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Assaying progesterone, estradiol and cortisol concentrations in hair of Père David deer hinds: an alternative way to reflect seasonality of steroid secretion

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ABSTRACT

The use of a hair hormone concentration assay is increasingly recognized as a useful and noninvasive technique for monitoring the endocrinological status of animals. However, few studies have focused on reproductive and stress hormones together. We used a chemiluminescent immunoassay to determine whether the progesterone, estradiol, and cortisol concentrations could be measured from hair and whether these hormone concentrations varied in different hair segments of captive Père David deer hinds. We found that progesterone, estradiol, and cortisol could be measured in the hair samples and that the progesterone concentration varied but the estradiol and cortisol concentrations did not among different hair segments. Contrary to the segmental decline in hair cortisol found in many studies, we found that progesterone concentration was higher near the tip than at the base of hair in Père David deer. This suggests that the variation in segmental hair steroid hormone concentration in seasonal molting animals may be mainly due to internal reproductive cycles and that hair steroid hormones may reflect long-term physiological changes and can thus be used for the conservation and management of wildlife.

Introduction

The development and use of noninvasive techniques for monitoring the physiological and evolutionary status of animals has been promoted over the last two decades. Several techniques have been used to monitor the endocrinological status of animals, including the measurement of hormones from feces, urine, saliva, hair, claws, ballen and feathers (Touma and Palme 2005; Davenport et al. 2006; Ashley et al. 2011; Baxter-Gilbert et al. 2014; Hunt et al. 2014; Romero and Fairhurst, 2016).

The extraction and preservation of hair samples are relatively simple, and the collection does not entail health hazards (Meyer and Novak 2012; Russell et al. 2012). Moreover, hair

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is a relatively stable medium that is known to incorporate blood-borne hormones during its active growth phase, which can last weeks to months (Gow et al. 2010; Stalder et al. 2012). Under optimal conditions, steroid hormones in hair may be detectable for years to centuries (Webb et al. 2010). Therefore, hair assays are increasingly used as a reliable way to measure hormones (Davenport et al. 2006; Meyer and Novak 2012).

Most studies on hair hormones have focused on the effect of long-term stress, disease progression and treatment efficacy on cortisol in humans, captive animals and wildlife (Dettenborn et al. 2012; Meyer and Novak 2012; Russell et al. 2012). Limited work has been done on detecting reproductive hormones in human hair (Thomson et al. 2009; Deshmukh et al. 2012), and on cattle hair (Duffy et al. 2009). Whether reproductive hormones would be deposited and could be measured in wild animal hairs remain unknown.

The assessment of hair hormone could reflect changes in hormones along hair growth period, which has been validated in many studies (Meyer and Novak 2012; Russell et al. 2012). Some studies have found a decrease in cortisol toward the distal end of the hair shaft (Kirschbaum et al. 2009; Gow et al. 2010; Dettenborn et al. 2012; Carlitz et al. 2015), whereas others have not found symmetric cortisol decrease toward distal segments of the hair in human or wild animals (Davenport et al. 2006; Macbeth et al. 2010; Carlitz et al. 2014; Hunt et al. 2014).

Père David deer (*Elaphurus davidianus*), rut and molt hair seasonally (Beck and Wemmer 1983). Steroid hormones in blood, urine, and feces of Père David deer have been studied (Monfort et al. 1991; Li et al. 2001, 2004, 2007). The different hair segments might reflect different biological periods. We used Père David deer to explore whether progesterone, estradiol, and cortisol could be measured in hair and whether these hormones varied in different hair segments.

Materials and methods

Study area and animals

The study was conducted at the Beijing Milu Park (39°07′N, 116°03′E), Beijing, China, where the average annual temperature is 13.1 °C, with a mean temperature of −3.4 °C in January and 26.4 °C in July; the average annual precipitation is approximately 600 mm. Père David deer were bred in a 60 ha enclosure; there were 56 adult and sub-adult stags and 62 hinds and one-year fawns. All stags and hinds had ear tags. Facial characteristics were used to identify individuals when ear tags were unavailable.

This study was conducted under animal research protocol IOZ-2015 and was approved by the Animal Welfare Ethics Review Committee of the Institute of Zoology, Chinese Academy of Sciences. We adhered to the "Guidelines for the Use of Animals in Research" published in Animal Behavior 1991.

Hair sample collection

Hair was collected from the neck/shoulder region of 10 Père David deer hinds in late May after parturition. This area was relatively uncontaminated from self-grooming. These 10 deer hind were chosen for similar age and body size. About 3.0 g of hair were shaved from each hind and sealed in individually labeled plastic bags during the regular feeding time. Samples were then transferred to the lab and stored at −20 °C until the laboratory analyses. Hair

samples were washed to remove surface contamination based on validated methods from the published literature (Davenport et al. 2006; Macbeth et al. 2010). We removed all foreign material from original hair samples and weighed them. Hair samples were washed three times (3 min per wash) with 2.0 ml of methanol per 100 mg of hair. We put each sample into a 50-ml tube, added 30 ml of isopropanol and vibrated the tube for three minutes to wash the sample again. We repeated this procedure twice. After discarding the isopropanol, samples were dried in a hood extractor at room temperature.

Steroids extraction and assay

Hair samples were carefully cut into five even segments, I, II, III, IV, and V, from the base to the tip of hair. For the progesterone and estradiol assay, previous method on measurement of steroid reproductive hormones in human hair was used (Yang et al. 1998). 50 mg of hair by segment were powdered using a mixer mill MP Fastprep-24 and put into a centrifuge tube (Tube A) and 2 ml of ether were added. This tube was vibrated for two minutes, kept at room temperature for one hour, and then centrifuged (15 min/4500 rpm). We collected the supernatant in a new tube (Tube B). We then added 1 ml of ether into Tube A, vibrated it for two minutes, kept it at room temperature for one hour, and then centrifuged it (15 min/4500 rpm). Again, we collected 0.6 ml of the supernatant and transferred it to Tube B. After manual vibration, the supernatant was dried at room temperature, and was reconstituted with 0.4 ml of phosphate buffer provided in the assay kit until assayed. For the cortisol assay, previous method on measurement of cortisol in the hair of rhesus macaques (*Macaca mulatta*) was used (Davenport et al. 2006). 50 mg of hair by segment was powdered using a mixer mill MP Fastprep-24 and put into a centrifuge tube (Tube A). After adding 1 ml of methanol, the tube was vibrated for 24 h and then centrifuged (15 min/4500 rpm). We then collected and transferred 0.6 ml of supernatant to a new tube (Tube B). After manual vibration, the supernatant was dried at room temperature, and was reconstituted with 0.4 ml of phosphate buffer provided in the assay kit until assayed.

Hair progesterone concentration was quantified using a commercially available immunoassay with chemiluminescent detection (CLIA; progesterone, estradiol, and cortisol *in vitro* diagnostic kits, Chengdu CapitalBio Independent Medical Laboratory, Chengdu China). Intra-assay and inter-assay coefficients of variation were less than 10% (10 assays). According to the manufacturer, the cross-reactivity of the antibody used for the progesterone kit is as follows: progesterone (100%), less than 0.1% for estradiol, estriol, and estrone, no detectable cross-reactivity with testosterone and cortisone. The approximate limit of detection of the CLIA kit (0.011 ng/ml) was determined by calculating the absorbance (mean – 2SD) of the zero standard provided with the CLIA kit based on 20 runs. Extraction efficiency was 95.16% ± 2.62% based on three determinations of recovery. Progesterone measured in serially diluted Père David deer hair extract was parallel ($r^2 = 0.992$, $P < 0.001$) with serially diluted progesterone standards provided with the CLIA kit.

Hair estradiol concentration was quantified using a commercially available immunoassay with chemiluminescent detection (CLIA, Chengdu CapitalBio Independent Medical Laboratory, China). Intra-assay and inter-assay coefficients of variation were less than 5% (20 assays) and 9% (18 assays), respectively. According to the manufacturer, the cross-reactivity of the antibody used for the estradiol kit is as follows: estradiol (100%), estriol (1.84%), corticosterone (0.001%), Dehydroepiandrosterone (0.005%), 17α-Hydroxyprogesterone

(0.005%), cortisol (0.001%), no detectable cross-reactivity with cortisone, estrone, progesterone, testosterone, dehydroepiandrostrerone sulfate, and prednisone. The approximate limit of detection of the CLIA kit (2.5 pg/ml) was determined by calculating the absorbance (mean – 2SD) of the zero standard provided with the CLIA kit based on 20 runs. Extraction efficiency was 94.72% ± 3.45% based on three determinations of recovery. Estradiol measured in serially diluted Père David deer hair extract was parallel ($r^2 = 0.996$, $P < 0.001$) with serially diluted estradiol standards provided with the CLIA kit.

Hair cortisol concentration was quantified using a commercially available immunoassay with chemiluminescent detection (CLIA, Chengdu CapitalBio Independent Medical Laboratory, China). Intra-assay and inter-assay coefficients of variation were less than 8 and 10% (6 assays), respectively. According to the manufacturer, the cross-reactivity of the antibody used for the cortisol kit is as follows: cortisol (100%), less than 0.50% for other steroids. The approximate limit of detection of the CLIA kit (0.02 ng/ml) was determined by calculating the absorbance (mean – 2SD) of the zero standard provided with the CLIA kit based on 20 runs. Extraction efficiency was 93.67% ± 3.64% based on three determinations of recovery. Cortisol measured in serially diluted Père David deer hair extract was parallel ($r^2 = 0.972$, $P < 0.001$) with serially diluted cortisol standards provided with the CLIA kit. Following the manufacturer's protocol, the immunoassay procedures were performed using a Maglumi 2000 Plus automatic analyzer from The New Industries Biomedical Engineering Co. Ltd. (Shenzhen, China).

Statistical analysis

Statistical analyses were done using SPSS V22 (SPSS Inc., Chicago, IL, U.S.A.). One-sample Kolmogorov–Smirnov test was used to verify normality. Levene's test was used to estimate the homogeneity of variances. When data failed the normality test, they were log-transformed for subsequent analysis. A Repeated Measures General Linear Model (GLM) with the Greenhouse–Geisser correction was used to compare hair steroid concentration among the five hair segments. Fisher's least significant difference pair-wise comparison was used to do the *post hoc* when there was significant difference in hair steroid concentration among the five hair segments. Significance was set at $P < 0.05$ for all tests.
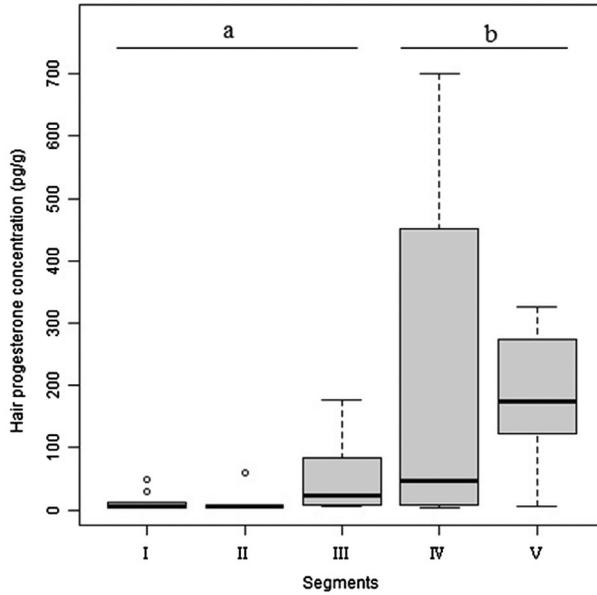
Results

### Difference in hair progesterone concentration among segments

There were significant differences in the hair progesterone concentrations among the five hair segments ($F_{1, 12} = 6.489$, $P = 0.020$; Figure 1). Post hoc test indicated that the progesterone concentrations in segments IV and V were significantly different from progesterone concentrations in segments I ($P = 0.001$ and 0.008), II ($P = 0.001$ and 0.007), and III ($P = 0.007$ and 0.036), while no significant difference could be found between progesterone concentrations in segments IV and V ($P = 0.504$), and among segments I, II, and III. The highest and lowest concentrations of hair progesterone were found in segments IV and II, respectively (Figure 1).

Differences in hair estradiol and cortisol concentration among segments

There was no significant difference in the estradiol ($F_{2, 22} = 2.057$, $P = 0.145$; Figure 2) or cortisol ($F_{1, 11} = 1.778$, $P = 0.213$; Figure 3) concentrations among different hair segments.
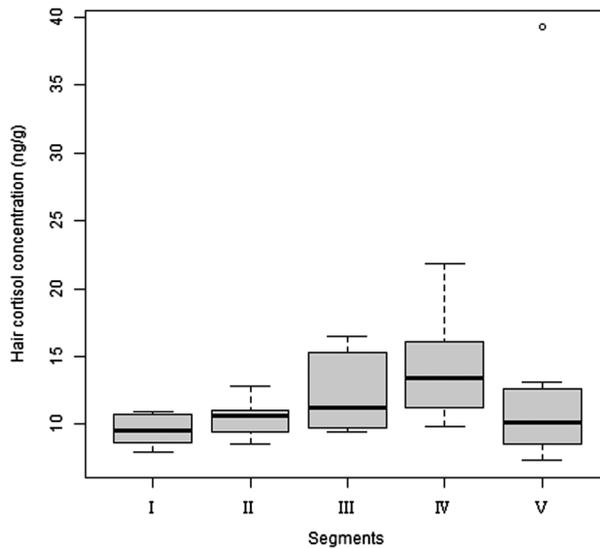
Figure 1. Concentration of progesterone in different hair segments of Père David deer hinds.
Notes: Different segments are indicated by roman numerals on the *x*-axis. The bold horizontal line represents the median, edges of the box are the 25th and 75th percentiles, and circles are outliers. The different letters at the top of the display indicated significant differences in hair progesterone concentrations among different segments.
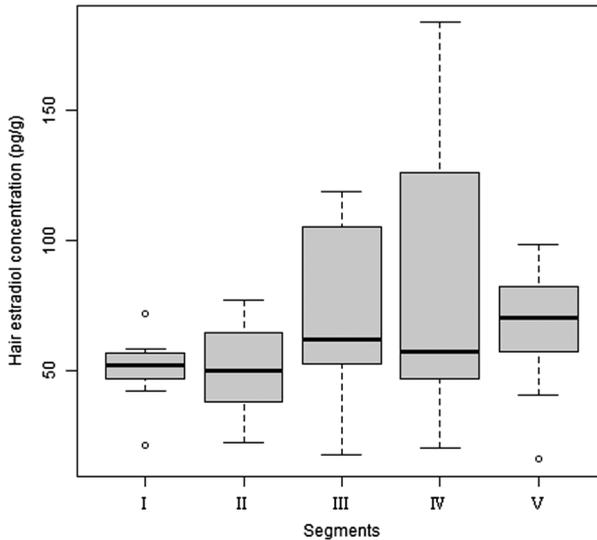
Figure 2. Concentration of estradiol in different hair segments of Père David deer hinds.
Notes: Different segments are indicated by roman numerals on the *x*-axis. The bold horizontal line represents the median, edges of the box are the 25th and 75th percentiles, and circles are outliers.

Discussion

We found that reproductive hormones were deposited and could be measured in deer hair and that the hormone steroid concentrations varied between hair segments. This indicated

Figure 3. Concentration of cortisol in different hair segments of Père David deer hinds.
Notes: Different segments are indicated by roman numerals on the *x*-axis. The bold horizontal line represents the median, edges of the box are the 25th and 75th percentiles, and circles are outliers.

that measuring reproductive hormones from deer hair could be a useful alternative to measuring serum or fecal hormones. Reproductive and stress hormones, such as testosterone, progesterone, estradiol, and cortisol have all been measured from blood, urine, and feces of Père David deer (Li et al. 2001, 2004, 2007). Hormones are thought to accumulate gradually as hair grows over an extended period of time (weeks to months); therefore, measuring the steroid hormones in hair could be used to monitor an animal's physiological responses to natural processes and potential long-term environmental changes (Koren et al. 2002; Macbeth et al. 2010; Meyer and Novak 2012).

Unlike previous studies, which found either no difference in the hormone concentrations or a decreasing hormone concentration along the hair shaft (Davenport et al. 2006; Kirschbaum et al. 2009; Gow et al. 2010; Macbeth et al. 2010; Dettenborn et al. 2012; Carlitz et al. 2014, 2015; Hunt et al. 2014), we found an increase in the concentration of progesterone from the base to the tip of hair. Previous studies mainly focused on cortisol, which is more sensitive to environmental stress, but the reproductive hormones that we measured are more sensitive to reproductive cycles, which may account for the differences found.

Steroids are thought to be incorporated into hair during the growth period via blood vessels, from the local synthesis of steroids in the follicle and/or from secretions from glands surrounding the hair follicle (Davenport et al. 2006; Pragst and Balikova 2006). Père David deer present a typical seasonality in reproductive behavior and sexual hormones (Beck and Wemmer 1983; Li et al. 2001). They molt twice a year. The spring molt occurs after the calving season in late May, and the autumn molt occurs after the rutting season in late September. Molt is a continuous process which lasts for about two months with different body parts are replaced gradually (Cowan and Raddi 1972). The hormones we measured indicated the physiological changes that occurred after autumn molt till spring molt in Père David deer (October to May).

The concentrations of luteinizing hormone, estradiol, progesterone and cortisol in blood, urine, and feces of Père David deer hinds change during their reproductive cycle (Curlewis et al. 1991; Li et al. 2001). The progesterone level is considered an indicator of pregnancy status in mammals, and the hair progesterone concentration differs between pregnant and non-pregnant red deer (*Cervus elaphus)* (Caslini 2013). The segment IV and V represented period right after the rutting season. The increase in the hair progesterone concentration might be due to early pregnancies in these deer.

Estradiol has been found to increase after impregnation in the Sichuan golden monkey (*Rhino pithecus roxellana*) (Yan et al. 2002), but other studies have found that estradiol increased before parturition in many ungulate species, including Père David deer (Plotka et al. 1977; Monfort et al. 1991; Li et al. 2001). Cortisol is mainly related to stress, but its secretion is very complicated and can be affected by many factors, including the external environment and other internal hormones (Li et al. 2007; Ashley et al. 2011; Carlitz et al. 2016). These might explain why estradiol and cortisol concentrations showed no significant difference in different hair segments in Père David deer.

To our knowledge, this is the first study on the variation in reproductive hormones in deer hair, and our results indicate this method could be helpful for monitoring the chronic level of hormones for long-term wildlife management. A number of studies have demonstrated that the hormone levels in hair reflect meaningful biological and ecological patterns (Macbeth et al. 2010, 2012; Bryan et al. 2013). We only determined hair steroid hormones in Père David deer hinds. Research on stags and other species is needed to further understand the variation in hormones between different hair segments and the effect of internal and external factors on these variations.

Geolocation information

The study was conducted at the Beijing Milu Park (39°07′N, 116°03′E), Beijing, China.

Acknowledgments

Disclosure statement

The authors report no conflicts of interest

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