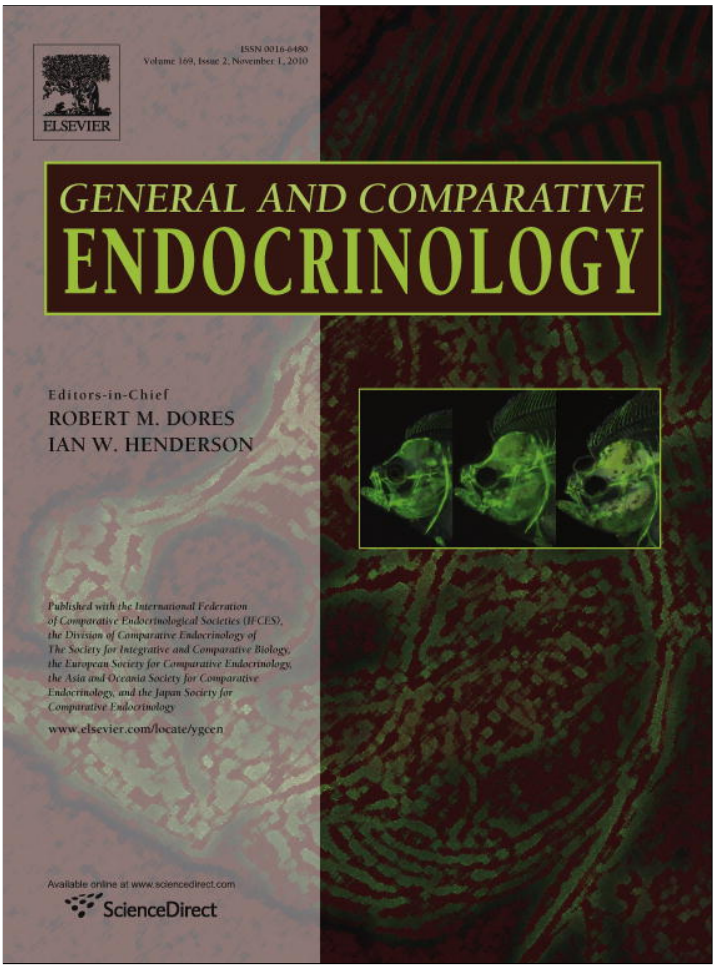

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Assessment of luteinizing hormone and prolactin immunoactivity in Asian and African elephant urine using assays validated for serum

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ABSTRACT

Analysis of serum hormones is useful for timing artificial insemination (Luteinizing hormone) and diagnosing pregnancy (prolactin) in elephants. However, these tests require blood collection, which is not tolerated by all animals, and is impractical for field studies. Thus, developing a means to obtain these measures noninvasively could improve species management. Matched urine and serum was collected from Asian and African elephants daily throughout the follicular phase and after administration of a GnRH analogue for LH determination, and in pregnant and nonpregnant females for prolactin analyses using immunoassays validated for elephant serum. Despite identifying robust increases in circulating hormone concentrations, no concomitant changes in urinary LH or prolactin immunoactivity was detected. Concentration of samples by centrifugal filtration or ethanol precipitation did not increase the ability to measure biologically relevant changes in endogenous urinary LH or prolactin immunoactivity. Sample matrix interference was ruled out following sufficient recovery of exogenous LH or prolactin added to samples, except for samples concentrated >35-fold where some interference was suspected. These results suggest that elephants either do not excrete native LH or prolactin in urine, or concentrations are too low to be measured accurately by standard immunoassay techniques that are valid for serum analyses. Thus, it does not appear feasible or economically viable to use these noninvasive tests for ovulation detection or for pregnancy diagnosis in elephants.

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1. Introduction

Much has been learned about the reproductive endocrinology of elephants in the past decade, and this has contributed significantly to improved breeding management (see reviews, Hodges, 1998; Brown, 2000, 2006). While most of the initial work was based on analyses of circulating hormones, more recent studies have shown the utility of urine and faecal steroid metabolite assessments for noninvasive monitoring of gonadal function in the elephant (Heistermann et al., 1997; Fiess et al., 1999; Czekala et al., 2003). In general, the elephant exhibits the longest estrous cycle of any mammal: 13–17 weeks in duration with a 4–6 week follicular phase and an 8–10 week luteal phase. Another unique aspect of the elephant estrous cycle is the occurrence of two LH surges about 20 days apart during the follicular phase (Kapustin et al., 1996; Brown et al., 1999). The anovulatory LH surge can be used to time artificial insemination and natural breeding attempts; however, the physiological function of this first surge is unknown.

After conception, circulating and excreted progestagens increase and remain elevated throughout the 20–22 months of pregnancy. Another hormonal indicator is a marked increase in prolactin immunoactivity after 5 months of gestation (Brown, 2000, 2006). The diagnostic advantage of prolactin is that only a single sample is needed after 5 months, compared to the longitudinal sampling that is necessary for pregnancy diagnosis with progestagens. Whereas progestagens can be measured in blood, urine and faeces, LH and prolactin have only been quantified in blood (Brown, 2000), and as such are impractical assessment tools for elephants that are not trained for routine blood collection or are free-ranging. This is especially true for LH, because characterizing the double surge requires daily blood sampling throughout most of the follicular phase.

Urinary protein hormone analyses have proven to have both research and clinical diagnostic value in numerous species. Both LH and prolactin are excreted in urine of humans and nonhuman primates and can be quantified using standard immunoassay techniques (LH – Miller and Soules, 1996; French et al., 1999: prolactin – Miller and Soules, 1996; Soltis et al., 2005; Schradin et al., 2003; Ziegler et al., 1996). LH also has been measured in the urine of several marine

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mammals, including bottlenose dolphins, white-sided dolphins and killer whales and is used to time artificial insemination (Robeck et al., 2004, 2005). Thus, the ability to measure protein hormones, like LH and prolactin, in elephant urine would be invaluable for assessing the reproductive status of intractable animals *in situ* and *ex situ*.

The objective of this study was to determine if LH or prolactin could be measured in elephant urine using immunoassay techniques developed and validated for elephant serum. To meet this objective, a number of sample treatments were employed to ensure data were not impacted by problems with urine dilution or matrix interference.

2. Materials and methods

2.1. Animals and sample collection

This project was approved by the SCBI-Institutional Animal Care and Use Committee (#07-11). Matched serum and urine from adult female Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants were used in this study. Females ranged from 20–37 years of age (mean 26 ± 5 years), and all exhibited normal ovarian cycles based on serum progestagen analyses. For all elephants, blood samples were collected from a leg or ear vein without sedation, centrifuged at 1500*g* within a few hours, and the serum stored at −20 °C until analysis. Urine samples were collected free-catch in a cup or tube, or off the enclosure floor. An aliquot of urine was treated with glycerol (7% v v^{-1}), centrifuged at 650*g* to remove debris and frozen at −20 °C. To characterize LH patterns, matched blood (7 mL) and urine (50 mL) were collected daily through at least one follicular phase per female (n = 3/species) to identify anovulatory and ovulatory LH surges. Samples also were collected from an additional female Asian elephant 1 h before and then at least twice daily for 1 week after GnRH agonist treatment (4 × 6 mg Deslorelin acetate implants, Peptech Animal Health, New South Wales, Australia) to assess GnRH-induced LH release. Longitudinal urine samples were analyzed for LH immunoactivity and the results compared to serum profiles. Urine samples were pooled for additional tests: (1) comparison of binding between serial 1:1 dilutions of urine (neat to 1:32 in assay buffer; 50 μl each added to the assay in duplicate) with standard binding curves (parallelism); and (2) mass recovery of added exogenous LH standard preparation (0.04–5.00 ng mL^{-1}).

To assess prolactin immunoactivity, matched blood and urine samples were collected during mid to late gestation (12–18 months; Brown and Lehnhardt, 1995) from a single pregnant Asian and African elephant (n = 10 samples each) and compared to samples from nonpregnant females (n = 10 samples each species). In addition to longitudinal analyses, urine pools (n = 3 pools from at least six samples collected at ~12, 15 and 18 months gestation) also were prepared for tests of parallelism (neat to 1:32) and mass recovery (0.31–40 ng mL^{-1}).

2.2. Hormone analyses

Serum samples were analyzed in duplicate for progestagen immunoactivity using a solid-phase ^{125}I progesterone radioimmunoassay (RIA) (Siemens Medical Diagnostic Solutions, Costa Mesa, CA) as previously described (Brown and Lehnhardt, 1995; Brown et al., 2004a,b). Elephant serum and urine samples were analyzed in duplicate for LH using a double-antibody RIA, validated for elephants, that relied on a monoclonal anti-bovine LH antiserum (518-B7; Coralie Munro, University of California, Davis), ovine ^{125}I-LH tracer and ovine LH standards (NIDDK-oLH-I-4; AFP8614B), and anti-mouse IgG second antibody (#M8645, Sigma Chemical Co., St. Louis, MO; 1:100) (Brown et al., 2004a,b). Serum and urine samples were analyzed in duplicate for prolactin using a validated assay for elephants that relied on an anti-human prolactin antiserum (NIDDK-anti-hPRL-3), ovine ^{125}I prolactin label and standards (NIDDK-oPRL-I-2), and sheep anti-rabbit IgG second antibody (PKC-SARGG, Pool D; 1:300 in buffer with 5% polyethylene glycol; Uniformed Services University, Bethesda, MD) (Brown and Lehnhardt, 1995).

Urine was diluted 1:10 for creatinine (Cr) analysis, and hormone data were expressed as ng mg Cr^{-1}. Assay sensitivities (based on 90% of specific maximum binding) were as follows: 0.039 ng mL^{-1} for the LH RIA, 0.20 ng mL^{-1} for the prolactin RIA, and 0.05 ng mL^{-1} for the progesterone RIA. For all assays, intra- and interassay coefficients of variation, based on analysis of high and low quality control samples in each assay, were <10% and <15%, respectively.

2.3. Urine sample treatments for LH assessment

2.3.1. Addition of glycerol before freezing

For all urine samples, glycerol (7% v v^{-1}) was added to an aliquot of urine before freezing as a cryoprotectant to stabilize the LH (Livesey et al., 1983; Ziegler et al., 1987); samples were frozen at −20 °C until analysis. To test the effect of glycerol, a 20-mL pool of Asian and African elephant urine was split into two 10-mL fractions, and one of the 10-mL fractions was spiked with 5 ng mL^{-1} exogenous LH standard. Each 10-mL aliquot was then divided into two 5-mL aliquots and glycerol (7% v v^{-1}) was added to one of the aliquots. Each 5-mL aliquot was then separated into two 2.5-mL aliquots and one was frozen at −20 °C and thawed 5 times while the other was maintained at 4 °C until analysis (within 1 day of treatment). Urine samples (neat or spiked, frozen once, cooled or repetitively frozen and thawed, with or without glycerol) were diluted 2-fold (neat to 1:128) and tested for parallelism against the LH standard curve. Accuracy and percent recovery tests were performed by adding an equal volume of urine to the LH standards (0.039–5 ng mL^{-1}) to determine if the urine matrix interfered with LH measurements.

2.3.2. Urine concentration

Urine was concentrated using Centricon Ultrafree-4 Centrifugal Filters (10,000 MW; 4-ml capacity; Millipore Corp., Billerica, MA, USA). Pools of urine collected in conjunction with anovulatory, ovulatory and Deslorelin-induced LH surges and were divided into aliquots for treatment with and without glycerol (7% v v^{-1}), and with and without addition of exogenous LH standard (5 ng mL^{-1}). For each treatment, 4 mL of urine were loaded into the tubes and centrifuged at 5000*g* for 60 min. The remaining concentrate (0–100 μL) was reconstituted to a total volume of 350 μL in phosphate assay buffer (pH 7.4). Each sample was analyzed in the LH RIA by comparing 2-fold (neat to 1:64) dilutions to the standard curve. A total of six trials were performed. When these trials revealed no significant binding inhibition, Centricon Plus-70 Centrifugal Filters (70-ml capacity; Millipore Corp.) were tested using 37–70 mL of urine, concentrated ~35-fold. Two LH peak and four baseline LH samples were processed using these larger capacity tubes.

Proteins in urine pools, with and without glycerol (7% v v^{-1}), and with and without addition of 5.0 ng mL^{-1} LH standard, were precipitated by adding increasing amounts of ethanol (ETOH) (1, 2, 3 and 4 mL) to 1 mL of urine. Urine/ethanol samples were vortexed for 30 s, snap frozen in liquid nitrogen and centrifuged at 2600*g* for 10 min. The precipitated protein pellet was re-suspended in 200 μl of buffer for a 5-fold concentration of sample. Samples were then diluted 2-fold (neat to 1:16) and analyzed in the LH RIA. Additional sets of daily urine samples (n = 7) around a natural LH surge (days −3 to +3, 0 = surge) were analyzed neat or precipitated with ETOH (1 mL urine to 5 mL ETOH).

2.3.3. Urine sample treatments for prolactin assessment

Matched urine and serum samples collected during gestation were analyzed for prolactin and compared with values in nonpregnant females. Glycerol (7% v v^{-1}) was added to samples before freezing. Urine pools of pregnant and nonpregnant urine ($n = 3$ each) also were subjected to ethanol precipitation (1 mL urine: 5 mL ETOH) and Centricon-4 centrifugal filtration, with and without addition of exogenous oPrl (20 ng mL^{-1}). Serial dilutions of concentrated and unconcentrated urine, with and without exogenous oPrl were tested for parallelism against the oPrl standard binding displacement curve. Accuracy and percent recovery tests were performed by adding an equal volume of urine to the prolactin standards (0.31–40 ng mL^{-1}) to determine if the urine matrix interfered with urinary prolactin measurements. Two additional samples each were processed with the Centricon-70 tubes for pregnant and nonpregnant urine.

2.4. Statistical analysis

Peak and baseline hormone concentrations were determined for each individual by an iterative process in which high values were excluded if they exceeded the mean plus 2 standard deviations (Brown et al., 1999). The highest concentration within a group of elevated samples was considered a peak. Baseline values were those remaining after all high values had been excluded. To calculate accuracy in neat urine samples, the amount observed (minus the background) versus the amount expected in each immunoassay was plotted using simple linear regression and expressed as r^2. The average percent recovery was calculated as the amount observed (minus the background) over the amount expected in the dilutions that bound closest 50% binding. Data were transformed before analysis to account for non-normality of data. Paired *t*-tests were employed to compare differences in average percentage recovery of exogenous LH added to urine before and after repeated freeze–thawing with or without glycerol. Nonpaired *t*-tests were used to assess mean differences between LH peak and baseline, and pregnant and nonpregnant sample immunoactivity. To determine the percent recovery of exogenous LH or prolactin added to samples before concentration treatment (ethanol precipitation, centrifugal filtration), concentrations in ng mL^{-1} were calculated from the dilution that bound closest to 50% binding and divided by the ng mL^{-1} in the unconcentrated sample. To test for parallelism in neat and concentrated urine samples, the percentage binding of the standard curve and serially diluted samples were compared using simple linear regression. Parallelism was considered significant ($P < 0.05$) when $r^2 > 0.90$ and $b > 0.50$. Data are expressed as the mean ± SEM.

3. Results

Analyses of daily serum samples during the follicular phase of the estrous cycle demonstrated clear elevations in LH above baseline (<1.0 ng mL^{-1}) corresponding to anovulatory and ovulatory surges in both Asian (9–20 ng mL^{-1}) and African (3–10 ng mL^{-1}) elephants, and within a day of Deslorelin implantation (16.8 ng mL^{-1}) in the Asian female. However, no corresponding changes in LH immunoactivity of neat urine samples were identified in longitudinal sample sets (e.g., Fig. 1). All samples bound labelled antigen at >90%, i.e., below the assay sensitivity. Urinary creatinine concentrations averaged 0.32 ± 0.31 mg mL^{-1}.

The addition of glycerol to urine before freezing also did not improve the ability of the LH RIA to detect LH immunoactivity in neat samples. Serial dilutions of neat urine (frozen, cooled or repetitively frozen and thawed) did not demonstrate significant parallelism with the standard curves in any assay, samples consistently remained >90% for all assays tested ($r^2 < 0.90$, $b < 0.50$; $p > 0.05$) (Fig. 2). Glycerol itself had no impact on measurements of exogenously added LH standard, based on tests of accuracy and parallelism ($r^2 > 0.90$, $b > 0.50$, $p < 0.05$) (Fig. 2). No degradation of LH immunoactivity was observed in response to repeated freezing and thawing of spiked urine samples. Quantitatively, the percent recovery of exogenous LH added to refrigerated urine (79.2 ± 2.7% and 82.4 ± 7.9%) did not differ ($p > 0.05$) from samples that were frozen and thawed five times (81.8 ± 1.4% and 88.8 ± 4.4%) with or without glycerol, respectively.

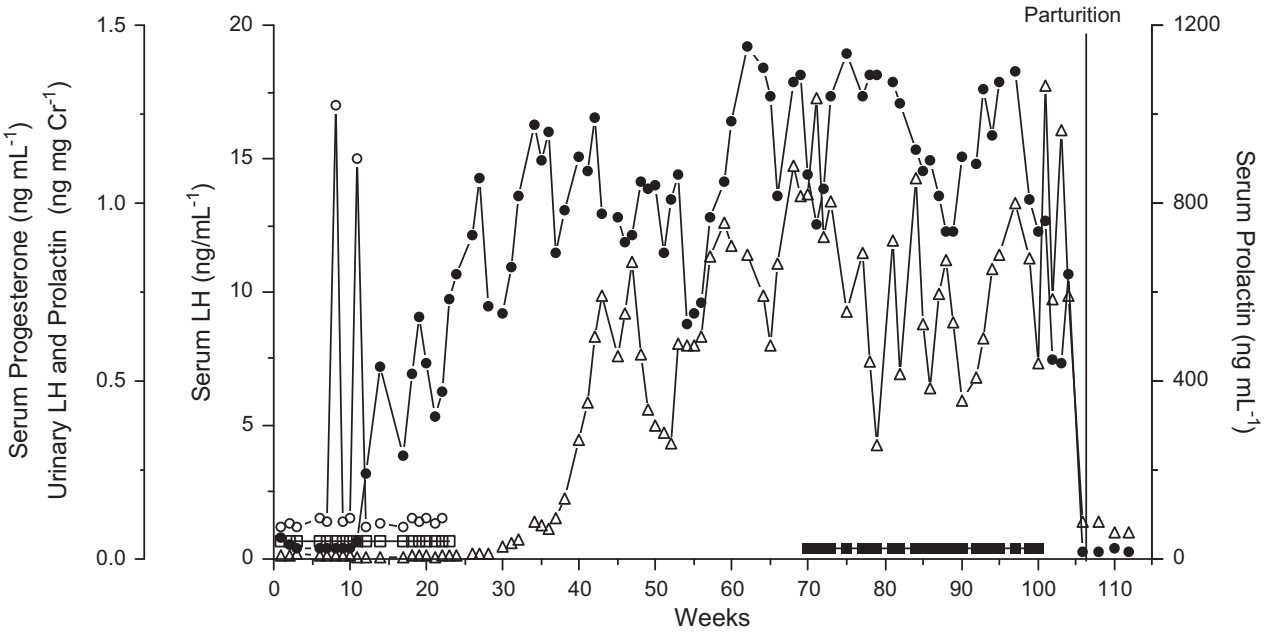

Fig. 1. Longitudinal profiles of serum progesterone (●), serum LH (○), urinary LH (□), serum prolactin (Δ) and urinary prolactin (■) in an Asian elephant during the estrous cycle and pregnancy. Urine samples for LH were collected throughout one follicular phase and corresponding two LH surges. Urine samples for prolactin were collected from 12 to 18 months of gestation. Week 0 represents the start of sample collection. A presumed ovulation occurred during Week 11.

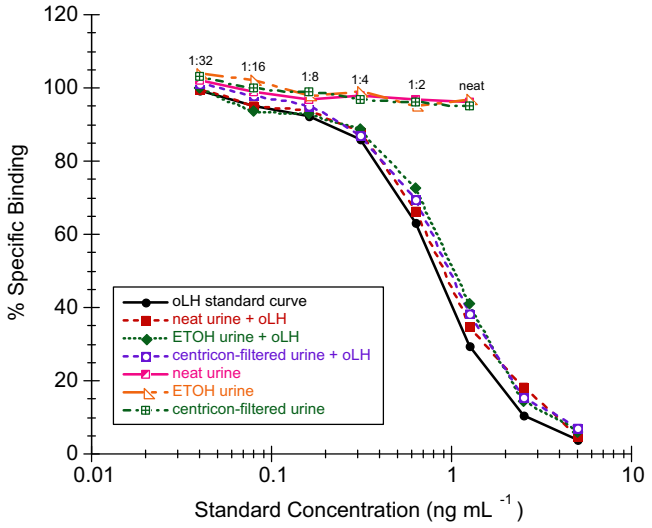

Fig. 2. Specific binding displacement curves for serial dilutions of elephant urine (neat to 1:32 dilution in buffer, 50 μL added to the assay at each dilution) analyzed straight and after concentration by ETOH precipitation or Centricon Ultrafree-4 centrifugal filtration. Specific binding curves were compared to that of the oLH standard curve and to neat and concentrated urine spiked with 5 ng mL^{-1} oLH.

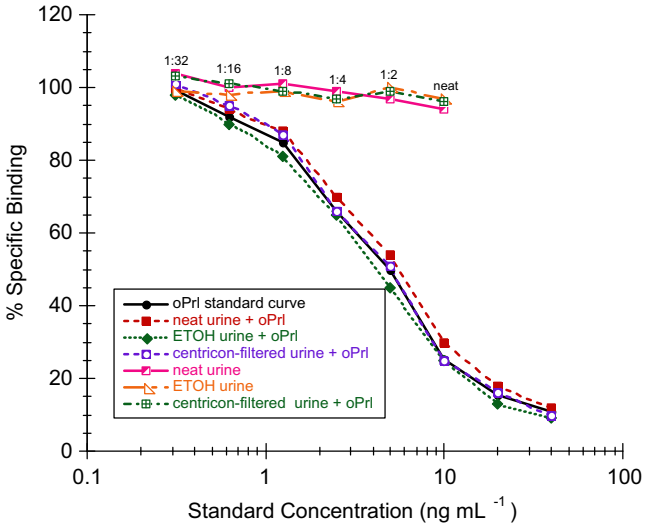

Fig. 3. Specific binding displacement curves for serial dilutions of elephant urine (neat to 1:32 dilution in buffer, 50 μL added to the assay at each dilution) analyzed straight and after concentration by ETOH precipitation or Centricon Ultrafree-4 centrifugal filtration. Specific binding curves were compared to that of the oPrl standard curve and to neat and concentrated urine spiked with 20 ng mL^{-1} oPrl.

Centrifugal filtration using Centricon Ultrafree-4 tubes resulted in about a 6- to 10-fold concentration of urine once the residual was reconstituted, and recovery of exogenous LH exceeded 90% for all spiked samples. However, a similar increase in LH immunoactivity in conjunction with known LH surges was not observed. Percent labelled antigen binding of neat, Centricon concentrated samples consistently remained >90% for all assays tested ($r^2 < 0.90$, $b < 0.50$; $p > 0.05$) (Fig. 2). For samples concentrated ~35-fold, some immunoactivity was observed, but concentrations did not correlate with physiological status. For these samples, urinary LH peak concentrations (0.23 ± 0.05 ng mg Cr^{-1}) did not differ from baseline (0.14 ± 0.04 ng mg Cr^{-1}) ($p > 0.05$), despite a 30-fold difference in serum concentrations (10.2 ± 0.34 ng mL^{-1} versus 0.29 ± 0.07 ng mL^{-1}).

Ethanol precipitation increased the concentration of LH in spiked urine in a quantitative manner (>90% recovery of added LH), but again did not reveal any LH immunoactivity in unspiked samples treated with ethanol (percent labelled antigen binding >90%) ($r^2 < 0.90$, $b < 0.50$; $p > 0.05$) (Fig. 2). There was no difference in the efficiency of ethanol precipitation of exogenously added LH for the varying concentrations of ETOH used.

Serum prolactin concentrations in pregnancy samples were about 100-fold higher than those observed in nonpregnant samples (4.9 ± 0.7 and 6.8 ± 1.6 ng mL^{-1}) for Asian (489.5 ± 32.6 ng mL^{-1}) and African (421.7 ± 23.6 ng mL^{-1}) elephants, respectively. Despite this significant difference in circulating concentrations, no differences in prolactin immunoactivity were detected in any elephant urine sample (e.g., Fig. 1), whether neat or concentrated by ETOH precipitation or centrifugal filtration. Again, labelled antigen binding for all concentrated urine samples was >90% of maximum binding in the prolactin RIA, with no displacement observed upon serial dilution ($r^2 < 0.90$, $b < 0.50$; $p > 0.05$) (Fig. 3). For samples concentrated using Centricon-70 tubes, results were similar to LH; immunoactivity was observed, but urine concentrations were low and did not correlate with pregnancy status (pregnant, 0.19 ± 0.03 ng mg Cr^{-1} versus nonpregnant, 0.34 ± 0.11 ng mg Cr^{-1}; $p > 0.05$), despite the marked difference observed in serum concentrations (443.2 ± 10.1 ng mL^{-1} versus 6.3 ± 0.6 ng mL^{-1}, respectively). There was no evidence of matrix interference, as addition of urine to prolactin standards did not alter the amount expected ($r^2 > 0.99$; $p < 0.05$), and recovery of added prolactin (20 ng mL^{-1}) to urine resulted in >90% recovery after RIA analysis.

4. Discussion

Despite the use of appropriate urine samples that corresponded to hormone increases in matched serum samples, and immunoassays that have been proven valid for measuring these hormones in elephant serum, there was no evidence of biologically relevant LH or prolactin immunoactivity in any of the Asian or African elephant urine samples. The LH 518-B7 antibody used in this study cross reacts with circulating LH of diverse species (Matteri et al., 1987). Serum and urine were collected during spontaneous and GnRH-induced LH surges, which were clearly identified in serum, but not in urine. By contrast, this antibody has proven effective in measuring LH concentrations in urine of other species, including callitrichids (Ziegler et al., 1993, 1996), bottlenose and white-sided dolphins (Robeck et al., 2005), killer whales (Robeck et al., 2004), giant pandas (Kersey, Monfort, Brown, unpubl.), manatees (Iske, Brown, unpubl.) and Przewalski horses (Collins, Flaggs, Brown, unpubl.). For prolactin, sampling took place during gestation, when circulating concentrations were up to 100-fold higher than concentrations in nonpregnant females. Furthermore, to increase the chances of recovering immunoactive hormone in urine, glycerol was added to samples before freezing as a cryoprotectant. This treatment was used because of positive results reported for human urine, where preservation with glycerol prevented loss of FSH and LH immunoactivity (Livesey et al., 1983; Saketos et al., 1994). For samples left untreated, significant loss (up to 80%) of FSH and LH activity were observed after only a few weeks of −20 °C storage. In this study, urine samples preserved with glycerol exhibited no more LH or prolactin immunoactivity than did untreated samples. Nevertheless, it was interesting that repeated freezing and thawing (5 times) of samples with or without glycerol did not cause a net loss in immunoactivity of exogenous LH standards added to elephant urine. Glycerol itself did not interfere with antigen/antibody binding, as determined by similar parallelism and mass recovery analyses of samples spiked with exogenous hormone standards to results from samples without glycerol.

Many studies have evaluated urinary protein hormones, like LH and prolactin, often using neat urine (e.g., Czekala et al., 1988; French et al., 1992, 1999; Ziegler et al., 1993; Miller and Soules, 1996; Robeck et al., 2004, 2005). However, when no immunoactivity was observed in neat samples, either in longitudinal samples or as a result of parallelism tests on urine pools, efforts were made to concentrate samples to ensure the problem was not due to sample dilution. The use of ethanol to concentrate urinary proteins was based on a process called "Cohn Fractionation" (Cohn et al., 1946), which was developed as a means to extract and recover albumin from blood plasma. The procedure relies on the different solubility of proteins to changes in pH, ethanol concentration, temperature and ionic strength. It is a gentle enough process that precipitated proteins retain their biological activity. Because the goal was to precipitate all proteins in urine rather than a specific subset, only a modified version of the ethanol protocol was used. This procedure proved to be very efficient at precipitating urinary proteins, which were then reconstituted in assay buffer at a 5-fold concentration. More than 90% of LH and prolactin added to urine before ETOH treatment was recovered in the precipitant pellet. But, this process did not improve the ability of the immunoassays to detect native excreted LH or prolactin.

The use of Centricon centrifugal filtration tubes to concentrate urine before protein hormone analysis was based on studies conducted in primates (Schradin et al., 2003; Keely and Faiman, 1994; Ziegler et al., 1996). Following essentially the same protocol, using tubes that concentrated urine ~6- to 10-fold, we failed to observe changes in urinary prolactin or LH immunoactivity that correlated with serum concentrations. Evidence that this method was effective in concentrating hormones was provided by the observation of significant recovery (>90%) of exogenously added standard preparations to samples before centrifugation. To further test that concentrated hormones were still too dilute, additional samples were concentrated ~50-fold using Centricon-70 tubes. Though some immunoactivity was detected, it was not biologically relevant as concentrations did not reflect changes in physiological status and were not correlated to serum levels, for either LH or prolactin. Given that differences in serum concentrations between high and low samples were about 30- and 100-fold different for LH and prolactin, respectively, the inability to detect even moderate differences in immunoactivity was discouraging.

The question now remains as to whether these protein hormones simply are not excreted in urine, they are present in extremely low concentrations, or they are not excreted in a form that is recognizable by the respective antibodies. Injection of rats with tritiated ovine LH indicated a rapid clearance from circulation (5-min half-life) by the kidneys without extensive degradation or dissociation into subunits before excretion (Ascoli et al., 1975). However, a small amount of LH was catabolised by the kidney and liver; perhaps this type of metabolism is more extensive in elephants. If subunit dissociation is occurring, the 518-B7 LH antibody would recognize only the beta subunit (Matteri et al., 1987), but some immunoactivity should have been detected. In studies of Asian elephants, urine was found to contain between 60 and 100 $\mu g\ mL^{-1}$ of protein, the majority of which was albumin present as both monomers (66 KDa) and dimers (132 kDa) (Rasmussen, 2001; Lazar et al., 2004). Another major group of proteins in elephant urine were in the 55 kDa range. In looking for lipocalins, which have a molecular mass of ~20 kDa, as possible protein carriers of elephant urinary pheromones, Lazar et al. (2004) reported an absence of proteins in this molecular weight range in the urine. This could explain the apparent lack of protein hormones in urine, as the molecular weight of elephant LH is 26 kDa (McFarlane et al., 1990) and that for elephant prolactin is 20 kDa (Li et al., 1987).

In summary, results of this study suggest that elephants do not excrete appreciable amounts of LH or prolactin in urine. For LH, no immunoactivity was observed in samples collected around spontaneous and GnRH-induced LH surges. Thus, only those elephants that are tolerant of daily blood collection can be considered good candidates for AI. An especially valuable tool would have been the ability to noninvasively diagnose pregnancy in elephants using a single sample analysis of urinary prolactin. Pregnancy diagnosis of wild elephants, where blood sampling is not possible, would permit evaluations of conception rates and pregnancy losses in different populations. For elephants living under captive conditions in range countries (e.g., timber or tourist industries), knowing that a female is pregnant could lead to improved management and welfare conditions through reduced work loads and improved nutrition. Unfortunately, increases in prolactin immunoactivity were not observed in mid- to late-pregnant elephants (12–18 months), despite a nearly 100-fold increase in circulating concentrations. Furthermore, techniques designed to concentrate samples (centrifugal filtration, ethanol precipitation) and protect proteins during freeze/thawing (glycerol) did not alter the ability of our assays to detect hormone immunoactivity in urine. These findings are disappointing as it means it is not possible at the current time to noninvasively monitor LH surges for timing breeding or diagnose pregnancy by prolactin using standard immunoassay analyses of urine samples.

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