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A novel method using hair for determining hormonal levels in wildlife

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Hormones influence behaviour, and are also influenced by behaviour. Monitoring their levels can therefore provide insights into the mechanistic aspects of behaviour. In male mammals for example, elevated levels of testosterone are associated with increased aggression and dominance (Creel et al. 1993, 1997; Mazur & Booth 1998) and in social mammals, levels of stress hormones (e.g. corticosterone, glucocorticoid and cortisol) are associated with rank (Sapolsky 1985; Creel et al. 1996, 1997). Research has associated hormone levels with different behaviours such as sexual, reproductive, courtship, parental, aggressive and feeding behaviours. Comparative tools for hormonal analysis provide insights into evolutionary theories based on behavioural aspects, such as reproductive suppression and the 'challenge hypothesis' (e.g. Creel et al. 1993).

In field studies, hormones are usually extracted from blood samples, or noninvasively from saliva, urine and faeces (Creel et al. 1992; Cavigelli 1999; Hirschenhauser et al. 1999; von Engelhardt et al. 2000). Samples derived from trapped or handled animals are problematical because stress may alter blood and urine hormonal levels (Creel et al. 1992). Additional problems with blood samples are that they are not always available, the amount that can be taken at a given time is limited, and various safety and ethical issues exist. Furthermore, blood and saliva must be transported cold or frozen, conditions that are sometimes difficult to obtain in the field (Yang et al. 1998). Urine and faeces samples are sometimes difficult to obtain from free-ranging animals that cannot be continuously observed, or from species that deposit in common latrines.

An alternative source for hormones may be found in hair, which can be collected noninvasively, and is already used to extract DNA (Woodruff 1993; Morin et al. 1994), trace metals, naturally occurring compounds and drugs (Wheeler et al. 1998). Hair is safe, readily available, and easy to store and transport. Hair sampling does not involve pain or possible infection, and the analysis is unaffected by the momentary stress of capture (Yang et al. 1998). Hair analysis may allow one to monitor hormonal changes over weeks or months (between moults; Maurel et al. 1986) by shaving off a patch of hair and resampling the newly grown hair. Hormonal hair analysis offers only a long-term profile, however, and is not suitable for monitoring hourly or daily (short-term) fluctuations in hormonal levels. It provides the resolution needed for studies of main behavioural trends, especially in stable hierarchical social systems. Hair has already been used to diagnose early pregnancy in cows by detection of progesterone (Liu et al. 1988), to detect oestradiol and testosterone in cattle (Gleixner & Meyer 1997) and anabolic steroid and corticosteroid abuse in athletes (Bowers & Segura 1996; Hold et al. 1999; Kintz et al. 1999; Cirimele et al. 2000). In humans, the levels of steroid hormones in hair do not vary significantly between different regions of the scalp (Wheeler et al. 1998). Oestradiol, progesterone and testosterone levels measured in healthy human adults' hair correlate significantly with the levels measured in their serum (Yang et al. 1998).

As an example of the utility of this method, we use data from our long-term study on rock hyrax, *Procavia capensis*. Observations of urination are rare in this species, and shared defecation sites do not allow individual identification of faeces. Extracting hormones from hair samples taken from live captured rock hyrax is an easy method, which enables reliable monitoring of long-term trends in hormonal changes despite the stress caused by trapping (Koren 2000).

Methods

Hormonal analysis

Between March and June 2000, we caught rock hyrax in live box traps, which were baited with vegetables and checked every 2 h. The hyrax were anaesthetized with ketamine hydrochloride (0.1 mg/kg) and individually marked with small animal commercial ear tags (no. 1005 size 1, National Band and Tag Co., Newport, Kentucky, U.S.A.) and light collars weighing 5 g. All handling was

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done in situ, and the hyrax were released after 90 min, once the anaesthesia had fully worn off, at the site of capture (Koren 2000). All adult males (N=10) in a 500-m section of the Arugot canyon in the Ein Gedi Nature Reserve were included in the study group. The animals are part of an ongoing long-term study.

We plucked between 7 and 20 mg of hair from each animal and placed it in a dry vial. In the laboratory, we weighed the hair accurately, minced it into 3–4-mm pieces with fine scissors, and placed it in a glass vial. Methanol was added and the vials were sonicated for 30 min and then incubated overnight at 50°C with gentle shaking. We then pipetted the methanol off into glass tubes and evaporated it to dryness under a stream of nitrogen. Each sample was reconstituted with phosphate-buffered saline (PBS; pH=7.0) and dispensed into the appropriate polyclonal hormone antiserum coated well of the ELISA (solid phase enzyme linked immunoassay) microplate, in duplicates. We measured the samples for immunoreactive hormones, using human serum or plasma testosterone and human saliva cortisol ELISA kits from ALPCO Diagnostics (Windham, NH, U.S.A.), according to the manufacturer's protocols.

Briefly, the microplate was incubated for 5 min at room temperature. Enzyme-conjugate was then added to each well, the plate was thoroughly mixed for 10 s, incubated at room temperature for 60 min and washed. After substrate solution was added the plate was incubated for 15 min at room temperature. Adding stop solution (2N HCl) stops the enzymatic reaction. The absorbance was read at 450 nm. It is inversely proportional to the concentration of hormone present. A linear relationship was found between log concentration and logit optical density. From the linear standard curve, we calculated the concentration of hormone in the unknown samples first in ng/ml and than transformed it into pg/mg hair. Standards are 0.2–16 ng/ml for testosterone, 2–80 ng/ml for cortisol. All standards and reagents are provided in the kit.

Technical specifications for the kits used are as follows. The lowest detectable level of testosterone that can be distinguished from the zero standard is 0.069 ng/ml and for cortisol is 1.14 ng/ml. Average interassay CV is 3.72%. Cross reactivity with other steroids is $\leq$1% for the testosterone kit. The cortisol kit cross-reacts with prednisolone (60%), corticosterone (29%), cortisone (3%) and other steroids ($\leq$1%). Extraction efficiency could not be determined for hair since spiking and retrieving methods are applicable only for exogenous hormones. Such an assessment indicated 93% efficiency for testosterone from cattle hair (Gleixner & Meyer 1997).

Ranking males

We observed rock hyrax in the field 5 days a week, for a total of 800 accumulated h. We use 10 × 42 binoculars and a telescope with × 50 and × 100 magnifications to observe agonistic interactions that involved display by one male hyrax and resulted in evasive action being taken by a second male. Dominance relationships within pairs (dyads) were placed in a matrix of encounters. In each interaction, the winner was placed in the row, and the loser in the column. We determined the hierarchy and assigned each individual a rank using the Batchelder–Bershad–Simpson (BBS) Scaling Method (Jameson et al. 1999) via a JAVA matrix applet, which was designed by Robert Huber (http://caspar.bgsu.edu/~software/Java/1Hierarchy.html). High-ranking individuals received a positive rank while low-ranking individuals received a negative rank. This scale reflects both the order of dominance rank and its magnitude for each animal relative to the rest of the group.

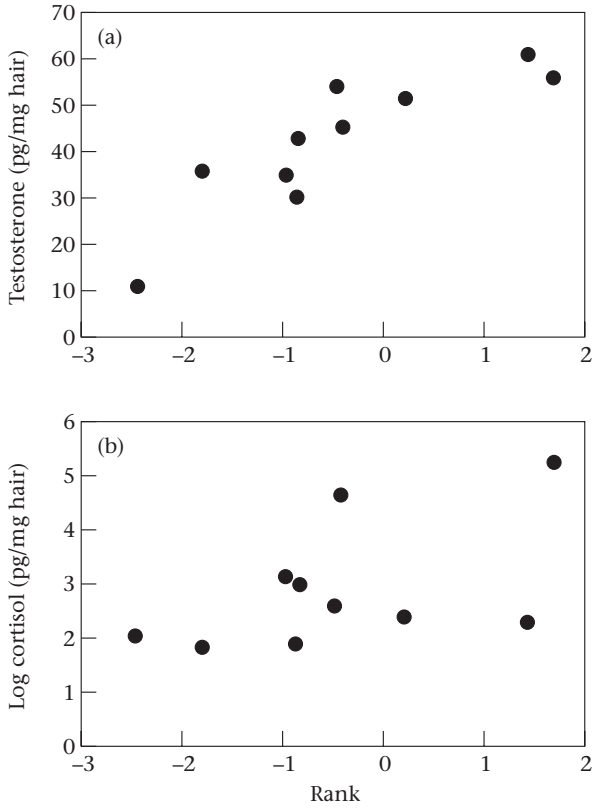

Figure 1. Correlations between rank and hormonal levels in male hyrax in Ein Gedi: (a) testosterone and (b) cortisol. Hierarchical ranking was determined with the Batchelder–Bershad–Simpson (BBS) scaling method.

Results and Discussion

To illustrate the utility of this method we examined the relationship between rank and hormonal levels in male hyrax at Ein Gedi. For 10 male hyrax, BBS ranks were calculated and plotted against the concentration of hormones extracted from their hair (Fig. 1). Spearman correlation indicated that rank was significantly associated with testosterone levels (r_S=0.903, t_8=5.95, P=0.0003). Rank and cortisol levels were not associated (r_S=0.539, t_8=1.81, P=0.108).

Very few field studies have compared androgen levels and social rank. In two cooperatively breeding birds (Australian magpies, *Gymnorhina tibicen*; and Harris hawk, *Parabuteo unicinctus*) and one mammal (dwarf mongoose, *Helogale parvula*), no relationship was found between

dominance and testosterone in males (Mays et al. 1991; Schmidt et al. 1991; Creel et al. 1993). Yet in four cooperatively breeding birds (white-browed sparrow-weavers, *Plocepasser mahali*; noisy bell-miners, *Manorina melanophrys*; Florida scrub jays, *Aphelocoma coerulescens*; and pied kingfishers, *Ceryle rudis*), rank is associated with testosterone levels in males (Reyer et al. 1986; Schoech et al. 1991; Wingfield et al. 1991; Poiani & Fletcher 1994). Our findings show that in the rock hyrax, a cooperatively breeding mammal, male rank is also associated with testosterone levels.

In two social carnivores (African wild dog, *Lycaon pictus*; and dwarf mongoose), dominant animals suffer from chronic stress (Creel et al. 1996, 1997), while in wild baboons, *Papio anubis*, it is the subordinate group members that are subjected to such stress (Sapolsky 1985). Social stress may depend on social organization, conditions and aggression within the group (Creel et al. 1997). The weak correlation we found between rank and the stress hormone cortisol may prove significant with a larger sample size.

Comparing hormonal levels in hair samples is an alternative, noninvasive method that can be used to sample a wide range of animals in the field. Hair can be collected stress-free from nesting or sleeping burrows, or by using sticky hair traps (as already used for obtaining hair samples for DNA analyses from bears; Woods et al. 1999). A large sample of hair can usually be taken from individual animals and can benefit the efficiency of both the DNA and hormonal extractions. Although hair growth patterns can be studied, growth rates may be affected by a number of intrinsic and extrinsic factors. Repeatedly shaving and resampling patches of hair from the same area can allow one to monitor hormonal levels over periods of weeks or months. Another advantage of this method is that hormonal extraction from hair is insensitive to the immediate stress afflicted by trapping and handling wild animals. In the future, similar protocols may be adjusted to extract hormones from feathers, scales and other animal products, from which DNA is already being extracted. Nevertheless, this method is not suitable for studies requiring short-term monitoring of hormonal levels. Traditional tools based on blood, faeces or urine would be more appropriate for such applications.

Although Yang et al. (1998) showed that testosterone levels in hair correlate significantly with serum concentrations (r=0.395), only 16% of the variability is explained by this correlation. This is not surprising since hormonal blood concentrations change rapidly, while levels reflected in hair represent accumulation over time. Nevertheless, individuals that have relatively high hormonal levels should have higher levels than others in both hair and blood samples. Consequently, hair testing is suitable for comparing individuals in behavioural studies focusing on long-term social trends.

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