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Research paper

Investigation of keratinase digestion to improve steroid hormone extraction from diverse keratinous tissues

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ABSTRACT

Monitoring the physiology of wild populations presents many technical challenges. Blood samples, long the gold standard of wildlife endocrinology studies, cannot always be obtained. The validation and use of non-plasma samples to obtain hormone data have greatly improved access to more integrated information about an organism's physiological state. Keratinous tissues like skin, hair, nails, feathers, or baleen store steroid hormones in physiologically relevant concentrations, are stable across decades, and can be used to retrospectively infer physiological state at prior points in time. Most protocols for steroid extraction employ physical pulverization or cutting of the sample, followed by mixing with a solvent. Such methods do produce repeatable and useful data, but low hormone yield and detectability issues can complicate research on small or rare samples. We investigated the use of keratinase, an enzyme that breaks down keratin, to improve the extraction and yield of corticosterone from vertebrate keratin tissues. Corticosterone content of keratinase-digested extracts were compared to non-keratinase extracts for baleen from three species of whale (blue, *Balaenoptera musculus*; bowhead, *Balaena mysticetus*; southern right, SRW; *Eubalaena australis*), shed skin from two reptiles (tegu lizard, *Salvator merianae*; narrow-headed garter snake, *Thamnophis rufipunctatus*), hair from arctic ground squirrel (AGS; *Urocitellus parryii*), feathers from Purple Martins (PUMA; *Progne subis*), and spines from the short-beaked echidna (*Tachyglossus aculeatus*). We tested four starting masses (10, 25, 50, 100 mg) for each sample; digestion was most complete in the 10 and 25 mg samples. A corticosterone enzyme immunoassay (EIA) was validated for all keratinase-digested extracts. In all sample types except shed skin from reptiles, keratinase digestion improved hormone yield, with PUMA feathers and blue whale baleen having the greatest increase in apparent corticosterone content (100% and 66% more hormone, respectively). The reptilian shed skin samples did not benefit from keratinase digestion, actually yielding less hormone than controls. With further optimization and refinement, keratinase digestion could greatly improve yield of steroid hormones from various wildlife epidermal tissue types, allowing more efficient use of samples and ultimately improving understanding of the endocrine physiology of wild populations.

1. Introduction

For more than 60 years, hormone analyses have been critical for studying fundamental physiological phenomena such as reproduction, metabolism, and stress in a wide variety of vertebrate taxa (Hane and Robertson, 1959; Higgs and Eales, 1973; Wingfield and Farner, 1975). In the early decades of comparative endocrinology, laboratory analyses focused almost exclusively on plasma samples. Plasma hormone concentrations are invaluable for studying the animal's current endocrine state, i.e., at a given day and time, and have proven essential for study of peptide/protein hormones. It has since become clear, however, that steroid and thyroid hormones are quantifiable in numerous other vertebrate sample types as well, such as saliva, earwax, blubber, urine, and feces, as well as keratinized cornified epidermal tissues like skin, hair, nails, feathers or baleen (Berkvens et al., 2013; Bortolotti et al., 2008; Hodges and Eastman, 1984; Hunt et al., 2017; Kellar et al., 2013; Ohl et al., 1999; Palme, 2019; Reslir et al., 1987; Sheriff et al., 2011; Trumble et al., 2013; Warnock et al., 2010). These alternative sample types can offer some advantages over plasma, e.g., integrating hormone concentrations over longer timeframes (hours to days or weeks), avoiding influences of capture stress and time-of-day, and improving field feasibility for endangered species or those species that are

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particularly difficult to capture or handle (Ashley et al., 2011; Berkvens et al., 2013; Cook, 2012; Cyr and Romero, 2008; Fernández Ajó et al., 2020; 2018; 2010;; Heimbürge et al., 2019; Hunt et al., 2014; Karpovich et al., 2020; Kersey and Dehnhard, 2014; Lübcker et al., 2020; Macbeth et al., 2012; Mingramm et al., 2019; Narayan, 2013; Ramos et al., 2018; Schwarzenberger, 2007; Wasser et al., 2010; Wasser and Hunt, 2005; Will et al., 2014).

Keratinized cornified epidermal tissues ("keratin tissues" hereafter), such as feather, fur, claw, baleen, reptilian shed skin, and spines have recently drawn particular attention. Many of these tissues are linear structures that extend from a growth zone embedded in well-vascularized dermis, accumulating hormones from circulating plasma as they grow. Once the tissue extends out of the growth zone, hormones are remarkably stable in the dry, firm keratin matrix; indeed, keratin tissues retain detectable hormones for decades or even millennia (Bechshøft et al., 2012; Bortolotti et al., 2009; Crain et al., 2021; Fairhurst et al., 2015; Kennedy et al., 2013; Koren et al., 2018; Webb et al., 2010). Thus, keratin tissues provide the ability to retrospectively assess the animal's endocrine state at some prior point in time when the tissue was grown. In the case of archived specimens, such tissues can enable study of past populations. Additionally, some keratin tissues grow slowly and continuously over years (guard hairs of some mammals, claw, vibrissae, baleen) such that the sample can be used to reconstruct months or even years of individual endocrine history (e.g., whale baleen (Fernández Ajó et al., 2020; 2018; 2017; 2016; 2014;; Hunt et al., 2018), hair/fur (Ashley et al., 2011; Bechshøft et al., 2012; Bryan et al., 2014; Cattet et al., 2014; Davenport et al., 2006; Macbeth et al., 2010), seal claw (Karpovich et al., 2020) and whiskers (Lübcker et al., 2020)). Thus, keratin tissues can enable retrospective investigation of long-term phenomena such as reproductive cycles, interactions between stress and reproduction, and life stage effects.

However, extraction and analysis of hormones in keratin-based samples present a multitude of technical considerations and validation issues (Berk et al., 2016; Berkvens et al., 2013; Bortolotti, 2010; Bortolotti et al., 2008; Freeman and Newman, 2018; Hunt et al., 2014; Romero and Fairhurst, 2016; Russell et al., 2015; Salaberger et al., 2016; Sheriff et al., 2011). Keratins are strong and durable structural proteins comprising two major classes - alpha keratins (found in the epidermis of all terrestrial vertebrates) and beta keratins (primarily found in crocodilians and birds). Extensive inter- and intramolecular disulfide bonds make both classes of keratins naturally insoluble in water and organic solvents (Wang et al., 2016). The strength, durability, and insolubility of the keratins result in long-lasting, robust tissue types with very low water content that can preserve hormones for years, but also present challenges when trying to extract hormones from the matrix in which they are held. A particular concern is extraction "yield" - amount of native hormone that can be recovered from the sample.

Hormone extraction methods for keratin tissues typically rely on mechanical disruption of the keratin matrix, i.e., either cutting samples into small fragments or abrading samples (e.g., with a rotary tool or drill) to create fine-grained powder, followed by mixing with an alcohol or other solvent to extract hormone (Bortolotti et al., 2008; Hunt et al., 2017; Koren et al., 2002). These methods are simple, cost-effective, and have proven to generate useful data, but it is presently unknown whether the majority of hormone is actually extracted from the sample. Conceivably, solvents may not penetrate to the center of keratin fragments (which vary in size and shape), potentially resulting in low or variable extraction efficiency and adding unwanted variation to data (Romero and Fairhurst, 2016). Additionally, certain hormones, notably the glucocorticoids and the thyroid hormones, are often at or near the limit of detectability in extracts produced with the above methods (Hunt et al., 2017; Karpovich et al., 2019). This, in turn, results in many samples with "undetectable" results, limits the number of analyses that can be employed on a given volume of extract, and imposes minimum sample mass limits that can be problematic when studying rare historic or small samples.

Alba and colleagues (2019) presented an attractive supplement to mechanical disruption that may improve hormone yield – keratinase digestion prior to extraction. Keratinase is a bacterial enzyme that breaks the disulfide bonds found in both alpha and beta keratins thus facilitating release of hormones contained within the physical matrix of keratin (Mazotto et al., 2011; Schallmey et al., 2004; Williams et al., 1990). Traditionally, keratinase is used to break down keratin waste (e.g. feathers, hair, hooves) produced by industrial poultry, beef, leather and wool operations (De Oliveira Martinez et al., 2020; Srivastava et al., 2020). When tested on chicken feathers containing radiolabelled corticosterone (CORT), keratinase (synthesized by the feather-digesting *Bacillus licheniformis*) digestion produced a pellet (with equal mass as the feather) and a liquid supernatant above. The pellet contained more radiolabelled hormone than the supernatant; however, solid-phase extraction (SPE) of just the supernatant produced reliable and repeatable data (Alba et al., 2019). To date, this method has been confirmed to produce detectable hormone in extracts from chicken feathers and pangolin scales (Alba et al., 2019; Blecher et al., 2021); however, no studies have directly compared hormone yield of keratinase digestion methods to non-keratinase methods, an essential step before researchers can determine the ideal extraction method for a given sample type.

The primary goal of this study was, therefore, to investigate keratinase digestion as a method for improving steroid hormone yield from a wide variety of keratin-based sample types from multiple wildlife species. Species and tissues tested include baleen from three species of whale (blue, *Balaenoptera musculus*; bowhead, *Balaena mysticetus*; southern right, SRW, *Eubalaena australis*), shed skin from two reptiles (tegu lizard, *Salvator merianae*; narrow-headed garter snake, *Thamnophis rufipunctatus*), hair from arctic ground squirrel (AGS; *Urocitellus parryii*), feathers from Purple Martins (PUMA; *Progne subis*), and spines from the short-beaked echidna (*Tachyglossus aculeatus*), a monotreme. These sample types were selected to represent a broad range of taxa and are also tissues previously shown to contain immunoreactive CORT (blue and bowhead whales (Hunt et al., 2017); SRW (Fernández Ajó et al., 2018); tegu (Zena et al., 2020); garter snake (Lauger, 2019); PUMA (Bortolotti et al., 2008); AGS, echidna; pers obs, Dillon, Hunt, Buck). We focused on CORT for this study as it is a hormone of particular interest for wildlife stress assessment and the major glucocorticoid of all non-mammals. Two mammalian taxa, rodents and cetaceans, were also included due to the fact that CORT is the primary glucocorticoid in all rodent tissues as well as cetacean baleen. For shed skin and whale baleen, sample types from different species were assessed as a preliminary investigation as to whether similar sample types from different species react similarly to keratinase digestion.

Specific objectives were to: (1) perform immunoassay validations of each sample type for keratinase-digested extracts, i.e., tests of parallelism and accuracy (Grotjan and Keel, 1996; Hodges, 1985; Sheriff et al., 2011); (2) compare hormone content of keratinase-digested extracts to non-keratinase-digested extracts to determine if digestion increases detectability; (3) compare hormone content of the supernatant and pellet to examine whether both must be extracted and whether SPE cartridges must be used; and (4) determine the minimum sample mass that produces high repeatability and low variability. Ultimately, we offer preliminary methodological guidance regarding the extraction and assay of a diverse set of keratin-based samples for hormone analyses.

2. Materials and methods

2.1. Keratinase digestion

We made a keratinase solution using a native *Bacillus licheniformis* keratinase (FEED-0001; Creative Enzymes, Shirley, NY, USA) and an alkaline phosphate buffered saline (PBS; pH 9.0). Keratinase (1 g) was added to 30 mL alkaline PBS and mixed until dissolved (Alba et al., 2019). Pools were created from excess keratinous samples collected for other studies, comprising 1 individual (whale baleen) to $\geq$ 3 individuals

(all other sample types). All samples were obtained under relevant permits (see Acknowledgments; type and preparation described in Table 1). Samples had previously been mechanically disrupted following routine protocols widely in use for each sample type, as follows: whale baleen was abraded into a fine powder using a Dremel (Fernández Ajó et al., 2018; Hunt et al., 2018; 2017; 2014), reptile sheds were snipped into 5 mm squares, while the squirrel hair, bird feather and echidna spines were chopped into ≤ 1 cm pieces. Four different sample masses were tested — 10, 25, 50 and 100 mg — selected to represent a range spanning the most commonly used sample mass for keratin tissues (typically 100 mg), and a commonly reported ~ 10–20 mg minimum threshold below which data variation increases and spurious "small sample effects" can arise. Each sample was well-mixed before testing to homogenize hormone content. After mixing, samples were weighed and aliquoted into 13x100 mm borosilicate glass tubes, with 10 mg aliquots in quintuplicate; 25 mg in triplicate; 50 mg in quadruplicate; and 100 mg in triplicate (limited sample restricted additional replicates). Weighed aliquots were then divided as follows: (1) **Kc group (keratinase followed by combined extraction and assay)** - aliquots digested with keratinase, followed by extraction and assay of combined supernatant + pellet (10, 25, 50, and 100 mg, all in singlicate). (2) **Ks group (keratinase followed by separate extraction and assay)** - aliquots digested with keratinase, followed by separate extraction and assay of supernatant and pellet. Additional replicates were added to the Ks group to enable closer examination of variation in pellet vs. supernatant, as follows: 10 mg in triplicate, 25 mg in singlicate, 50 mg in duplicate, 100 mg in singlicate, for all sample types. (2) **No-keratinase group (NK)** - one set of aliquots (10, 25, 50, and 100 mg in singlicate) extracted with methanol without keratinase treatment.

For Kc and Ks samples, keratinase treatment utilized 3 mL keratinase solution added to sample tubes, vortexing for 30 s, and placing in a shaking water bath for five days (45 °C). Tubes were visually inspected and photographed to assess degree of digestion every 24 h until day 5, at which point tubes were removed from the water bath (as recommended by Alba et al. 2019). Shrinking or disappearance of visible sample particles was interpreted as an indicator of the effectiveness of keratinase digestion (Abdel-Fattah et al., 2018; Alba et al., 2019; Jin et al., 2017; Tiwary and Gupta, 2012).

2.2. Extraction post-keratinase digestion

Kc (combined extraction and assay of supernatant + pellet) and NK groups: Each sample tube received 4 mL of absolute methanol and was placed on a multi-tube vortexer overnight at room temperature (Glas-Col Large Capacity Mixer, speed set on 65; Glas-Col, Terre Haute, IN, USA). After extraction, sample tubes were centrifuged for 15 min at 1056 g to separate the pellet and supernatant (i.e., the methanol hormone extract). Supernatant was collected in a clean, labeled 13x100 mm borosilicate glass tube and dried in a ThermoSavant SpeedVac Concentrator (model SDP121P; Thermo Fisher Scientific, Waltham, MA, USA) at 35 °C and stored at −80 °C.

Ks group (separate extraction and assay of supernatant and pellet): Sample tubes were centrifuged for 15 min at 1056 g to separate the keratinase digestion pellet and supernatant. Supernatant was collected for SPE (Newman et al., 2008). Briefly, DI water was added to the supernatant to bring the volume to 10 mL. The SPE cartridge (Bond Elut-C18OH, 500 mg, 3 mL; Agilent Technologies, Santa Clara, CA, USA) was placed in a vacuum manifold, primed with ethanol, and washed twice with DI water. Diluted supernatant was added and the cartridge was then washed again with DI water. Finally, the bound hormone was eluted with 90% methanol into a 12x75 mm borosilicate glass tube and dried as described above. Separately, the remaining pellet was combined with 4 mL of absolute methanol, vortexed overnight, extracted and dried as described above.

Table 1
Keratin-based samples used for testing efficacy of keratinase digestion for hormone extraction.

Sample type	Scientific name	Common name	Sample preparation
Baleen	*Balaenoptera musculus*	Blue whale	Powdered
Baleen	*Balaena mysticetus*	Bowhead whale	Powdered
Baleen	*Eubalaena australis*	Southern right whale	Powdered
Feather	*Progne subis*	Purple Martin	≤ 1 cm pieces
Hair	*Urocitellus parryii*	Arctic ground squirrel	≤ 1 cm pieces
Shed skin	*Thamnophis rufipunctatus*	Narrow-headed garter snake	Chopped, ≤ 5 mm pieces
Shed skin	*Salvator merianae*	Tegu lizard	Chopped, ≤ 5 mm pieces
Spine	*Tachyglossus aculeatus*	Short-beaked echidna	≤ 1 cm pieces

2.3. Assay

One day prior to assay, all samples, regardless of extraction method, were resuspended in 500 uL assay buffer (buffer X065, Arbor Assays), shaken for 1 h, and then stored at 4 °C overnight (Newman et al., 2008). All samples were diluted 1:2 (based on pilot trials) with assay buffer to keep results near 50% bound on the assay standard curve, the area of greatest precision (Ezan and Grassi, 2000; Grotjan and Keel, 1996), and assayed for immunoreactive CORT content using a commercially available enzyme immunoassay kit (kit #K014; Arbor Assays, Ann Arbor, MI, USA). This kit was developed by the manufacturer to assay non-plasma wildlife-derived sample types and had previously passed parallelism and accuracy validations for non-keratinase-digested extracts for all sample types and species in this study (Braga et al., in prep; Fernández Ajó et al., 2018; Hunt et al., 2017; Lauger, 2019; Zena et al., in review; hair and feather, unpublished results). Samples and standards were run in duplicate and an internal control (i.e., a reference standard of known concentration) was run on all plates. The manufacturer's assay protocol was followed with no modifications. Sample order was randomized within and across assay microplates to minimize influence of plate position on intra- or inter-assay variation. Samples were rerun if the coefficient of variation (CV) between duplicates exceeded 10%. Additional QA/QC included rerun of the full assay if standard curves had more than two anomalous standards (CV > 10%) or optical density outside of normal bounds (this did not occur) or if the internal control deviated > 10% from the expected value. Intra-assay and inter-assay precision for this assay in our laboratory average 2.1% and 12.2%, respectively; the manufacturer's reported detection limit is 16.9 pg/mL. For cross-reactivities and other assay details, see Hunt et al. (2017).

2.4. Assay validations

To ensure the keratinase digestion did not interfere with assay performance, tests of parallelism and accuracy were conducted for the supernatant and pellet extracted together and separately, for a total of 3 sets of validations for each sample type/species combination (Grotjan and Keel, 1996; Hodges, 1985; Sheriff et al., 2011). The parallelism test involves assay of serial dilutions (1:2 – 1:128) of a pooled extract alongside a standard curve, to assess whether the assay antibody has good binding affinity with the hormone of interest in the sample. The test of parallelism can also be used to determine the appropriate dilution (approx. 50% binding) for the sample. The slopes of the sample serial dilution line and the standard curve should be statistically indistinguishable (i.e., the lines are parallel), with the linear portion of the sample serial dilution curve spanning a wide range of percent-bounds (ideally at least 20–80% bound). Such a finding would indicate that the sample behaves in a way that is immunologically similar to the standard and can be measured proportionally (Ezan and Grassi, 2000; Grotjan and Keel, 1996). The accuracy test, assaying a standard curve

spiked with pooled sample, measures potential interference from other substances contained in the sample ("matrix effect"; Grotjan and Keel, 1996) that could lead to an erroneous over- or underestimate of the actual hormone concentration. Acceptable accuracy (i.e., little to no interference or matrix effect) was defined as a straight line with an $R^2 >$ 0.95 and a slope between 0.7 and 1.3 (ideal slope = 1).

2.5. Statistical approach

For parallelism, percentage of antibody bound (%B/B0) was plotted vs log(relative dose), with an F-test employed to compare the slopes of the linear portions of both binding curves (serially diluted extract and assay standards). For accuracy, linear regression was used to assess slope and linearity of observed vs. expected dose. Effectiveness of keratinase digestion was assessed with visual cues (a decrease in size or change in appearance of K samples compared to NK samples) and comparison of absolute concentration of apparent CORT across aliquots of the same well-mixed sample (i.e., higher concentrations interpreted as better extraction yield). All data are presented as means ± SEM and all differences considered significant at $P < 0.05$.

3. Results

3.1. Effect of sample mass on keratinase digestion

After 5 days, all sample types and masses incubated in the keratinase solution experienced some level of digestion, indicated by a decrease in the size of the solid particles of the sample and/or a change in appearance, namely, a white powdery substance was present in the bottom of all K sample tubes, but was present in greater amounts in the samples where the sample particles had largely disappeared (10 and 25 mg; Fig. 1). At higher masses (50 and 100 mg), however, the K samples did not appear as visually distinct from NK samples, which we interpreted as evidence of little to no keratinase digestion (Fig. 1).

3.2. Kc group - keratinase supernatant and pellet extracted combined

When the supernatant and pellet were extracted combined (Kc group), apparent immunoreactive CORT levels (per g of sample) were higher in Kc samples as compared to NK samples, in every sample type and species, and at every mass (Supplementary Fig. 1). Generally, the difference in apparent concentrations was greater at the lower masses (10 and 25 mg; over 1200% more) than at the higher masses (50, 100 mg; 700% more; Supplementary Fig. 1). Unfortunately, tests of parallelism and accuracy failed (parallelism slopes were significantly different; $P < 0.05$; accuracy slope > 1.3 and/or relationship was nonlinear in the central portion of the curve) across all sample types tested, with the exception of squirrel hair (parallel between 19% and 58% binding, $p = 0.1837$; accuracy slope = 1.240), suggesting a strong matrix effect when supernatant and pellet are extracted together (Fig. 2, Supplementary Fig. 2, Supplementary Table 1).

3.3. Ks group - keratinase supernatant and pellet extracted separately

When the supernatant and pellet were extracted separately (Ks group), immunoreactive CORT levels were detectable in all extracts, with acceptable accuracy and parallelism in all cases (Figs. 3 and 4, Tables 2 and 3, Supplementary Figs. 3–9). There were no significant differences between slopes of the CORT standard curve and extract serial dilutions (Fig. 3, Table 2). Assay accuracy was acceptable for extracts tested, showing a linear relationship ($R^2 > 0.99$) between expected and observed immunoreactive CORT concentration and slopes within the desired range of 0.7 – 1.3 (Fig. 4, Table 3).

When compared to NK samples, the relative improvement in yield of immunoreactive CORT for Ks samples varied with species and sample type, and also varied between supernatant vs. pellet (results from 10 mg samples shown in Fig. 5; see Supplementary Information for results from other sample masses). With the notable exception of the reptiles, extracting the supernatant separately from the pellet after keratinase digestion (Ks group) substantially improved yield. In most sample types (including hair, monotreme spine, snake skin, and all three samples of baleen), yield from supernatant was from 30% to 183% higher in Ks than NK samples. For the samples of baleen from the three species of whale, the increase (Ks supernatant vs. NK) averaged 69%. In two sample types (feather and hair), yield more than doubled compared to NK samples, with the greatest increase in yield (377%) occurring in the feather. In contrast, extraction of the Ks pellets did not always yield more apparent

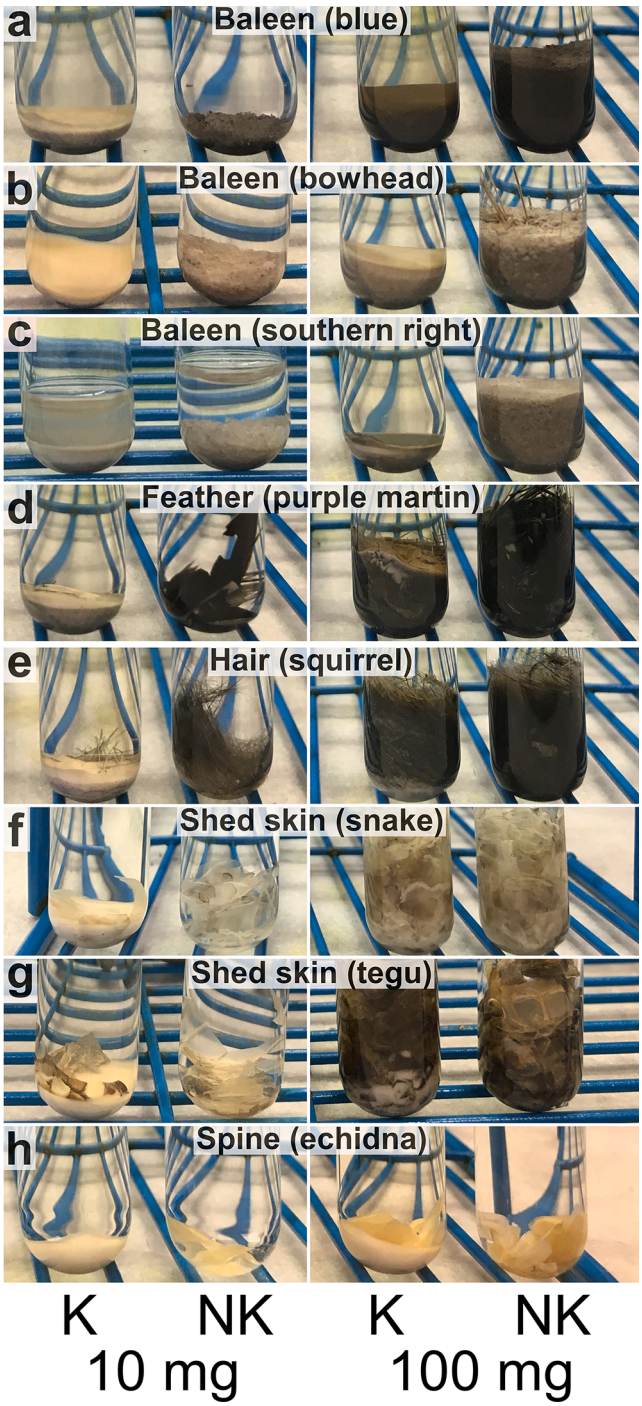

Fig. 1. Keratin tissues from eight different species digested with keratinase for five days (K) or untreated (NK) at 10 and 100 mg. In all cases, lower sample masses resulted in greater visually apparent digestion.

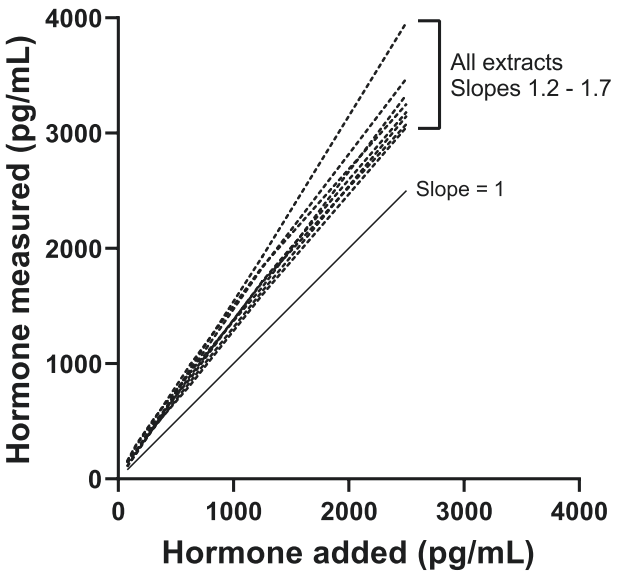

Fig. 2. Accuracy tests for keratinase-digested extracts (supernatant & pellet extracted together; Kc group). All but one extract (squirrel hair slope = 1.240) exceeded maximum acceptable slope of 1.3, meaning corticosterone cannot be measured in these extracts with mathematical accuracy.

hormone than the NK samples. The feather and blue whale pellets had 100% and 66% more immunoreactive hormone, respectively, than NK samples. The hair and bowhead whale pellets yielded approximately the same amount of hormone found in the NK samples, while the echidna Ks pellet actually yielded 53% less hormone than the NK samples. Interestingly, neither snake nor tegu shed skin samples benefited from keratinase digestion (Fig. 5). The snake Ks supernatant had approximately the same apparent concentration of immunoreactive CORT as snake NK samples, while the snake Ks pellet yielded only 55% of the hormone found in snake NK samples. The tegu Ks pellet and supernatant yielded<10% and 30% immunoreactive CORT, respectively, compared to tegu NK samples.

4. Discussion

The primary objective of this study was to perform initial validations of keratinase digestion for improved steroid hormone extraction in a wide variety of keratin-based sample types across a range of vertebrate taxa. We focused on CORT due to its ubiquity as the dominant adrenal glucocorticoid in non-mammalian vertebrates as well as some mammals (all tissues from rodents, and baleen from mysticete whales), as well as it being a hormone of particular interest for assessment of effects of acute and chronic stress in wildlife. Though this first study could not include all possible sample types, we were able to test a range of five vertebrate epidermal sample types (shed skin, feather, hair, baleen, spine) from eight species comprising several different taxa (reptiles, birds, placental mammals and a monotreme). These samples also spanned a range of keratin classes, with the mammalian samples (squirrel hair, whale baleen, echidna spine) assumed to be predominantly alpha-keratin, the single avian sample (Purple Martin feather) predominantly beta-keratin, and the squamate samples (shed skin from tegu lizard and garter snake) assumed to be mixed alpha and beta. To our knowledge, this work represents the first time the keratinase digestion technique has been directly compared to non-keratinase methodology with regard to hormone yield. Our results demonstrate that multiple keratinized tissue types of varying masses can be digested with keratinase, extracted, and ultimately provide repeatable estimates of hormone content using a commercially available EIA that was successfully validated for all sample types and species. Generally, hormone yield from keratinase-digested supernatant was superior compared to non-keratinase methodology for all samples except reptilian skin.

4.1. Digestion

Although little is known about the mechanism of deposition of hormones in keratinous tissues, it is clear that steroid and thyroid hormones are present in all vertebrate keratin tissues so far tested (Berkvens et al., 2013; Bortolotti et al., 2009; 2008;; Heimbürge et al., 2019; Hunt et al., 2014; Koren et al., 2002; Warnock et al., 2010; Will et al., 2019). Previously published studies (using non-keratinase methodology) report generally good agreement between hormone content in keratin tissues grown at certain times vs. the animals' known physiological state at those times (e.g., progesterone profiles in whale baleen matching known pregnancies (Hunt et al., 2016); hair cortisol content correlating with stress exposure during the hair growth period (Malcolm et al., 2013); etc.). This suggests that hormones, along with many other compounds (e.g., heavy metals, toxicants, stable isotopes), are deposited as the tissue grows and are thereafter held within the stable keratin matrix. This matrix is strong, durable, and impervious to common proteolytic enzymes and thus, does not readily release the compounds contained within it. The standard protocol for non-keratinase analyses has been to mechanically disrupt the keratin matrix by cutting or powdering and then use a solvent to extract hormones. This method has often produced useful data; however, in some cases, low hormone detectability in the resulting extract has limited application of the technique for certain research questions, particularly when available sample masses are small. Our results show that including a keratinase digestion step may increase liberation of hormones from the keratin, making them more readily available for extraction (Alba et al., 2019).

Sample mass strongly affected apparent hormone yield. We tested a range of sample masses that represent practical utility in the laboratory, ranging from 100 mg of dried sample (recommended by early hair and feather literature), to 10 mg, the mass at which spurious "small sample effects" and increased variation often begin to affect apparent hormone content (Berk et al., 2016; Hayward et al., 2010; Lattin et al., 2011). In this study, the smaller mass aliquots (10 and 25 mg) seemed to digest more readily for most sample types. The 50 and 100 mg samples were not effectively digested and thus the apparent hormone concentration was equal to or lower than the NK samples (Figs. 1 and 5). In sum, keratinase digestion appeared most effective at sample masses $\leq$ 25 mg, at which samples were digested effectively while still retaining good repeatability across replicates.

Keratinase protocols could potentially be adjusted to increase digestion of the keratin. Keratinase enzymes differ widely in their digestion efficacy and optimal conditions for full activity. A recent review discussed the characteristics of 26 different keratinases and the optimal operating parameters spanned a wide range of pH values (6.5 – 11) and temperatures (30 to 75 °C) (Srivastava et al., 2020). We chose to use a temperature and pH that fell within the safe operating limits for researchers, equipment, and hormone stability, but further investigation into pH and temperature protocols may improve keratinase digestion. Additionally, digestion efficacy of the 26 keratinases varied depending on the substrate. Given the benefit to industrial poultry operations (where the intended application is simply breakdown of feather waste), it is not surprising that most enzymes have been tested only on domestic chicken and duck feathers, most of which are boiled and/or subjected to high pressure to facilitate digestion. Human hair and wool have proven more resistant to degradation (Srivastava et al., 2020). Due to concerns of preserving integrity of the hormones contained within the keratin matrix, we did not employ pressure or boiling pre-treatments here, but it would be informative to systematically test such protocols in future studies.

Melanin content of samples may also affect efficacy of enzymatic digestion of keratin. There is evidence that the presence of melanin, at least in feathers, inhibits bacterial keratinolytic activity (Goldstein et al., 2004; Gunderson et al., 2008). Melanin in keratinous tissues serves

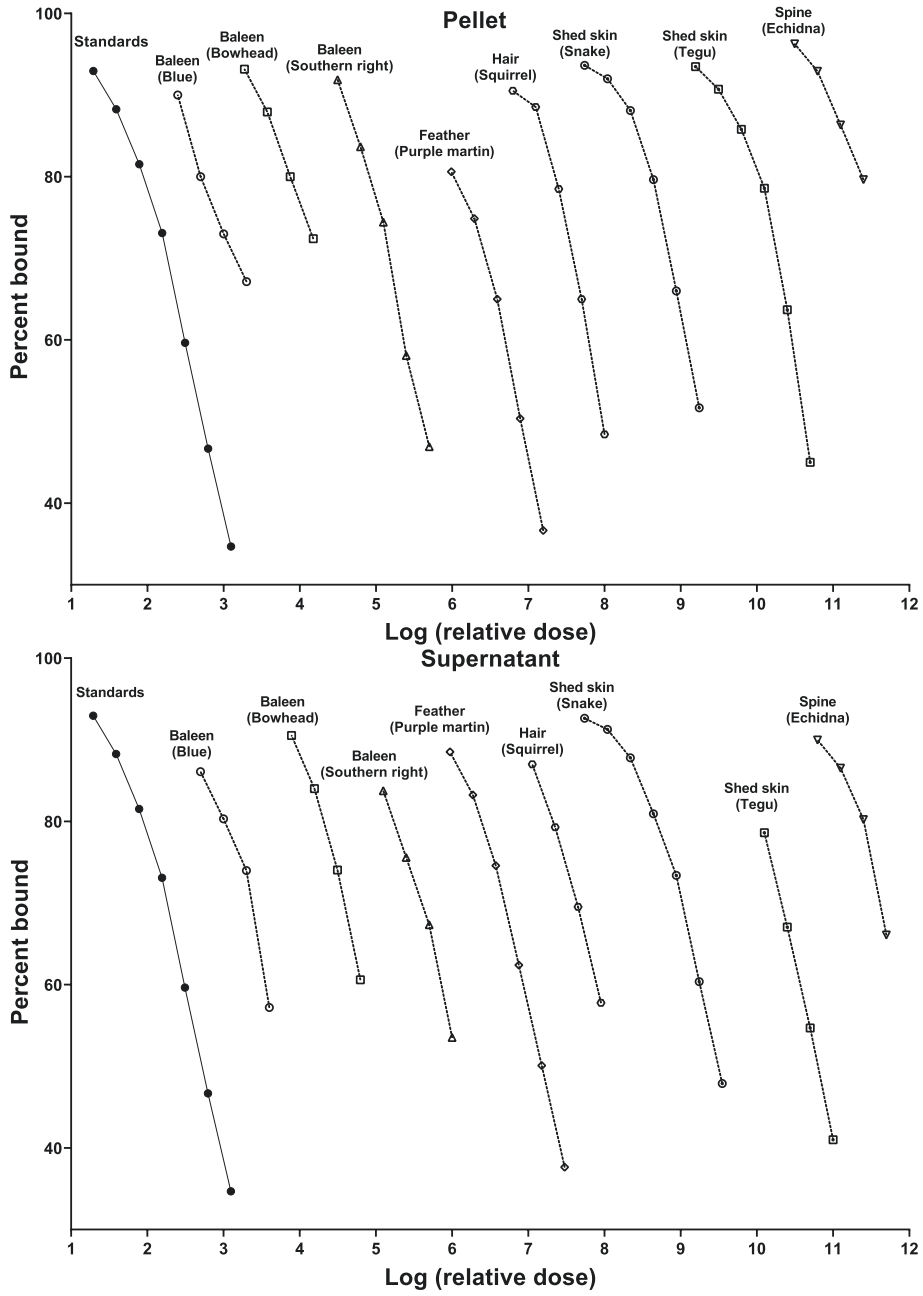

Fig. 3. Serially diluted keratinase-digested extracts from 8 different species (pellet and supernatant extracted and assayed separately; Ks group) are parallel to the corticosterone standard curve (closed circles). All p-values > 0.1.

many purposes: increasing hardness, decreasing wear and abrasion, signaling, crypsis, and photoprotection (Cordero and Casadevall, 2020; Riley, 1997). With the exception of the snake shed, all of our samples contained melanin (i.e., appeared dark). Thus, nonmelanized samples (white hair, white feather, etc.) may react differently to keratinase digestion.

4.2. Extraction post-keratinase

After keratinase digestion, we found that some immunoreactive hormone remains in the pellet while some moves into the aqueous supernatant. Extracting the entire digestion product - supernatant and pellet combined - would seem to be the most labor-efficient method and could, theoretically, capture all immunoreactive hormone. Unfortunately, we saw significant matrix effects in our EIA validations of combined supernatant and pellet, presumably caused by compounds present in the digestion mixture (Fig. 2, Supplementary Fig. 2, Supplementary Table 1). Only a single EIA protocol was studied here, selected due to its excellent prior performance with a wide variety of vertebrate keratin sample types (baleen: Fernández Ajó et al., 2018; Hunt et al., 2017; Rolland et al., 2019; feather: Weimer et al., 2018; hair: Jarcho et al., 2019; Uarquin et al., 2016; Waterhouse et al., 2017; Woolsey et al., 2015; shed snake skin: Lauger, 2019; shed tegu skin: Zena et al., in review; echidna spines: Braga et al., in prep). EIAs are presently the most widely used and cost-effective technique for quantification of hormones in wildlife samples; however, other quantification techniques such as RIA, HPLC or mass spectrometry approaches may be more robust to interference from matrix effects (Skrzipczyk and Verdier, 2013).

Pellet and supernatant can, however, be analyzed separately. Alba et al. (2019) found the pellet of white chicken feathers contained the majority (80%) of radiolabeled CORT, with the other 20% in the supernatant. However, when we extracted and assayed the pellet and

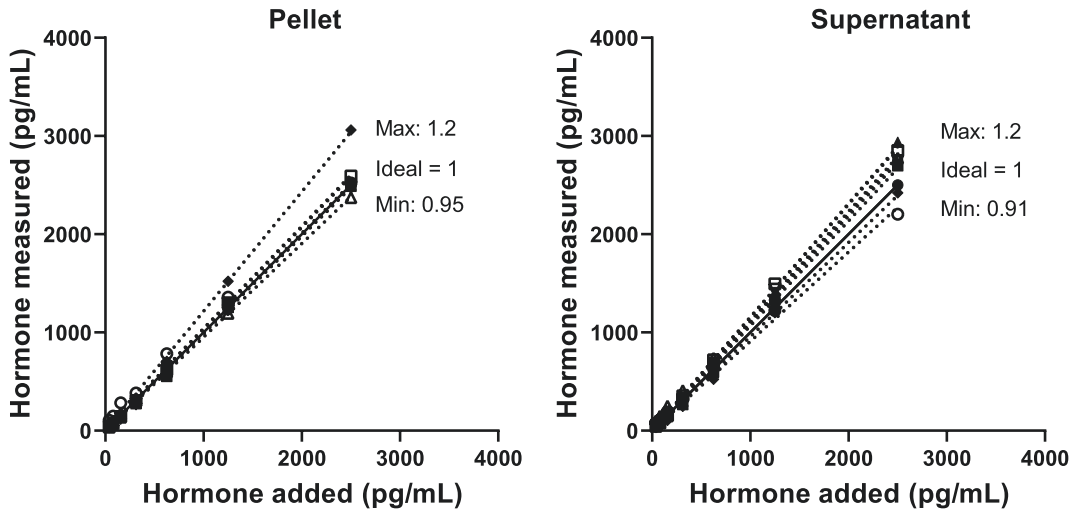

Fig. 4. Results of assay accuracy ('matrix effect') tests for keratinase digested extracts (pellet and supernatant extracted and assayed separately; Ks group) of 5 sample types from 8 different species spiked with known amounts of hormone. A straight line with a slope between 0.7 and 1.3 (ideal = 1) is preferred, indicating hormone can be measured with good mathematical accuracy across a range of concentrations, i.e., despite the presence of unusual sample matrix.

Table 2
F-test results for corticosterone immunoassay parallelism for keratinase-digested extracts of five sample types from eight species. For each sample type and species, supernatant and pellet were extracted and assayed separately, using solid phase extraction for supernatants and methanol extraction for pellets (Ks group). Each parallelism test compared slopes of the linear portions of the binding curves of assay standards vs. serial diluted extract.

Sample type	Species	Pellet	Supernatant
Baleen	Blue whale	$F_{1,6} = 0.5214$ $P = 0.4974$	$F_{1,7} = 0.04017$ $P = 0.8469$
Baleen	Bowhead whale	$F_{1,6} = 1.069$ $P = 0.3410$	$F_{1,7} = 0.01439$ $P = 0.9079$
Baleen	Southern right whale	$F_{1,8} = 0.01056$ $P = 0.9207$	$F_{1,7} = 1.912$ $P = 0.2093$
Feather	Purple Martin	$F_{1,8} = 0.03029$ $P = 0.8662$	$F_{1,10} = 2.860$ $P = 0.1217$
Hair	Arctic ground squirrel	$F_{1,8} = 0.2285$ $P = 0.6454$	$F_{1,7} = 2.529$ $P = 0.1558$
Shed skin	Narrow-headed garter snake	$F_{1,8} = 0.05828$ $P = 0.8153$	$F_{1,9} = 0.8689$ $P = 0.3756$
Shed skin	Tegu lizard	$F_{1,8} = 0.02224$ $P = 0.8851$	$F_{1,7} = 2.347$ $P = 0.1694$
Spine	Short-beaked echidna	$F_{1,6} = 3.187$ $P = 0.1245$	$F_{1,6} = 0.2641$ $P = 0.6257$

Table 3
Linear regression results of corticosterone immunoassay accuracy tests ('matrix effect' tests) for keratinase-digested extracts from five sample types from eight different species. Supernatant and pellet were extracted and assayed separately, using solid phase extraction for supernatants and methanol extraction for pellets (Ks group). Slope of observed vs expected dose (acceptable range of slope = 0.7–1.3) and coefficient of determination (r^2; ideally close to 1.00) of the linear regression line is shown.

Sample type	Species	Pellet	Supernatant
Baleen	Blue whale	Slope = 1.178 $r^2 = 0.9990$	Slope = 1.010 $r^2 = 0.9996$
Baleen	Bowhead whale	Slope = 1.023 $r^2 = 0.9989$	Slope = 1.100 $r^2 = 0.9993$
Baleen	Southern right whale	Slope = 0.9524 $r^2 = 0.9999$	Slope = 1.116 $r^2 = 0.9996$
Feather	Purple Martin	Slope = 1.041 $r^2 = 0.9978$	Slope = 0.9094 $r^2 = 0.9978$
Hair	Arctic ground squirrel	Slope = 1.002 $r^2 = 0.9990$	Slope = 1.088 $r^2 = 0.9990$
Shed skin	Narrow-headed garter snake	Slope = 1.044 $r^2 = 0.9993$	Slope = 1.153 $r^2 = 0.9993$
Shed skin	Tegu lizard	Slope = 0.9959 $r^2 = 0.9995$	Slope = 1.106 $r^2 = 0.9991$
Spine	Short-beaked echidna	Slope = 1.230 $r^2 = 0.9995$	Slope = 0.9822 $r^2 = 0.9990$

supernatant of our samples separately, the supernatant generally contained more hormone than the pellet, and indeed contained more hormone than matched NK extracts (Fig. 5, Supplementary Figs. 10 and 11). However, the variability among supernatant replicates exceeded that of the pellet replicates (Supplementary Figs. 10 and 11), and the SPE extraction method used for the supernatant is time-consuming and expensive. Nonetheless, assay of post-keratinase supernatants with SPE appears a viable approach that could markedly increase hormone yield for many sample types.

4.3. Assay validations and yield

When compared to control NK samples not subjected to keratinase digestion, all but the tegu shed skin supernatant extract yielded higher apparent hormone levels. As might be expected, the Ks pellet extracts did not consistently yield more hormone than the NK extracts due to hormone being liberated into the supernatant (Fig. 5, Supplementary Figs. 10 and 11). However, the pellet from Purple Martin feathers and blue whale baleen still had twice the apparent hormone found in the no keratinase controls. It is unclear why these particular sample types had so much more accessible hormone in the pellet than other samples. Also unclear is why similar sample types from different species reacted differently to keratinase digestion, as was the case for the three baleen samples and the two shed skin samples (Fig. 5, Supplementary Figs. 10 and 11). Species differences, individual variation, sex differences, and/or the age of the sample could all contribute to such variation. Furthermore, only one hormone, CORT, was investigated and it is possible that other steroid or thyroid hormones may show different patterns in yield across and within sample types and species.

4.4. Recommendations

Keratinase digestion is a promising addition to hormone extraction protocols designed for keratinous tissues. We showed that keratinase can digest epidermal samples from a wide variety of vertebrate taxa. Still, hormone extraction efficiency is not always improved as compared to non-digested samples, particularly for larger mass (>25 mg) samples,

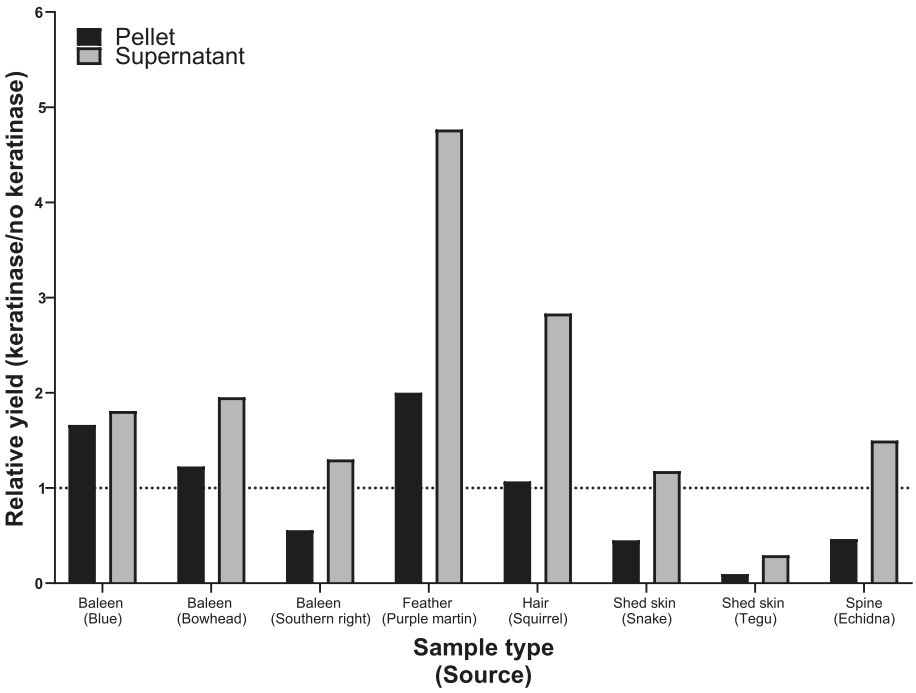

Fig. 5. Yield of corticosterone from keratinase-treated samples (Ks group; 10 mg aliquots) relative to no keratinase treatment. The dotted line indicates yield equal to no keratinase treatment.

which may require a modification of our enzymatic digestion protocol. With the specific keratinase enzyme we tested (*Bacillus licheniformis* keratinase, FEED-0001; Creative Enzymes), we recommend using 25 mg or less of sample to ensure efficient digestion. In all keratinase-digested samples except tegu shed skin, SPE of the post-digestion supernatant resulted in greater hormone recovery than undigested samples. However, if a study requires a more high-throughput method than SPE, we found that the pellet from hair, baleen powder, and feather digestions can be extracted using a simple methanol protocol and produces data with low variability and high repeatability (Fig. 5 and Supplementary Fig. 10). Because hormone yield of shed skin from the reptiles tested did not increase from keratinase digestion, the time and effort spent digesting these samples preclude recommending keratinase digestion of shed-skin at present (summarized in Fig. 6).

Keratin tissues have proven to have great utility for retrospective analysis of the endocrine state of past times in the animal's life,

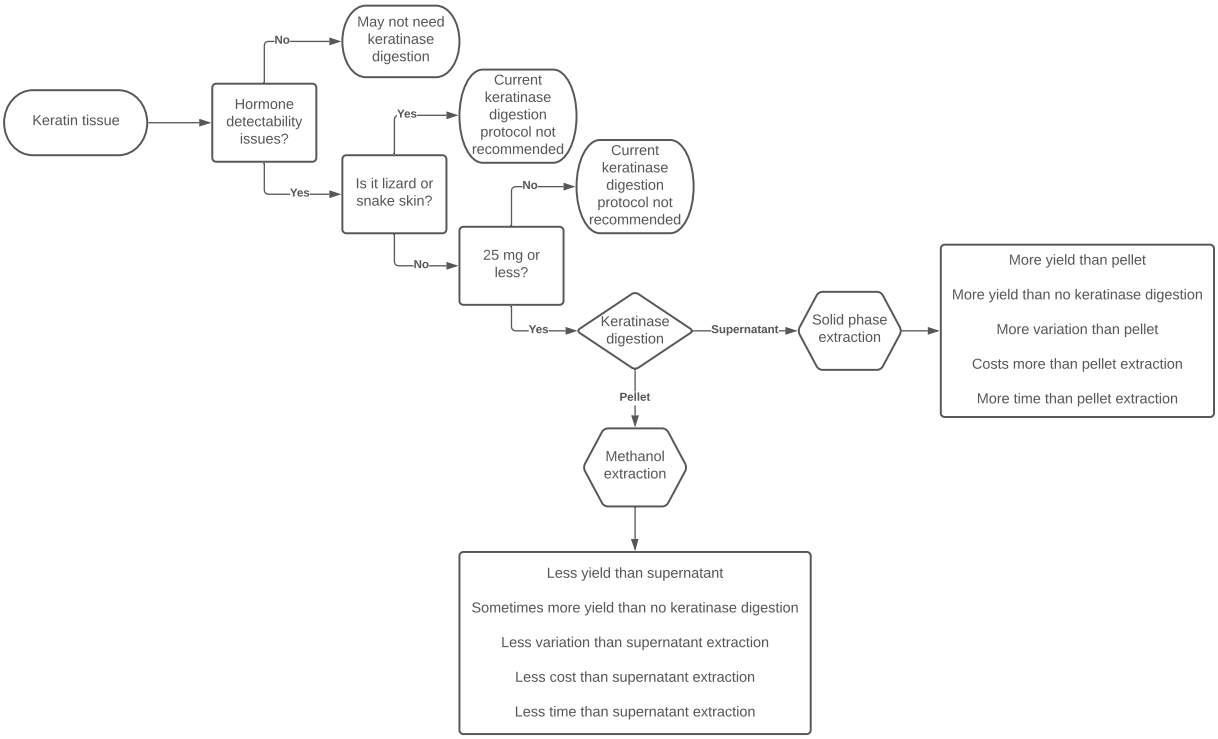

Fig. 6. Summary of recommendations and findings regarding the current keratinase digestion protocol.

reconstruction of individual longitudinal profiles across months or years, assessment of chronic changes in hormones (e.g., discrimination of chronic stress from acute stress events), and comparison of past populations to present-day populations. The widespread occurrence of keratin tissues across vertebrate taxa, the availability of numerous linear "time series" sample types (e.g., baleen, long hairs, seal claws), and the existence of sample archives in natural history museums make these samples potentially informative for a variety of research questions. Moreover, many of these tissues can be collected in either non– or minimally-invasive fashion, an attribute that is desirable for many field biology applications and studies. The keratinase digestion protocol described here has the potential to improve hormone yield from such samples, which may be particularly useful in cases of low hormone detectability or small sample mass. This initial study does not address all validation issues, i.e., the keratinase digestion technique should still be further optimized, tested and refined, but data presented here show that it is effective for increasing hormone yield from at least four keratinous sample types of mammals and birds - feather, hair, baleen and spine. Testing of other steroids, as well as the thyroid hormones, will be necessary to determine the broader applicability of this method. Future studies should also assess variation in yield within and between individuals of the same species, as in this first study we only assessed samples that were aggregated from several individuals per species. Ultimately, this protocol may allow for more efficient use (more data from less mass) of rare samples collected from wildlife and may enable increased ability to address questions involving retrospective assessment of endocrine state across time.

CRediT authorship contribution statement

Danielle Dillon: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Alejandro Fernández Ajó:** Investigation, Resources, Writing - review & editing. **Kathleen E. Hunt:** Resources, Writing - review & editing. **C. Loren Buck:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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