Measuring cortisol in hair and saliva from dogs: coat color and pigment differences

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Abstract

Cortisol concentrations are frequently measured from a variety of sources including blood, saliva, urine, and feces to quantify stress in dogs. However, a need still exists for less intrusive collection methods in domestic animals and for more efficient means of measuring basal cortisol. The objectives of the present study were to minimize restraint for saliva sampling, to validate hair for basal cortisol measurement in dogs, and to determine concentrations of cortisol within the hair shaft and in relation to hair color. Using food luring, 79% of dogs required no restraint for saliva collection. Salivary and hair cortisol concentrations were positively correlated ($P < 0.001$), thus validating hair as a medium for basal cortisol quantification. Black dogs had less cortisol than nonblack dogs ($P = 0.039$) in hair, but not saliva. Across dogs, the average amount of cortisol did not differ between proximal and distal hair sections ($P = 0.348$). However, for 7 of the 9 dogs, more cortisol was present in the distal portions of the hair. We observed a difference in cortisol concentrations among hairs of different colors from individual dogs ($P = 0.001$). From the same 7 × 7 cm ischiatic patch from the same dog, black (eumelanin) hairs were consistently lower in cortisol than yellow (pheomelanin) hairs, and cortisol concentrations of agouti hairs were intermediate. This is the first evidence that hair of different colors might sequester cortisol differently.

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1. Introduction

Cortisol is at the center of an interconnected web of physiological, behavioral, and developmental functions and responses. Cortisol has long been considered a reliable physiological measure of the stress response in both domestic mammals (cows [1], goats [2], guinea pigs [3], horses [4], pigs [5], rats [6], and sheep [7]) and wild mammals (Antechinus [8], Macaca [9], Odocoileus [10], Peromyscus [11], Procavia [12], Puma [13], Saimiri [14], and Spermophilus [15]). In the short term, stress is adaptive and helps individuals cope with emerging situations, but over the long term, stress is maladaptive [16]. Overexposure to glucocorticoids owing to malfunction or long-term (chronic) hyperactivation of the stress system can damage the body, impair growth and development, and elicit endocrine, metabolic, autoimmune, or psychiatric disorders [17].

Cortisol is a key component of the stress response. Measuring cortisol usually requires invasive procedures, such as venous collection of blood, which may significantly disrupt behavior. Animal welfare concerns have contributed to the development of noninvasive cortisol sampling methods, each with its own strengths and weaknesses.

Cortisol concentrations are regularly assessed through blood [18,19], saliva [20–22], urine [23], and
feces [24]. However, each sampling medium has constraints. Blood and saliva provide instantaneous views of cortisol concentrations. The restraint required for blood collection can be stressful, thus raising cortisol concentrations. Saliva sampling is generally “noninvasive,” yet current methods employ mild to moderate restraint. Saliva absorption materials can be flavored with beef stock to make them more palatable, but this method yields inconsistent cortisol results, and citric acid is sometimes added to increase salivation but is not well tolerated by dogs [25].

Urine and feces provide measures of cortisol over a short time period, but using urine and feces has limitations. Both urine and fecal samples are less sensitive to acute variability in cortisol concentrations than blood or saliva [23,26,27] and assess an unknown or variable accumulation of cortisol. Cortisol takes approximately 3 h to reach maximum concentration in urine [26] and would take longer to reach maximum concentration in feces. Urine samples can be difficult to collect, resulting in incomplete data sets [23]. Corticosteroids may not be uniformly distributed within fecal samples and may be inversely related to fecal output [11]. Excretion patterns are variable [27], and fecal samples collected in the field risk cross-contamination.

Hair is the newest sampling medium for cortisol measurement. Hair is increasingly used to measure both basal and chronically elevated hormone concentrations as compared to the instantaneous samples obtained from blood and saliva or the short-term view provided by urine and feces [22,24,28]. Hair is of particular interest because the hair follicle is a local source of cortisol [29]. However, cortisol measured in the hair may originate either directly from the hair follicle or systemically from the hypothalamic-pituitary-adrenal axis. Hair may also function as a storage area for cortisol. Because species differ in cortisol rhythms and secretion as well as the stress response, hair sampling requires validation for each species.

Little is known about how cortisol is stored or circulates within the hair shaft. Cortisol appears to degrade over time in human hair, in which significantly lower cortisol concentrations are detected in distal hair segments [30]. Yet no difference was found between proximal and distal hair segments in rhesus monkeys [22].

Mammals have developmental, physiological, and biochemical similarities with respect to glucocorticoid and pigment production. The biochemistry of pigment control involves melanocyte-stimulating hormone (MSH, a derivative of pro-opiomelanocortin) and melanocortin receptors. Similarly, the biochemistry of cortisol control involves adrenocorticotropin hormone (ACTH, also a derivative of pro-opiomelanocortin) and melanocortin receptors. Thus, control of both pigment and cortisol involves the same families of hormones and the same family of receptors. The product of the agouti gene (one of the major coat color genes) is an antagonist of MSH at its melanocortin receptor [31], but agouti may also interact with other melanocortin receptors. Consequently, control of both coat color and cortisol may be influenced by the product of the agouti gene. Research on agouti and nonagouti deer mice (Peromyscus) revealed a difference in corticosteroid concentrations with differences in coat color [11].

The predictions of our study were 4-fold: (1) that food luring would minimize the restraint required for saliva collection; (2) that hair-cortisol concentrations would correlate with concurrent salivary-cortisol concentrations and thus validate hair sampling as a medium for measuring basal cortisol in dogs, Canis familiaris; (3) that cortisol concentrations would not vary significantly between proximal and distal segments of the hair shaft in dogs, as found in macaques but not humans; and (4) that cortisol and coat color would be correlated in dogs, as observed in Peromyscus.

2. Materials and methods

The Smith College Institutional Animal Care and Use Committee approved this research protocol.

2.1. Dogs

The subjects were 48 dogs ranging in age from 9 mo to 11 y whose owners volunteered them for the research. The group included 23 Labrador retrievers (LRs) and 25 German shepherd dogs (GSs), with 28 females (6 intact, 22 spayed) and 20 males (3 intact, 17 neutered). All subjects were pet dogs except for an 8 y-old spayed female LR and a 6 y-old neutered GS, which were working guide dogs living in the home. The dogs ranged in weight from 24 to 48 kg. Two of the female yellow LRs had recently weaned puppies. Household status was defined as whether a dog lived alone (single-dog) or with one or more other dogs (multidog). Fifteen of the dogs lived in single-dog households, whereas 33 of the dogs lived in multidog households.

All dogs were sampled in their own homes, and no changes to their normal routine or diet were required. Dogs in the home environment may offer a means to
measure “basal” cortisol values not obtainable in “chronic stress” environments such as kennels or shelters. Basal values reflect natural daily rhythms as well as everyday stressors encountered by pet dogs living in the home, including the daily activity of the family, changes in diet or weather, illnesses, trips to the veterinarian, social interactions with family members and/or other animals, exercise, and so on.

2.2. Hair and saliva sampling

All sampling occurred between May and October 2008. Hair was collected using a shave and re-shave method: about 50 cm² of old hair was removed from the ischiatic region by shaving close to the skin with commercially available pet grooming clippers on day 1 for each dog, then a sample of new hair (about 35 cm²) was collected within the same region before complete re-growth (6 to 12 wk), being careful not to cut any old hair. Hair samples were stored in aluminum foil and kept in a −20 °C freezer until time of assay. For each dog, 5 representative hairs were chosen from the old hair sample. Hair lengths were measured to the nearest mm and averaged for each dog. Hair re-growth was recorded as a percentage of the uncut hair length.

Saliva was collected between 1:00 and 6:30 PM on day 1 and approximately every 2 wk for 12 wk (a total of 7 samples per dog). Between 0.06 mL and 1.50 mL of saliva was absorbed by swabbing inside a dog’s cheeks and mouth with a 7-cm piece of cotton rope (Salimetrics, State College, PA, USA) while encouraging salivation by allowing the dog to sniff at treats in the experimenter’s closed hand. Saliva was extracted from the cotton swab with a 5-mL needleless syringe (Fisher Scientific, Hampton, NH, USA) into 2-mL cryovials (Fisher Scientific), frozen at −20 °C to facilitate the separation of particulate matter, and stored until time of assay (1 to 6 mo). To assess the ease of our novel saliva collection method, we recorded the amount of restraint required for the initial saliva collection as follows: no restraint (the dog participated voluntarily in collection), mild restraint (gently holding the dog’s collar), or medium restraint (holding the dog’s head or opening the dog’s mouth). In addition, saliva collection time was recorded for this initial sampling. None of the dogs had previous experience with saliva sampling.

2.3. Cortisol extraction from hair

Following Davenport et al. (2006) [22], hair samples were washed, dried, and ground into a powder. Approximately 250 mg of hair was washed twice in 5 mL of isopropanol by gentle rotation for 3 min. Hair was dried at room temperature for approximately 5 d, then ground to a fine powder with a Retsch ball mill (mixer mill MM200; 10-mL stainless grinding jar; single 12-mm stainless steel grinding ball) for 5 min at 30 Hz.

Cortisol was extracted from the powdered hair. One mL of methanol was added to 50 mg of powdered hair and incubated at room temperature with slow rotation for 24 h. After spinning in a microcentrifuge for 30 sec, a 0.6-mL aliquot was taken from the top, and this aliquot was dried using a vacuum centrifuge (Savant DNA Speed Vac NNA110). The dried extract was reconstituted with either 0.2 or 0.4 mL of phosphate buffer from the cortisol assay kit. We recommend use of 0.2 mL buffer, as this amount more often brings samples into the range of the kit standards. The buffer volumes were accounted for in the final analysis.

2.4. Cortisol determination

Cortisol concentrations from saliva and reconstituted hair were assessed using the Salimetrics EIA (enzyme immunoassay) kit for salivary cortisol (Salimetrics, State College, PA, USA). This kit was chosen for 2 reasons: (1) previous use for salivary cortisol assay in dogs [20,25]; and (2) previous use for cortisol assay in hair [22]. Whenever possible, all 7 saliva samples from each dog were run on a single plate. Samples were assayed in duplicate, and duplicate samples with a coefficient of variance (CV) >10% were re-run until a CV <10% was achieved. If an insufficient volume of saliva was available to run in duplicate, a single well was used (n = 7). If a sample ran out before achieving a CV <10%, then all results were averaged (n = 22). The mean intra- and interassay CVs were 6.1% and 14.6%, respectively. Intra- and interassay CVs were calculated using cortisol concentrations from the kit standards. The interassay CV for the 112 samples that were run on more than 1 plate was 13.7%.

In addition, 8 samples were serially diluted and compared with predicted dilution results to check for parallelism and confirmed recovery of cortisol from dog hair (a representative serial dilution is in Fig. 1).

2.5. Proximal and distal hair sections

To determine whether cortisol concentrations varied along the length of the hair shaft, hundreds of individual hairs from 9 dogs (4 GSs, 5 LRs) were cut approximately in half into proximal and distal sections. These samples were then analyzed following the hair assay procedures outlined above. Statistical comparisons were made between proximal and distal samples within subjects. As
frequent hair washing might deplete cortisol more in the distal section of the hair, we asked owners approximately how often they bathed their dogs.

2.6. Cortisol and coat color

To investigate the relationship between cortisol and coat color in dogs, we compared agouti (sable or black and tan) and nonagouti (black) GSs. As hair grows, melanocytes can switch between the synthesis of the pigments eumelanin and pheomelanin in response to a paracrine signaling molecule, agouti protein [32]. The resulting banded phenotype is known as agouti or wild-type. In contrast, nonagouti hairs have only eumelanin and are thus completely black. German shepherd dogs are one of 2 breeds in which a uniformly black coat color is always caused by the recessive nonagouti genotype [33].

Melanistic (all black) animals may also result from mutations to other coat color genes, such as the unique K locus in dogs [34]. The K^B allele causes a dominant inheritance of uniformly black coat color, as seen in LRs. To assess the effect of coat color regardless of the genetic basis of that coat color, we included black (eumelanin) and yellow (pheomelanin) LRs and divided all dogs into groups of either black (eg, black GSs and black LRs) or nonblack (eg, agouti GSs and yellow LRs), regardless of breed.

To determine whether cortisol differences were present at the hair level versus the coat-color level, we used only GSs. Many sable or black and tan GSs have not only banded hairs, but also solid pheomelanin and/or solid eumelanin hairs. Individual hairs of agouti GSs vary in the relative amounts of pheomelanin and eumelanin: some hairs are entirely yellow (all pheomelanin), and some hairs are banded with both pigments in different proportions (agouti). Hairs from each of 4 GSs were separated into 3 color categories: eumelanin, pheomelanin, or agouti. Color categories were defined as follows: eumelanin hairs were 100% black; pheomelanin hairs were 100% yellow/red; and agouti hairs were hairs with a ratio of eumelanin to pheomelanin that was no greater than 70:30 and no less than 30:70. The color categories were not pooled across dogs, and the statistical analysis controlled for individual dogs. Only hair from a single shaving was used for each dog. Hair from 5 other GSs was sorted but did not yield enough hairs in the different color categories for analysis. Even for the 4 dogs that had sufficient hair in different color categories, we were not able to obtain the full volume of hair normally used for assay for every color category. Adjustments were made in the grinding time in proportion to the amount of sample available.

2.7. Statistical analysis

Statistical analyses were conducted using Minitab, version 15 (Minitab Inc., State College, PA, USA; release date January 31, 2007). Two-sample t tests were used for pairwise comparisons of categorical variables (eg, breed, sex, neuter status) with hair or salivary cortisol. Paired t tests were used to compare cortisol concentrations in the 41 dogs with old and new hair samples as well as cortisol concentrations in distal versus proximal hair segments of 9 dogs. Regression analysis was used for comparisons of continuous variables (eg, age, weight) with hair or salivary cortisol. The data come from 2 sources: owner-derived data (eg, age, sex, weight) and experimenter-measured data (cortisol concentrations in hair and saliva). Unless otherwise indicated, results are presented as mean ± SD. For each individual, new hair cortisol concentrations were compared to the average cortisol concentration of concurrent saliva samples. General linear models (GLM) compared cortisol with pigment type and with hair segment (proximal vs distal). In each case, pigment type (agouti, black, yellow, 2 df) or hair segment (proximal, distal, 1 df) was one factor and individual dog (7 df) was the second factor.

Although the generalized linear model (GLM) for the amount of cortisol in the proximal versus distal hair segment was not significant, we noticed that 7 of the 9 dogs had more cortisol in the distal segment than the proximal segment of their hair. We used a binomial test to see whether this bias (7 of 9 dogs) was significant. Thus, the continuous data on cortisol amounts in prox-
imal versus distal hair segments were assessed by GLM, but the count data on number of dogs with a directional difference in cortisol in the segments was assessed by the binomial test.

Analyses were done with and without cortisol outliers, but most results are reported only for analyses without outliers. Outliers were excluded until box plots revealed no points above or below the whiskers. Cutoffs for outliers were as follows: mean salivary cortisol >0.318 μg/dL, old hair cortisol >27.1 pg/mg, and new hair cortisol >21.7 pg/mg. After removing outliers, the cortisol data were normally distributed (Anderson Darling Normality tests). Correlations between hair and salivary cortisol concentrations included only saliva samples that were concurrent with the entire period of hair regrowth.

3. Results

Overall, cortisol concentrations were extremely variable among dogs and more variable in saliva than in hair. For the 315 saliva samples and 94 hair samples, coefficients of variability were 166% and 72%, respectively. Cortisol concentrations from new hair samples had the most extreme outliers. Without outliers, the adjusted mean cortisol concentrations were as follows: salivary cortisol for 45 dogs: 0.156 ± 0.061 μg/dL (range = 0.070–0.318 μg/dL, CV = 39%); old hair cortisol for 47 dogs: 12.63 ± 5.45 pg/mg (range = 4.56–27.09 pg/mg, CV = 43%); new hair cortisol for 42 dogs: 10.88 ± 3.85 pg/mg (range = 3.42–21.69 pg/mg, CV = 35%). For the 41 dogs with cortisol data for both old and new hair, cortisol concentrations were slightly greater for old hair (11.96 vs. 10.69 pg/mg, P = 0.049).

3.1. Salivary cortisol collection

Dogs readily accepted the new saliva sampling method, as illustrated by the low level of restraint needed and the minimal collection time. In fact, of the 48 dogs sampled, 79% required no restraint whatsoever, whereas 13% needed mild restraint, and only 8% required medium restraint. Saliva collection time averaged 58 ± 25 s (range = 30–120 s, n = 48). After removing 2 outliers, collection time was shorter with less restraint (P = 0.01). Collection time averaged 49.6 s (range = 30–100 s, n = 36) with no restraint, 64.2 s (range = 55–90 s, n = 6) with mild restraint, and 80.0 s (range = 30–120 s, n = 4) with medium restraint.

3.2. Hair collection

The shave (old hair) and re-shave (new hair) collection method provided adequate amounts of hair for cortisol analysis. Approximately 250 mg of hair is desirable for cortisol analysis, but 150 mg is sufficient. The initial sampling provided a surplus of hair from all dogs. Of the 47 dogs available for re-shaving, only 2 did not provide the desired amount (155.8 mg and 241.9 mg), and one had not regrown a sufficient amount of hair by week 12 (all 3 samples were from LRs). Hair was re-shaved at a mean time of 8.6 wk (range = 6–12, SD = 1.2) and at a mean of 82% regrowth (range = 15–100%, SD = 15). Thus, the hair collection method provides enough hair for cortisol assay in these breeds after approximately 9 wk of regrowth.

Old hair length differed significantly between breeds (t = 6.61, P < 0.001, df = 42). Mean hair length in GSs (4.3 cm, range = 1.6–6.8 cm) was about twice that in LRs (2.3 cm, range = 0.9–3.8 cm).

3.3. Validating cortisol measurement in hair

A major objective of this study was to further validate the use of hair for measuring cortisol in dogs. Cortisol concentrations from concurrent saliva and hair samples were positively correlated (new hair cortisol = 6.46 + 27.8 [salivary cortisol], r = 0.48, P = 0.001, n = 42, Fig. 2).

3.4. Cortisol and individual subject attributes

Individual attributes (age, sex, breed, weight, neuter status, household status, and coat color) were compared to one another. Pairwise tests yielded 2 significant re-

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**Fig. 2.** Scatter plot and regression line for new hair cortisol concentration (pg/mg) versus salivary cortisol concentration (μg/dL). New hair is hair shaved after a regrowth period of 6–12 wk. The equation for the regression line is new hair cortisol = 6.46 + 27.8 (salivary cortisol), P = 0.001, n = 42.
relationships among individual subject attributes. First, females weighed 14% less on average (mean = 32.5 kg) than males (mean = 37.9 kg, \( t = -4.16, P < 0.001, df = 44 \)). Second, on average, intact dogs were 2.4 y younger (mean = 3.3 y) than neutered dogs (mean = 5.7 y, \( t = -2.75, P = 0.014, df = 16 \)).

Salivary and hair cortisol concentrations were also assessed versus individual attributes. Pairwise comparisons revealed no significant differences with respect to individual attributes and either salivary or new hair cortisol, but there were 2 significant results for old hair cortisol. First, dogs living alone had old hair cortisol concentrations 36% lower (mean = 9.0 pg/mg) than dogs living in multidog households (mean = 14.2 pg/mg, \( t = -3.90, P < 0.001, df = 38 \)) and second, black dogs had old hair cortisol concentrations 24% lower (mean = 10.7 pg/mg, \( t = 2.13, P = 0.039, df = 39 \)).

3.5. Cortisol in proximal and distal sections of the hair shaft

Although the amount of cortisol across dogs did not differ between proximal and distal hair segments (paired \( t \) test, \( P = 0.348 \), Fig. 3), the number of dogs with more cortisol in the distal segment was significant (7 of the 9 dogs, \( P = 0.002 \)). One dog with high cortisol was eliminated from the figure and the paired \( t \) test. If this outlier were included, the significance of the paired \( t \) test would be \( P = 0.211 \). No pattern with respect to breed, sex, or coat color was evident.

3.6. Cortisol and hair color

Results of our comparison between agouti, eumelanin, and pheomelanin hairs within individuals revealed a relationship between hair color and cortisol concentration (partial \( r = 0.47, P = 0.001 \)). Eumelanin (black) hairs were consistently lower in cortisol than pheomelanin (yellow) hairs. Cortisol concentrations of agouti hairs were intermediate (partial \( r = 0.47, P = 0.001 \)). Pheomelanin hairs from dog D had an insufficient mass for analysis.

A survey of dog owners revealed that bathing was no more frequent than twice/mo. Dogs were washed between 0 to 24 times/y, with a mean of 3 baths/y. On average, GSs were bathed 6 times more frequently (mean = 6 baths/y, range = 0–24) than LRs (mean = 1 bath/y, range = 0–5). Bathing frequency was not correlated with the difference in cortisol in proximal versus distal hair segments (regression: \( P = 0.944 \)).

4. Discussion

The objectives of the present study were to improve cortisol sampling methods, to validate hair relative to saliva for measuring basal cortisol, to determine characteristics of cortisol within the hair shaft, and to assess cortisol with respect to coat and hair color.

The first objective of this research was to minimize the restraint needed for saliva collection in dogs. Traditional saliva collection, though considered nonstressful, still necessitates some restraint of the head and some handling of the mouth, neither of which is vol-
untarily accepted by most dogs. Using hidden treats, we rarely had to restrain any of the subjects and never for more than 2 min. Not surprisingly, the less restraint we used, the less time was needed for saliva collection. Cortisol concentrations are unaffected by handling for up to 4 min [35], and all sampling took place well within this time frame. Thus, saliva was collected with minimal restraint and completed quickly enough to avoid elevating the cortisol concentration.

Use of a hidden treat meant that salivation could be induced without flavoring the collection material, thus avoiding unreliable results [25]. Food luring was a nonstressful collection method, as demonstrated by the dogs’ voluntary participation, and was also easy enough for owners to perform on their own. Saliva has long been used as a valid measure of cortisol concentrations, and this successful new food luring technique makes collecting saliva in the home environment even more convenient.

The second objective of this study was to establish the validity of hair for measuring basal cortisol concentrations in dogs. Basal concentrations of cortisol are responsible for priming some of the organism’s homeostatic mechanisms for action [36]. Unlike many other domestic and wild species, dogs may display an episodic pattern of cortisol secretion [37] and significant individual circadian variability [38]. Though point samples (saliva, blood) are collected within a narrow time frame in this and most other cortisol studies, individual concentrations may vary regardless of stress or time of day. Hair sampling within the dog’s home provides a means of measuring long-term or basal cortisol secretions that are less sensitive to individual circadian patterns, momentary stressors, or the chronic stress of shelters. Hair and salivary cortisol concentrations were positively correlated in the home environment. This finding confirms hair as a valid medium to evaluate basal cortisol secretion in dogs. As cortisol concentrations in old and new hair did not differ, a single shaving would be sufficient for assessing cortisol concentrations and comparing them across breeds, sexes, ages, and so on.

Hair has many advantages for cortisol measurement. Shaving is well tolerated by most domestic species. Although shaving may require some restraint or sedation of wild animals such as wolves, hair would be easy to collect during routine capture and release. However, relatively large volumes of hair are required for assay, thus small animals such as hamsters may not be good candidates for this sampling. Hair sampling allows comparisons of baseline cortisol with individual traits such as temperament or social status [28]. In addition, the accumulation of hormones over precise time periods can be measured with a timed shave and regrowth period, and thus, hair sampling obviates the need for repeated blood sampling. Hair sampling could have broad applications in kennels, shelters, laboratories, or anywhere that long-term cortisol concentrations need to be assessed.

Hair cortisol concentrations obtained in this study were higher than in previous work on dogs [24]. We report a mean first hair cortisol concentration of 12.6 pg/mg and second hair cortisol concentration of 10.9 pg/mg, whereas Accorsi et al [24] report a mean cortisol concentration of 2.10 pg/mg. This difference may be because powdering of hair resulted in a 3.5-fold increase in cortisol recovery over the chopping method used by Accorsi et al [22]. In addition, other differences in assay methods—such as enzyme immunoassays versus radioimmunoassay and differences in study subjects such as breed, size, and age—may account for these discrepancies.

Our study included 2 of the most popular breeds in the United States, according to 2008 American Kennel Club breed registration statistics [39]. Though these are both large breeds, they are otherwise phenotypically very different. Differences in initial hair length and in growth rates between the 2 breeds, as well as substantial individual variability, were consistent with other studies on dog hair regrowth [40,41]. Mean hair length in GSs was almost twice as long as in LRs. Owners attested to the more frequent shedding in GSs than LRs, which would suggest differences in growth rates and hair growth stages between the 2 breeds. Both breeds in this study required a similar amount of time to achieve nearly complete hair regrowth despite the differences in original hair length. The rapid regrowth observed in the ischiatic (hip) region of dogs in our study is in agreement with previous work [40].

In terms of measuring cortisol in hair, the hair sampling area and regrowth time period outlined above provide sufficient hair for cortisol assay. Although old- and new-growth hair did not differ in cortisol concentrations in our study, the environments of the animals also did not change in any systematic manner. If one were to use hair to measure the effect of a stressor, we would recommend monitoring or marking the sample area to avoid inadvertent collection of old-growth hair because of significant variation in hair growth rates across breeds and individuals.

Analysis of cortisol and individual subject attributes of age, sex, breed, weight, neuter status, and household status yielded 1 significant relationship: dogs living
alone had significantly lower basal cortisol (in old hair samples) than did dogs living in multidog households. This finding is in agreement with reports of cortisol response in thunderstorm-phobic dogs to a simulated thunderstorm: dogs living without other dogs had lower baseline cortisol concentrations but a more significant change post-stressor than dogs living in multidog households [20]. Combined with our results, the higher baseline cortisol seen in dogs from multidog households suggests that living with other dogs is more stressful day to day than living without other dogs, but living with other dogs may have a physiological protective factor for dealing with certain stressors.

Although all 3 cortisol measurements (saliva, old hair, and new hair) were significantly correlated, only the old hair measure revealed an effect of household status. This finding may be owing to differences in variability among samples; new hair was the least variable after removing outliers for analysis, whereas old hair was the most variable.

Previous investigations of cortisol concentrations also reported no significant relationship between cortisol and sex or age [21,23]. Other studies of dogs also found no significant differences in cortisol concentrations among breeds [21], and no relationship between cortisol concentration and neuter status [42]. Thus, our results confirm these studies and reveal no significant effects of age, breed, weight, or neuter status on cortisol in either hair or saliva.

The third objective of this study was to determine whether cortisol concentrations vary significantly along the length of the hair shaft in dogs. This objective was achieved by comparing cortisol concentrations in proximal and distal sections of the hair shaft. Little is known about the possible leaching effects that washing and sunlight exposure may have on hair over time and throughout the length of the hair shaft. No difference in cortisol concentrations was evident between the proximal and distal ends of rhesus macaque hair [22], but a study on women by Kirschbaum et al [30] reported a decline in cortisol concentration throughout the length of hair from scalp to tip. We found no significant relationship between cortisol concentration and position along the hair shaft in a species subjected to variable washing frequencies. However, more dogs (7 of 9) had larger concentrations of cortisol in the distal portions of their hair than in the hair closer to the skin, the opposite of what would be expected if cortisol were leached from the hair as it aged.

The fourth and last objective was to assess the relationship between cortisol and coat color or pigment. We found differences in cortisol in relation to coat color and pigment. Across all subjects, black dogs had less cortisol than nonblack dogs, suggesting a coat color-cortisol relationship. In addition, within an individual dog eumelanin (black) hairs had less cortisol than pheomelanin (yellow) hairs, with agouti (banded) hairs intermediate.

The literature suggests 2 possible reasons for the black coat color and individual black hairs having less cortisol than yellow hairs. Given that glucocorticoids are involved in stress-associated hair growth inhibition [43] as well as melanocyte development and differentiation [44,45], differences in cortisol in different pigment types or coat colors may be related to different control mechanisms. Second, hair may be a storage vehicle for cortisol. In general, yellow hair has less pigment than black hair [46,47], thus yellow hair may have more room for glucocorticoids than black hair.

Interestingly, the pigment differences we found are within the same animal, not across breeds or coat colors. The differences between black, yellow, and agouti hairs within an individual dog do not translate to differences between breeds (LRs vs GSs) or coat colors within a breed (yellow vs black LRs, agouti vs black GSs). We expected to find coat color differences within GSs but not black and yellow LRs, because fecal glucocorticoid concentrations differed in agouti vs nonegouti deer mice (animals that differ only at the agouti coat color locus and otherwise were genetically identical [11]). Agouti and black GSs differ at the agouti locus, although the animals also differ at other loci, unlike the laboratory deer mice. Also, agouti GSs, unlike agouti deer mice, have all-black hairs and all-yellow hairs, as well as banded agouti hairs. As hair follicles have a local functional equivalent of the HPA axis and synthesize cortisol [29,48], local production of cortisol within hairs could be responsible for the differences between hairs with different pigment compositions, but differences in cortisol production across individuals may obscure differences across individual hairs. The genetic control that produces yellow, black, or agouti hairs differs in LRs vs GSs. Unfortunately, the biochemical mechanism, which produces all-black or all-yellow hairs in addition to the agouti-striped hair all in the same GS, is not known.

In summary, (1) food luring minimizes the restraint required for saliva collection; (2) hair cortisol concentrations do correlate with concurrent salivary cortisol, and thus hair is a valid medium for measuring basal cortisol in dogs; (3) distal hair segments more often have higher cortisol than proximal segments in contrast
to both macaques and humans; and (4) within an individual dog, black hairs have less cortisol than yellow hairs, and across all dogs, black dogs have lower cortisol in their hair than nonblack dogs.

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