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Enzyme Immunoassay Analysis for Androgens in Polar Bear (*Ursus maritimus*) Urine Using Enzyme Hydrolysis

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Abstract

Validation of androgen enzyme immunoassays (EIA) using purified antiserum with a high cross-reactivity for testosterone and 5α-dihydrotestosterone in polar bear urine (PBU) has previously been reported. However, the cross-reactivity of this antiserum with urinary testosterone metabolites was not determined. Therefore, our objective was to perform high performance liquid chromatography (HPLC) analysis of PBU to describe the major immunoreactive androgens and metabolites present, and to determine if enzymatic hydrolysis (EH) of PBU prior to EIA would improve our detection of androgen secretion during different reproductive states. EH of urinary conjugated steroids was performed using ß-glucuronidase-arylsulfatase. Three buffer treatments were tested to determine if buffer type and/or pH negatively influenced EIA parameters. Due to lower matrix interference, PBS (pH 5.0) was selected for subsequent EH. Androgen concentrations of neat and hydrolyzed PBU assayed directly and after extraction were highly correlated ($r \geq 0.954$, $p < 0.012$). HPLC supported these findings, whereby the fraction of neat urine eluting at the same time as the known testosterone-glucuronide standard demonstrated high immunoreactivity after, but not before, EH. Profiles of a breeding and a non-breeding female using parallel fecal, neat PBU, and hydrolyzed PBU samples displayed comparable patterns of androgen secretion, but discrete changes in androgen concentrations were better detected using the latter. These results indicate that accurate analysis of urinary androgen concentrations in the polar bear can be achieved following EH without an extraction step, thus saving substantial time which may critically influence the success of natural or assisted breeding management decisions.

Key Words: enzyme hydrolysis, androgens, polar bear, *Ursus maritimus*, enzyme immunoassay

Introduction

The current population of polar bears (*Ursus maritimus*) in North American zoos is not self-sustaining primarily due to the low numbers of breeding animals, low reproductive success, and high neonate mortality (Meyerson & Mucha, 2014). The current Species Survival Plan (Association of Zoos and Aquariums [AZA] Bear Taxon Advisory Group, 2009) includes basic reproductive biology research to improve natural breeding efforts and the sustainability of this population. Assisted reproductive technologies, such as artificial insemination (AI), could provide an alternative resource when natural mating attempts fail or for institutions housing single sex populations. To achieve success with AI, an extensive understanding of the species' basic reproductive biology, particularly of folliculogenesis and ovulation, is necessary.

The reproductive biology of female polar bears is characterized by a seasonal breeding period, induced ovulation, and delayed implantation (Derocher, 2012). In female polar bears, androgens and not estrogens appear to be the main steroid hormone metabolites excreted in feces in association with estrus and mating events (Stoops et al., 2012). Similar observations have also been reported in the female sun bear (Schwarzenberger et al., 2004). Furthermore, in wild polar bears, no differences in concentrations of 17β estradiol were observed in plasma between single females and females with cubs or yearlings (Haave et al., 2003), or in serum of lactating or non-lactating females or females with or without accompanying males (Gustavson et al., 2015). Validation of

androgen (testosterone [T]) enzyme immunoassays (EIAs) for fecal extracts (Stoops et al., 2012) and neat polar bear urine (PBU) (Steinman et al., 2012) has been previously described. In a study of captive females, fecal T concentrations increased during the breeding season, with brief spikes observed around estrus and mating events (Stoops et al., 2012). Although fecal collection is easier to achieve in current zoological husbandry settings, the processing of samples for hormone analysis is quite labor intensive and does not allow for rapid sample assessment. Alternatively, urine does not require this extensive processing, and the faster assessment time (≤ 1 d vs 3 to 5 d) is more useful when management decisions need to be made quickly for timing of natural mating or assisted reproductive procedures.

Profiles of neat urine show a strong correlation with fecal hormone concentrations in the polar bear (Steinman et al., 2012). The T antiserum utilized cross-reacts with T (100%) and 5α-dihydrotestosterone (DHT; 57%); however, the cross-reactivity of this antiserum with T metabolites commonly found in mammalian urine, such as T-glucuronide and T-sulfate, has not been reported. Therefore, we hypothesized that enzymatic hydrolysis of urine to deconjugate androgen metabolites leaving the parent hormone, T, prior to EIA may provide a better index of urinary androgen secretion in the polar bear. Enzymatic hydrolysis of urinary conjugated steroids using β-glucuronidase-arylsulfatase followed by steroid extraction has been used in other species (pig: Jones & Erb, 1968; primate: Muller & Lipson, 2003). However, sample extraction requires an additional processing step that not all laboratories may have the equipment to perform, and the extraction step would increase the turn-around time needed to perform the assay. The objective of this study was to determine if incorporation of enzyme hydrolysis, with or without an extraction step, into the existing EIA methodology leads to an improvement for measuring urinary androgens via increased detection of testosterone conjugates in the polar bear.

Methods

Animals

Two nulliparous adult female polar bears were included in this study. Female 1 (Studbook #1041, 18 y) was housed with a non-proven (never sired a cub), intact adult male at the San Diego Zoo for the duration of the study period (February through May 2012). Natural breeding occurred from 21 April through 8 May, but the female did not produce cubs. This female has experienced estrus, bred, and ovulated in prior breeding seasons as demonstrated by fecal androgen and progestagen activity (Stoops et al., 2012). Female 2 (Studbook #1051, 19 y) was a non-breeding female, housed with another, unrelated female at SeaWorld San Diego during her study period from January through April 2013.

Sample Collection

Urine samples were aspirated from a previously cleaned and dried enclosure floor using a new syringe (Monoject; Henry Schein, Melville, NY, USA). Samples were placed into a sterile, 15 ml polypropylene tube (BD Falcon; ThermoFisher, Waltham, MA, USA), refrigerated immediately, then aliquoted into multiple 2 ml tubes (Sarstedt, Nümbrecht, Germany) and stored at either -20 or -80° C until analysis. Fecal samples were collected from the enclosure floor into a sterile Whirlpak bag (Whirlpak; Nasco, Modesto, CA, USA) and stored frozen (-20 or -80° C) until lyophilization, steroid extraction, and analysis using previously published methods (Stoops et al., 2012). All samples were collected as part of routine husbandry procedures for the polar bear. All procedures described within were reviewed and approved by each institution's Animal Care and Use Committee and were performed in accordance with the U.S. Animal Welfare Act for the care of marine mammals.

Creatinine Assay

Urine samples were assayed for creatinine (Cr) to standardize sample concentration. Samples were diluted with distilled water (1:100, v:v) and analyzed using a colorimetric assay as described previously (Taussky, 1954). Samples with a Cr concentration below 0.2 mg Cr/ml sample were considered too diluted and not analyzed further. Hormone concentrations were indexed by Cr concentration, and results were expressed as ng of hormone per mg of Cr excreted.

High Performance Liquid Chromatography (HPLC)

HPLC was performed to isolate the immunoreactive androgens and metabolites present in female PBU. An HPLC machine (Beckman System Gold Programmable Solvent Module 125 and Model 168 Diode Array Detector; Beckman Instruments, Brea, CA, USA) was used along with a reverse phase HPLC column (C18, 4 μm, 3.9 × 150 mm, Nova-Pak®; Waters Corporation, Milford, MA, USA). A pool of urine was filtered and concentrated using a SPICE C18 cartridge (Analtech, Newark, DE, USA) to remove contaminants and the eluant dried down. Samples were reconstituted in 0.15 ml methanol (HPLC Grade; ThermoFisher), were resuspended by vortexing, and then 0.05 ml was

loaded onto the column. A gradient of 20 to 50% acetonitrile: water/acetic acid (90/10 v:v, HPLC Grade; Sigma Aldrich, St. Louis, MO, USA) at a flow rate of 1 ml/min over a 60-min run time (0.5 ml fractions collected every 30 s) was used to separate androgens. Known T, T-glucuronide (T-gluc), T-sulfate (T-sulf), androstenedione (A), dihydroepiandrosterone (DHEA), and DHT standards (1 mg/ml) were run prior to each sample to determine where each eluted (as detected by the diode array detector). Because all known standards eluted prior to 30 min, only fractions for the first 30 min of the run were collected and analyzed (fractions 1 to 60). Fractions were evaporated to dryness, reconstituted in 0.25 ml of 0.2 M PBS with 0.1% bovine serum albumin (pH 7.0, EIA buffer), and then stored at -20° C until hormone analysis using the T EIA. The reconstituted fractions were split into two batches and analyzed by EIA: one batch was assayed neat (fractions reconstituted in 0.2 M PBS only), and the second batch underwent enzyme hydrolysis (see methods below) and then was analyzed by EIA. The percentage of immunoreactivity for each peak (a peak was defined as an elevation above baseline that accounted for more than 10% of the total immunoreactivity) was determined by dividing the observed hormone concentration for each immunoreactive peak by the total immunoreactivity (the sum of the observed hormone concentration for all HPLC fractions) measured using the EIA (described below). Sample immunoreactivity, as detected by the EIA, was then compared to the retention times of the known standards to identify the presumptive androgens and metabolites.

Enzyme Hydrolysis

The enzyme β-glucuronidase-arylsulfatase (βG) from *Helix pomatia* (Roche Diagnostics, Mannheim, Germany) was used for enzyme hydrolysis (EH). According to the directional insert for the βG, optimal enzymatic activity occurs during a pH between 4.5 and 6.2, with a 1 M acetate buffer recommended for usage. However, the optimum EIA buffer is typically phosphate-buffered saline (PBS; pH 7.0 to 7.4). Previous studies on urinary EH for T analysis, using gas chromatography and not immunoassays, have utilized both PBS and acetate-based (AB) buffers with a pH range of 6.9 to 7.0 and 5.2 to 5.5 for each buffer type, respectively (see review in Gomes et al., 2009). To determine if buffers and/or pH negatively influenced EH and EIA parameters, βG (0.02 ml) was added to known concentrations of T (range, 12 to 0.046 ng/ml) reconstituted in three different buffers at a final volume of 0.42 ml: (1) 1 M AB, pH 5.0 (AB-5.0); (2) 0.2 M PBS, pH 7.0 (PBS-7.0; PBS comprised of sodium phosphate, monobasic [20%], and dibasic [30%]); and (3) 0.2 M PBS, pH 5.0 (PBS-5.0). As a negative control, βG added to neat buffer of the three treatments described above was also assayed.

Enzyme hydrolysis was adapted from previously published methods (Muller & Lispon, 2003). Briefly, 0.1 ml of urine was added to 0.3 ml of PBS-5.0 and 0.02 ml βG. The samples were incubated in a 37° C water bath for 16 h and assayed immediately following the incubation. For subsequent EH, after the selection of the optimal buffer, a second negative control (blank) comprised only of the optimal buffer and βG was also included in each hydrolysis batch to check for possible matrix interference with the EIA. To demonstrate that the combination of buffer and βG had no negative effect on T EIA parameters when assayed, the negative control was expected to have an optical density (OD) similar to the zero standard OD for the T EIA. Therefore, the minimum recovery (OD of negative control divided by the OD of the zero standard for the EIA) for the second negative control for each hydrolysis batch was set at 85%, and any batch that had a control < 85% recovery was repeated.

Urinary Hormone Extraction

Following EH, hormones were extracted from a subset of samples using previously published methods (Patton et al., 1999). Diethyl ether (5 ml; Acros Organics, ThermoFisher) was added to hydrolyzed samples (0.1 ml urine in 0.3 ml PBS-5.0 and 0.02 ml βG) and then vortexed for 2 min. Samples were flash frozen in a methanol-dry ice bath, and the supernatant was decanted into a 16 × 100 mm glass tube (ThermoFisher). Samples were dried down under air, reconstituted in 1 ml EIA buffer, and then assayed.

Fecal Hormone Extraction

Fecal samples were processed and hormones extracted as previously described (Stoops et al. 2012). Briefly, 0.1 g of the freeze-dried sample was used for extraction by shaking overnight with 3 ml of 90% ethanol. Samples were then centrifuged (1,000 g for 15 min), and the supernatant was decanted and stored at -20° C until EIA analysis.

Enzyme Immunoassay

The same polyclonal antiserum (R156/7; C. Munro, University of California at Davis, USA) was used for both fecal and urinary androgen analysis and was previously validated and described for use in the polar bear (neat urine: Steinman et al., 2012; fecal extracts: Stoops et al., 2012). Antiserum against the emulsified immunogen, T-6-carboxymethyloxime/bovine serum albumin, was produced in one male

New Zealand rabbit. Purification of the gamma globulin fraction of the anti-T antiserum was achieved via ammonium sulfate precipitation, and nonspecific anti-bovine serum albumin antiserum was removed by equivalence zone adsorption as previously described (Munro & Stabenfeldt, 1984). The enzyme conjugate (C. Munro) was made by coupling the horse radish peroxidase to the carboxymethyloxime on the 3 position of the T molecule using a mixed anhydride method (Munro & Stabenfeldt, 1984).

Briefly, for urine, T concentrations were measured using a single antibody, direct EIA. Aliquots of 0.05 ml sample (neat urine, hydrolyzed urine) and standards (range: 12 to 0.046 ng/ml) were added to a 96-well microtiter plate (Maxisorp, ThermoFisher) coated with the aforementioned polyclonal antiserum (1:20,000) along with a T-horse-radish peroxidase enzyme conjugate (1:15,000). Following a 2 h incubation, the plate was washed, 0.1 ml substrate solution (azino-bis-3ethyl benzthiazoline-6-sulfonic acid in citrate buffer, Sigma Aldrich) was added to all wells, and the plate was placed on a plate shaker (400 rpm; MidSci, Valley Park, MO, USA) for 20 to 30 min. The plate was read in a microplate reader (Model 680; BioRad, Hercules, CA, USA) at 405 nm with a 540 nm reference filter. Cross-reactivities of the T antiserum are 100% testosterone, 57% 5α-dihydrotestosterone, 0.27% androstenedione, 0.04% androsterone and DHEA, and < 0.05% with other tested analytes (Dloniak et al., 2004); it was not reported if T-gluc or T-sulf were among these other tested analytes. Assay sensitivity was 0.046 ng/ml, and intra-assay variation was < 10%. Inter-assay variation for two quality controls binding at 30 and 70%, and a low biological control made from a pool of urine was 4.2, 12.8, and 11.9%, respectively ($n = 20$).

Because the cross-reactivity of T-gluc and T-sulf has not been reported, to determine the cross-reactivity of these metabolites with the R156/7 antiserum, a range of samples containing serially diluted concentrations (5,000 to 0.61 ng/ml) of each metabolite standard in EIA buffer were created and analyzed by EIA. The hormone concentration observed (ng/ml) for each metabolite at ~50% binding was then divided by the known concentration of standard added (also at 50% binding) to determine the percent cross-reactivity for each standard.

To determine the efficacy of the urinary EH process and whether or not a hormone extraction step was required following EH, concentrations of neat, hydrolyzed, and hydrolyzed then extracted urine ($n = 10$ samples) were compared for samples collected during the peri-copulatory period of Female 1 and during the non-breeding season for Female 2 ($n = 5$ samples). Hormone profiles of hydrolyzed urine and fecal extracts for both females during the breeding season were also compared.

Results

For the in-house T EIA, the cross-reactivities of T-gluc and T-sulf in EIA buffer containing differing amounts of each metabolite were 0.47 and 0.12%, respectively ($n = 4$). Among the three EH buffers tested, PBS-5.0 displayed the lowest matrix interference as well as high recovery of standard and negative controls performed within expectations (Table 1; Figure 1), and was used for all subsequent EH.

EIA of neat urine fractions separated by HPLC demonstrated the presence of two large immunoreactive peaks that co-eluted with T and DHT (36.3 and 23.9% of the total immunoreactivity, respectively). T-gluc, T-sulf, A, and DHEA constituted 3.5, 0.5, 1.4, and 0.5% immunoreactivity in neat fractions, respectively (Figure 2). After EH of the HPLC fractions, the fraction representative of T-gluc (fraction 17) yielded the highest immunoreactivity in the EIA (26.6%), indicating the majority of conjugated T was in the glucuronide form and, therefore, did not cross-react with the antibody until after deconjugation of the glucuronide moiety during EH, leaving the parent

Table 1. Percent binding of pure buffers, linear regression, correlation (r), r-squared (r^2), and recovery of standards in each buffer type

Buffer type	Mean (± SE) % bound blank[a]	Linear regression	r	r^2	Mean (± SE) % recovery of standard[a]
PBS-5.0[b]	88.4 ± 0.6	$y = 0.89x + 1.90$	0.998	0.996	99.1 ± 14.8
PBS-7.0	85.8 ± 1.2	$y = 0.87x + 4.71$	0.992	0.983	93.8 ± 19.1
AB-5.0	64.5 ± 2.3	$y = 0.64x + 1.96$	0.992	0.984	170.9 ± 58.3

[a] The number of observations for the blank % bound and the standards in buffer tested were 16 and 8, respectively, and were tested in four total assays.

[b] PBS-5.0 was selected as the preferred buffer for further EH analysis.

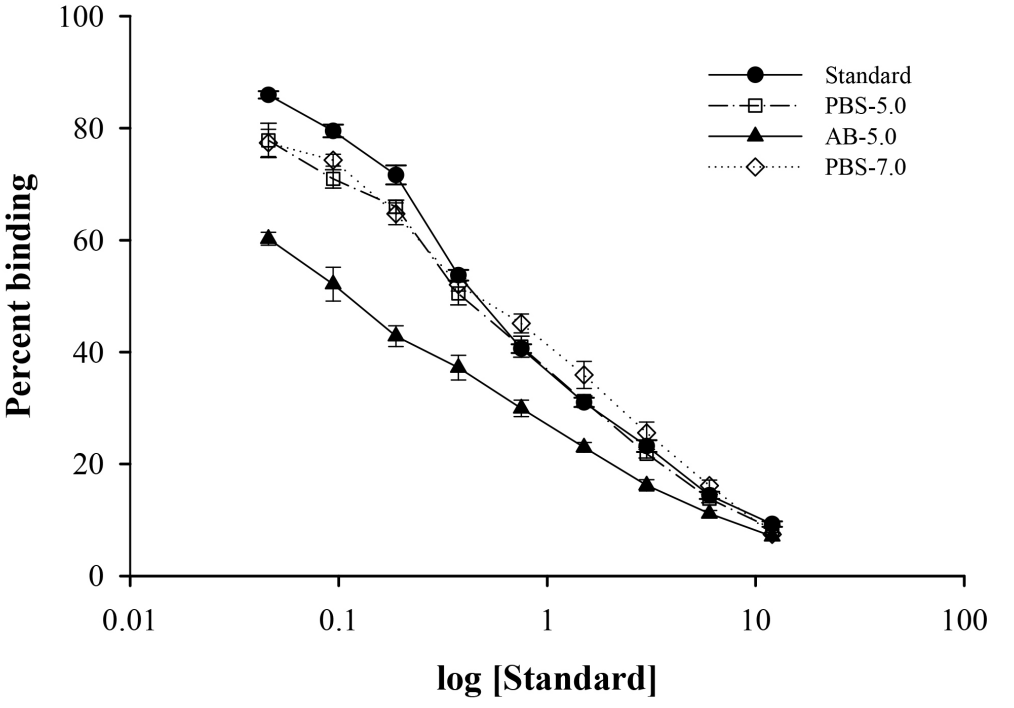

Figure 1. Comparison of standards reconstituted in different buffers. The log of the standards (x-axis) and percent bindings (y-axis, mean ± SE) for PBS-5.0 (open squares, dashed line), AB-5.0 (closed triangles, solid line), and PBS-7.0 (open diamonds, dotted line) were compared to the non-treated, normal standard (closed circles, solid line).

hormone, T. This T-gluc immunoreactive peak, after EH, was followed by smaller peaks that co-eluted with T (8.7%) and DHT (5.7%). Fractions, after EH, collected at the elution time-points for T-sulf, A, and DHEA had low immunoreactivity (1.8, 0.5, and 0.7%, respectively; Figure 2). The HPLC analysis and EH procedures described herein demonstrated that T-gluc is a predominant androgen metabolite in PBU under our experimental conditions.

Urinary androgen profiles for the breeding female (Female 1; Figure 3a) were highly correlated across all three urine sample groups—neat, hydrolyzed, and hydrolyzed then extracted ($r \geq 0.954$, $p < 0.01$). Hydrolyzed urine concentrations were ~sevenfold higher compared to neat urine and ~twofold higher than hydrolyzed then extracted urine. For the non-breeding female (Female 2), urinary T concentrations were also highly correlated across all three sample groups ($r \geq 0.975$, $p < 0.01$) and compared with hydrolyzed urine; concentrations were ~fourfold lower for neat urine and ~two and a half-fold lower for hydrolyzed then extracted urine (graphical data not shown). Overall, fecal and hydrolyzed urine demonstrated similar patterns during the breeding season for Female 1 (Figure 3b) and Female 2 (Figure 3c). However, peak urinary T during the copulatory period for Female 1 occurred 2 d prior to peak fecal T concentrations (1 May and 3 May, respectively).

Discussion

This study is the first to report analysis of androgens in PBU using EH; it confirms previous reports that androgens are the predominant excreted steroid hormone associated with estrus and breeding in this species (Steinman et al., 2012; Stoops et al., 2012; Gustavson et al., 2015). Interestingly, in wild polar bears sampled during the breeding season, serum T was undetectable in all but one female analyzed (Gustavson et al., 2015). However, androstenedione and DHEA were found to be the major androgens in plasma (Gustavson et al., 2015). The role of androstenedione and DHEA and their association with testosterone in the female polar bear estrous cycle is unknown, but future research should examine the potential importance of these androgens.

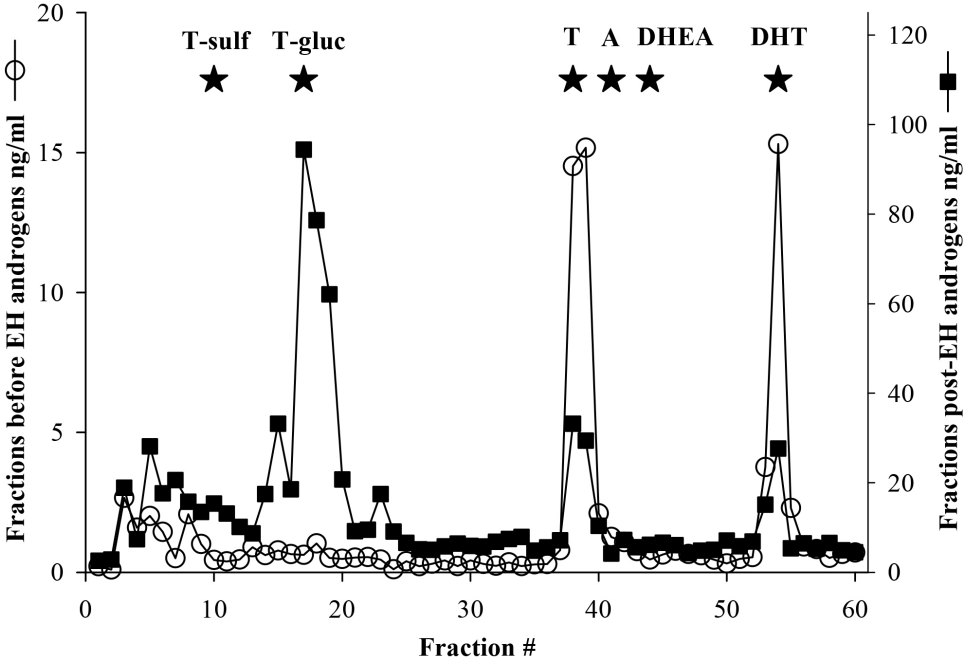

Figure 2. Immunoreactivity of neat urine fractions separated by HPLC with (closed squares) and without (open circles) subsequent enzyme hydrolysis. Immunoreactivity was measured using EIA with antiserum, with high cross-reactivity for T and DHT and low for T-sulf and T-gluc. Elution time points of known standards (T-sulf, T-gluc, T, A, DHEA, and DHT) are indicated by stars.

Our results also demonstrate that EH of PBU without a subsequent extraction step increased detectable urinary androgen concentrations compared to previously described methods for measuring urinary androgens, with concentrations after EH sevenfold greater compared to neat urine. Although PBS was determined to be the optimal buffer for the combination of EH and EIA in the present study, a potential limitation exists in that PBS can inhibit arylsulfatase activity resulting in only deconjugation of the glucuronide and not the sulfate moiety (Roche Diagnostics, directional insert). Thus, the EH procedure described herein may not result in complete hydrolysis of conjugated androgens in PBU, and it may be that only T-gluc is hydrolyzed and then detected by the antiserum as opposed to both T-gluc and T-sulf. Furthermore, pH of the EH buffer may also play a role in the hydrolysis efficiency of the enzyme. Deconjugation of the glucuronide moiety using enzyme derived from *H. pomatia* is best achieved at a pH between 4.5 and 5.0, whereas sulfatase activity is optimal at a pH > 6.2 (Gomes et al., 2009). There is no consensus in the literature regarding how to overcome these issues, and no universal method for EH has been recommended (Gomes et al., 2009).

In the anti-doping field and the World Anti-Doping Agency, where much of the attention to proper androgen conjugate analyses occurs, most of the focus for EH is on steroids with the glucuronide conjugate moiety (Gomes et al., 2009). In circumstances in which the sulfate conjugate moiety is of interest, the current best practices recommendations are for analyses of sulfated conjugates to be accomplished by liquid chromatography/mass spectrometry (Gomes et al., 2009), which was outside the scope of the present study. Despite these limitations, EH of urine in PBS without extraction was sufficient to allow for an increase in the concentration of androgens measured by EIA as evidenced by HPLC analysis, which showed that the predominant immunoreactive peak in urine co-eluted with T-gluc after but not before EH (Figure 2). This is the first report of identification of androgens and their metabolites present in polar bear excreta using HPLC. Again, we do not know if the absence of EIA immunoreactivity of the fraction collected at the T-sulf elution point following EH indicates that conjugation with sulfotransferases (Gower, 2010) is not a major pathway for testosterone metabolism and excretion in the polar bear or was an effect of the

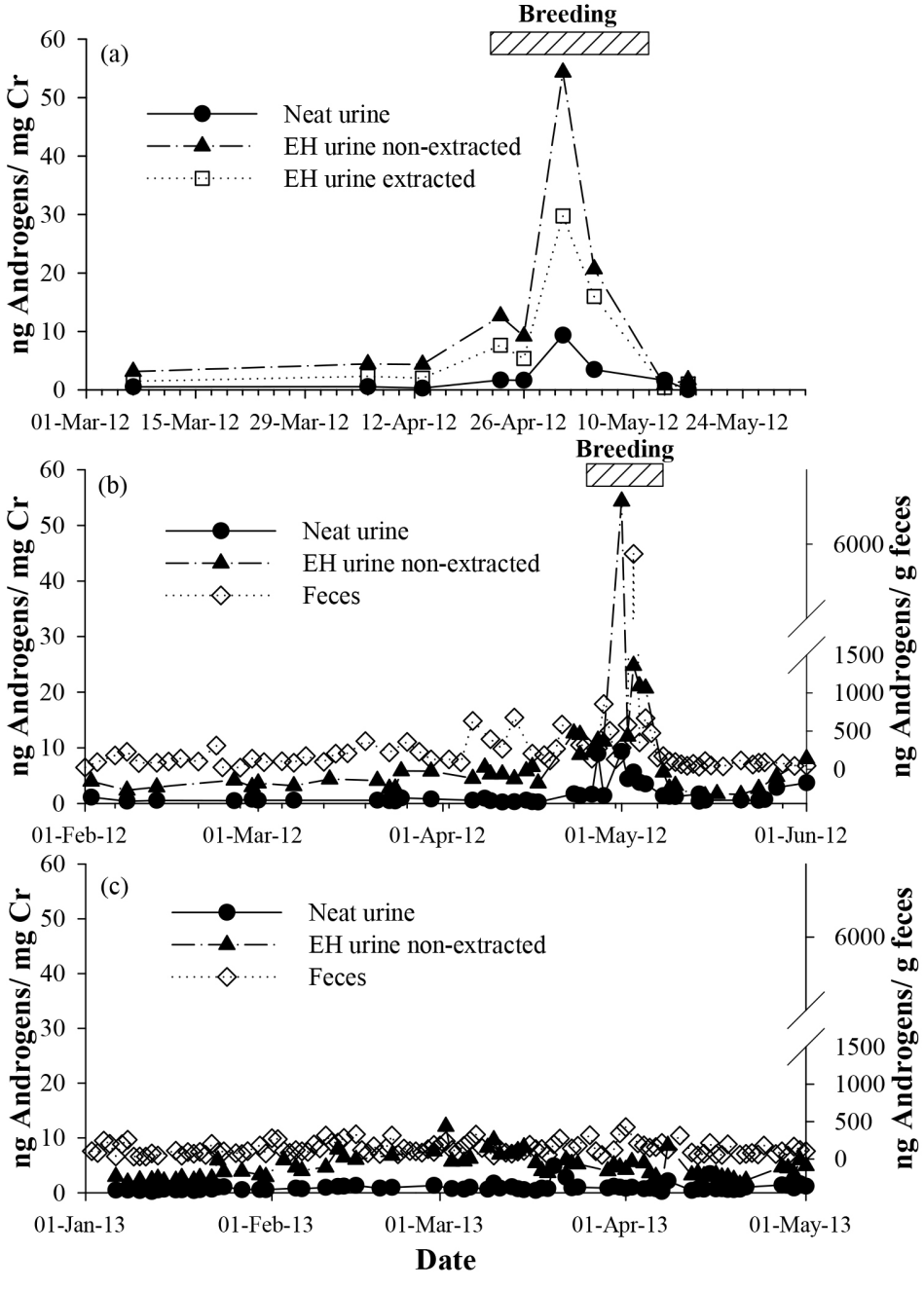

Figure 3. (a) Comparison of androgen concentrations in neat (closed circles, solid line), enzyme hydrolysis (EH) non-extracted (closed triangles, dashed line), and EH extracted (open squares, dotted line) urine in Female 1 during the breeding season; horizontal bar represents days Female 1 bred with a male. (b) Comparison of androgen concentrations in neat urine (closed circles, solid line), EH non-extracted urine (closed triangles, dashed line), and fecal extracts (open diamonds, dotted line) for Female 1 from February through May 2012; horizontal bar represents days Female 1 bred with a male. (c) Comparison of androgen concentrations in neat urine (closed circles, solid line), EH non-extracted urine (closed triangles, dashed line), and fecal extracts (open diamonds, dotted line) for Female 2 from January through April 2013; Female 2 was housed with a non-related female.

buffer type or pH utilized during EH. Nonetheless, we conclude that even if only T-gluc was hydrolyzed, our methodology still leads to improved measurements of total T.

Another factor to consider for EH procedures is the biological enzyme source (i.e., bacterial or animal origin). It has been reported that βG sourced from *H. pomatia* also has 3β-hydroxysteroid activity and may convert androstenediol to testosterone during EH affecting the true quantification of testosterone (Venturelli et al., 1995; Gomes et al., 2009). However, this activity appears to be isolated to one manufacturer of βG (Sepracor) and varied from batch to batch (Venturelli et al., 1995). In the present study, βG was supplied by Roche Diagnostics and, although this product was not tested in the aforementioned study, the lack of increased immunoreactivity of neat urine HPLC fractions after they were hydrolyzed (with the exception of fraction 17 which was identified as T-gluc) indicates that this βG product probably does not contain 3β-hydroxysteroid activity or, at maximum, does but at insufficient quantities to have an effect on the androgen measures presented herein.

The potential of AI and assisted reproductive technologies to increase reproductive success in the captive polar bear population is limited if we do not have an understanding of the basic reproductive biology of this species. This study adds to the existing information of the estrous cycle of the polar bear and describes improvements to existing hormone analyses methodologies. Our results demonstrate that EH of PBU without a subsequent extraction step does improve urinary androgen measures in this species, and results can be obtained within ~20 h of sample collection. This time savings in comparison with the current fecal androgen assay is critical when developing AI protocols and will aid in breeding management decisions that need to be made quickly.

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