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TECHNICAL REPORT

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Investigation of techniques to measure cortisol and testosterone concentrations in coyote hair

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Long-term noninvasive sampling for endangered or elusive species is particularly difficult due to the challenge of collecting fecal samples before hormone metabolite desiccation, as well as the difficulty in collecting a large enough sample size from all individuals. Hair samples may provide an environmentally stable alternative that provides a long-term assessment of stress and reproductive hormone profiles for captive, zoo, and wild mammals. Here, we extracted and analyzed both cortisol and testosterone in coyote (*Canis latrans*) hair for the first time. We collected samples from 5-week old coyote pups (six female, six male) housed at the USDA-NWRC Predator Research Facility in Millville, UT. Each individual pup was shaved in six different locations to assess variation in concentrations by body region. We found that pup hair cortisol ($F_{5,57.1} = 0.47$, $p = 0.80$) and testosterone concentrations ($F_{5,60} = 1.03$, $p = 0.41$) did not differ as a function of body region. Male pups generally had higher cortisol concentrations than females (males = 17.71 ± 0.85 ng/g, females = 15.48 ± 0.24 ng/g; $F_{1,57.0} = 5.06$, $p = 0.028$). Comparatively, we did not find any differences between male and female testosterone concentrations (males = 2.86 ± 0.17 ng/g, females = 3.12 ± 0.21 ng/g; $F_{1,60} = 1.42$, $p = 0.24$). These techniques represent an attractive method in describing long-term stress and reproductive profiles of captive, zoo-housed, and wild mammal populations.

KEYWORDS
Canis latrans, cortisol, hair, mammals, testosterone

1 | STATEMENT OF THE PROBLEM

Steroid extraction techniques using noninvasive sampling have become widely appreciated and implemented for a multitude of research programs and taxa (Goymann, 2012; Palme, 2005). Nonetheless, there are a few shortcomings with using feces or urine samples to categorize animal steroid profiles. Steroid metabolites are highly sensitive to ambient environmental conditions (e.g., weather, temperature, humidity) and begin to degrade within hours of the focal individual defecating (Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011; Touma and Palme, 2005). Variation in an individual animal's diet can also greatly impact the concentration of glucocorticoid metabolites excreted (Goymann, 2012; Millspaugh & Washburn, 2004). Finally, steroid metabolites from each fecal or urine sample represent a small window of time (urine: 30 min–3 hr; feces: 6–24 hr) more susceptible to normal daily and seasonal variation (Millspaugh & Washburn, 2004), as well as acute and transient stressors (Sheriff et al., 2011).

Hair samples potentially provide a viable noninvasive alternative for several reasons. First, hair hormones represent a broad average of concentrations over months to years allowing researchers to quantify long-term or chronic stress profiles of individuals (Meyer & Novak, 2012; Sheriff et al., 2011; Stalder & Kirschbaum, 2012). Second, hair steroids are stable at room temperature and do not require long-term storage at below freezing temperatures (Meyer & Novak, 2012; Stalder & Kirschbaum, 2012). Third, hair hormone concentrations are less sensitive to ambient environmental conditions as are other noninvasive sample media (Stalder & Kirschbaum, 2012). Finally, previous work in domestic and captive populations demonstrated covariation among hair cortisol concentrations and behavioral indices suggesting some biological relevancy to hair hormonal measures (Laudenslager, Jorgensen, Grzywa, & Fairbanks, 2011; Ouschan,

Kuchar, & Möstl 2013; Siniscalchi, McFarlane, Kauter, Quaranta, & Rogers, 2013).

Hair extraction methods are not without their own drawbacks, however, and those considerations must be accounted for in the study design. Though hair samples are relatively stable with refrigeration, a few studies have suggested that prolonged exposure to UV radiation can alter cortisol concentrations in hair (Carlitz, Kirschbaum, Stalder, & van Schaik, 2014; Wester, van der Wulp, Koper, de Rijke, & van Rossum, 2016). This may especially be the case for distal hair segments that are exposed to UV light more frequently than base hairs (Stalder & Kirschbaum, 2012). Collection methodologies must therefore, appropriately sample or homogenize hair samples so as not to bias endocrine results (Manenschijn, Koper, Lamberts, & Van Rossum, 2011). Sebaceous and sweat glands surrounding the hair follicle also secrete hormones that may affect stored hormones in hair from the bloodstream (Manenschijn et al., 2011; Stalder & Kirschbaum, 2012). Further, differences in mechanical irritation (e.g., brushing, rubbing, rolling) within and between individuals can alter cortisol concentrations across different body regions (Salaberger et al., 2016), resulting in variable results according to the location the hair was collected (Manenschijn et al., 2011; Stalder & Kirschbaum, 2012). Moreover, the rate of hair growth is variable within and across taxa, with inequitable growth in regions such as the scalp or back (Manenschijn et al., 2011). For all these reasons, it is important to perform multiple validations across a range of mammalian taxa to fully understand the biological relevancy of hair hormones.

In an effort to increase the number of taxa observed, we present an extraction methodology that integrates previously established techniques (Bennett & Hayssen, 2010; Bryan, Adams, Invik, Wynne-Edwards, & Smits, 2013; Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006; Ouschan et al., 2013) with standardized protocols and procedures commonly utilized by zoological institutions (Brown, Walker, & Steinman, 2004; Santymire, Freeman, Lonsdorf, Heintz, & Armstrong, 2012; Schell, Young, Lonsdorf, & Santymire, 2013) in the coyote (*Canis latrans*). Our secondary goal in doing so was to make hair hormonal analyses broadly applicable to non-model organisms. Further, our techniques build on advancements from recent studies in domestic canids (Bennett & Hayssen, 2010; Bryan et al., 2013; Ouschan et al., 2013; Siniscalchi et al., 2013), captive nonhuman primates (Carlitz et al., 2014, 2016; Davenport et al., 2006; Dettmer, Rosenberg, Suomi, Meyer, & Novak, 2015; Fairbanks et al., 2011; Kapoor, Lubach, Ziegler, & Coe, 2016; Laudenslager et al., 2011; Tennenhouse, Putman, Boisseau, & Brown, 2017; Wester et al., 2016; Yamanashi, Morimura, Mori, Hayashi, & Suzuki, 2013), and humans (Meyer & Novak, 2012; Staufenbiel, Penninx, Spijker, Elzinga, & van Rossum, 2013; Ullmann et al., 2016; Vaghri et al., 2013; Yang, Lan, Meng, Wan, & Han, 1998). We performed our newly-formed methods on coyote (*Canis latrans*) hair samples collected from pups (5 weeks old), to (1) assess potential variation in concentrations as a function of body region similar to previous studies (Carlitz et al., 2014; Macbeth, Cattet, Stenhouse, Gibeau, & Janz, 2010; Siniscalchi et al., 2013) and (2) determine any sex-related differences in concentrations. To date, only a few studies observing hair hormones in wild species exist (Koren et al., 2006; Macbeth et al., 2010), and only recently have hair reproductive hormones been quantified in either human (Ullmann et al., 2016; Wang, Moody, & Shirtcliff, 2016) or nonhuman species (Arnon, Hazut, Tabachnik, Weller, & Koren, 2016; Dettmer et al., 2015; Kapoor et al., 2016). To that end, we present both cortisol and testosterone concentrations.

2 | DESCRIPTION OF THE PROCESS

2.1 | Study group and sample collection

We observed 12 coyote pups ($n = 6$ female, 6 male) in 2014 at the United States Department of Agriculture —National Wildlife Research Center (NWRC)—Predator Research Facility in Millville, UT, a captive research facility. Pups were from three different litters and were housed in 1,000 m^2 outdoor "clover" pens standard for coyote family units (i.e., parents and pups). We shaved each pup in six distinct locations: above tail, abdomen, hips, mid-back, neck, and shoulders. 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). For each location, we shaved a 4 cm area and stored samples in a plastic bag, and experimenters that handled pups also wore gloves to reduce further potential for cross-contamination. Bags were then stored and maintained at room temperature until they were processed.

2.2 | Extraction and enzyme immunoassays (EIA)

To extract hormones from our hair samples, we washed the hair with 5.0 ml of 90% methanol (methanol:distilled water) and agitated on a mixer (Glas-col, Terre Haute, Indiana) for 1 min at setting 50 (Bryan et al., 2013). 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 2–3 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 s intervals; Omni International, Kennesaw, GA). We then weighed out 0.02 ± 0.005 g of pulverized hair into pre-labeled 16 × 125 mm plastic tubes. Pulverized hair was then combined with 2 ml of 90% methanol, vortexed briefly, and agitated on the Glas-col mixer for 4 hr (setting 50). Tubes were later centrifuged for 15 min at 1,500 rpm at 10°C, the supernatant was poured into clean plastic tubes, and then dried down under forced air and a hot-water bath (60°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 4× concentrated extract. These samples were briefly vortexed, then sonicated for 20 min before analysis.

We analyzed hair cortisol using a previously described cortisol enzyme immunoassay (Santymire et al., 2012; Schell et al., 2013). Polyclonal cortisol antiserum (R4866) and horseradish peroxidase (HRP) ligands 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, Margulis, & Santymire, 2011). Testosterone horseradish peroxidase and polyclonal antiserum were used at 1:30,000 and 1:10,000, respectively. Polyclonal testosterone antiserum (R156/7) and HRP were also provided by C. Munro.

We biochemically validated the enzyme immunoassays by demonstrating parallelism between binding inhibition curves of hair extract dilutions (eight times concentrated-1:4, $n = 6$ dilutions: 1:4, 1:2, neat, 2× concentrated., 4× conc., and 8× conc.), the cortisol standard ($R^2 = 0.986$), and the testosterone standard ($R^2 = 0.983$). In addition, we found a significant percent recovery (>90%) of exogenous cortisol (1:4; $y = 1.413\times - 9.511$, $R^2 = 0.995$) and exogenous testosterone (1:4; $y = 1.108\times -7.469$, $R^2 = 0.962$) added to pooled hair extracts. Assay sensitivity for cortisol and testosterone enzyme immunoassays were 1.95 pg/well and 2.3 pg/well, respectively, and intra- and inter-assay coefficient of variation was <10% for all enzyme immunoassays.

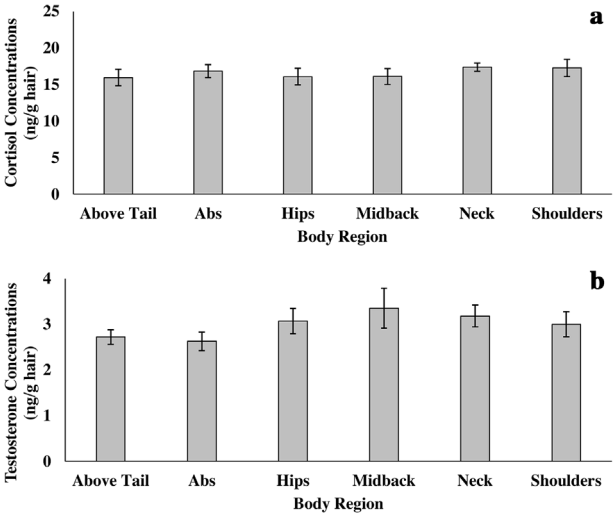

FIGURE 1 Mean (± S.E.M.) hair cortisol (a) and hair testosterone (b) concentrations of 5-week old pups ($N = 12$) from six different shaved body regions

2.3 | Statistical analyses

We used linear mixed models (LMMs) to assess differences in cortisol and testosterone as a function of body region. Sex and body region were set as fixed effects in our models, along with the interaction term between the two factors. Animal identity was nested in litter identity to account for familial or genetic effects. Consequently, models were fit with a random slope and intercept (i.e., animal ID | litter ID). All analyses were conducted in R version 3.3.0 (R Core Team, 2016). Linear mixed models were performed using the lmer function from "lme4" (Bates, Maechler, & Bolker, 2012) and "lmerTest" (Kuznetsova, Brockhoff, & Christensen, 2013) packages. Normality for all data was determined using Shapiro–Wilk testing. We report data as mean ± S.E.M.

3 | DEMONSTRATION OF EFFICACY

Pup cortisol ($F_{5,57.1} = 0.47$, $p = 0.80$) and testosterone concentrations ($F_{5,60} = 1.03$, $p = 0.41$) did not differ according to body region (Figure 1a,b). Average cortisol concentrations were greater for male versus female pups (males = 17.71 ± 0.85 ng/g, females = 15.48 ± 0.24 ng/g; $F_{1,57.0} = 5.06$, $p = 0.028$; Figure 2). We did not find any differences in testosterone concentrations between male and female pups (males = 2.86 ± 0.17 ng/g, females = 3.12 ± 0.21 ng/g; $F_{1,60} = 1.42$, $p = 0.24$; Figure 2). There were no significant interaction effects between body region and sex for pup cortisol ($F_{5,57.1} = 0.79$, $p = 0.56$) or testosterone concentrations ($F_{5,60} = 1.68$, $p = 0.15$).

4 | DISCUSSION

This represents the first study to quantify hormone concentrations in coyote hair. Moreover, we observed several trends in steroid concentrations associated with sex. We did not find any hormone concentration differences for cortisol or testosterone associated with various body regions of coyotes (Figure 1), unlike studies in other taxa that have comparable sample sizes (Bryan et al., 2013; Carlitz et al., 2014; Ouschan et al., 2013; Yamanashi et al., 2013). Other studies have reported regional concentration differences in adult individuals (Carlitz et al., 2014; Siniscalchi et al., 2013; Yamanashi et al., 2013), not necessarily in infants or juveniles (although see Dettmer et al., 2015; Laudenslager, Jorgensen, & Fairbanks, 2012). Because our design was limited to pups, the same methodological process is needed for adult coyotes to determine if the lack of difference across body regions is consistent in coyotes or unique to young individuals. Adrenal and gonadal function vary greatly from infancy to adulthood in mammals (Reeder & Kramer, 2005). Given that gonadal tissues are not fully developed at the infancy stage, there may have been reduced testosterone production in pups resulting in the absence of sex-related differences in hair concentrations. Moreover, cortisol concentration differences between the sexes may be an artifact of developmental

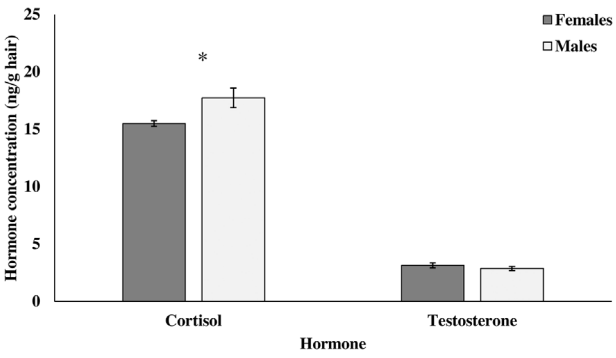

FIGURE 2 Mean (± S.E.M.) differences of hair cortisol and testosterone concentrations between male and female coyote pups ($N = 6$ males, 6 females). Asterisks indicate statistical significance ($p < 0.05$)

differences in adrenal glands between the sexes (Bale & Epperson, 2015). Granted, sex-related differences in stress physiology usually manifest around puberty (Panagiotakopoulos & Neigh, 2014), and our coyotes are unlikely to experience that maturation process until later in development. In any case, these hypotheses provide a platform to address potential sex and age-related differences in hair hormone concentrations in the future.

We found testosterone concentration levels within the hair of pups particularly surprising. Hair concentrations ranged from 1.7 to 7.8 ng/g, which is approximately the same as circulating serum concentrations of male adult coyotes during the breeding season (3.31 ± 0.09 ng/ml; Minter & DeLiberto, 2008). There may be several explanations for these trends. These results may likely reflect cumulative deposition of hormones in hair. In their study, Minter and DeLiberto (2008) collected blood samples from male coyotes at single time points, whereas the hair from our pups represents an accumulation of testosterone over a 5-week period. Alternatively, parental licking and grooming may have deposited adult testosterone via saliva. Both fathers and mothers orally clean their pups' back and hindquarters regularly in the first 5 weeks of life (Bekoff & Wells, 1986; Schell, 2015). The highest testosterone concentrations were found in the mid-back, shoulders, and hips regions (Figure 1b), suggesting this transfer mechanism may be possible. Hair samples were thoroughly washed, however, reducing the potential for exogenous androgens to have influenced our results. Certainly, more work is necessary to describe how internal and external environmental constructs influence hair concentrations.

We believe our study provides a preliminary framework that reveals potential opportunities to explore more multifaceted questions about long-term steroid concentrations in coyotes. For instance, future empirical work should determine if there are positive associations among hair hormone concentrations and other sample types (e.g., saliva, urine, feces, blood) to further biologically validate hair concentrations. In addition, evidence of behavioral associations with coyote hair steroids may broaden our understanding of how long-term stress profiles correspond with personality traits (Davenport et al., 2006; Laudenslager et al., 2011; Siniscalchi et al., 2013) and environmentally-induced plasticity (Carlitz et al., 2014; Fairbanks et al., 2011). Further, systematic testing of hair snares, barbed wire, and similar mechanical products will be necessary to determine which apparatus is best suited to procure the amount of hair needed for analyses (Macbeth et al., 2010). By shaving individuals, we have found a sufficient pulverized mass (0.02 ± 0.005 g) to effectively measure steroid hormones that can be extrapolated to other systems. For carnivores in particular, it may be important to consider the behavioral biology of the study species to procure the maximal amount of shed hair. For example, baited hair snares may be best suited for coyotes because baiting elicits a rub-roll response on the snare itself increasing the likelihood of obtaining the necessary amount of hair needed (Kimball et al., 2000). Here we have provided the first step in validating hair hormone concentrations for coyotes and have consequently expanded the number of non-model taxa that investigate hair as a viable sample medium. We hope that future research will build off these results to expand our understanding of wildlife physiology.

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