04	703.21	1.54	1434.19	1.33
1143	M	6	2012	3639.08	1485.13	-2.45	1029.41	-3.54	634.74	-5.73	1710.98	-2.1
1141	M	7	2012	2312.55	1387	-1.67	2726.63	1.18	1206.39	-1.92	1472.97	-1.6
1331	M	8	2014	2707.23	981.55	-2.76	486.54	-5.56	1443.03	-1.88	1020.11	-2.7
1333	M	9	2014	1246.69	358.78	-3.47	420.7	-2.96	406.13	-3.07	1082.64	-1.2
1347	M	10	2014	3154.17	2019.29	-1.56	538.78	-5.85	1110.89	-2.84	523.99	-6
1301	M	11	2014	846.23	957.33	1.13	1769.07	2.09	546.2	-1.55	795.3	-1.1
1351	M	12	2014	3879.78	299.46	-13	1087.95	-3.57	890.86	4.36	522.69	-7.4
1311	M	13	2014	1259.78	991.11	-1.27	308.5	-4.08	249.76	-5.04	562.29	-2.2
1361	M	14	2014	700.08	382.52	-1.83	950.91	1.36	NA	NA	452.85	-1.6

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