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RESEARCH ARTICLE

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Validation and measurement of physiological stress and reproductive hormones in wolf hair and claws

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Abstract

The use of keratinized tissues (e.g., hair, claws) to investigate physiological effects of environmental and anthropogenic stressors in free-ranging wildlife populations has increased because these tissues retain steroid hormones during growth and are relatively easy to collect and store in the field. We measured reproductive and stress-related steroid hormones in wolves (*Canis lupus ligoni*; $n = 31$) captured on Prince of Wales Island, Alaska, USA, during 1993–1994 and 2012–2014, representing periods of time when both wolf harvest and densities ranged from high to moderate. We validated enzyme immunoassay kits to measure steroid hormone concentrations in wolf guard hair, undercoat hair, and claw tip samples. Progesterone, testosterone, and cortisol were extracted and measured in the 3 keratinous tissues from wolves of different age class, sex, residency status, and collection periods. Within each tissue type, progesterone and testosterone were positively correlated (guard hair, $r = 0.59$, $P = 0.003$; undercoat hair, $r = 0.55$, $P = 0.011$; claws, $r = 0.62$, $P \leq 0.001$) and cortisol concentrations were not related to either reproductive hormone. We were able to measure hormone concentrations in archived keratinous tissues collected up to 25 years earlier to assess stress and reproductive activity in historical samples.

Our study validates a method for measuring steroid hormones in hair, and for the first time, continuously growing claws in wolves. Measurement of hormone concentrations in keratinous tissues may aid in the assessment of reproductive activity and physiological stress responses in wolf populations over long-term time periods (i.e., decades) to enhance conservation efforts of an important apex predator.

KEYWORDS
Alexander Archipelago wolves, *Canis lupus*, claws, cortisol, hair, progesterone, testosterone

When confronted with a stressor, animals employ a rapid cascade of both behavioral and physiological changes (Landys et al. 2006, Busch and Hayward 2009). One change, commonly used as a measure of physiological stress, is the release of glucocorticoid hormones (Sapolsky et al. 2000, Möstl and Palme 2002), specifically cortisol in carnivores, from the adrenal cortex (Romero 2004, Reeder and Kramer 2005, Koren et al. 2012). To increase chances of survival in the short term (i.e., hours to days), cortisol initiates a temporary increase in energy production by stimulating lipolysis and increasing blood glucose levels, at the expense of processes that are not required for immediate survival, commonly known as the fight or flight response (Romero and Butler 2007, Gormally and Romero 2020). However, when glucocorticoids, including cortisol, are chronically elevated for weeks to months, negative consequences can occur, such as decreases in immune response, reproduction (Dobson and Smith 2000, Sapolsky et al. 2000, Sheriff et al. 2009), and growth (Möstl and Palme 2002, Gormally and Romero 2020). The reproductive hormones progesterone and testosterone are frequently used to assess reproduction in female and male canids, respectively (Walker et al. 2002, Kim et al. 2007, Barja et al. 2008), but may also be useful in assessing social structure and potential stressors, such as human harvest, in wolves (Bryan et al. 2015). Therefore, examining paired cortisol, progesterone, and testosterone concentrations in free-ranging wildlife populations may offer insight into the effects of environmental stressors or changes in reproductive patterns.

Traditional methods for monitoring reproduction and physiological stress in wolves includes assessing cortisol and progesterone concentrations in blood, fecal, or urine samples (McLeod et al. 1996, Walker et al. 2002, Creel 2005, Eggermann et al. 2013, Molnar et al. 2015). Blood and feces reflect hormone concentrations over a short timeframe (i.e., hours to days; Cook 2012) and longitudinal monitoring requires repeated sampling, which is often not feasible in free-ranging wildlife. Further, blood collection requires animal capture and handling which can cause immediate elevation of stress-related hormones (Cook et al. 2000, Romero 2004), which skews baseline physiological states. Therefore, tissues that incorporate steroid hormones over a longer timeframe would be better suited for analyzing the effects of environmental or biological factors in wildlife populations.

In recent years, several canid studies measured hormone levels in hair to investigate reproduction and physiological stress (Bryan et al. 2015, Grigg et al. 2017, Mesarcova et al. 2017, Schell et al. 2017, Koren et al. 2019). Hair incorporates steroid hormones from the blood stream, and to a lesser extent, steroids that are locally metabolized in skin glands and hair follicles (Slominski and Wortsman 2000; Bamberg et al. 2004, 2005; Greff et al. 2019). Steroid hormones are deposited into the hair over the growth period, thus providing a beneficial method for assessing physiological responses in wildlife to long-term stressors and potential impacts on reproduction or social structure (Bryan et al. 2013, Schell et al. 2017). Wolf guard hair begins growing after the annual molt during late spring through late fall (Young and Goldman 1944, Darimont and Reimchen 2002) and undercoat hair grows from late summer to late fall (Castelló and Sillero-Zubiri 2018). Thus, steroid hormone concentrations in wolf hair are related to circulating concentrations between late spring or summer through the fall when hormones are

incorporated into the growing hair. Claws, another keratinous tissue, also contain steroid hormones and have been used to examine stress associated with premature birth in dogs (Veronesi et al. 2015), stress associated with proximity to roads in turtles (Baxter-Gilbert et al. 2014), and reproductive status in mammals (Karpovich et al. 2020, Crain et al. 2021; M. J. Keogh, Alaska Department of Fish and Game [ADF&G], unpublished data). Claws contain material deposited continuously during growth, providing a tissue to track physiological information over months to years (Ethier et al. 2010). Accordingly, examinations of the concentrations of cortisol, progesterone, and testosterone in guard hair, undercoat hair, and claw tips would provide a suite of information about the endocrine state of an individual over time as the hormones in each tissue were deposited during different timeframes (Palme et al. 2005, Sheriff et al. 2011, Vilela et al. 2020).

For our study we used Alexander Archipelago wolves (*Canis lupus ligoni*) on Prince of Wales Island (Figure 1) as a focal population to measure stress-related and reproductive hormones in wolves captured during 1993–1994 and

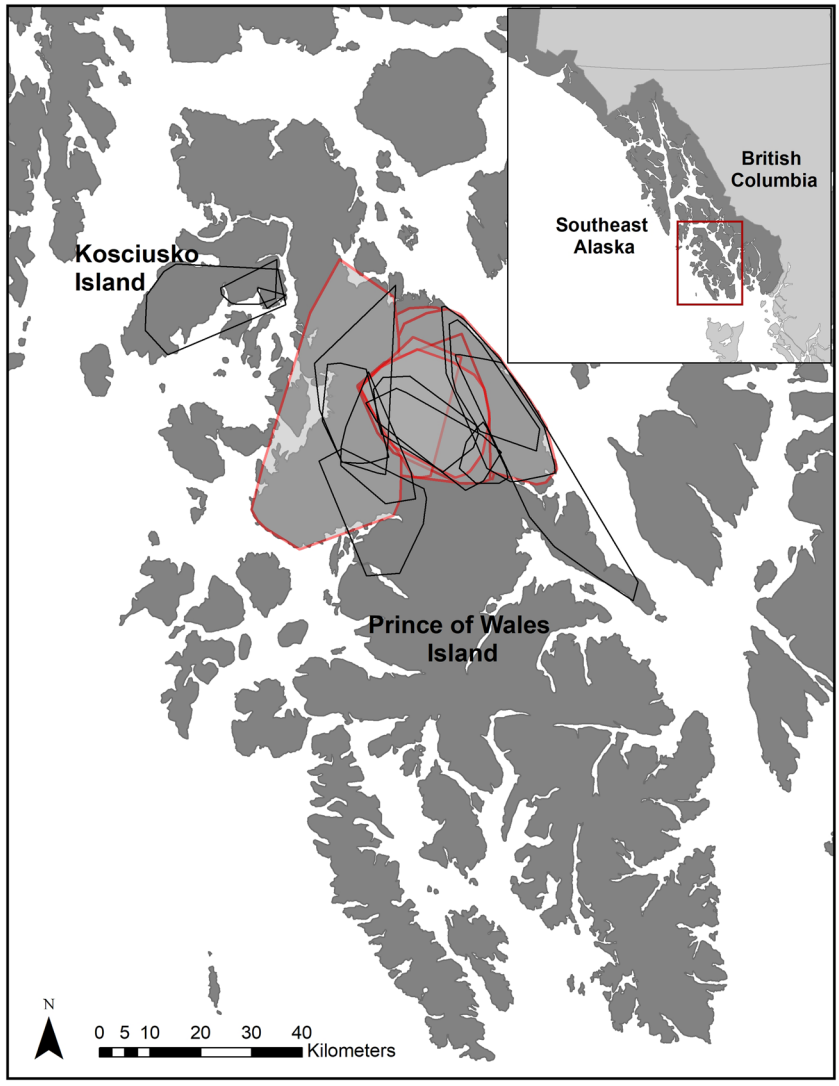

FIGURE 1 Minimum convex polygon home ranges of radiocollared wolves in study area during 1993–1994 (black outline) and 2012–2014 (red outline), Prince of Wales Island, Alaska, USA.

2012–2014, encompassing an era when human-caused wolf mortality has ranged from high to moderate, respectively, and has influenced wolf densities (Person et al. 1996, Person and Russell 2008, Roffler et al. 2019). Since the mid-1990s, wolves on Prince of Wales Island have been the focus of conservation efforts including multiple considerations for listing under the Endangered Species Act (U.S. Fish Wildlife Service 2016) and recommendations from the multi-agency Wolf Technical Committee, restricting wolf harvest, and protection of wolf dens to avoid disruption of reproductive activities (Wolf Technical Committee 2017). Even with current conservation efforts, anthropogenic activities such as hunting, trapping, and logging may result in increased stress and changes in reproductive patterns in Prince of Wales Island wolves; therefore, verification and application of methods to measure physiological stress and reproductive activity could provide a better understanding of the environmental factors that influence this population.

The main goals of our study were as follows: (1) validate enzyme immunoassay (EIA) kits for cortisol, progesterone, and testosterone extracts from wolf guard hair, undercoat hair, and claw tip samples, (2) assess concentrations of each hormone by individual wolf sex, age class, residency status, and decade sampled, and (3) explore relationships among hormones and tissue types.

STUDY AREA

Prince of Wales is the largest island (6,670 km^2) in the southern portion of the southeastern Alaska Archipelago (Figure 1). Prince of Wales Island contains rugged mountains up to 1,160 m, multiple watersheds, and large tracts of temperate rain forests dominated by Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*) at elevations below 600 m (Alaback 1982). Old-growth forest was distributed in a matrix interspersed with even-aged forest stands at different successional stages resulting from clear-cut logging. Annual precipitation ranged from 130 to 400 cm mostly as rain with intermittent snow during the winter months, and historic mean temperatures ranged from 1–12°C. Approximately 4,800 km of roads were built throughout Prince of Wales Island to facilitate logging, with the greatest road densities in the northern portion of the island (0.49–1.04 km/km^2; Person and Russell 2008).

We sampled wolves on Prince of Wales Island during 2 periods (1993–1994 and 2012–2014) distinguished by differences in wolf densities and harvest rates. The wolf population was estimated during autumn 1994 for Prince of Wales and adjacent Kosciusko Island (6,808 km^2) at $\hat{N} = 269$ (SE = 80; Person et al. 1996), with corresponding wolf densities of 39.5 wolves/1,000 km^2, and an extrapolated wolf population estimate of $\hat{N} = 356$ for Game Management Unit (GMU) 2 (9,6069 km^2), which encompassed Prince of Wales Island and the surrounding island complex (Figure 1). Wolf abundance was not estimated for GMU 2 again until a noninvasive DNA-based monitoring method was implemented in autumn 2013. Wolf abundance had declined since the early 1990s (2013, $\hat{N} = 221$ wolves [95% CI = 130–378]; 2014, $\hat{N} = 89$ wolves [95% CI = 49.8–159.4]), as had wolf densities (2013, $\hat{N} = 24.5 \pm 6.8$ wolves/1,000 km^2; 2014, $\hat{N} = 9.9 \pm 3.0$ wolves/1,000 km^2; Roffler et al. 2019).

Wolf harvest was consistently high during the 1990s and reported harvest in 1992 ($n = 105$) and 1993 ($n = 100$) represented a period of the second-highest annual wolf harvests in GMU 2 since harvest data has been recorded (ADF&G, unpublished data). Wolf harvest was lower during 2012–2014 ($\bar{x}$ = annual harvest = 66, min–max = 52–89).

METHODS

Animal handling and sample collection

We captured wolves using modified or padded leghold traps set along the road system with a variety of commercially produced lures and canid urine used as attractants (Caven's, Pennock, MN, USA; Carman's, New Milford, PA, USA). We immobilized restrained wolves with either tiletamine HCl and zolazepam HCl (7 mg/kg),

or a combination of ketamine (200 mg/ml concentration, 4–6 mg/kg) and medetomidine (20 mg/ml concentration, 0.08–0.15 mg/kg) administered with a jab pole. We recorded sex and age category for each wolf (subadult <2 years, adult ≥2 years), which was determined by palpating the epiphyseal process on the front legs (Rausch 1967). We collected hair samples from the shoulder area with scissors from 23 wolves and by plucking from 2 wolves. We collected approximately 0.5 cm of material from the tip of each claw with dog nail clippers. Hair and claw tips were stored in resealable plastic bags or paper envelopes at room temperature until processed. In total, 31 claw tip, 23 guard hair, and 25 undercoat samples were used in our study (Table 1).

We fitted each captured wolf with a very high frequency radio collar containing mortality sensors (Mod 500, Telonics, Inc., Mesa, AZ, USA) during 1993–1994 (Person and Russell 2008), or a spread-spectrum, Global Positioning System (GPS) radio collar (Mod 4500, Telonics, Inc.) during 2012–2016 (Roffler et al. 2018). We classified wolves as residents or nonresidents based on GPS location data of individual wolves in relation to other marked wolves and existing wolf pack home range territories. Resident wolves were associated with other wolves in the pack home range and did not permanently leave the pack territory during the time they were monitored (Ballard et al. 1997). Nonresident wolves included wolves making extraterritorial forays (temporary movements outside of home range that are markedly separate from their previous locations; Ballard et al. 1997, Burch et al. 2005), or dispersal events (permanent movement away from natal pack home range, not remaining in one place for >14 days, or not exhibiting fidelity to a home range (Apollonio et al. 2004, Person and Russell 2008).

We calculated home ranges for individual wolves using 95% minimum convex polygons (MCP; Mohr and Stumpf 1966). We included individual wolves with a minimum of 30 independent locations (Fuller and Snow 1988, Burch et al. 2005) and used the rhr package (Signer and Balkenhol 2015) in R (v. 4.0.3, R Core Team 2021). Two-tailed t-tests ($\alpha = 0.05$) were used to compare differences in wolf home range sizes between decades and resident and nonresident wolves.

TABLE 1 Mean ± standard deviation, min–max, and sample size for concentrations of cortisol, progesterone, and testosterone (reported picogram [pg] of hormone per milligram [mg] of sample) extracted from wolf hair (guard hair and undercoat) and claws, 1993–1994 and 2012–2014, Prince of Wales Island, Alaska, USA. A female wolf (NA-2) with missing age class and residency status information is included in the results but was excluded from analyses including age class and residency status.

	Cortisol	Progesterone	Testosterone
Claw			
All wolves	3.2 ± 1.1 (1.4–6.8), *n* = 31	21.8 ± 6.4 (12.3–46.1), *n* = 31	4.9 ± 1.6 (2.5–9.5), *n* = 31
Male	3.1 ± 0.8 (2.1–4.4), *n* = 13	20.2 ± 3.4 (15.5–27.4), *n* = 13	4.7 ± 1.2 (3.0–6.6), *n* = 13
Female	3.3 ± 1.3 (1.4–6.8), *n* = 18	22.8 ± 7.8 (12.3–46.1), *n* = 18	5.0 ± 1.8 (2.5–9.5), *n* = 18
Guard Hair			
All wolves	6.9 ± 3.7 (1.6–20.6), *n* = 23	45.4 ± 20.1 (22.1–104.8), *n* = 23	9.3 ± 8.59 (0.7–32.7), *n* = 23
Male	6.2 ± 2.1 (3.0–10.3), *n* = 11	43.7 ± 23.3 (22.1–104.8), *n* = 11	5.4 ± 2.5 (2.2–9.4), *n* = 11
Female	7.6 ± 4.8 (1.6–20.6), *n* = 12	47.9 ± 17.6 (23.6–75.6), *n* = 12	12.9 ± 10.5 (0.7–32.7), *n* = 12
Undercoat			
All wolves	8.2 ± 5.5 (3.5–23.8), *n* = 25	65.6 ± 52.0 (19.8–205.4), *n* = 23	9.8 ± 10.7 (1.2–50.6), *n* = 25
Male	6.5 ± 1.4 (4.8–8.9), *n* = 11	59.7 ± 53.7 (19.8–205.4), *n* = 11	5.9 ± 4.1 (1.8–16.1), *n* = 11
Female	9.6 ± 7.0 (3.5–23.8), *n* = 14	70.9 ± 52.3 (24.5–198.0), *n* = 12	12.8 ± 13.3 (1.2–50.6), *n* = 14

Sample preparation

We separated guard hair and undercoat hair and removed the follicles from each hair shaft for the 2 plucked samples. We cleaned hair samples using similar methods used for bear hair (Macbeth et al. 2010). We removed visible contaminants with forceps and washed hair samples 5 times for approximately 30 seconds with ~10 mL of methanol to remove any remaining surface contaminants, oils, or additional steroid hormones derived from glands in the skin (Greff et al. 2019). While still damp, we cut the hair into small pieces (<3 mm) with surgical scissors and dried overnight at room temperature.

We sonicated claw tips in deionized water for 30 minutes, removed dirt and other visible surface contaminants using forceps and a probe, then sonicated for an additional 30 minutes. To remove any remaining surface oils or contaminants, we vigorously agitated claw tips in a 2:1 chloroform:methanol solution for 10–30 seconds then wiped with tissues wetted in the solution until no visible discoloration was evident (Karpovich et al. 2020). We dried claw samples overnight at room temperature and then cut each tip into smaller pieces with either a small chisel or dog nail clipper.

Hormone extractions and analysis

We weighed 20 mg subsamples from each tissue into polypropylene tubes (Type I, Sarstedt®, Sarsted Inc., Newton, NC, USA). Samples were powdered at 30 KHz in a mixer mill (Retsch® MM400, Retsch Inc., Newtown, PA, USA) with two 5-mm steel ball bearings for 15 minutes for hair samples and 20 minutes for claw samples. We extracted hormones following previously described methods (Karpovich et al. 2020, Keogh et al. 2020). Briefly, we added 1 mL 100% methanol to the powdered sample and rotated slowly on a benchtop rotator (13 rpm) for 24 hours at room temperature. We centrifuged samples at 10,500 g, 10°C for 13 minutes and transferred the supernatant to a new polypropylene tube. We added 0.3 mL methanol to the pellet, agitated, centrifuged, and then combined supernatant with the previously collected supernatant. Methanol extracts were stored at ≤−20°C until assayed. We transferred a subsample of the methanol extract to borosilicate glass tubes, dried extract under forced air, and reconstituted in hormone-specific assay buffer from the EIA kits. All samples were run in duplicate, and all assays included a standard curve, non-specific binding wells, blank wells, and 2 controls. Intra-assay coefficients of variability averaged 6.8% for cortisol ($n = 75$), 2.7% for progesterone ($n = 74$), and 5.1% for testosterone ($n = 76$). Inter-assay coefficients of variability were 11.9% for cortisol ($n = 4$), 4.8% for progesterone ($n = 4$), and 7.6% ($n = 4$) for testosterone.

We validated commercially available EIA kits (Arbor Assays®, Ann Arbor, MI, USA) for cortisol (K003), progesterone (K025), and testosterone (K032) using pooled extracts by sex from each tissue type (guard hair, undercoat, claw tip) through standard practices including parallelism, dilution linearity, and accuracy. We used serially diluted sample extract pools to compare sample linearity and parallelism to the standard curves (Hunt et al. 2014). Parallelism results were graphed as percentage of antibody bound vs. logarithm of relative dose and curves were compared to the standard curve from each assay. We assessed the matrix effect by combining standards with an equal volume of the diluted hormone pool. The diluted hormone pool with and without standards added were then assayed as unknowns alongside the standard curve. We plotted results as the observed vs. the expected concentrations and assessed the slope and y-intercept.

Data analysis

We analyzed data with Systat 13 (Systat Software, Inc., Point Richmond, CA, USA). We assessed normality of data with probability plots. We used a natural log transformation on all hormone concentrations to allow conformation

to a normal distribution. We assessed 2 separate models: model 1) sex, age class (adult vs. subadult), and the interaction term sex*age class as categorical variables, and model 2) decade (1990s, 2010s), residency status (resident vs. nonresident), and the interaction term decade*residency status as categorical variables. For each model, we used a stepwise general linear model with an iterative process of comparing the full model with all terms to reduced models, which included only terms with a $P \leq 0.10$. We report only the final models. Information about age and residency status was absent for one female wolf (NA-2; Appendix A, Table A1), thus she was excluded from the sex and age class general linear models. We used Pearson correlations to assess the relationships between hormones (cortisol, progesterone, and testosterone) within each tissue as well as assessing the relationship between guard hair and undercoat concentrations for each hormone. We used a general linear model with repeated measures Bonferroni post hoc for multiple comparisons to assess the concentrations of each hormone in wolves with paired samples from all 3 tissues. We reported means ± SD, and results were considered statistically significant at $\alpha = 0.05$.

RESULTS

Animal handling and sample collection

We sampled 31 wolves, 23 during 1993–1994 and 8 during 2012–2014 (Appendix A, Table A1). Minimum convex polygon home ranges were smaller ($t_{14} = 2.14$, $P = 0.043$) for resident wolves ($\bar{x} = 283$ km^2, SE = 48 km^2, $n = 12$) than for nonresident wolves ($\bar{x} = 524$ km^2, SE = 124 km^2, $n = 4$) during 1993–1994. Because of the limited sample size we restricted the comparison of home ranges between decades to resident wolves only. Minimum convex polygon home ranges were larger ($t_{17} = 2.11$, $P = 0.013$) for wolves during 2012–2014 (mean = 490 km^2, SE = 51 km^2, $n = 7$) than during 1993–1994.

Validation of steroid hormones in tissues

The slopes of serially diluted methanol extracts, for each tissue type and sex, were visually inspected. Results were plotted as the percentage bound vs. the logarithm of relative dose, and the slopes were compared for parallelism of the linear portion of the curve. Further, the slopes for the observed vs. expected combined standard-pool concentrations for all EIAs for accuracy tests demonstrated a good fit for the y-intercept and the slope of the expected dose alone ($R^2 \geq 0.972$), demonstrating no matrix effect (Appendix B, Table B1).

Claw hormone concentrations

All 3 hormones were measurable in all 31 claw samples (Table 1). There were 30 claw samples with complete information available for comparisons between sexes and the 1990s and 2010s. Hormone concentrations measured in male and female claw samples were similar (progesterone $F_{1,27} = 0.682$, $P = 0.416$; testosterone $F_{1,27} = 0.001$, $P = 0.971$; and cortisol $F_{1,27} = 0.326$, $P = 0.573$; Table 1). Cortisol concentrations were greater in adults compared to subadults ($F_{1,27} = 5.871$, $P = 0.021$; Figure 2), whereas progesterone ($F_{1,27} = 0.214$, $P = 0.645$) and testosterone ($F_{1,27} = 1.065$, $P = 0.311$) concentrations did not differ between adult and subadult wolves. The interaction terms were not retained in any of the models.

Cortisol, progesterone, and testosterone concentrations did not differ between resident and nonresident wolves ($P \geq 0.224$) or between claw samples collected in the 1990s and 2010s ($P \geq 0.159$) and the interaction term was not retained in any of the models. In the claw samples ($n = 31$), progesterone concentrations were positively correlated with testosterone ($r = 0.624$, $P \leq 0.001$), whereas cortisol concentrations were not related to progesterone ($r = 0.325$, $P = 0.074$) or testosterone concentrations ($r = -0.067$, $P = 0.719$).

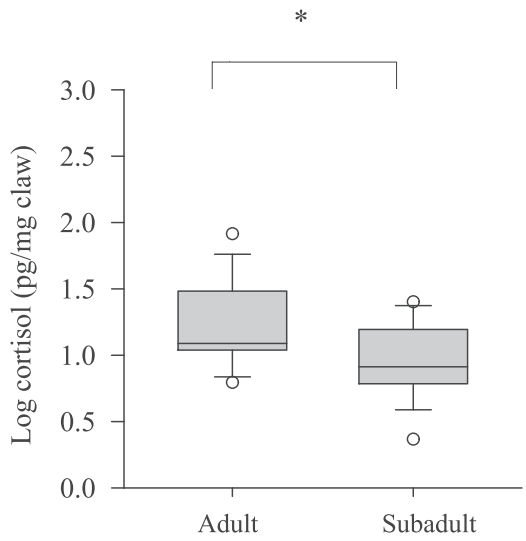

FIGURE 2 Concentration of log cortisol (pg/mg) in claw samples from adult ($n = 15$) and subadults ($n = 15$) wolves during 1993–1994 and 2012–2014, Prince of Wales Island, Alaska, USA. One female wolf (NA-2) had an unknown age class and was not included in the analysis. Grey box includes data between the 25th and 75th percentile, with a line within each box denoting the median, the whiskers are the error bars (10 and 90% percentile), and open circles are outliers. An asterisk (*) denotes a significant difference between groups.

Guard hair hormone concentrations

There were no differences between adult ($n = 11$) and subadult ($n = 11$) wolves for the 3 hormones measured in guard hair ($P \leq 0.369$), nor was there an influence of sex for the 3 hormones ($P \leq 0.241$). However, there was a significant sex*age class interaction for progesterone concentrations ($F_{1,18} = 8.176$, $P = 0.010$).

There were 23 guard hair samples with cortisol, testosterone, and progesterone concentrations available for comparison between collection period (1990s, 2010s) and 22 available for comparisons of residency status (resident, nonresident). Mean and standard deviations of cortisol concentrations did not differ between samples collected in the 1990s (6.8 ± 4.0 pg/mg) compared to the 2010s (7.4 ± 2.5 pg/mg; $F_{1,19} = 0.943$, $P = 0.344$), nor was there a difference between resident (7.0 ± 2.4 pg/mg) and nonresident wolves (6.9 ± 2.5 pg/mg; $F_{1,19} = 0.273$, $P = 0.607$). Concentrations of reproductive hormones in guard hair differed between samples collected in the 1990s compared to samples collected in 2010s; however, only 4 guard hair samples were available from the 2010s. Guard hair progesterone concentrations were greater in 1990s (49.9 ± 19.8 pg/mg) compared to 2010s (27.0 ± 6.8 pg/mg; $F_{1,19} = 9.412$, $P = 0.006$, Figure 3A). Testosterone concentrations were also greater in 1990s (10.6 ± 8.8 pg/mg) compared to 2010s (3.4 ± 3.1 pg/mg; $F_{1,19} = 5.901$, $P = 0.025$; Figure 3B). In guard hair ($n = 23$), cortisol concentrations were not correlated with testosterone ($r = 0.222$, $P = 0.310$) or progesterone ($r = -0.048$, $P = 0.827$), whereas testosterone was positively correlated with progesterone ($r = 0.590$, $P = 0.003$).

Undercoat hair hormone concentrations

Due to small samples, it was not possible to measure all 3 hormones in every undercoat sample (Table 1). There was no difference between adults and subadults for the 3 hormone concentrations in undercoat samples

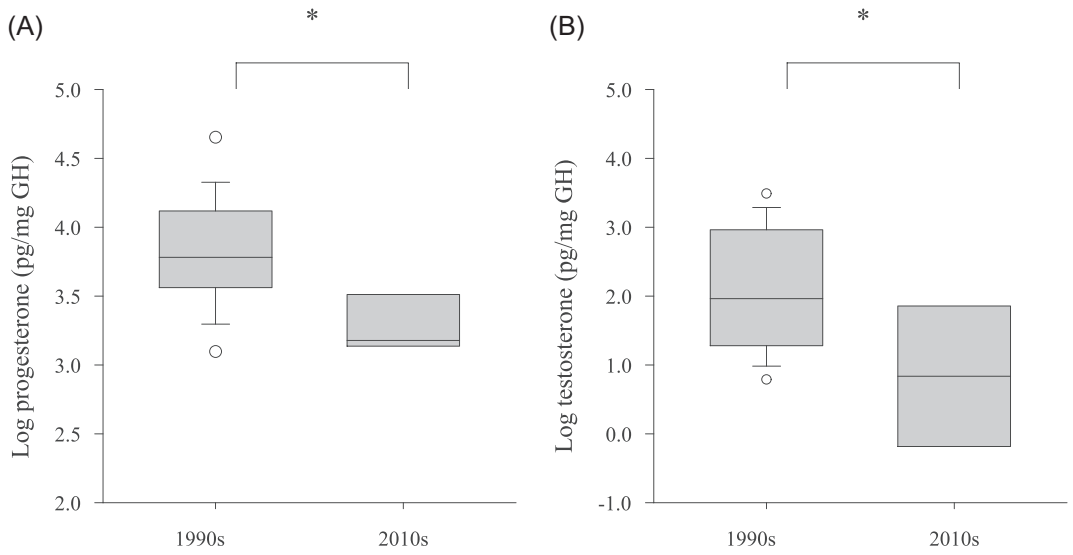

FIGURE 3 Concentrations of (A) log progesterone and (B) log testosterone (pg/mg guard hair) for guard hair samples collected from wolves during 1993–1994 ($n = 18$) and 2012–2014 ($n = 4$), Prince of Wales Island, Alaska, USA. One female wolf (NA-2) had an unknown residency status and was not included in the analysis. Grey box includes data between the 25th and 75th percentile, with a line within each box denoting the median, the whiskers are the error bars (10 and 90% percentile), and open circles are outliers. Asterisk (*) denotes significant difference between groups.

($P \geq 0.425$) nor was there a difference between male and female wolves ($P \geq 0.223$) or the sex*age class interaction term ($P \geq 0.105$). Cortisol concentrations ($F_{1,22} = 28.25$, $P \leq 0.001$) were lower in the 1990s ($n = 19$) compared to samples collected in the 2010s ($n = 5$; Figure 4A) and residency and the interaction term were not retained in the model. Progesterone concentrations ($F_{1,20} = 6.233$, $P = 0.021$; Figure 4B) were greater in undercoat samples collected in the 1990s ($n = 19$) compared to the 2010s ($n = 4$) and residency and the interaction term were not retained in the model. Testosterone concentrations were greater in the 1990s compared to the 2010s ($F_{1,22} = 4.575$, $P = 0.044$; Figure 4C) and residency status and the interaction term were not retained in the model. In undercoat, cortisol concentrations were not correlated with testosterone ($r = -0.211$, $P = 0.312$) or progesterone ($r = -0.195$, $P = 0.374$) while testosterone was positively correlated with progesterone ($r = 0.545$, $P = 0.011$).

Comparisons among tissues

Cortisol concentrations differed among guard hair, undercoat, and claw samples ($F_{2,44} = 47.223$, $P = 0.001$; Table 1). Post hoc analysis found no difference between concentrations in guard hair and undercoat samples ($P = 0.586$), whereas cortisol concentrations in claw samples were lower compared to guard hair ($P = 0.001$) and undercoat ($P = 0.001$) samples (Figure 5A). Similar results were found for progesterone ($F_{2,40} = 43.158$, $P = 0.001$) with no difference between the 2 hair types ($P = 0.303$) and claw samples having significantly lower concentrations than both guard hair ($P = 0.001$) and undercoat ($P = 0.001$; Figure 5B). There was no difference in testosterone concentrations among the 3 tissue types ($F_{2,44} = 1.441$, $P = 0.248$; Figure 5C).

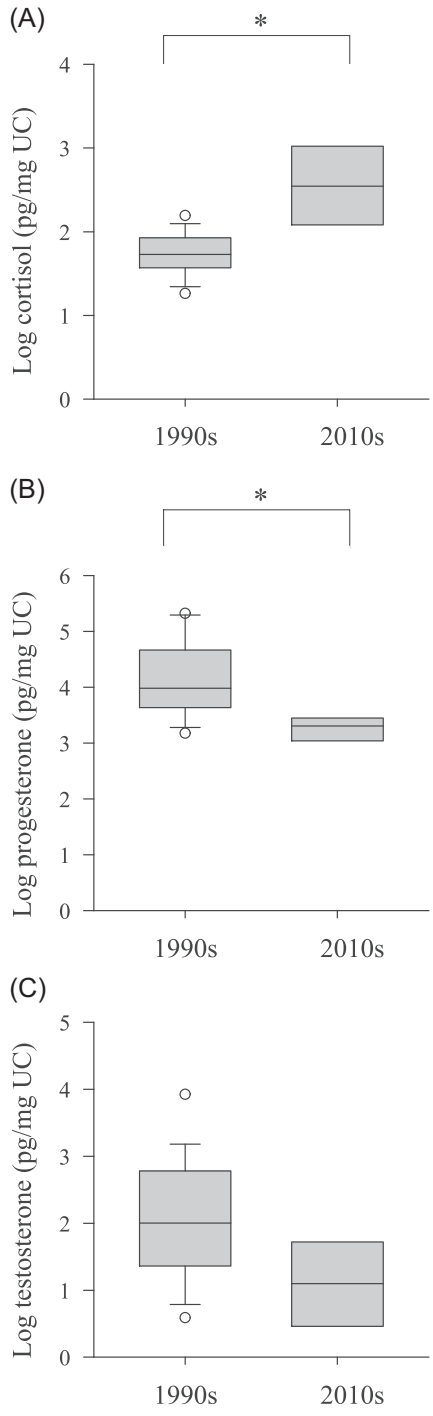

FIGURE 4 Concentrations of (A) log cortisol, (B) log progesterone, and (C) log testosterone (pg/mg undercoat [UC]) for undercoat samples collected from wolves during 1993–1994 and 2012–2014, Prince of Wales Island, Alaska, USA. Grey box includes data between the 25th and 75th percentile, with a line within each box denoting the median, the whiskers are the error bars (10 and 90% percentile), and open circles are outliers. Asterisk (*) denotes significant difference between groups.

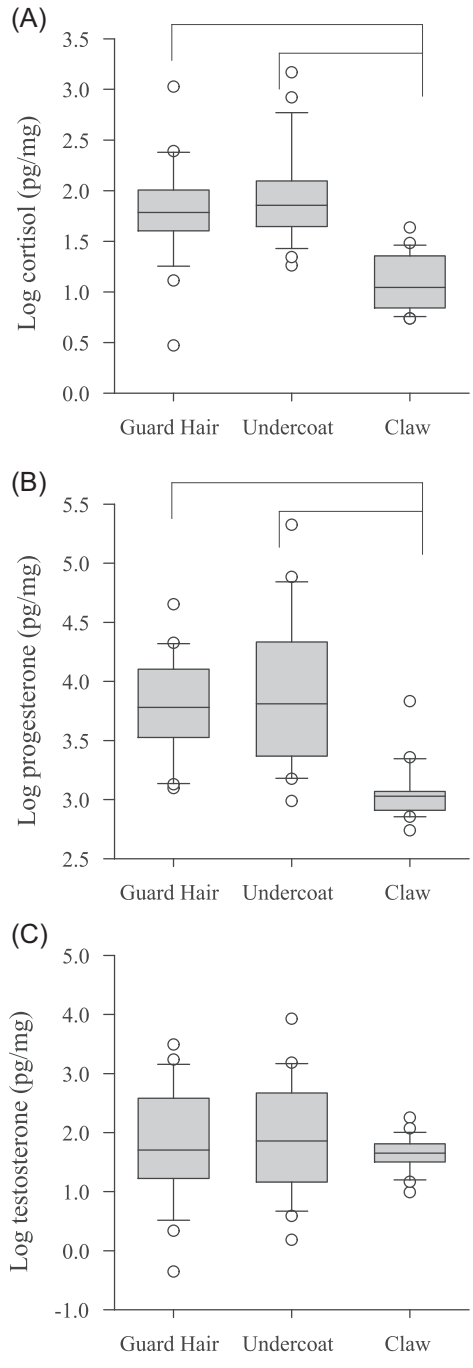

FIGURE 5 Concentrations of (A) log cortisol (n = 23), (B) log progesterone (n = 21), and (C) log testosterone (n = 17) for paired guard hair, undercoat, and claw samples collected from wolves on Prince of Wales Island, Alaska, USAGrey box includes data between the 25th and 75th percentile, with a line within each box denoting the median, the whiskers are the error bars (10 and 90% percentile), and open circles are outliers. Asterisk (*) denotes significant difference between groups.

DISCUSSION

We successfully measured reproductive and stress-related steroid hormones in wolf guard hair, undercoat hair, and claw samples using commercially available EIA kits. Progesterone, testosterone, and cortisol concentrations could be measured in the 3 keratinous tissue types, including wolves of different age class, sex, and residency status. Within each tissue type, progesterone and testosterone were positively correlated, and cortisol concentrations were not related to either reproductive hormone. Although other studies have measured stress-related or reproductive hormones in claws of other species (Baxter-Gilbert et al. 2014, Karpovich et al. 2020, M. J. Keogh, ADF&G, unpublished data), our study is the first attempt to measure hormones in wolf claws. Additionally, we measured hormones in archived tissue samples collected from 4–25 years ago, demonstrating the potential to assess changes in stress and reproductive hormone concentrations in populations over decades (Bechshøft et al. 2012*a*, Hunt et al. 2016, Crain et al. 2021).

We found no difference between male and female wolves for the 3 hormones we measured in either hair (guard hair, undercoat) or claw samples. Two males in our study (a nonresident adult [AM63] and a resident subadult [JM66]) had unexpectedly high progesterone concentrations measured in guard hair and undercoat hair which was comparable to or exceeded the highest female concentrations. Bryan et al. (2013) similarly had outliers ($n = 4$) in cortisol and/or testosterone concentrations greater than 2 standard deviations in hair samples collected from grizzly bears (*Ursus arctos*) and suggested that future studies should explore outliers for potential physiological stress.

A possible reason for the high progesterone concentrations in the hair from the male wolves is that circulating concentrations during an acute stress response can increase due to extragonadal sources (Båge et al. 2000). However, we did not expect to capture acute stressors in our samples due to the timeframe of hair and claw growth represented in each sample (i.e., months) which would capture chronic rather than acute stress (Kellar et al. 2013, Beaulieu-McCoy et al. 2017). Furthermore, in our study, the cortisol concentration for both males in guard hair and undercoat hair were near the population mean concentrations. Our findings suggested that the high progesterone concentrations in the 2 males were not associated with cortisol concentrations. Elevated progesterone in male rodents has been linked to increased sexual prowess (Witt et al. 1994, Andersen and Tufik 2006, Alvarenga et al. 2010) and decreased aggression (Erpino and Chappelle 1971, Fraile et al. 1988, Schneider et al. 2003). Therefore, it is possible that the unusually high progesterone concentrations measured in the hair of 2 male wolves are related to behaviors associated with the complex social and sexual hierarchy in wolf packs (Mech and Boitani 2003, Packard 2003).

The lack of a difference in keratin tissue cortisol concentrations between male and female wolves in our study was consistent with results from other studies. For example, Bryan et al. (2013, 2015) found no difference in hair cortisol concentrations between males and females in wolves or grizzly bears. Similarly, no differences between sexes were detected in concentrations of fecal glucocorticoid concentrations (Sands and Creel 2004, Molnar et al. 2015) in free-ranging wolves, or in cortisol concentrations measured in urine from captive wolves (McLeod et al. 1996).

In our study reproductive hormone concentrations between males and females were not different, in contrast to higher testosterone concentrations in males compared to females measured in wolf hair (Bryan et al. 2015). As Bryan et al. (2015) suggested, the sex differences in testosterone in wolf hair was likely not related to reproduction, because wolf hair is not grown during the breeding season. Rather, the sex differences in testosterone may be related to social stress due to unstable social structure (or pack stability) associated with increased human-caused mortality and increased breeding efforts of subordinate wolves (Bryan et al. 2015). The high progesterone concentrations in the 2 male wolves in our study, supports measuring all steroid hormones in both male and female wolves, which may add to our understanding of the role these hormones in the complex social structure of wolves.

Although we did not detect differences in steroid hormone concentrations between male and female wolves, we did detect some variation based on age class, residency status, and the decade when samples were collected; a

longitudinally focused study is needed to further explore these patterns. Cortisol concentrations measured in claws from adults were higher than subadult wolves, indicating a relationship with age and stress-related hormones in our study area. In wolves, social-dominance hierarchy is structured by age within each sex and older wolves are more dominant (i.e., age-graded dominance hierarchy; Zimen 1982, Packard 2003). Elevated basal glucocorticoid levels have been documented in dominant individuals in cooperatively breeding species (Creel 2005), including wolves (Sands and Creel 2004, Barja et al. 2008). In addition, an increase in cortisol metabolites measured in wolf feces was associated with reproductive activity during proestrous (Molnar et al. 2015), breeding, and denning seasons (Creel 2005, Eggermann et al. 2013). On average wolves are sexually mature at 22 months (Seal et al. 1979, Paquet and Carbyn 2003). Thus, adults would be more likely to compete for breeding opportunities and experience a corresponding increase in cortisol. The lack of difference between subadult and adult cortisol concentrations measured in guard and undercoat hair may be explained by differences in growth period, with guard hair and undercoat completing the annual growth cycle in the late fall which does not include the winter breeding season. In contrast, the longer and continuous growth of mammalian claws (Ethier et al. 2010, Jasmine et al. 2018) means the tips may contain hormones deposited during the winter breeding season when cortisol is expected to be higher (Molnar et al. 2015).

Testosterone concentrations were higher in the 1990s than in the 2010s in wolf guard hair and undercoat hair. Testosterone levels are not only associated with reproduction, but are also influenced by social competition, especially in fitness-related conflicts (Bryan et al. 2013). During the 1990s sample collection period, higher wolf densities and smaller individual home ranges would have led to increased competition and territorial conflict. Opportunities for aggression increase along the periphery of pack territories, and more wolves are killed by other wolves in this zone (Mech and Boitani 2003). In addition to higher wolf densities, the higher wolf harvest rates during the 1990s could have influenced testosterone, similar to patterns in Nunavut and the Northwest Territories where higher testosterone concentrations in wolves were attributed to social disruption due to elevated human-caused mortality (Bryan et al. 2015). Although high testosterone levels were associated with increased rates of aggression and breeding activity in male African wild dogs (*Lycaon pictus*; Creel et al. 1997), testosterone measured in wolf hair is more likely to be a result of social stress due to increased interactions among individuals in the absence of a stable social hierarchy. Wolf hair growth occurs during spring through autumn, thus incorporating hormones during the pup rearing season but not during the breeding season in late winter (Bryan et al. 2015).

Progesterone concentrations were higher in the 1990s than in the 2010s in wolf guard hair and undercoat hair, possibly reflecting increased reproductive activity (Packard et al. 1983, Packard et al. 1985, Bryan et al. 2015), as progesterone increases throughout the wolf gestation period and would be elevated when hair was grown (Sha et al. 2010). Wolves respond to large-scale reductions in wolf densities from increased harvest or predator control programs by increasing their reproductive rates (Fuller et al. 2003, Schmidt et al. 2017). Levels of wolf harvest were high on Prince of Wales Island during the 1990s, and the average annual mortality rate of radiocollared wolves was 55% during 1993–1995 (Person and Russell 2008). Removal of wolves from a pack may lead to social disruption and even pack dissolution, especially when one or both breeding wolves are removed (Borg et al. 2015). Additionally, social disruption is associated with higher levels of stress in social species (Gobush et al. 2008, Molnar et al. 2015). Although wolf packs typically have one pair of breeding wolves, multiple reproducing wolves may occur, particularly in cases when the breeding wolves have been removed (Packard et al. 1983, Mech and Boitani 2003, Ausband et al. 2017). Therefore, higher progesterone concentrations in the 1990s could have resulted from an increase in reproductive activity as a response to consistently high levels of harvest.

Using keratinous tissues to measure steroid hormone concentrations may be beneficial because they accumulate information regarding physiological states representing monthly (i.e., hair) to years (i.e., claws) timeframes. In addition, hair may be collected noninvasively (e.g., from hair snare devices), and both tissues (hair and claws) may be convenient to collect opportunistically from live-captured or harvested animals. We found hormone

concentrations in claws were significantly lower than in hair samples, likely due in part to these tissues representing hormone concentrations from different timeframes during tissue growth. Furthermore, hair and claw samples are stable and do not require low temperature or chemical preservation for long term storage. Here, we demonstrated the utility of using archived keratinous tissues that had been collected 25 years prior to quantify wolf stress-related and reproductive hormones. The results of our study suggested there are potential management benefits from using historical samples to evaluate population-level changes in reproductive and stress-related steroid hormones due to environmental stressors or changes in reproductive activity over periods of time. Further, additional physiological parameters such as dietary stable isotopes, trace minerals, or contaminants can be determined in both hair and claw tissues and are incorporated during growth and are therefore temporally linked to hormone concentrations in the same tissue allowing for exploring potential stressors (Bechshøft et al. 2012*b*, 2015; Crain et al. 2021; M. J. Keogh, ADF&G, unpublished data).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

Capture and handling procedures conformed to guidelines established by the ADF&G Animal Care and Use Committee (ACUC #2012–028 and #2014–15) and the American Society of Mammalogists (Sikes and Gannon 2011).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from ADF&G. Restrictions apply to the availability of these data, which were used under license for this study. Data are available gretchen.roffler@alaska.gov with the permission of ADF&G.

REFERENCES

Alaback, P. B. 1982. Dynamics of understory biomass in Sitka spruce- western hemlock forests of Southeast Alaska. Ecology 63:1932–1948.

Alvarenga, T. A., M. L. Andersen, and S. Tufik. 2010. Influence of progesterone on sexual performance in male rats. The Journal of Sexual Medicine 7:2435–2444.

Andersen, M. L., and S. Tufik. 2006. Does male sexual behavior require progesterone? Brain Research Reviews 51:136–143.

Apollonio, M., L. Mattioli, M. Scandura, L. Mauri, A. Gazzola, and E. Avanzinelli. 2004. Wolves in the Casentinesi Forests: insights for wolf conservation in Italy from a protected area with a rich wild prey community. Biological Conservation 120:253–264.

Ausband, D. E., M. S. Mitchell, and L. P. Waits. 2017. Effects of breeder turnover and harvest on group composition and recruitment in a social carnivore. Journal of Animal Ecology 86:1094–1101.

Båge, R., M. Forsberg, H. Gustafsson, B. Larsson, and H. Rodríguez-Martínez. 2000. Effect of ACTH-challenge on progesterone and cortisol levels in ovariectomised repeat breeder heifers. Animal Reproduction Science 63:65–76.

Ballard, W. B., L. A. Ayres, P. R. Krausman, D. J. Reed, and G. Steven. 1997. Ecology of Wolves in Relation to a Migratory Caribou Herd in Northwest Alaska. Wildlife Monographs 135:3–47.

Bamberg, E., A. Aichinger, and G. Mitteregger. 2004. In vitro metabolism of dehydroepiandrosterone and testosterone by canine hair follicle cells. Veterinary Dermatology 15:19–24.

Bamberg, E., A. Aichinger, and G. Wünsch. 2005. In vitro metabolism of progesterone by canine hair follicle cells. Veterinary Dermatology 16:153–155.

Barja, I., G. Silván, and J. C. Illera. 2008. Relationships between sex and stress hormone levels in feces and marking behavior in a wild population of Iberian wolves (*Canis lupus signatus*). Journal of Chemical Ecology 34:697–701.

Baxter-Gilbert, J. H., J. L. Riley, G. F. Mastromonaco, and J. D. Litzgus. 2014. A novel technique to measure chronic levels of corticosterone in turtles living around a major roadway. Conservation Physiology 2:1–9.

Beaulieu-McCoy, N. E., K. K. Sherman, M. L. Trego, D. E. Crocker, and N. M. Kellar. 2017. Initial validation of blubber cortisol and progesterone as indicators of stress response and maturity in an otariid; the California sea lion (*Zalophus californianus*). General and Comparative Endocrinology 252:1–11.

Bechshøft, T. Ø., A. E. Derocherc, E. Richardson, P. Mislan, N. J. Lunn, C. Sonne, R. Dietz, D. Janz, and V. L. St. Louis. 2015. Mercury and cortisol in Western Hudson Bay polar bear hair. Ecotoxicology 24:1315–1321.

Bechshøft, T. Ø., F. F. Riget, C. Sonne, R. Letcher, D. Muir, M. Novak, E. Henchey, J. Meyer, I. Eulaers, and V. Jaspers. 2012*a*. Measuring environmental stress in East Greenland polar bears, 1892–1927 and 1988–2009: What does hair cortisol tell us? Environment International 45:15–21.

Bechshøft, T. Ø., C. Sonne, R. Dietz, E. W. Born, D. C. G. Muir, R. J. Letcher, M. A. Novak, E. Henchey, J. S. Meyer, B. M. Jenssen, et al. 2012*b*. Associations between complex OHC mixtures and thyroid and cortisol hormone levels in East Greenland polar bears. Environmental Research 116:26–35.

Borg, B. L., S. M. Brainerd, T. J. Meier, and L. R. Prugh. 2015. Impacts of breeder loss on social structure, reproduction and population growth in a social canid. Journal of Animal Ecology 84:177–187.

Bryan, H. M., C. T. Darimont, P. C. Paquet, K. E. Wynne-Edwards, and J. E. G. Smits. 2013. Stress and reproductive hormones in grizzly bears reflect nutritional benefits and social consequences of a salmon foraging niche. PLoS ONE 8:e80537.

Bryan, H. M., J. E. G. Smits, L. Koren, P. C. Paquet, K. E. Wynne-Edwards, and M. Musiani. 2015. Heavily hunted wolves have higher stress and reproductive steroids than wolves with lower hunting pressure. Functional Ecology 29:347–356.

Burch, J. W., L. G. Adams, E. H. Follmann, and E. A. Rexstad. 2005. Evaluation of wolf density estimation from radiotelemetry data. Wildlife Society Bulletin 33:1225–1236.

Busch, D. S., and L. S. Hayward. 2009. Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142:2844–2853.

Castelló, J. R., and C. Sillero-Zubiri. 2018. Canids of the world: wolves, wild dogs, foxes, jackals, coyotes, and their relatives. Princeton University Press, Princeton, New Jersey, USA.

Cook, N. J. 2012. Review: minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. Canadian Journal of Animal Science 92:227–259.

Cook, C. J., D. J. Mellor, P. J. Harris, J. R. Ingram, and L. R. Matthews. 2000. Hands-on and hands-off measurements of stress. Pages 126–146 *in* G. P Moberg and J. A. Mench, editors. The Biology of Animal Stress. Basic Principles and Implications for Animal Welfare. CAB International, New York, New York, USA.

Crain, D. D., S. A. Karpovich, L. Quakenbush, and L. Polasek, 2021. Using claws to compare reproduction, stress and diet of female bearded and ringed seals in the Bering and Chukchi seas, Alaska, between 1953–1968 and 1998–2014. Conservation Physiology 9:coaa115.

Creel, S. 2005. Dominance, aggression, and glucocorticoid levels in social carnivores. Journal of Mammalogy 86:255–264.

Creel, S., N. M. Creel, M. G. L. Mills, and S. L. Monfort. 1997. Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. Behavioral Ecology 8:298–306.

Darimont, C. T., and T. E. Reimchen. 2002. Intra-hair stable isotope analysis implies seasonal shift to salmon in gray wolf diet. Canadian Journal of Zoology 80:1638–1642.

Dobson, H., and R. F. Smith. 2000. What is stress, and how does it affect reproduction? Animal Reproduction Science 61: 743–52.

Eggermann, J., J. Theuerkauf, B. Pirga, A. Milanowski, and R. Gula. 2013. Stress-hormone levels of wolves in relation to breeding season, pack size, human activity, and prey density. Annales Zoologici Fennici 50:170–175.

Erpino, M. J., and T. C. Chappelle. 1971. Interactions between androgens and progesterone in mediation of aggression in the mouse. Hormones and Behavior 2:265–272.

Ethier, D. M., C. J. Kyle, T. K. Kyser, and J. J. Nocera. 2010. Variability in the growth patterns of the cornified claw sheath among vertebrates: implications for using biogeochemistry to study animal movement. Canadian Journal of Zoology 88:1043–1051.

Fraile, I. G., B. S. McEwen, and D. W. Pfaff. 1988. Comparative effects of progesterone and alphaxalone on aggressive, reproductive and locomotor behaviors. Pharmacology Biochemistry and Behavior 30:729–735.

Fuller, T. K., L. D. Mech, and J. F. Cochrane. 2003. Wolf Population Dynamics. Pages 161–191 *in* L. D. Mech and L. Boitani, editors. Wolves: behavior, Ecology, and Conservation. University of Chicago Press, Chicago, Illinois, USA.

Fuller, T. K., and W. J. Snow. 1988. Estimating winter wolf densities using radiotelemetry data. Wildlife Society Bulletin 16: 367–370.

Gobush, K. S., B. M. Mutayoba, and S. K. Wasser. 2008. Long-term impacts of poaching on relatedness, stress physiology, and reproductive output of adult female African elephants. Conservation Biology 22:1590–1599.

Gormally, B. M. G., and L. M. Romero. 2020. What are you actually measuring? A review of techniques that integrate the stress response on distinct time-scales. Functional Ecology 34:2030–2044.

Greff, M. J. E., J. M. Levine, A. M. Abuzgaia, A. A. Elzagallaai, M. J. Rieder, and S. H. M. van Uum. 2019. Hair cortisol analysis: an update on methodological considerations and clinical applications. Clinical Biochemistry 63:1–9.

Grigg, E. K., B. Marie Nibblett, J. Q. Robinson, and J. E. Smits. 2017. Evaluating pair versus solitary housing in kennelled domestic dogs (*Canis familiaris*) using behaviour and hair cortisol: a pilot study. Veterinary Record Open 4:1–14.

Hunt, K. E., N. S. Lysiak, M. J. Moore, and R. M. Rolland. 2016. Longitudinal progesterone profiles in baleen from female North Atlantic right whales (*Eubalaena glacialis*) match known calving history. Conservation Physiology 4:1–9.

Hunt, K. E., R. Stimmelmayr, C. George, C. Hanns, R. Suydam, H. Brower, and R. M. Rolland. 2014. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (Balaena mysticetus). Conservation Physiology 2:cou030.

Jasmine, F., C. Antonella, F. Massimo, P. Alberto, and V. M. Cristina. 2018. The usefulness of claws collected without invasiveness for cortisol and dehydroepiandrosterone (sulfate) monitoring in healthy newborn puppies after birth. Theriogenology 122:137–143.

Karpovich, S. A., L. A. Horstmann, and L. K. Polasek. 2020. Validation of a novel method to create temporal records of hormone concentrations from the claws of ringed and bearded seals. Conservation Physiology 8:coaa073.

Kellar, N. M., J. Keliher, M. L. Trego, K. N. Catelani, C. Hanns, J. C. C. George, and C. Rosa. 2013. Variation of bowhead whale progesterone concentrations across demographic groups and sample matrices. Endangered Species Research 22:61–72.

Keogh, M. J., A. Gastaldi, P. Charapata, S. Melin, and B. S. Fadely. 2020. Stress-related and reproductive hormones in hair from three north Pacific otariid species: steller sea lions, California sea lions and northern fur seals. Conservation Physiology 8:coaa069.

Kim, Y., A. J. Travis, and V. N. Meyers-Wallen. 2007. Parturition prediction and timing of canine pregnancy. Theriogenology 68:1177–82.

Koren, L., H. Bryan, D. Matas, S. Tinman, Å. Fahlman, D. Whiteside, J. Smits, and K. Wynne-Edwards. 2019. Towards the validation of endogenous steroid testing in wildlife hair. Journal of Applied Ecology 56:547–561.

Koren, L., D. Whiteside, Å. Fahlman, K. Ruckstuhl, S. Kutz, S. Checkley, M. U. Dumond, and K. Wynne-Edwards. 2012. Cortisol and corticosterone independence in cortisol-dominant wildlife. General and Comparative Endocrinology 177:113–19.

Landys, M. M., M. Ramenofsky, and J. C. Wingfield. 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. General and Comparative Endocrinology 148: 132–149.

Macbeth, B. J., M. R. L. Cattet, G. B. Stenhouse, M. L. Gibeau, and D. M. Janz. 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): considerations with implications for other wildlife. Canadian Journal of Zoology 88:935–949.

McLeod, P. J., W. H. Moger, J. Ryon, S. Gadbois, and J. C. Fentress. 1996. The relation between urinary cortisol levels and social behaviour in captive timber wolves. Canadian Journal of Zoology 74:209–216.

Mech, L. D., and L. Boitani. 2003. Wolf Social Ecology. Pages 1–34 *in* L. D. Mech and L. Boitani, editors. Wolves: Behaviour, ecology, and conservation. University of Chicago Press, Chicago, Illinois, USA.

Mesarcova, L., J. Kottferova, L. Skurkova, L. Leskova, and N. Kmecova. 2017. Analysis of cortisol in dog hair—a potential biomarker of chronic stress: a review. Veterinarni Medicina 62:363–376.

Mohr, C. O., and W. A. Stumpf. 1966. Comparison of methods for calculating areas of animal activity. The Journal of Wildlife Management 30:293–304.

Molnar, B., J. Fattebert, R. Palme, P. Ciucci, B. Betschart, D. W. Smith, and P.-A. Diehl. 2015. Environmental and intrinsic correlates of stress in free-ranging wolves. PLoS ONE 10:e0137378.

Möstl, E., and R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology 23:67–74.

Packard, J. M. 2003. Wolf Behavior: reproductive, Social, and Intelligent. Pages 35–65 *in* L. D. Mech and L. Boitani, editors. Wolves: behaviour, ecology, and conservation. University of Chicago Press, Chicago, Illinois, USA.

Packard, J. M., L. D. Mech, and U. S. Seal. 1983. Social influences on reproduction in wolves. Pages 78–85 *in* L. N. Carbyn, editor. Wolves in Canada and Alaska: their status, biology and management. Canadian Wildlife Report Series 45. Edmonton, Alberta, Canada.

Packard, J. M., U. S. Seal, L. D. Mech, and E. D. Plotka. 1985. Causes of reproductive failure in two family groups of wolves (*Canis lupus*). Zeitschrift für Tierpsychologie 68:24–40.

Palme, R., S. Rettenbacher, C. Touma, S. M. El-Bahr, and E. Möstl. 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Annals of the New York Academy of Sciences 1040:162–171.

Paquet, P. C., and L. N. Carbyn. 2003. Gray wolf (*Canis lupus*) and Allies. Pages 482–510 *in* G. A. Feldhammer, B. C. Thompson, and J. A. Chapman, editors. Wild mammals of North America: biology, management, and conservation, 2nd ed. John Hopkins University Press, Baltimore, Maryland, USA.

Person, D. K., M. Kirchhoff, V. Van Ballenberghe, G. C. Iverson, and E. Grossman. 1996. The Alexander Archipelago wolf: a conservation assessment. U.S. Department of Agriculture Forest Service General Technical Report PNW-GTR-384, Pacific Northwest Research Station, Portland, Oregon, USA.

Person, D. K., and A. L. Russell. 2008. Correlates of mortality in an exploited wolf population. The Journal of Wildlife Management 72:1540–1549.

R Core Team. 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rausch, R. A. 1967. Some aspects of the population ecology of wolves, Alaska. American Zoologist 7:253–265.

Reeder, D. M., and K. M. Kramer. 2005. Stress in free-ranging mammals: integrating physiology, ecology, and natural history. Journal of Mammalogy 86: 225–235.

Roffler, G. H., D. P. Gregovich, and K. R. Larson. 2018. Resource selection by coastal wolves reveals the seasonal importance of seral forest and suitable prey habitat. Forest Ecology and Management 409:190–201.

Roffler, G. H., J. N. Waite, K. L. Pilgrim, K. E. Zarn, and M. K. Schwartz. 2019. Estimating abundance of a cryptic social carnivore using spatially explicit capture–recapture. Wildlife Society Bulletin 43:31–41.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution 19:249–55.

Romero, L. M., and J. M. Butler. 2007. Endocrinology of Stress. International Journal of Comparative Psychology 20:89–95.

Sands, J., and S. Creel. 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. Animal Behaviour 67:387–396.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55–89.

Schell, C. J., J. K. Young, E. V. Lonsdorf, J. M. Mateo, and R. M. Santymire. 2017. Investigation of techniques to measure cortisol and testosterone concentrations in coyote hair. Zoo Biology 36:220–225.

Schmidt, J. H., J. W. Burch, and M. C. MacCluskie. 2017. Effects of control on the dynamics of an adjacent protected wolf population in interior Alaska. Wildlife Monographs 198:1–30.

Schneider, J. S., M. K. Stone, K. E. Wynne-Edwards, T. H. Horton, J. Lydon, B. O'Malley, and J. E. Levine. 2003. Progesterone receptors mediate male aggression toward infants. Proceedings of the National Academy of Sciences 100:2951–2956.

Seal, U. S., E. D. Plotka, J. M. Packard, and L. D. Mech. 1979. Endocrine correlates of reproduction in the wolf. I. Serum progesterone, estradiol and LH during the estrous cycle. Biology of Reproduction 21:1057–1066.

Sha, W., Z. Hong-hai, and L. Chen. 2010. Fecal progesterone and estradiol changes during the breeding season in captive female wolf. Journal of Forestry Research 21:367–372.

Sheriff, M. J., B. Dantzer, B. Delehanty, R. Palme, and R. Boonstra. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166:869–887.

Sheriff, M. J., C. J. Krebs, and R. Boonstra. 2009. The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. Journal of Animal Ecology 78:1249–1258.

Signer, J., and N. Balkenhol. 2015. Reproducible home ranges (rhr): a new, user-friendly R package for analyses of wildlife telemetry data. Wildlife Society Bulletin 39:358–363.

Sikes, R. S., and W. L. Gannon. 2011. Guidelines of the American Society of Mammalogists for the use of wild mammals in research. Journal of Mammalogy 92:235–253.

Slominski, A., and J. Wortsman. 2000. Neuroendocrinology of the skin. Endocrine Reviews 21:457–487.

U.S. Fish Wildlife Service. 2016. Endangered and threatened wildlife and plants; 12-month finding on a petition to list the Alexander Archipelago wolf as an endangered or threatened species. 81 Federal Register 435. U.S. Fish Wildlife Service, Anchorage, Alaska, USA.

Veronesi, M. C., A. Comin, T. Meloni, M. Faustini, A. Rota, and A. Prandi. 2015. Coat and claws as new matrices for noninvasive long-term cortisol assessment in dogs from birth up to 30 days of age. Theriogenology 5:791–796.

Vilela, S., A. Alves da Silva, R. Palme, K. E. Ruckstuhl, J. P. Sousa, and J. Alves. 2020. Physiological stress reactions in red deer induced by hunting activities. Animals 10:1003.

Walker, S. L., W. T. Waddell, and K. L. Goodrowe. 2002. Reproductive endocrine patterns in captive female and male red wolves (*Canis rufus*) assessed by fecal and serum hormone analysis. Zoo Biology 21:321–335.

Witt, D. M., L. J. Young, and D. Crews. 1994. Progesterone and sexual behavior in males. Psychoneuroendocrinology 19:553–562.

Wolf Technical Committee. 2017. Interagency wolf habitat management program: recommendations for Game Management Unit 2. Management Bulletin R10-MB-822. U.S. Department of Agriculture Forest Service, U.S. Department of the Interior Fish and Wildlife Service and Alaska Department of Fish and Game. <https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/fseprd537975.pdf>. Accessed 13 Jan 2018.

Young, S. P., and E. A. Goldman. 1944. Wolves of North America. Dover Publications, New York, New York, USA.

Zimen, E. 1982. A wolf pack sociogram. Pages 238–261 *in* F. H. Harrington and P. C. Paquet, editors. Wolves of the world: perspectives of behavior, ecology, and conservation. Noyes Publications, Park Ridge, New Jersey, USA.

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APPENDIX A: CAPTURED WOLVES AND CHARACTERISTICS

TABLE A1 Wolves included in this study by sex, age class, status, and year captured, Prince of Wales Island, Alaska, USA.

Wolf ID	Sex	Age	Status	Year
AF78	F	Adult	Resident	1993
JM76	M	Subadult	Resident	1993
YF82	F	Subadult	Nonresident	1993
YF80	F	Subadult	Resident	1993
YF74	F	Subadult	Resident	1993
AF73	F	Adult	Nonresident	1993
YF77	F	Subadult	Resident	1993
YF81	F	Subadult	Resident	1993
AM75	M	Adult	Resident	1993
AM62	M	Adult	Resident	1993
AM59	M	Adult	Nonresident	1993
JM79	M	Subadult	Resident	1993
AM64	M	Adult	Nonresident	1993
JM61	M	Subadult	Resident	1993
NA-2	F	Unknown	Unknown	1993
AF57	F	Adult	Resident	1994
JM66	M	Subadult	Resident	1994
AM63	M	Adult	Nonresident	1994
AM80	M	Adult	Resident	1994
AF82	F	Adult	Resident	1994
JM64	M	Subadult	Resident	1994
AF62	F	Adult	Resident	1994
JF65	F	Subadult	Resident	1994
AF430	F	Adult	Resident	2012

TABLE A1 (Continued)

Wolf ID	Sex	Age	Status	Year
AM310	M	Adult	Resident	2012
AF270	F	Adult	Resident	2012
AM260	M	Adult	Resident	2012
AF255	F	Subadult	Resident	2012
JF465	F	Subadult	Resident	2012
JF495	F	Subadult	Resident	2012
201401	F	Subadult	Nonresident	2014

APPENDIX B: ENZYME IMMUNOASSAY ASSAY KIT VALIDATION

TABLE B1 Results for hormone concentration parallelism and accuracy tests for cortisol, progesterone, and testosterone extracted from wolf hair (guard hair and undercoat) and claws, 1993–1994 and 2012–2014, Prince of Wales Island, Alaska, USA.

Hormone	Sex	Tissue	Parallelism slopes and fit	Accuracy slopes and fit
Testosterone	F	Hair	Standard slope: −13.76, $R^2 = 0.9907$ Sample pool slope: −14.91, $R^2 = 0.9992$	y = 1.0100x + 369.11 $R^2 = 0.9873$
		Claw tips	Standard slope: −14.61, $R^2 = 0.9960$ Sample pool slope: −15.40, $R^2 = 0.9999$	y = 0.9981x + 309.00 $R^2 = 0.9939$
	M	Hair	Standard slope: −14.33, $R^2 = 0.9964$ Sample pool slope: −15.41, $R^2 = 0.9994$	y = 1.0783x + 453.13 $R^2 = 0.9855$
		Claw tips	Standard slope: −14.33, $R^2 = 0.9964$ Sample pool slope: −15.28, $R^2 = 0.9995$	y = 0.9220x + 747.48 $R^2 = 0.9428$
Cortisol	F	Hair	Standard slope: −22.69, $R^2 = 0.9903$ Sample pool slope: −24.09, $R^2 = 0.9987$	y = 0.9541x − 50.73 $R^2 = 0.9884$
		Claw tips[a]	Standard slope: −21.72, $R^2 = 0.9852$ Sample pool slope: −22.68, $R^2 = 0.9994$	y = 1.0293x − 210.38 $R^2 = 0.9892$
	M	Hair	Standard slope: −20.69, $R^2 = 0.9944$ Sample pool slope: −22.55, $R^2 = 0.9994$	y = 1.0444x + 16.37 $R^2 = 0.9938$
		Claw tips	Standard slope: −20.69, $R^2 = 0.9944$ Sample pool slope: −22.43, $R^2 = 0.9998$	y = 1.0655x − 99.43 $R^2 = 0.9967$
Progesterone	F	Hair	Standard slope: −27.27, $R^2 = 0.9951$ Sample pool slope: −29.21, $R^2 = 0.9951$	y = 0.9368x − 155.17 $R^2 = 0.9994$
		Claw tips	Standard slope: −27.84, $R^2 = 0.9906$ Sample pool slope: −28.41, $R^2 = 0.9913$	y = 0.8517x − 69.04 $R^2 = 0.9968$
	M	Hair	Standard slope: −23.28, $R^2 = 0.9917$ Sample pool slope: −24.81, $R^2 = 0.9995$	y = 0.9636x + 92.70 $R^2 = 0.9825$
		Claw tips	Standard slope: −23.28, $R^2 = 0.9917$ Sample pool slope: −25.06, $R^2 = 0.9921$	y = 1.0553x − 54.81 $R^2 = 0.9872$

[a]Included 2 individuals of unknown sex.