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Research Article

Anuran Gender Identification by Fecal Steroid Analysis

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This study tested the hypothesis that steroid hormone metabolites can be measured in anuran feces and their concentrations used to identify the sex of adults. Fecal samples from American toads, *Bufo americanus*, and boreal toads, *B. boreas boreas*, were extracted using ethyl acetate, and the concentrations of estradiol, progesterone and testosterone metabolites were measured by enzyme immunoassays with antibodies commonly used to evaluate steroid hormone concentrations in mammalian species. In American toads, mean testosterone metabolite concentrations ($P < 0.05$) between males (224.3 ± 15.5 ng/g feces) and females (80.7 ± 10.6 ng/g), but estradiol and progesterone metabolite concentrations did not. In contrast, estradiol immunoreactivity differed ($P < 0.05$) between male (19.0 ± 1.8 ng/g) and female (48.3 ± 6.3 ng/g) boreal toads. Progesterone and testosterone metabolite concentrations did not differ. Fecal hormone metabolite analysis offers a promising noninvasive approach to gender identification in anuran amphibians. However, the group of metabolites differentiating gender may not be consistent among species. Zoo Biol 25:35–46, 2006. © 2005 Wiley-Liss, Inc.

Keywords: hormone; gender determination; amphibians; toads; testosterone; progesterone; estrogen

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INTRODUCTION

Declines in amphibian species and abundance have been described since the late 1960s [Pechmann and Wilbur, 1994] and substantial research has been conducted to determine the cause. Characteristics of amphibian physiology and behavior, such as dependence on water and site fidelity for breeding may be contributing to failed re-colonization of previously decimated areas [Blaustein et al., 1993]. Therefore, many of the most critically endangered species have been placed in captive breeding programs to produce self-sustaining populations that offer a reservoir of animals for re-establishing wild populations once environmental problems have been resolved [Young et al., 2000; Rowson et al., 2001; Roth and Obringer, 2003]. Breeding amphibians in captivity, however, is often challenging.

One of the most basic hurdles to amphibian captive breeding programs is determining the gender of individuals within monomorphic species so that males and females can be properly paired for mating. When gender cannot easily be differentiated based on external characteristics, more invasive and often stressful techniques are used and can actually result in the loss of reproductive success [Lasley and Kirkpatrick, 1991]. For example karyotype analysis requires a tissue sample biopsy from the animal in question. In addition to being highly invasive, only 4% of amphibian species that have been examined with this method have sex chromosomes that can be distinguished from one another [Hayes, 1998]. A noninvasive, reliable method for determining gender would be an improvement over current, invasive or unreliable methods.

An alternative to invasive methods of sexing amphibians that has been used in other classes of vertebrates is the comparison of steroid hormone metabolite concentrations excreted by the animal. Steroid hormones can be excreted in either the urine or the feces, and fecal steroids have been used for sex determination in monomorphic birds and mammals. For example, Berkovitz et al. [1978] used droppings from two dimorphic birds, *Gallus gallus murghi* and *Nymphicus hollandicus*, to develop an assay for distinguishing sex in several avian species based on ratios of testosterone and estradiol. Kubokawa et al. [1992] found that the sex of giant pandas (*Ailuropoda melenoleuca*) can be identified based on relative amounts of androgen metabolites excreted in their feces. The adaptation of these methods to amphibians would provide a noninvasive alternative for determining gender.

This is the first study in amphibians to evaluate steroid hormones derived from feces as a means of distinguishing gender. Using two sexually dimorphic anuran species, the specific goals of this study were to: (1) determine if testosterone, progesterone and estradiol metabolites could be measured in anuran fecal samples; (2) evaluate the accuracy of using hormone metabolite concentrations to determine gender and; (3) test the interspecific consistency of this technology.

MATERIALS AND METHODS

Animal Maintenance and Sample Collection

American toads (Bufo americanus)

A population of 13 adult American toads housed at the Cincinnati Zoo and Botanical Garden's Center for Conservation and Research of Endangered Wildlife

(CREW) was used for this study. American toads have a typical range of the eastern United States extending into Canada. They breed seasonally from March to May. The toads used in this study were obtained through hand collections in Northern Kentucky and Southern Ohio between the years of 2000–2002. Although specific ages are not available for these amphibians, all toads had produced gametes in previous reproductive studies; therefore all toads were considered adult.

Toads were housed individually in 16 × 11 × 6 inch plastic tubs. Water was provided ad lib and they were fed 2–3 times/week a diet of 6-week old crickets (Flucker Cricket Farm, Baton Rouge, LA) supplemented with ground vitamins (One Daily Women's- Kroger, Co., Cincinnati, OH). All toads were kept under a light cycle of 10L:14D and constant temperature (25°C). Fecal samples were collected daily from September through May and stored individually in plastic bags at −20°C until hormone extraction; samples ranged from 1.5–4.0 g in weight.

Boreal toad (Bufo boreas boreas)

A population of 77 captive-reared boreal toads was sent from the Colorado Division of Wildlife to the Cincinnati Zoo CREW facility in October of 2001. This species is much smaller, has a typical range from New Mexico to Wyoming, and breeds from May to June; it is considered endangered. Boreal toads were housed in groups of up to eight toads in 20 × 14 × 8 inch plastic tubs. Each tub contained animals of the same sex and from the same clutch of eggs hatched in 2001. Males were known to be mature because spermatozoa were collected from all males during a concurrent breeding study. Maturity of female boreal toads was assumed but unproven. The toads were given water ad lib and fed 4–6 times/week a diet of 4-week old crickets (Flucker) dusted with vitamin supplement (Kroger). Boreal toads were kept under lighting conditions that mimicked a normal daylight cycle and constant temperature of 25°C.

Tanks were checked daily for feces. Fecal samples from specific individuals (when the source of feces could be identified visually) and pooled samples from same-sex tanks were stored separately at −20°C in labeled plastic bags until hormone extraction. Group samples from boreal toads typically ranged from 0.3–1.0 g in weight whereas individual samples typically weighed between 0.2–0.4 g.

Hormone Extraction

All reagents used for hormone extractions and media for hormone assays were purchased from Sigma Chemical Company (St. Louis, MO). Thawed American toad samples were mixed by stirring and an aliquot was removed for extraction. Aliquots (0.5 g) were extracted once with ethyl acetate (ratio 1:20 w/v; g of wet fecal material:ml of ethyl acetate) by vortexing diluted samples and allowing them to mix by rocking for one hour at room temperature. Ethyl acetate was used as an extraction solvent to ensure polar and nonpolar metabolites were obtained from feces. Because boreal toad fecal samples were small, the entire sample, ranging in mass from 0.1–1.0 g, was used for hormone extraction. Boreal toad samples were extracted with ethyl acetate at a 1:10 ratio (w/v), vortexed and allowed to mix by rocking 1 hr at room temperature.

After mixing, samples for both species were centrifuged for ten minutes at 500 × *g*. Aliquots of solvent were removed from each tube, evaporated to dryness, reconstituted in 1.0 ml of assay buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, NaCl, and

1% BSA; pH 7.0), and stored at −80°C until evaluated in hormone assays. Known amounts of authentic testosterone, progesterone, and estradiol were added to some fecal samples before extraction to establish extraction efficiency. This resulted, in some cases, in extraction efficiencies of >100%.

Hormone Assays

Enzyme immunoassays (EIA) were used to quantify steroid hormones according to procedures previously published in detail [Munro and Stabenfeldt, 1984; Gudermuth et al., 1998]. The Clinical Endocrinology Lab at the University of California at Davis provided the polyclonal antibodies, progesterone CL425, which cross reacts over 50% with most 4-pregnene- and 5-α pregnan-metabolites; estradiol R4972, which cross reacts 3.3% with estrone and ≤1% with all other metabolites; and testosterone R156/7, which cross reacts ≤1% for any hormone other than dihydrotestosterone or testosterone. The Clinical Endocrinology Lab at the University of California at Davis also provided horseradish peroxidase-conjugated hormones used in these studies. Briefly, assay plates were coated with diluted antibody (progesterone, 1:6,000; estradiol, 1:10,000; testosterone, 1:10,000) and incubated overnight; standards (progesterone, 7.8–500 pg/well; estradiol, 3.9–250 pg/well; testosterone, 3.125–200 pg/well), blanks and samples (dilution = 1:5 for progesterone; 1:10 for estradiol; 1:30 for testosterone) were added to wells (progesterone, 50 µl; estradiol, 20 µl; testosterone assay plates, 100 µl). Testosterone plates were incubated overnight at 4°C with standards, blanks, and samples before conjugated hormone was added. Fifty micrograms of the horseradish peroxidase conjugated to native steroid hormones were added for each assay plate (dilution = progesterone, 1:60,000; estradiol, 1:50,000; testosterone, 1:60,000) and plates were incubated 2 hr at room temperature. Assay plates were incubated at room temperature for up to 60 min in the presence of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS-$(NH_4)_2$) diluted in citrate buffer (citric acid-anhydrous, pH 4.0). The absorbance ($\lambda = 405$ nm) of each well of the hormone assay plates was determined using a Dynex MRX automatic microplate reader (Dynex Technologies) and a standard curve was used to quantify hormone levels. Hormone metabolite concentrations are presented as ng hormone equivalent/g feces.

Hormone assays were validated by confirming parallelism between absorbance graphs of serially diluted standards and pooled extracts. Additionally, extracts of crickets with and without vitamin supplements, obtained using the methods described previously, were tested for parallelism to determine if the source of the hormones in the toad feces was dietary in origin.

Statistical Analysis

All statistical analyses were carried out using SYSTAT version 10 software. For American toads, a power analysis was carried out initially to determine if the number of toads available for this study was adequate to reliably report differences between male and female toads.

The quantities of each hormone measured in American toad and boreal toad feces were analyzed by an analysis of variance (ANOVA) with month and sex as factors. In addition, each hormone was analyzed as a separate dependent variable. Ratios of testosterone/estradiol, progesterone/testosterone, and progesterone/

estradiol were also analyzed as separate dependent variables. Significance was assessed where appropriate using Student's *t*-test; a $P<0.05$ was considered significant.

RESULTS

Assay Validations

Recognition of the hormone metabolites in the extract by the antibody was indicated by the fact that the added hormone displaced the conjugated form of the native hormone from the antibody in a consistent, proportional manner (Fig. 1). Parallelism between serially diluted American and boreal toad fecal extracts and hormone standards was demonstrated for all antibodies tested, but results only for estradiol are presented in Figure 1. For each species, several extraction ratios were evaluated and that showing the most consistent change in native hormone displacement was used. For the American toad this ratio was 1:20 w/v; g of wet fecal material:ml of ethyl acetate and for the boreal toad this ratio was 1:10 w/v; g of wet fecal material:ml of ethyl acetate.

Previous studies in wild baboons have found that diet does not significantly affect hormone measurements in feces [Wasser et al., 1994]. Data from the cricket extraction and analyses conducted in this study support that finding. Serially diluted cricket extracts demonstrated no parallelism for any of the three antibodies tested confirming that crickets were not contributing to the hormone metabolites measured in toad feces (Fig. 1).

Extraction efficiency (Materials and Methods) for the added steroids in American toad fecal material was 135%, 82%, and 56% for testosterone, estradiol,

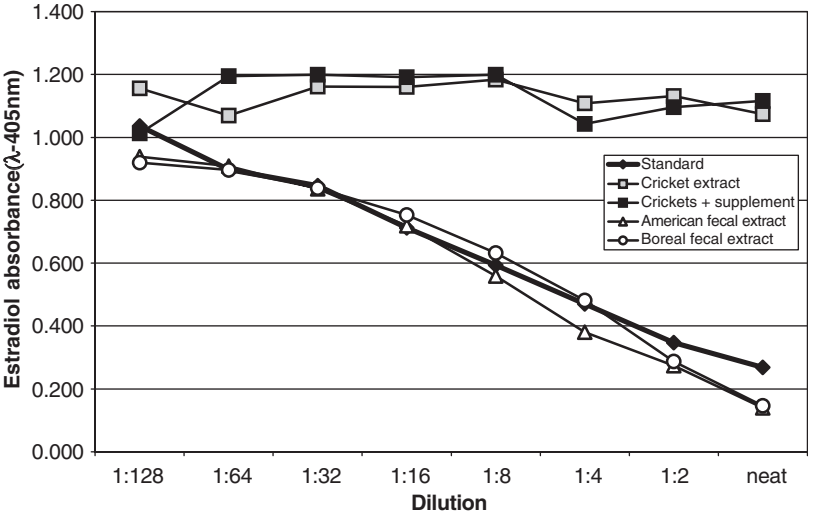

Fig. 1. Validation of the anti-estradiol antibody. Estradiol standard and extracts of American toad feces (extract 1:20 with ethyl acetate), boreal toad feces (extracted 1:10 with ethyl acetate), and crickets with or without vitamin supplement were serially diluted and incubated with the anti-estradiol antibody in a standard EIA. Vertical axis plots absorbance at 405 nm as a function of extract dilution.

and progesterone respectively. For boreal toad samples the extraction efficiency was 126% for testosterone, 119% for estradiol, and 49% for progesterone.

American toad

Hormone metabolite values obtained over the entire sampling period were averaged to obtain a grand mean hormone concentration for each toad. Female American toads excreted slightly higher concentrations of estradiol metabolites than males (Table 1), but these values did not differ statistically ($P > 0.05$). Progesterone values were not significantly different between sexes and the overall values were so low that they approached the lower limits of the assay. ANOVA showed that the amount of fecal extract binding to the testosterone antibody was different ($P < 0.05$) between the two sexes; fecal testosterone metabolite levels in males was almost three times that of females. Plots of the 95% confidence intervals (CI) about the means of testosterone and estradiol metabolites for the American toad show the differences between the sexes (Fig. 2).

In addition, available estradiol and testosterone metabolite levels were calculated as monthly averages and analyzed by ANOVA to confirm that any differences between the sexes were consistent across seasons. Estradiol metabolite concentrations fluctuated throughout the year in both males and females, and consistent gender differences were not observed. In contrast, with the exception of the winter months (December and January) where variability was high and the

TABLE 1. Steroid immunoreactivity in American toad feces[a]

	n	Estradiol	Progesterone	Testosterone
Female	6	107.7 ± 25.6	8.3 ± 1.5	80.7 ± 10.6[b]
Male	7	89.4 ± 12.3	6.6 ± 1.5	224.3 ± 15.5[b]

[a]All hormone concentrations are mean ± SE (ng/g feces).
[b]Denotes significant differences ($P < 0.05$, Student's *t*-test).

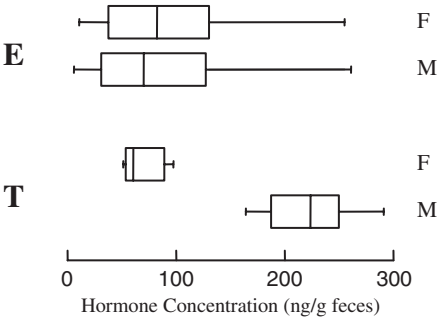

Fig. 2. Confidence intervals for concentrations of fecal steroids from the American toad using antibodies to estradiol (E) and testosterone (T). Center bars represent mean hormone concentrations for each sex, boxes represent 1 SD from the mean, and the horizontal line spans the length of the 95% CI for that sex (males, $n = 7$; females, $n = 6$).

sample numbers low, testosterone metabolites remained higher in males than in females over the collection period (Fig. 3).

Boreal toad

Hormone metabolite concentrations in individual samples (taken when animals were separated for feeding) and pooled tank samples within sex did not differ ($P > 0.05$). Therefore, data were pooled for the final analysis. Unlike the American toads, boreal toad males and females did not differ ($P < 0.05$) in testosterone metabolite concentrations. Likewise, there were no gender differences ($P > 0.05$) in the concentrations of progesterone excreted. Only estradiol immunoreactivity was significantly different for boreal toad males and females ($P < 0.05$). Females excreted more than twice the concentration of estradiol metabolites compared to males (Table 2). Figure 4 shows the 95% CI for concentrations of estradiol and testosterone metabolites excreted by the boreal toads during this interval.

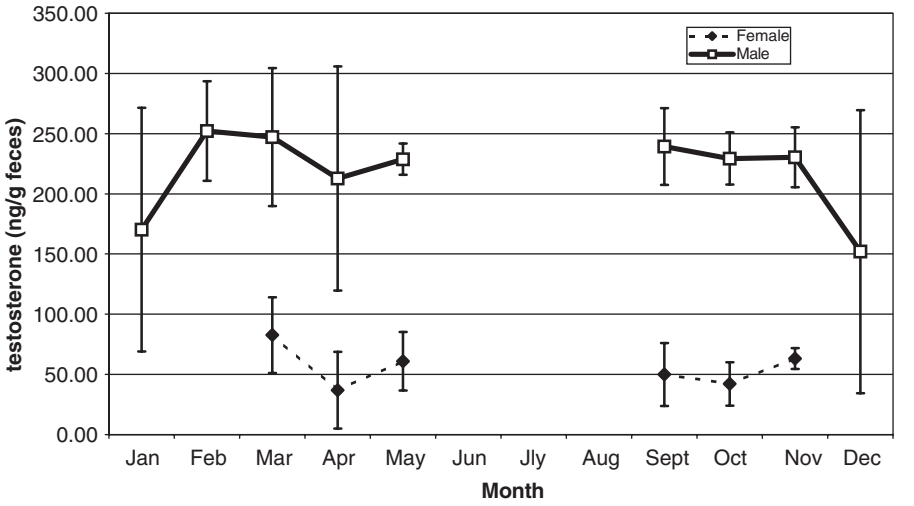

Fig. 3. Monthly testosterone concentrations in the feces of male and female American toads. Data are presented as the mean hormone concentration ± SEM. Samples were not available from female toads for the months of January and June. Males, $n = 5$/month; females, $n = 3$/month.

TABLE 2. Steroid immunoreactivity in boreal toad feces[a]

	n	Estradiol	Progesterone	Testosterone
Female	12	48.3 ± 6.3[b]	3.6 ± 0.3	29.9 ± 3.6
Male	16	19.0 ± 1.8[b]	3.0 ± 0.3	45.2 ± 7.6

[a]Samples were collected from August–November in 2002 and June–August in 2003. Hormone concentrations are expressed as means ± SE (ng/g feces).
[b]Denotes significant difference ($P < 0.05$, Student's *t*-test).

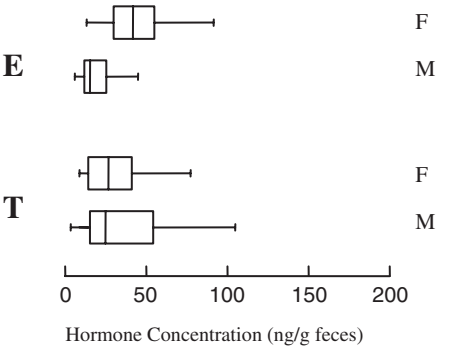

Fig. 4. Confidence intervals for concentrations of fecal steroids from the boreal toad using antibodies to estradiol (E) and testosterone (T). Center bars represent mean hormone concentrations for each sex and the horizontal line spans the length of the 95% CI for that sex. Samples collected from June–November. Males, $n = 16$; females, $n = 12$.

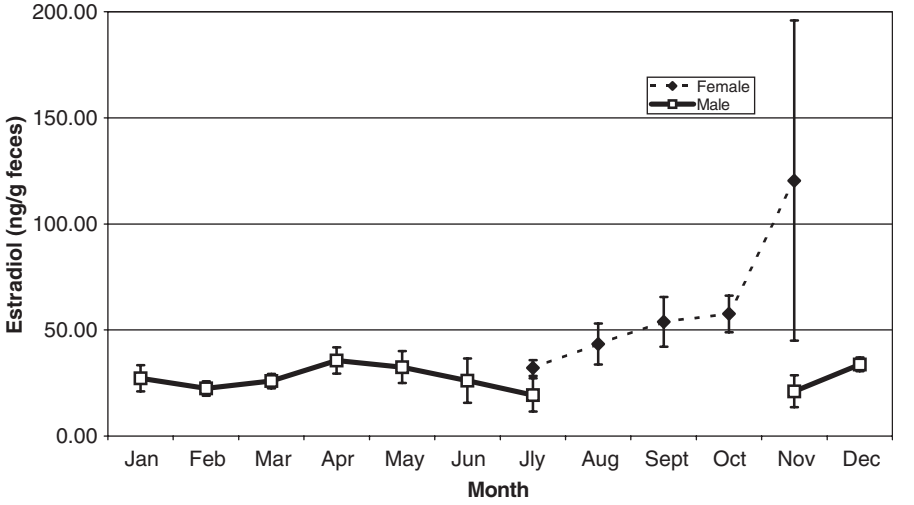

Fig. 5. Monthly averages of testosterone and estradiol metabolite concentrations in the feces of male and female boreal toads over the period when collections were taken. Data are presented as the mean hormone metabolite concentration $\pm$ SEM from both pooled and individual samples. Males, $n = 8$; females, $n = 8$ (except for November, $n = 5$).

Like the American toads, boreal toads varied in the concentrations of hormone metabolites they excreted over the sampling intervals. Gender differences in the concentration of testosterone metabolites were absent (data not shown). Because male and female samples were collected during different months, it was not possible to directly compare steroid metabolite levels in boreal toads each month. However, males excreted relatively constant amounts of estradiol metabolites over time whereas estradiol immunoreactivity was slightly but consistently higher in females (Fig. 5).

DISCUSSION

This study is the first report of fecal hormone metabolite analysis in amphibians and provides encouraging data supporting the use of this noninvasive technology for sex determination in anurans. Specific hormone metabolites excreted by the toads were not identified in this study, nor are there existing published reports of steroid metabolites excreted in the feces of any amphibian. Previous studies of amphibian hormones have focused on androgens with testosterone or 5α-dihydrotestosterone described as the major plasma androgen in different amphibian species [Kime and Hews, 1978; Delgado et al., 1989; Canosa and Ceballos, 2002]. Because there are many possible ways amphibians process steroid hormones, it is difficult to predict the identities of metabolites they excrete, and one may anticipate species differences in the identity of secreted hormone metabolites. Regardless, the testosterone, estradiol and progesterone antibodies and EIA protocols, well established for mammalian and bird species [Munro and Stabenfeldt, 1984; Lasley and Kirkpatrick, 1991; Bamberg et al., 1991; Gudermuth et al., 1998], seemed to be effective for measuring steroid metabolite immunoreactivity in toads. Hormone metabolites derived from feces in other species often include more than a single form [Shille et al., 1990; Lasley and Kirkpatrick, 1991; Goymann et al., 1999] and it is unknown if the immunoreactivity detected in toad feces was derived from multiple metabolites or a single primary metabolite.

Ultimately, the goal of this study was to determine if sex-specific differences in hormone metabolite excretion in captive amphibians could be used to distinguish males from females. Because captive animals are often maintained in controlled environments, this study was carried out under laboratory conditions. In the American toad, it seems that fecal testosterone metabolite concentrations clearly can be used to determine the sex of adult animals. Furthermore, because there was no overlap in the 95% CI surrounding mean testosterone concentrations for males and females (Fig. 2), one would expect this method of gender identification to be accurate. In contrast, estrogen or progesterone metabolite concentrations would not be useful for determining the gender of American toads because there is no statistical difference between males and females. In general, variation within individuals was high in raw measurements of testosterone, estradiol, and progesterone.

To confirm that distinguishing gender based on differences in hormone metabolite immunoreactivity is applicable to more amphibian species than just the American toad and under different captive conditions, feces from male and female boreal toads were also evaluated for testosterone, progesterone and estradiol metabolite concentrations. In support of our findings in American toads, gender identification by fecal hormone metabolite analysis may also be possible for the boreal toad. However, the differentiating hormone does not seem to be testosterone. In fact, testosterone metabolite concentrations differed little between the sexes in this toad species. Although female boreal toads excrete more estrogen metabolites than their male counterparts, the 95% CI does overlap, suggesting that this technique may be only marginally effective in distinguishing between the sexes in this species. Likewise, direct comparisons should be made monthly in this species to verify the effectiveness of this method over time. Further research on the boreal toad is needed to confirm whether or not this technique is reliable in identifying gender.

TABLE 3. Ratio of progesterone metabolite levels to fecal sex hormone metabolite levels in two anuran species

Sex	*n*	Progesterone:Estradiol	Progesterone:Testosterone
Bufo americanus			
Female	6	0.12 ± 0.04	0.12 ± 0.02[a]
Male	7	0.09 ± 0.02	0.03 ± 0.01[a]
Bufo borealis borealis			
Female	12	0.07 ± 0.01[a]	0.13 ± 0.02
Male	16	0.27 ± 0.04[a]	0.10 ± 0.02

[a]Denotes significant differences ($P < 0.05$, Student's *t*-test).

Previous studies distinguishing gender based on fecal hormone metabolite concentrations relied on differences in hormone metabolite ratios as well as absolute differences in estradiol, progesterone, and testosterone metabolites between males and females. Velloso et al. [1998] found that the ratio of progesterone/testosterone was best for distinguishing between males and females for their study of maned wolves with 80% of randomly selected samples being correctly identified during anestrus and estrus, and 100% correctly identified during the luteal phase. In the present study, progesterone metabolites exhibited minimal variation between sexes in both species and varied less than estradiol or testosterone metabolites over time. In light of the differences of the sex-determining hormone between American and boreal toads, and the anticipated variations of testosterone or estradiol metabolites over time, ratios of progesterone metabolites to those of testosterone and or estradiol might be a more accurate predictor of gender than the absolute quantities of sex hormone metabolites alone. When expressed in this manner (Table 3), the ratio of progesterone metabolites to those of sex hormones were as good a predictor of gender as were concentrations of testosterone or estradiol metabolites alone. In our study, one benefit of using ratio analysis was increased uniformity among the values, reducing problems potentially caused by seasonal changes in sex hormone levels or metabolites.

Hormone metabolite measurement variability is a concern when using hormone metabolite immunoreactivity and low sample sizes to determine the sex of an animal. Hirschenhauser et al. [2000] found that in domestic geese, measurements of fecal metabolites were much more variable than those of plasma hormones. One source of variability may be differences in fecal production [Bamberg et al., 1991]. Variation in excretion rates in conjunction with temporal differences in circulating hormone levels could exacerbate hormone metabolite concentration variability. Atkins et al. [2002] found that in blue-tongued lizards fecal production varied by season, and that in times of reduced feces production, fecal testosterone metabolite concentrations were higher. Additionally, length of sample storage before analysis can increase variation in hormone measurements. For example, an increase in variation was directly linked to time of sample storage when measuring hormones in baboons [Lynch et al., 2003]. Sources of variability were considered and precautions taken to minimize their effects on analyses of metabolite concentrations. Individual samples were averaged by month to reduce sample-to-sample and sampling frequency differences for each toad. Additionally, to reduce variability

within an individual sample, feces were mixed before extraction according to the guidelines recommended by Millspaugh and Washburn [2003]. The amount of storage time at −20°C was not controlled, however, and may have affected the immunoreactivity. Future work should take these problems into consideration when designing experiments by determining production and excretion rates of feces and setting a maximum storage time before samples are extracted. Furthermore, several samples from each individual should be analyzed and averaged to avoid misleading results due to an unusually concentrated or dilute sample.

This study is the first to use hormone metabolites extracted from feces to distinguish gender in amphibians. The method shows promise in that gender differences are present throughout the year despite expected fluctuations in circulating sex steroids due to breeding seasonality. Their use in distinguishing gender in amphibians, however, must be determined on a species by species basis as the differentiating hormone can vary. For each species it will be necessary to identify the differentiating hormone before employing this methodology to predict gender. Further, based on the variability of hormone metabolite concentrations in this study, future applications of this technique should rely on multiple samples to determine gender of an individual amphibian.

CONCLUSIONS

Fecal steroid metabolites can be measured in toads using enzyme immunoassays and antibodies previously employed for monitoring fecal steroid metabolites in mammals. Gender determination based on fecal steroid metabolite concentrations seems possible in adult anurans and the hormone metabolite differentiating gender may differ across species.

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