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REVIEW

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Towards the validation of endogenous steroid testing in wildlife hair

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Abstract

1. Hair analysis is emerging as a popular tool to examine stress and reproduction hormone levels in wild mammals. The reliability of this approach, however, depends on an understanding of steroid hormone incorporation into hair as well as appropriate validations.
2. We reviewed studies that have examined steroid hormones in wildlife hair with the goal of summarizing the analytical, physiological, and biological evidence that this approach is meaningful. Accordingly, we differentiated among validations aimed at evaluating the reliability of the analytical method versus those designed to assess whether hormone levels in hair reflect physiologically meaningful processes in the target species.
3. Our literature survey revealed that endogenous steroids have been examined in hair from 40 species of nonhuman animals across seven mammalian classes. Although the majority (85%) of 72 studies reported analytical validations of the method, physiological validations have only been reported for five species. Moreover, results of physiological validations were inconsistent among studies. This highlights the need for further research, carefully designed to differentiate between the multiple purported models of steroid incorporation into hair in species with different types of hair and different hair growth patterns.
4. To complement our review of published studies, we present new data supporting a positive relationship between levels of the steroid, cortisol, in hair and blood across eight mammalian species. In addition, we present novel results from a laboratory-based study showing variable hair growth in genetically identical laboratory mice that were kept under controlled conditions.
5. *Synthesis and applications*. Collectively, this Review reveals substantial progress towards the validation of stress hormone assays in hair from a variety of wildlife species. Further validations of reproductive steroids, combined with appropriate physiological validations, would expand the potential applications of hair analyses

Lee Koren and Heather Bryan contributed equally to this work.

in wildlife research. As a key example, physiological data can provide mechanistic insights into species' responses to change and may therefore contribute to conservation planning.

KEYWORDS
cortisol, endogenous steroids, hair, hair growth, hormone levels, reproductive hormones, stress, validations

1 | INTRODUCTION

Measures of stress and reproductive steroid hormones can provide valuable information about the behaviour, physiology, and ecology of wildlife. Although many hormones are involved in the stress and reproductive endocrine pathways, the most common steroid hormones investigated for wildlife research include cortisol and corticosterone as measures of hypothalamic–pituitary–adrenal axis activity, and testosterone, progesterone, and estradiol as measures of hypothalamic–pituitary–gonadal axis activity. Historically, blood was the primary sample type used to examine steroid hormones in humans and other species. Starting in the late 1980s, field studies began using faeces and urine to measure steroid hormone levels in wild animals, with examples including the measurement of reproductive steroids in yellow baboon (*Papio cynocephalus cynocephalus*) faeces (Wasser, Monfort, & Wildt, 1991) and dwarf mongoose (*Helogale parvula*) urine (Creel, Creel, Wildt, & Monfort, 1992). Hair emerged as a sample type for measuring endogenous steroids in domestic animals in the mid-1990s (Gleixner & Meyer, 1997), and was first applied in wildlife by Koren et al. (2002).

Hair, faeces, and urine can often be collected less invasively (i.e., without capture and handling wildlife) compared with blood (Cook, 2012; Kersey & Dehnhard, 2014; Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). Although hormone concentrations in blood change rapidly over the course of a few minutes in response to short-term external or internal conditions, these less invasive sample types typically reflect endocrine activity over longer periods (minutes to months). In comparison with blood, these other sample types are therefore less influenced by short-term stressors (such as capture and handling) and are assumed to be more meaningful indicators of long-term physiological states and processes.

Compared with other noninvasive sample types, hair has several properties that make it advantageous or complementary. Although steroids are often excreted as metabolites in urine and faeces, steroids remain intact in hair, which facilitates analysis. In addition, hair is practical for logistical reasons as it can be stored in paper envelopes at room temperature for long periods. Steroid hormones in hair appear to be stable for months to years or more (Macbeth, Cattet, Stenhouse, Gibeau, & Janz, 2010), presenting opportunities to sample archival specimens in museums or other collections (e.g., Bechshøft et al., 2012; Bryan et al., 2015; Koren, Matas, et al., 2018).

Despite numerous logistical advantages, the interpretation of steroid hormone levels in hair is complicated by uncertainties around the mechanisms by which hormones enter hair. In general, hair is thought to reflect a long-term, integrated measure of steroid hormones that are incorporated into growing hair when steroids diffuse from the blood vessel that feeds the hair follicle into the follicle itself (Meyer & Novak, 2012). However, steroid hormones can also be incorporated into hair from tissues surrounding the growing follicle, glandular secretions such as sebum produced and secreted near the base of hair follicles, and by external substances such as saliva, urine, or faeces (Kintz, Villain, & Cirimele, 2006; Pragst & Balikova, 2006). The mechanism of steroid binding to hair is not fully understood, but involves associations with melanin and keratin proteins (Kintz et al., 2006). Since steroids must diffuse across plasma membranes to be incorporated into hair, or may be produced locally within the follicle or surrounding glands, steroids bound to albumin or to binding globulins are unlikely to be represented in hair. Therefore, hair steroids are generally thought to reflect the biologically active steroid forms (i.e., free, unbound (Siiteri et al., 1982)). Investigators routinely wash hair with the goal of removing steroid hormones deposited from glandular and external sources. Despite wash procedures, steroid hormones within hair may change over time in relation to short-term biological events even when hair is not growing (Cattet et al., 2014, 2017). For example, testosterone and progesterone in grizzly bear (*Ursus arctos*) hair varied over time in response to reproductive events, including during periods when hair was in a quiescent phase (Cattet et al., 2017). A further complication is that steroid hormones produced locally within the hair follicle can be incorporated into hair (Ito et al., 2005; Keckeis et al., 2012). Although the relative contributions of different sources are unknown, these findings suggest that hormones in hair reflect a combination of long-term steroid levels accumulated during hair growth and shorter-term levels from other sources. An additional challenge relates to differences in hair growth rate within and among individuals, which may further complicate the interpretation of hair hormone levels in relation to biological events. For example, hormone levels may differ by body region of the same individual (e.g., Carlitz, Kirschbaum, Miller, Rukundo, & van Schaik, 2015; Fourie et al., 2016).

Several review papers summarize the general methodology, advantages, and outstanding issues related to analysis of steroids in hair, with a particular focus on analysis of cortisol in human hair (Burnard, Ralph, Hynd, Hocking Edwards, & Tilbrook, 2017; Kintz et al., 2006; Meyer & Novak, 2012; Pragst & Balikova, 2006; Russell, Koren, Rieder, & Van Uum, 2012; Srogi, 2006). Here, our aim is to review the progress towards applying this approach in wild mammals.

Specifically, the review evaluates the analytical, physiological, and biological validation of determining endogenous steroids in wildlife hair. Since domestic animals are often considered models for wildlife, at least during initial testing and validation stages, the literature review also included studies on domestic animals. To complement the literature review, we present new data on the relationship between hair and serum cortisol concentrations in eight wildlife species, and on variability in hair growth patterns among genetically identical laboratory mice under controlled conditions. Our findings reveal that considerable progress has been made towards the validation of steroid hormones in wildlife hair, and that further validation of steroids other than cortisol, along with appropriate physiological validations, would expand potential applications of steroid hair testing in wildlife research.

2 | MATERIALS AND METHODS

2.1 | Literature search

We searched Thomson Reuters Web of Science using the key words; hair, steroids, analysis, testosterone, cortisol, corticosterone, estradiol, oestrogens, glucocorticoids, wildlife, and testing. Additional research papers that were referenced within or cited by these papers were added to achieve a comprehensive summary of all wildlife species in which hair testing was used to measure endogenous steroids. We included articles that described original methods or that reported endogenous steroid levels in hair from wild and domestic mammals so that as many unique species, steroids, and methodological combinations as possible were represented in Table 1. Specifically, we included multiple papers per species and steroid if a different method was used or a different validation performed.

For each study, we summarized the analytical and physiological validations, as well as the biological results. We defined analytical validations broadly as those that assess the reliability of analytical procedures, including the accuracy and precision of measurements (Cekan, 1975). We further classified the analytical validations reported in each paper as: (a) recovery, which is often used as a proxy for accuracy and is done by spiking a known amount of analyte into a sample and comparing the amount recovered with the amount added, (b) specificity, which reflects the ability of an assay to measure the compound of interest and is often evaluated by comparing reactivity of an assay to non-target compounds expected to be present in the sample, (c) linearity of the dilution, which evaluates the linearity of measurements over the target range of values using standards of known concentration, (d) parallelism, which compares the linearity of a diluted experimental sample with that of standards, and (e) repeatability, which evaluates the similarity among multiple measurements of the same sample, and is often reported as intra- and inter-assay coefficients of variation.

We classified physiological validations as those used to evaluate the ability of an analytical approach or sample type to reflect physiological responses in the target population. Physiological validations include the suppression or stimulation of steroid hormones followed by analysis to determine how well the expected endocrine changes were reflected in hair samples. For example, a common procedure is to inject study animals with a synthetic form of adrenocorticotropic hormone (ACTH), which mimics the one that is normally produced by the pituitary gland and stimulates glucocorticoid production by the adrenal cortex. A similar procedure may be performed for physiological validation of reproductive steroids using gonadotropic releasing hormone (GnRH). We considered the administration of radio-labelled steroids, which may be used to further clarify the routes, timing, and dynamics of steroid entry into hair, as an additional form of physiological validation. We defined biological validations as manipulations designed to alter steroid hormone levels in hair (e.g., relocations or altered social conditions), and biological results as correlations between steroid hormone levels and variables related to the ecology or life history of the study species.

2.2 | Associating hair and serum cortisol

Serum and hair samples from individual males and non-pregnant females over the age of 1 year were obtained opportunistically from ongoing sampling protocols. From each animal, a single hair and blood sample were obtained on the same day. We do not have information on the time of day that paired hair–blood samples were obtained, and since different methods were used to capture, restrain, and anaesthetize animals, the time to serum collection varied between the species. None of the animals received steroid or contraceptive medications. Samples from Amur (Siberian) tigers (*Panthera tigris altaica; N* = 10), moose (*Alces alces; N* = 11), and Przewalski's wild horses (*Equus ferus przewalskii*; *N* = 27) were obtained from animals kept at the Calgary Zoo. White-tailed deer (*Odocoileus virginianus*; *N* = 10) samples were collected from captive animals held at the Specialized Livestock Research Facility, Western College of Veterinary Medicine, University of Saskatchewan, Canada. Reindeer (*Rangifer tarandus tarandus*) samples (*N* = 11) were collected from captive animals held at the University of Calgary Faculty of Veterinary Medicine, Canada. The remaining species were sampled from wild animals, including muskoxen (*Ovibos moschatus*; *N* = 23), grizzly bears (*Ursus arctos; N* = 43) from Nunavut, Canada, and rock hyrax (*Procavia capensis*; *N* = 12) from Israel. Serum (50–100 μl) from each individual was used for the pooled species sample. Details on the serum samples and cortisol quantification are published in (Koren, Whiteside, et al., 2012). Hair sample pools were washed twice for 3 min with double distilled (dd) water on an orbital shaker and left to dry overnight. Next, they were washed twice with isopropanol (Fisher Scientific; Fair Lawn, NJ, USA) while shaking. Hair samples were ground to powder in a ball mill (Retsch mixer mill MM 200 with 10 ml stainless steel grinding jars and one 12 mm stainless steel grinding ball per jar). Pooled hair (200 mg) from each species was weighed into a glass vial. Methanol (Optima-grade; Fisher Scientific; Fair Lawn, NJ, USA) was added in excess to each sample so that

hair powder was submersed completely, and 20 μl of deuterium labelled internal standard cortisol-9,11,12,12-d_4 (C/D/N Isotopes Inc.; Pointe-Claire, QC, Canada) was added. Samples were sonicated for 30 min and incubated overnight at 50°C. The next day, samples were protein precipitated with 1 ml of 89 mg/ml $ZnSO_4 \cdot 7H_2O$ and 9 ml of methanol, vortexed, and then incubated at −20°C for 15 min. Next, 140 μl were placed into a vial into which 800 μl of water was added. Solid Phase Extraction and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) conditions followed (Koren, Ng, Soma, & Wynne-Edwards, 2012). We used Spearman's rank correlation to test for an association between cortisol immunoreactivity in serum and hair (JMP 12.1.0, SAS Institute Inc., 2015).

2.3 | Mouse model for hair growth rate and corticosterone concentrations

One hundred and sixteen ICR (CD-1® outbred) mice, 58 males and 58 females (Envigo, Rehovot, Israel), aged 6 weeks were used in this study. Mice were housed in same-sex sibling pairs in intraventilated (IVC) cages in a controlled environment (22 ± 2°C, 35–55% humidity) with a 12 hr light: dark cycle. Irradiated food pellets (Altromin 1324IRR; for decontamination) and filtered acidified water (pH 3.2; to control bacterial growth) were supplied ad libitum. After 2 weeks of acclimation, the hair on the backs of the mice (neck to base of the tail) was clipped with an Aesculap small animal clipper (ISIS model, Braun Vetcare, Suhl, Germany)—over a patch of 6 cm (*L*) × 3 cm (*W*). Hair regrowth relative to unclipped hair around the patch was visually assessed by a skilled technician following 6 and 9 weeks and expressed as a per cent of hair regrowth length, compared with the surrounding hair (categorized as 0%, 25%, 50%, 75%, 100% regrowth). Full grown hair was assigned 100% growth, 50% hair regrowth meant that hair within the patch had regrown to 50% of the length of surrounding hair, and a completely shaven hair patch, with the skin showing, was considered 0%. Ethical approval for this study was granted by the Bar Ilan Animal Use and Ethics Committee (protocol number 54-11-2014). In each hair sample, following 6 and 9 weeks, we measured hair corticosterone following extraction and detection procedures described in Arnon, Hazut, Tabachnik, Weller, and Koren (2016). Corticosterone was measured using commercial enzyme-linked immunosorbent assays (ELISA; item no. ADI-900-097; ENZO Biochem, USA). The same kit (and antibody) was formerly distributed by R&D Systems and by Assay Designs. This assay has been procedurally and biologically validated for noninvasive samples from other species. The manufacturers reported that cross-reactivity for the corticosterone kit was 28.6% for deoxycorticosterone, 1.7% for progesterone, and less than 0.28% for all other steroids. The kit, designed for human samples, was validated for mouse hair by showing parallelism between serial dilutions of mouse hair extracts and the standard curves that were provided with the kit. Linear range for corticosterone was between 5 and 45 mg of hair, corresponding to 800–20,000 pg/ml of corticosterone standard.

3 | RESULTS

3.1 | Literature search

Our literature search revealed > 67 publications between 1997 and 2017 that examined endogenous steroids in hair from nonhuman mammals. These studies included hair from 40 different species and seven mammalian orders (Table 1). Of these studies, 60% focused on captive or free-living wildlife and the remainder on domestic animals. Some of the studies included several steroids, resulting in 72 unique species and steroid entries (Table 1). Most studies (82%) quantified only glucocorticoids, primarily cortisol.

3.1.1 | Variation in hair analysis methods

Although all studies extracted steroids using an organic solvent (primarily methanol), our review revealed variation in other aspects of the methodology for analysing endogenous steroids in nonhuman hair (Figure 1). For example, wash procedures ranged from none, to several wash steps with one or more soaps or organic solvents. To prepare hair for steroid extraction, all methods used one or more physical approaches to break the hair structure using sonication, mincing with scissors, and/or pulverizing with a ball mill, with the exception of one study that performed extractions on whole hair (Di Francesco et al., 2017). During extraction of steroids from hair, all studies used high temperatures (50°C), except for a few that performed extractions at room temperature (Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006; Dettmer, Novak, Suomi, & Meyer, 2012; Feng et al., 2011), and one that performed extractions at 4°C (Di Francesco et al., 2017). Following extraction, 64% of the 72 cases used enzyme immunoassays (EIA), 22% used radioimmunoassays (RIA), and 3% used luminescence immunoassays (LIA) to quantify steroids. The remaining cases used LC-MS/MS (*N* = 8), high-performance liquid chromatography (HPLC) combined with EIA (*N* = 1), or gas chromatography tandem mass spectrometry (GC-MS/MS) (*N* = 1). Studies that employed immunoassay-based approaches (i.e., EIA, RIA, or LIA) reported no sample clean-up, or simple sample clean-up using filtration or centrifugation. Of studies that quantified steroids using chromatographic approaches (i.e., LC-MS/MS, HPLC-EIA, or GC-MS/MS), one or more clean-up steps with liquid–liquid extraction, solid phase extraction, or filtration were required.

3.1.2 | Analytical and physiological validations

Sixty-one of the 72 papers (85%) reported analytical validations, including linearity, parallelism, intra- and inter-assay variability, and recovery efficiency. Eight studies performed a physiological validation such as an ACTH challenge, or a steroid administration. Results from the five studies that performed ACTH challenges were inconsistent among studies (see Supporting Information Table S1). Although we found no studies that administered GnRH to examine the consequences of experimentally elevated hypothalamic–pituitary–gonadal axis on hormone levels in hair, several have administered synthetic

TABLE 1 Concentrations of steroids and assay validations in the hair of domestic and wild species

Taxon	Reference	*N*	Detection method	Steroid	Concentration[a]	Validations
Artiodactyla						
Camel (*Camelus*)	Shah, Haddow, Ibrahim, Sheikh, and Alhemeiri (2017)	30	LC-MS/MS	Cortisol	R: 31–935	A: CV, L, PII, Re, S
Barren-ground caribou (*Rangifer tarandus caribou*)	Ashley et al. (2011)	12	EIA	Cortisol	Neck: 4.2 ± 0.4[b] Shoulder: 1.5 ± 0.1 Rump: 1.4 ± 0.1	A: CV, PII, Re P: ACTH challenge B: body region, correlation with faeces
Reindeer (*Rangifer tarandus granti*)	Ashley et al. (2011)	12	EIA	Cortisol	Neck: 2.0 ± 0.1[b] Shoulder: 3.0 ± 0.2 Rump: 2.3 ± 0.2	A: CV, PII, Re P: ACTH challenge B: body region, correlation with faeces
	Carlsson et al. (2016)	10	EIA	Cortisol	0.7 ± 0.9[b]	B: body region, correlation with faeces, infection, new or old hair growth
Domestic cattle, Holstein breed (*Bos taurus*)	Burnett et al. (2015)	118	EIA	Cortisol	Min: 9.8 ± 0.0 Max: 12.6 ± 0.0	A: CV B: clinical disorders, pregnancy status
	Comin, Peric, et al. (2012)	229	RIA	Cortisol	R: 0.8–20.4	A: CV, PII
	Gonzalez-de-la-Vara et al. (2011)	12	RIA	Cortisol	Min: 12.2 ± 1.6[g] Max: 114.5 ± 14.4	A: CV, PII P: ACTH challenge B: age
	Nielen et al. (2006)	6	LC-MS/MS	Testosterone esters Boldenone esters	R: 2–5 R: 2–5	A: CV, L, Re, S P: administration of testosterone derivatives and boldenone undecylenate
	Tallo-Parra, Manteca, Sabes-Alsina, Carbajal, and Lopez-Bejar (2015)	17	EIA	Cortisol	Min: 1.4 ± 0.0[g] Max: 3.9 ± 0.1	A: CV, L, PII, Re B: correlation with faeces
	Tallo-Parra et al. (2017)	24	EIA	Cortisol	0.5 ± 0.2[i]	A: CV, L, PII, Re P: ACTH challenge B: correlation with blood
Domestic cattle breeds, Brown Swiss and Swiss Fleckvieh (*Bos taurus*)	Braun, Michel, Baumgartner, Hassig, and Binz (2017)	27	LC-MS/MS	Cortisol	R: 0.7–0.9	B: season, pregnancy, illness
Dorcas gazelle (*Gazella dorcas*)	Salas et al. (2016)	19	EIA	Cortisol	3.5 ± 1.9	A: CV, PII, Re B: social
Muskoxen (*Ovibos moschatus*)	Di Francesco et al. (2017)	150	LC-MS/MS	Cortisol	R: 3.5–48.9	A: CV B: season, sex, year of collection
Domestic goat (*Capra hircus*)	Endo et al. (2017)	12	EIA	Cortisol	2.2 ± 0.5[b]	A: CV, PII, Re P: ACTH challenge
Himalayan tahr (*Hemitragus jemlahicus*)	Lovari, Pellizzi, Boesi, and Fusani (2009)	40	RIA	Testosterone	17.5 ± 3.8[c]	A: CV B: age
Domestic sheep, breed Corriedale (*Ovis aries*)	Nejad, Lohakare, West, and Sung (2014)	9	EIA	Cortisol	Sum: 2.6–2.8	A: CV B: correlation with blood, neutrophils, water consumption, and restriction

(Continues)

TABLE 1 (Continued)

Taxon	Reference	*N*	Detection method	Steroid	Concentration[a]	Validations
Domestic pig (*Sus scrofa*)	Bacci et al. (2014)	25	RIA	Cortisol	Min: 5.2 ± 0.5 Max: 20.1 ± 1.0	A: CV, PII, Re B: pregnancy and lactation, season
	Casal et al. (2017)	14	EIA	Cortisol	19.3±0.6	A: CV, L, Re B: chronic stress
Red deer (*Cervus elaphus*)	Caslini et al. (2016)	174	RIA	Cortisol	M: 4.8 ± 0.7 F: 5.8 ± 0.6	A: L, PII, Re B: age, density, season
Perissodactyla						
Domestic horse (*Equus ferus caballus*)	Boyer, Garcia, Popot, Steiner, and Lesieur (2007)	1	GC-MS/MS	Testosterone	2.4 ± 2.1	A: Re P: testosterone administration B: body region
	Comin, Veronesi, et al. (2012)	102	RIA	Cortisol	R: 4.3–156.2	A: CV B: age, sex
Carnivora						
Canada lynx (*Lynx canadensis*)	Terwissen, Mastromonaco, and Murray (2013)	3	EIA	Cortisol	R: 3.0–7.8[b]	A: CV, PII, Re P: ACTH challenge B: age, sex
	Terwissen et al. (2014)	70 69 72	EIA	Testosterone Oestrogens Progesterone	R: 2.8–7.7[e] R: 2.8–13.8 R: 2.4–6.6	A: CV, PII, Re B: age, sex
Domestic cat (*Felis silvestris catus*)	Accorsi et al. (2008)	27	RIA	Cortisol	3.3 ± 0.3	A: CV, PII, Re, S B: correlation with faeces
	Finkler and Terkel (2010)	29	RIA	Cortisol	11.0 ± 0.5[c]	B: aggression
	Terwissen et al. (2014)	5 4 10	EIA	Testosterone Oestrogens Progesterone	5.2 ± 5.3[e] 1.4 ± 1.0 4.4 ± 3.0	A: CV, PII, Re P: progestin administration B: reproductive state
Coyote (*Canis latrans*)	Schell et al. (2017)	12	RIA	Cortisol Testosterone	M: 17.7 ± 0.9 F: 15.5 ± 0.2 M: 2.9 ± 0.2 F: 3.1 ± 0.2	A: CV, PII, Re B: body region, sex
Domestic dog (*Canis familiaris*)	Accorsi et al. (2008)	29	RIA	Cortisol	2.1 ± 0.2	A: CV, PII, Re, S B: correlation with faeces
	Bennett and Hayssen (2010)	42	EIA	Cortisol	10.9 ± 0.6[g]	A: CV, PII, Re
	Bryan, Adams, Invik, Wynne-Edwards, and Smits (2013)	7	EIA + LC-MS/MS	Cortisol	11.6 ± 0.1	A: CV, PII, R
	Piva, Liverani, Accorsi, Sarli, and Gandini (2008)	1	EIA	Cortisol	9.8 ± 11.4	None
	Roth, Faresjö, Theodorsson, and Jensen (2016)	46	RIA	Cortisol	Min: 6.8[b] Max: 40.3	A: CV B: interactions with humans, lifestyle, season
	Siniscalchi, McFarlane, Kauter, Quaranta, and Rogers (2013)	14	RIA	Cortisol	Min: 180.8 ± 21.5[f] Max: 264.9 ± 26.4	A: CV, S B: reactivity to acoustic stimuli
	Veronesi et al. (2015)	165	RIA	Cortisol	43.0 ± 18.8[i]	A: CV B: correlation with claws
Gray wolf (*Canis lupus*)	Bryan et al. (2015)	152	EIA	Cortisol Testosterone Progesterone	R: 4.8–40.4 R: 3.3–15.1 R: 12.8–53.3	A: CV, PII, Re B: hunting pressure

(Continues)

TABLE 1 (Continued)

Taxon	Reference	*N*	Detection method	Steroid	Concentration[a]	Validations
European badger (*Meles meles*)	Agnew et al. (2016)	25	EIA	Cortisol	0.4 ± 2.9[h]	A: CV, L B: turbine sound
American black bear (*Ursus americanus*)	Bryan et al. (2014)	59	EIA	Cortisol Testosterone	M: 6.5 ± 4.1[b] F: 7.7 ± 2.2 M: 6.4 ± 4.4 F: 6.6 ± 1.0	A: CV, L, PII, Re B: diet, sex
Asiatic black bear (*Ursus thibetanus*)	Malcolm et al. (2013)	46	EIA	Cortisol	16.7 ± 1.4[b]	A: CV, L, PII, Re B: relocation
	Lafferty et al. (2015)	64	EIA	Cortisol	R: 0.5–35.1	A: CV, Re B: diet, sex, social environment
Grizzly/brown bear (*Ursus arctos*)	Bourbonnais et al. (2013)	304	EIA	Cortisol	R: 0.2–23.7	B: anthropogenic disturbance
	Bryan et al. (2014)	54	EIA	Cortisol Testosterone	M: 7.8 ± 5.4[b] F: 8.5 ± 6.0 M: 5.8 ± 4.6 F: 5.0 ± 2.2	A: CV, L, PII, Re B: diet, food availability, sex
	Cattet et al. (2014)	303	EIA	Cortisol	0.9	B: capture, handling, and restraint
	Cattet et al. (2017)	94	EIA	Testosterone Progesterone Estradiol Cortisol	R: 1.2–25 R: 1.0–16.4 R: 0.004–0.022 R: 0.1–2.8	B: reproductive state
	Macbeth et al. (2010)	151	EIA	Cortisol	Md, *R*: 2.8[g], 0.6–43.3	A: CV, PII, Re B: age, body region, capture method, season, sex
	Sergiel et al. (2017)	12	EIA	Cortisol	1.3 ± 0.7[b]	A: CV
Polar bear (*Ursus maritimus*)	Bechshøft et al. (2012)	96	EIA	Cortisol	R: 4.0–29.4	A: CV B: age, persistent organic pollutants, sex, year of collection
	Bechshøft et al. (2015)	378	EIA	Cortisol	M: 0.7 ± 0.5 F: 0.8 ± 0.6	A: CV, PII, Re B: age, fat, mercury exposure
	Macbeth et al. (2012)	185	EIA	Cortisol	R: 0.2–2.3	A: CV, PII, Re
Rodentia						
Eastern chipmunk (*Tamias striatus*)	Martin and Reale (2008)	23	EIA	Cortisol	7.8 ± 1.3[b]	B: behavioural reaction, habituation
Domestic guinea pig (*Cavia porcellus*)	Keckeis et al. (2012)	8	EIA	Cortisol Cortisone	Not reported	P: injection of ^{3}H-cortisol
House mouse (*Mus musculus*)	Yu et al. (2015)	19	LC–MS/MS	Cortisol	Min: 39.3 ± 7.9 Max: 76.2 ± 13.3	A: CV, Re B: correlation with blood and brain, chronic stress
Root vole (*Microtus oeconomus*)	Książek, Zub, Szafrańska, Wieczorek, and Konarzewski (2017)	209	EIA	Corticosterone	Not reported	A: CV, L, PII, Re B: body mass, survival, immunocompetence, metabolic rate
Brown rat, breed Sprague–Dawley (*Rattus norvegicus*)	Yu et al. (2015)	22	LC-MS/MS	Cortisol	Min: 5.0 ± 0.6 Max: 19.6 ± 3.5	A: CV, Re B: correlation with blood and brain, chronic stress
Lagomorpha						
American pika (*Ochotona princeps*)	Waterhouse et al. (2017)	49	EIA	Corticosterone	8.5 ± 0.6	A: PII, Re B: body size, correlation with blood and faeces, sex

(Continues)

TABLE 1 (Continued)

Taxon	Reference	*N*	Detection method	Steroid	Concentration[a]	Validations
Domestic rabbit, breed New Zealand white rabbit (*Oryctolagus cuniculus*)	Peric et al. (2017)	19	RIA	Cortisol	1.2 ± 0.1	A: CV B: anxiety
	Trocino et al. (2014)	456	RIA	Corticosterone	13.9	A: CV, PII, Re B: housing
Primates						
Ring-tailed lemur (*Lemur catta*)	Tennenhouse et al. (2017)	34	EIA	Cortisol Testosterone Estradiol	Not reported	A: CV, PII B: affiliation, aggression
Gray mouse lemur (*Microcebus murinus*)	Rakotoniaina et al. (2017)	171	LC–MS/MS	Cortisol	18.8 ± 3.0[c]	B: body mass, survival
Common marmoset (*Callithrix jacchus*)	Clara, Tommasi, and Rogers (2008)	11	EIA	Cortisol	6.3 ± 1.2[a]	A: CV, Re B: mobbing
Guinea baboon (*Papio hamadryas papio*)	Fourie and Bernstein (2011)	19	EIA	Cortisol	Min: 1610 ± 18[e] Max: 3190 ± 71	A: CV, PII, Re B: age, phylogenetic
Chacma baboon (*Papio ursinus*)	Fourie et al. (2016)	6	EIA	Cortisol	M: 600 ± 150[b] F: 1150 ± 300	A: PII, Re B: age, sex
Vervet (*Chlorocebus aethiops*)	Fairbanks et al. (2011)	226	EIA	Cortisol	52.9 ± 0.8[b]	A: CV B: heritability of cortisol
	Fourie et al. (2015)	72	EIA	Cortisol	Min: 101 430 ± 434 Max: 180 100 ± 754	A: CV, PII, Re B: human disturbance
Rhesus macaque (*Macaca mulatta*)	Davenport et al. (2006)	20	EIA	Cortisol	110.3 ± 10.2	A: CV, PII B: prolonged stress
	Dettmer et al. (2012)	61	EIA	Cortisol	Min: 130 ± 0[b,i] Max: 191 ± 13	A: CV B: anxiety
	Feng et al. (2011)	66	RIA	Cortisol	Min: 0.9 ± 0.1[b,d] Max: 4.8 ± 0.3	B: maternal separation
	Hamel et al. (2017)	145	EIA	Cortisol	60 ± 1.7	A: CV B: reactivity
	Meyer, Novak, Hamel, and Rosenberg (2014)	9	EIA	Cortisol	75.8 ± 14.0	A: CV
	Novak et al. (2014)	99	EIA	Cortisol	Min: 47.7 ± 5.1[b] Max: 115.9 ± 15	A: CV B: hair loss
Orangutan (*Pongo* spp.)	Carlitz, Kirschbaum, Stalder, and van Schaik (2014)	56	LIA	Cortisol	19.3 ± 5.5	A: CV B: age, body region, sex, stress
Chimpanzee (*Pan troglodytes*)	Carlitz et al. (2015)	48	LIA	Cortisol	R: 0.2–31.5	A: CV
Hyracoidea						
Rock hyrax (*Procavia capensis*)	Koren and Geffen (2009)	233	EIA	Cortisol Testosterone Estradiol Androstenedione	69.4 ± 2.4[c] 23.7 ± 11.9 1090 ± 90 16.3 ± 0.7	A: CV, L, PII B: age, morphometric parameters, social status

Note. EIA: enzyme immunoassay; LIA: luminescence immunoassay; RIA: radioimmunoassay; HPLC: high-performance liquid chromatography; LC-MS/MS-liquid chromatography tandem mass spectrometry; GC-MS-MS: gas chromatography tandem mass spectrometry; F: female; M, male; A: analytical validation; Re: recovery; PII: parallelism; CV: coefficient of variation; L: linearity; S: specificity; P: physiological validation; ACTH: adrenocorticotropic hormone; B: biological correlations.

[a]Concentrations of pg/mg hair were reported as mean ± standard error (*M* ± *SE*) unless otherwise indicated: median and range (Md, *R*), or range (*R*). Decimals were rounded off to the nearest one tenth. [b]Data extracted from published figures using Web Plot Digitizer (https://automeris.io/WebPlotDigitizer). [c]Data provided by authors. [d]The concentration was reported in μg/dl. [e]Various age classes (between 12 and 72 months in guinea baboons, 6 months–12 years for cats, and 0.5–2.5 years in vervets). [f]The concentration was reported in pM/g. [g]Hair colour was tested. [h]Concentration reported in μg/dl per mg. [i]Data for pup.

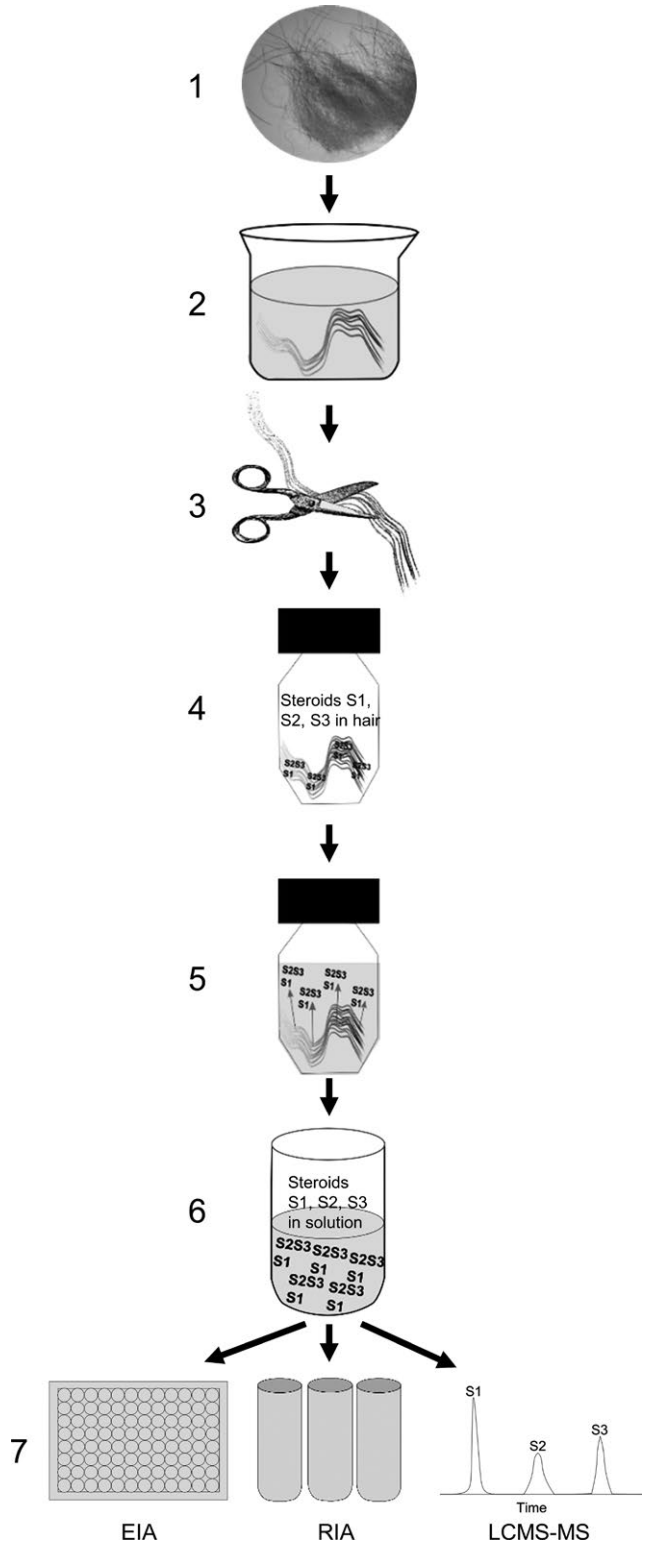

FIGURE 1 Procedure for analysis of steroid hormones in hair from wild and domestic animals. Typical steps include (1) initial hair prep (e.g., removal of follicles, sorting guard hairs from underfur), (2) washing (e.g., 1–3× with water and 1–3× with isopropanol), (3) cutting or grinding (optional), (4) weighing hair into a vial, (5) extraction of steroids from hair using a solvent (typically methanol), (6) separation of extract from hair matrix by centrifugation, and (7) analysis using EIA, RIA, or LC-MS/MS. Clean-up steps (e.g., solid phase extraction) may be required prior to analysis (not shown)

reproductive hormones followed by subsequent measurements of hair hormones. Results of these interventions have also been mixed.

3.1.3 | Biological validations and applications

Several studies have performed interventions to manipulate chronic levels of cortisol in hair. Hair cortisol was elevated following relocation to new enclosures or altered social conditions in rhesus macaques (*Macaca mulatta*) (Davenport et al., 2006; Dettmer et al., 2012), domestic rabbits (*Oryctolagus cuniculus*) (Peric et al., 2017), domestic pigs (*Sus scrofa domesticus*) (Casal, Manteca, Peña, Bassols, & Fàbrega, 2017), chimpanzees (*Pan troglodytes*) (Yamanashi et al., 2016), and Asiatic black bears (*Ursus thibetanus*) (Malcolm et al., 2013). To evaluate the biological relevance and predictive potential of reproductive hormones in hair, several studies have compared differences between sexes, age classes, and different stages of the reproductive cycle. Comparisons between sexes have revealed higher hair testosterone in males than females in some studies (grizzly bears Bryan, Darimont, Paquet, Wynne-Edwards, & Smits, 2013; wolves [*Canis lupus*] Bryan et al., 2015) but not others (rock hyraxes [*Procavia capensis*] Koren, Mokady, & Geffen, 2006; grizzly bears Cattet et al., 2017; black bears [*U. americanus*] Bryan, Darimont, Paquet, Wynne-Edwards, & Smits, 2014; coyotes [*Canis latrans*] Schell, Young, Lonsdorf, Mateo, & Santymire, 2017; ring-tailed lemurs [*Lemur catta*] Tennenhouse, Putman, Boisseau, & Brown, 2017; lynx Terwissen, Mastromonaco, & Murray, 2014). As an alternative to testosterone, progesterone or the progesterone–oestrogen ratio may be more useful in distinguishing between sexes (Cattet et al., 2017; Terwissen et al., 2014). Between reproductive classes, testosterone, progesterone, and estradiol were found to reflect key reproductive events (breeding season, parturition) in grizzly bears (Cattet et al., 2017). In addition, hormone profiles from hair were found to be useful in categorizing age classes in grizzly bears (Cattet et al., 2018) but were less promising for lynx (Terwissen et al., 2014).

Hair testing has been applied in wildlife to investigate numerous aspects of physiological ecology, including relationships among hormone levels and fitness proxies. For example, lower probability of survival was associated with higher hair cortisol in grey mouse lemurs (*Microcebus murinus;* Rakotoniaina et al., 2017) as well as higher hair corticosterone in root voles (*Microtus oeconomus*; Książek et al., 2017). Other studies have used hair cortisol to investigate the effects of social status (Koren & Geffen, 2009; Koren et al., 2006), social density (Dettmer, Novak, Meyer, & Suomi, 2014; Grigg, Nibblett, Robinson, & Smits, 2017; Salas et al., 2016), human disturbance (Agnew, Smith, & Fowkes, 2016; Bourbonnais, Nelson, Cattet, Darimont, & Stenhouse, 2013; Ewacha, Roth, Anderson, Brannen, & Dupont, 2017; Fourie et al., 2015; Lyons, Mastromonaco, Edwards, & Schulte-Hostedde, 2017), hunting (Bryan et al., 2015), diet (Bryan, Darimont, et al., 2013; Lafferty, Laudenslager, Mowat, Heard, & Belant, 2015), resource availability (Bryan et al., 2014), parasitism (Carlsson, Mastromonaco, Vandervalk, & Kutz, 2016), season (Di Francesco et al., 2017), and climate variability (Bechshøft et al., 2013; Fardi, Sauther, Cuozzo, Jacky, & Bernstein, 2018; Macbeth, Cattet, Obbard, Middel, & Janz, 2012).

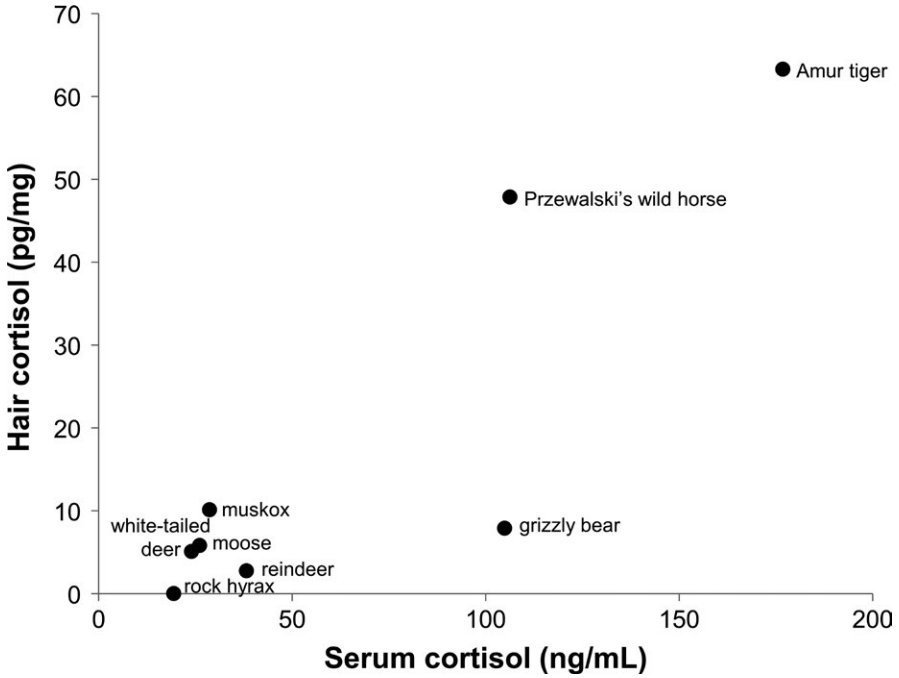

FIGURE 2 The relationship between hair and serum cortisol in pools from eight wild mammalian species: moose (*N* = 11), white-tailed deer (*N* = 10), muskox (*N* = 23), reindeer (*N* = 11), Amur tiger (*N* = 10), grizzly bear (*N* = 43), Przewalski's wild horse (*N* = 27), and rock hyrax. (*N* = 12). Serum cortisol data have been previously published (Koren, Whiteside, et al., 2012)

3.2 | Associating hair and serum cortisol

Cortisol in serum and hair samples pooled from the same individuals of eight species was well correlated (r_s = 0.81 and the two-tailed value of p = 0.01; Figure 2). In other words, species with high hair cortisol also showed relatively high circulating cortisol.

3.3 | Mice hair growth rate and corticosterone concentrations

To understand the variability in hair growth rates among individuals, we shaved laboratory mice and recorded hair regrowth rates relative to unshaven hair (Koren, Bryan, et al., 2018). Six weeks after the initial shave, the mean hair growth in 116 mice was 77.7% (±34.8% *SD*) of the surrounding hair. Three weeks later, at 9 weeks, hair regrowth averaged 88.2% (±25.3% *SD*) of surrounding hair. The total range of hair growth was 0%–100% in both time points; however, the proportion of hair regrown in the first 6 weeks predicted the proportion regrown at 9 weeks (R^2 = 0.53; N = 116; p < 0.01), suggesting an overall consistent rate of hair growth over time within individuals. However, no association was found between corticosterone at the time hair was shaved (0) and 9 weeks later, nor corticosterone and the proportion of hair regrown at 6 and 9 weeks. Further, since mice received similar treatment before and after the shaving, corticosterone values were not different (paired t test; p = 0.72) between 0 and 9 weeks.

4 | DISCUSSION

Though steroid hair testing has been developing for wider use over the past decade, our literature search showed that most wildlife and domestic animal studies only measured cortisol in hair. The validation of other steroids, such as testosterone, may provide additional information on topics such as reproductive ecology (Bryan et al., 2014), social status (Koren et al., 2006), potential growth (Mouritsen et al., 2014), and social behaviours (e.g., Finkler & Terkel, 2010; Salas et al., 2016).

Although our literature search showed that the basic methodology used to measure steroids in wildlife hair is similar among studies, it also highlighted procedural variations in the initial washing of hair, breakup of hair prior to extraction, extraction temperature, and detection method. Decisions regarding the procedural variation to use at each step of the analysis for hair hormones depend on the chemistry behind each step, and the biology and related hair structure of a particular species. For example, although most studies cut, pulverize, and sonicate hair and perform extractions at high temperatures, Di Francesco et al. (2017) found that a cold extraction on whole hair performed best on muskoxen qiviut. This example highlights the importance of validating extraction methods for each species, as interspecific differences in hair characteristics influence the optimal procedure.

The majority of studies used antibody-based methods such as RIAs or EIAs to measure hormones in wildlife hair. Although immunoassays are more practical for most ecologists, since they are easier to set up, need less expensive instruments, require less complicated sample clean-up, and are more sensitive (thus needing less hair), they are not as specific as LC-MS/MS, since antibodies may cross-react to variable degrees with non-target compounds. To address this issue, Koren and Geffen (2009) compared testosterone measurements in hair using LC-MS/MS and EIA and found a strong correlation (r = 0.84, p < 0.01), suggesting that testosterone immunoreactivity measured by EIA was proportional to the actual testosterone levels in hair. A similar approach of confirming the presence of a steroid through initially testing by LC-MS/MS with subsequent analysis by

immunoassay (Bryan, Adams, et al., 2013; Koren, Matas, et al., 2018) should be applied more widely in other studies of wildlife hair.

Overall, we found that most (85%) studies reported results from standard analytical validations, which are often required by journals that publish endocrine assay results. Therefore, the high prevalence of analytical validation results from hair studies likely reflects adherence to accepted standards. An important caveat, however, is that recovery is not possible to accurately assess in hair. Exogenous steroids can be spiked into a solvent surrounding the hair, and extracted at different stages of the detection process, but they cannot be spiked into the hair itself, unlike liquids such as blood.

Differences in findings of physiological studies could relate to differences in methodology, including the frequency, formulation, and dosage of ACTH injections, the timing of hair sampling relative to ACTH injections, and hair collection approach (e.g., whether hair was shaved before and during the experiment). In addition, interspecific differences in hair growth and timing of the experiment relative to hair growth cycles would clearly affect results. Alternatively, or concomitantly, differences in results of ACTH challenges could relate to the mechanism by which steroids are incorporated into hair. Keckeis et al. (2012) injected radio-labelled cortisol into rabbits on three successive days and found that little of the radio-labelled steroid was detected in hair. The authors interpreted this finding as evidence that steroids in hair are produced locally by the hair follicle, rather than by diffusion from the blood vessel that feeds the growing hair follicle. An alternate possibility is that hormones in hair reflect short-term changes in hormone levels due to incorporation of steroids from glandular secretions onto the hair shaft (Cattet et al., 2017). This would explain discrepancies in studies that found no evidence of cortisol elevation several days after an ACTH challenge, but did find elevated cortisol in the circulation on the same day (Endo, Yamane, Rahayu, & Tanaka, 2017).

Regardless of the causes, different findings among physiological validation studies suggest that further work, including the development of standardized protocols, are needed to confidently interpret steroid hormone levels in hair. Importantly, studies must be carefully designed to differentiate among different possible routes of steroid incorporation into hair (i.e., accumulation over time from the blood vessel that feeds the growing hair follicle, incorporation from steroids produced locally in the hair follicle, and deposition from glands that would reflect short-term, circulating steroid hormone levels). Interspecific differences in hair growth patterns, hair structure, and physiology also should be considered during the study design. As a key example of research designed to address these issues, Cattet et al. (2017) provided novel insight into the complexity of steroid incorporation into grizzly bear hair by comparing steroid hormone levels in actively growing versus quiescent hair, and by comparing hair with and without follicles. Moreover, only a few studies (e.g., Cattet et al., 2018) have examined the incorporation of steroids other than cortisol into hair, including progesterone, oestrogen, and testosterone. Additional studies focused on physiological validation of these other hormones are needed, and could be done using GnRH challenges.

An important part of validating hair hormone assays is to relate hormone levels in hair with those in samples known to reflect physiological processes. Accordingly, we found that hair and serum cortisol were well correlated among eight species. Since we were sample limited, and the LC-MS/MS required a large amount of hair to extract a detectable amount of steroid, we pooled samples from multiple individuals of the same species. Thus, one or more individuals with unusually high or low cortisol could have influenced the results. Analysis of samples from multiple individuals from a broader diversity of species could reveal whether our findings hold true both within and among taxa, as empirical studies have shown a weak, significant association between circulating and hair steroids within individuals of the same species (e.g., Koren et al., 2006; Tallo-Parra et al., 2017). Since blood and hair reflect different steroid states (bound vs. free) and time periods (momentary vs. integration over several minutes to months), the relationship between measured concentrations in the two matrices is not expected to be strong. Nonetheless, we found a significant relationship that is consistent with that of Fourie and Bernstein (2011), who found that hair cortisol reflected known intra- and interspecific differences in primates. Our finding therefore provides additional evidence that hair reflects taxonomic differences in circulating cortisol and may therefore be a valuable sample type for comparative studies.

Finally, hair testing for steroids still presents multiple challenges. For example, individual variation in hair growth rate from genetically identical mice was vast, ranging from 0% to 100%. The coarse categorization we used for hair growth may have impeded detection of a relationship between corticosterone and hair growth. Nonetheless, a plausible explanation is that this finding reflects the stable conditions that mice experienced. To minimize potential effects of different hair growth rates on steroid hormone concentrations in hair, study designs would ideally collect hair that is grown during known time frames, through shaving a predefined location on an animal's body, allowing hair to grow during a known period, and then sampling the same patch through re-shaving. This approach, however, is rarely possible for studies of free-ranging wildlife, and would not be feasible for noninvasive studies where hair is collected without handling wildlife. For free-ranging wildlife, an understanding of hair growth patterns, and their relationship with hormone incorporation into hair, could be gained first using similar captive animals or domestic animal models, although hair growth rates likely differ between wildlife in captivity and in nature and vary with diet and nutritional status. Information about hair growth should then be extrapolated carefully during the design and data interpretation of studies on free-roaming wildlife.

Despite outstanding challenges, our literature review and data show that hair provides an attractive source of hormonal information that can be applied to a breadth of wildlife studies. Biological applications (Table 1) reveal that hair testing has been used to examine physiological responses to numerous ecological and biological processes. The wide array of applications underscores the potential benefit of further validation studies, in terms of expanded opportunities for conducting non- or minimally invasive physiological

research on wild mammals as well as possible applications in conservation (Cooke & O'Connor, 2010).

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AUTHORS' CONTRIBUTIONS

L.K., H.M.B, J.S., and K.E.W.E. conceived the ideas; L.K. and H.M.B. designed methodology; A.F., D.W., S.T., and L.K. collected the data; D.M. and L.K. analysed the samples; H.M.B. and D.M. led the literature search; L.K. and H.M.B. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data for the mice study are available via the Dryad Digital Repository https://doi.org/10.5061/dryad.fq13rf7 (Koren, Bryan et al. 2018).

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SUPPORTING INFORMATION

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