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ABSTRACT

Successful maintenance of captive species relies on propagation of new generations. One challenge to successful propagation of captive amphibian species is the accurate determination of an animal's sex, because many species of amphibians lack phenotypic differences between males and females. Genotype analysis and laparoscopy can be used to identify sex in some species, but these methods are invasive, which may lead to problems such as disease that could hinder reproduction.

This project utilizes steroid hormone metabolites extracted from feces to identify the sex of adult anuran amphibians. Feces from the American toad, *Bufo americanus*, and the boreal toad, *Bufo boreas boreas*, were extracted using ethyl acetate and the concentrations of hormone metabolites were measured using enzyme immunoassay. For the American toad significant differences were found between males and females in the average concentration of testosterone metabolites they excreted as determined by enzyme immunoassay. For the boreal toad significant differences between the sexes occurred in the average amount of estradiol immunoreactivity they excreted. The use of hormone metabolites derived from feces to distinguish sex is an improvement over current, invasive methods to sex amphibians, although the group of metabolites showing gender differences is not consistent from species to species.

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INTRODUCTION

Descriptions of declines in amphibian species and abundance have been described since the late 1960s (Pechmann and Wilbur, 1994). Many of these accounts were initially dismissed because of insufficient long-term population information. In 1994 the Declining Amphibian Population Task Force (DAPTF) was formed to investigate these claims, and in 1997 they concluded that declines in amphibian diversity and abundance were not part of natural population fluctuations and were a cause for concern (Roth and Obringer, 2003).

Since this time there has been a great deal of effort put into determining the causes of amphibian disappearances. While likely causes such as habitat destruction, exposure to increased amounts of UV-A and UV-B radiation, and infections by novel pathogens probably all play a role in the declines (Young et al, 2000), no real solutions have been proposed to stop wild populations from dying off. In addition, characteristics of amphibian physiology and behavior, such as dependence on water and site fidelity for breeding may prevent successful re-colonization of already decimated areas (Blaustein et al, 1993).

As such, many of the most critically endangered species have been placed in captive rearing programs with the intent of reintroduction into the wild (Young et al, 2000). However, wild populations continue to dwindle and it becomes increasingly difficult to obtain clutches of eggs for captive rearing; therefore the long-term survival of many species requires that the animals be bred in captivity. Self-sustaining captive populations, therefore, offer a reservoir for threatened species that could be used to re-

establish wild populations once environmental problems have been resolved (Rowson et al. 2001).

Species with monomorphic sexes provide a particular challenge to captive breeding of amphibians. The basic breeding strategy of pairing males and females is made difficult in these species because sex cannot be easily differentiated. Genetic sex determination and laparoscopy have been used successfully with amphibians to identify sex, however these techniques are invasive and of limited use (Hayes, 1998 and Roth and Obringer, 2003). Hormone ratios derived from feces have been used in other vertebrates to differentiate between males and females (Bercovitz et al., 1978, Kubokawa et al, 1992, and Velloso et al, 1998), and may be a way to distinguish sex in amphibians. Using feces to obtain hormones would be advantageous because it would be noninvasive and applicable to a broad range of amphibian species.

In order to develop a reliable method to identify sex, males and females must be verified by means independent of hormones. The species that will be used in this study, *Bufo americanus* and *Bufo boreas boreas*, have dimorphic secondary sexual characteristics providing a way to validate sexual identity without sacrificing animals. Males of both these species display dark, roughened skin on the inner digits of their forelimbs and are capable of vocalization. Females do not possess this skin and are incapable of producing sound. It should be noted that not all bufonids are sexually dimorphic (Noble, 1954). The use of sexually dimorphic species to develop a method of fecal hormone analysis provides continuing feedback as to the fidelity of this method in distinguishing sex.

The goal of this study is to determine if the sex of adult male and female toads can be predicted by the amounts and ratios of androgens, estrogens and progestagens in their feces. Specifically, this project will determine whether concentrations of excreted androgens are higher in males and concentrations of excreted estrogens and progestagens are higher in females. The key question is whether or not hormone metabolites extracted from feces and assayed quantitatively can reliably predict sex in amphibians. This study tests the hypothesis that concentrations of excreted androgens are higher in feces from male toads and excreted estrogens and progestagens are higher in feces from females.

LITERATURE REVIEW

Amphibian sex determination

There are three orders of amphibians: Anura, which include frogs and toads, Caudata, which are mainly salamanders, and Gymnophiona, which are caecilians. Many species of amphibians, particularly in the Anuran order of amphibians, display phenotypic differences between males and females, or sexual dimorphism. In these species males typically display roughened calluses, or nuptial pads, on the inner digits of their forelimb or near the junction of the upper arm and the rest of the body (Duellman and Trueb, 1986). Nuptial pads appear at sexual maturity and are used to aid in clasping the female for mating (Duellman and Trueb, 1986 and Noble, 1954). These pads are typically present year-round allowing captive animals to be assigned sex with minimal handling. Other anuran amphibians, such as the toad *Bufo vulgaris*, do not possess these traits. In addition, non-anuran amphibians mostly found in the Caudata order, do not display overt sexual dimorphism year-round. These species may display sexual dimorphism only during the breeding season when males exhibit swollen glands around their cloaca or they may lack sexual dimorphism altogether. Even in species that typically display seasonal sexual dimorphism in the wild, those same characteristics may not be displayed when in a captive situation (Roth and Obringer, 2003). Therefore other methods must be employed to determine sex.

It is important to know the sex of these animals in order to execute basic breeding strategies and maintain populations in captivity, however chronic stress can result in the loss of reproductive function (Lasley and Kirkpatrick, 1991). Invasive techniques add to

stress caused by captivity because they require restraint of the animal, can potentially cause pain, and may lead to disease. Genotype analysis is one such example. This technique commonly involves looking at the morphologies of chromosomes in the metaphase stage of cell division, requiring a tissue sample from the animal in question. Not only is this method of determining sex invasive, but only 4% of amphibian species that have been examined with this method have sex chromosomes that can be distinguished from one another (Hayes, 1998) making the use of genotype analysis extremely limited. In large amphibians, such as the Japanese giant salamander (*Andrias davidianus*), researchers have used a laparoscope inserted into the animal's abdomen to view the gonads in order to determine sex (Roth and Obringer, 2003). As these examples show, current methods of distinguishing sex in many monomorphic amphibian species are stressful and ineffective for broad numbers of species. A noninvasive method for determining sex would decrease the risks involved in sexing these amphibians and may lead to greater reproductive success by reducing the stress associated with gender identification.

Benefits of Fecal Hormones

An alternative to genotype analysis and laparoscopy that has been used in other classes of vertebrates is to determine the amounts of steroid hormone metabolites excreted by the animal in order to identify sex. Steroid hormones can be excreted in either the urine or the feces. Fecal steroids have been used for sex determination in monomorphic birds and mammals, estrus and pregnancy determination in a variety of mammalian species, and to increase the success of fertility procedures such as artificial insemination (Schwartzenberger et al., 1996 and Walker et al., 2002). While many of

these procedures are not applicable to amphibians directly, the use of hormone metabolite ratios derived from feces for distinguishing between males and females would be highly desirable as a noninvasive alternative to the present ways sex is distinguished in monomorphic amphibians.

Feces offer several advantages as a source of hormones. One major benefit is that metabolite concentrations can be 2 to 4 times that of the parent steroid found in blood (Lasley and Kirkpatrick, 1991), allowing detection of hormones that are of low concentrations in blood. Also, fecal hormones represent a sum measure of plasma hormones over time (Goymann et al., 1999) preventing changes in hormone levels caused by experimental conditions from being missed due to sampling error. Fecal metabolites are stable for a relatively long period of time making their collection and storage practical for a captive environment (Lasley and Kirkpatrick, 1991). Finally, feces can be collected without contact between the animal and the investigator preventing stress to the animal and protecting both the collector and the subject from harm (Lasley and Kirkpatrick, 1991).

Hormone Biosynthesis and Metabolism

It is useful to know the roles of steroid hormones and how they are metabolized in order to understand how they can be used to predict sex. Steroid hormones are lipid soluble chemical messengers used to control functions within an organism. The three major categories of steroid hormones that are responsible for controlling sexual development and reproductive events are the androgens, such as testosterone, which are commonly associated with the development and maintenance of male characteristics, and the estrogens and progestagens that are commonly associated with the expression of

female characteristics. In general, these types of hormones are produced in the testes for males, the ovaries for females, and in the adrenals of both sexes, using cholesterol that is either stored in the cell or circulating in the blood (Norris, 1980) as a precursor.

In the first step of steroid hormones biosynthesis, cholesterol is converted to pregnenolone (Canosa et al., 1998). Steroid hormones can then be synthesized through two possible pathways, the 4-ene pathway or the 5-ene (Canosa and Ceballos, 2001). The enzymes and products produced by these pathways are conserved among vertebrate species with minor changes in the properties of the enzymes resulting in some variations (Canosa et al., 1998). Steroid hormones secreted into the blood are modified in the liver into steroid sulfates or glucuronides, which makes them more soluble in blood but also reduces their biological activity (Norris, 1980). These modified forms of the hormones can then diffuse into the bile from biliary capillaries in the liver and enter the small intestine to be eliminated in the feces (Sperber, 1959 and Morrow et al, 2002). Some of these same metabolites may also be reabsorbed and excreted through the kidneys (Bamberg et al., 1991).

The amounts of hormone metabolites excreted in the urine and the feces vary from species to species; in some, hormones are voided preferentially in one form or the other. For example, Schwartzenberger et al. (1996) report that in many mammalian species the main route of estrogen excretion is in the urine, while in the maned wolf 97% of testosterone metabolites are excreted in the feces (Velloso et al., 1998). In amphibians the main excretory routes of hormones are not known. While Brem et al. (1988) has shown that aldosterone metabolites are produced by the toad urinary bladder; urine collections are not practical for amphibians because many species are aquatic and

hormones would be diluted into their environment. The use of feces to obtain steroid hormones from amphibians allows hormone concentrations to be measured without stress to the animal and is practical for use in both aquatic and terrestrial species of amphibian.

Identification of Amphibian Hormones

There are no to date studies that identify the steroid hormone metabolites amphibians excrete in their feces. A few examples of steroid hormone metabolites that have been reported from feces in other vertebrate species include estradiol conjugated to sulfates and glucuronides, estrone sulfate and glucuronide, unconjugated forms of these hormone metabolites, 5α-dihydrotestosterone (DHT), conjugated androgen metabolites, and pregnanediol-3-glucuronide (Shille et al., 1990, Lasley and Kirkpatrick, 1991, and Goymann et al, 2002).

There are several studies in amphibians that have identified androgens synthesized by the testes. Muller (1977), for example, incubated testes fragments with different precursor hormones and found that in urodele amphibians the major testicular product is testosterone. Delgado et al. (1989) used testosterone in their studies of *Rana perezi* because their research suggested it was the dominant plasma androgen in this species. Conversely, the major androgen products in other male anuran amphibians have been found to be (DHT) and other 5α androgens along with lower amounts of testosterone (Kime and Hews, 1978 and Canosa and Ceballos, 2002). Studies of the toad *Bufo arenarum* have found that androgen biosynthesis proceeds mainly through the 5-ene pathway, however there are differences in a few of the side products produced from those of other species using this pathway (Canosa and Ceballos, 2002). Studies using *Rana esculenta* suggest that it may rely on the 4-ene pathway for synthesizing androgens

(Canosa and Ceballos, 2001). Because there are many possible ways amphibians produce steroid hormones, it is difficult to predict the identities of metabolites and one may anticipate species differences in the identity of secreted hormone metabolites.

The steroid products excreted in feces are generally considered to be conjugated forms of hormone metabolites, however Wasser et al. (1994) and Velosso et al. (1998) found a higher percentage of free hormones than conjugated metabolites in mammalian feces. The identification of hormone metabolites is important because antibodies that have been made against native hormones may or may not recognize hormone metabolites. Because biological samples contain known and unknown metabolites, the polyclonal antibodies used for EIA typically recognize many closely related compounds. This allows antibodies to a single hormone to cross-react with similar metabolites. For example, the testosterone antibody used in this study cross-reacts 57% with DHT, a testosterone metabolite that shares many structural similarities with testosterone (Norris, 1980).

To identify specific hormone metabolites in feces, previous studies have used high-pressure liquid chromatography (HPLC) (Wasser et al., 1994 and Walker et al., 2002). In HPLC, chemical properties of each hormone determine how quickly it will pass through a column of coated beads. The time it takes for each hormone to pass through the medium in the column and be detected is consistent for a given solvent allowing peaks that co-elute with a standard to be identified. Compounds that do not match the standard are identified as more or less polar than the closest hormone standard (Walker et al., 2002). This lack of specificity in identification is one disadvantage of HPLC, but can be overcome by using a broad number of compounds in the standard or by collecting

fractions from the HPLC and resolving unknown compounds with a mass spectrometer (Chang and Weng, 1994).

Summary

Steroid hormone metabolites from feces have been used successfully in a variety of species to identify sex. Similarities in hormone biosynthesis and hormone products between species offer hope that this technique may be effective at identifying sex in amphibians as well. The use of feces as a source of hormones for this group would allow sex to be determined for these animals without resorting to stressful procedures that could impair reproductive function; therefore, using a noninvasive technique for determining sex may lead to greater reproductive success with captive amphibians.

For this study, feces were collected from two species of sexually dimorphic anuran amphibians in the bufonid family and assayed for hormone metabolite concentrations using antibodies developed against native hormones. There are several reasons that bufonids are appropriate for this initial study. First, bufonids are largely terrestrial making the collection of feces convenient because it is unlikely the feces will be diluted or washed away. Second, bufonids comprise a large number of closely related yet diverse species making an assay developed for one species likely to be applicable to a number of other species (Noble, 1954). Finally, many endocrine studies have been done with this family meaning others have used it as a model for amphibian studies (Idler, 1972, Itoh et al., 1990, and Pankak-Roessler and Norris, 1991).

The use of sexually dimorphic species will allow assay results to be confirmed without sacrificing animals. Differences in fecal hormone metabolite concentrations between males and females might be expected to show that males excrete high

concentrations of testosterone metabolites and females excrete high concentrations of estrogen metabolites. If there are significant differences in the levels of androgens and/or estrogens excreted by males and females, these differences may enable sex to be identified in animals that do not possess obvious phenotypic gender differences.

METHODS

Animal Maintenance and Sample Collection

American Toads (*Bufo americanus*)

A population of 13 adult American toads (*Bufo americanus*) housed at the Cincinnati Zoo and Botanical Gardens' Center for Conservation and Research of Endangered Wildlife (CREW) was used for this study. These animals were obtained through hand collections in Northern Kentucky and Southern Ohio between the years of 2000 and 2002. Toads were housed individually in 16x11x6 inch plastic tubs. Water was provided *ad libitum* and animals were fed 2-3 times per week a diet of 6-week old crickets (Flucker) supplemented with ground vitamins (One Daily Women's- Kroger). All American toads were kept under a light cycle of 10L: 14D and constant temperature (25°C). Tanks were checked daily for feces. Feces were collected and stored in plastic bags in a –20°C freezer until hormone extraction. American toad fecal samples ranged from 1.5g to 4.0g in weight.

Boreal Toad (*Bufo boreas boreas*)

A population of 77 captive reared boreal toads was sent from the Colorado Division of Fish and Wildlife to the Cincinnati Zoo CREW facility in October of 2001. Boreal toads were housed in groups of up to 8 toads in 20x14x8 inch plastic tubs. Each tub contained animals of the same sex and from the same clutch of reared eggs. The toads were given water *ad libitum* and fed 4-6 times per week a diet of 4-week old crickets (Flucker) dusted with vitamin supplement. Boreal toads were kept under lighting conditions that mimicked a normal daylight cycle and constant temperature of 25°C

except for the months of December through April when 50 toads (30 females and 20 males) were placed in a breeding study. Toads in the breeding study were placed under hibernation conditions using plastic tubs with layers of moistened pea gravel, charcoal, sand, and peat moss and put into a Percival environmental chamber set to a constant temperature of 4°C and 24 hour darkness. Animals were checked for body condition and tanks moistened every two weeks during this period.

During the month of May as part of the breeding study, all toads except for a small subset of males were injected with gonadotropin hormones. Females received one IP injection of either 300IU human corionic gonadotropin (hCG) or 6μg Leutinizing Hormone Releasing Hormone (LH-RH-(Sigma product #-L4513)). These injections were followed two days later by 6μg of LH-RH. Males received either one or two IP injections of LH-RH. The two LH-RH injections for males were two days apart.

Boreal toad tanks were checked daily for fecal samples for one year. However, no samples were collected from females and males in the breeding study between the months of December and April. Multiple fecal samples from individual tanks were pooled, these samples were stored in the same plastic bag and labeled as a group sample. In addition, fecal samples from individual toads were obtained opportunistically when fecal samples could be assigned identity visually. Individual samples were stored in separate bags labeled with the individual's identification number. All samples were stored at –20°C until hormone extraction. Boreal toad group fecal samples typically ranged from 0.3g to 1.0g in weight while individual samples typically weighed between 0.2 and 0.4g.

Hormone Extraction

Frozen samples were allowed to thaw to room temperature prior to extraction. American toad fecal samples were mixed by stirring with a weighing spatula to homogenize the distribution of steroid metabolites in the feces, and an aliquot of the mixed feces was removed for extraction. Fecal aliquots were extracted once with ethyl acetate (ratio 1:20 grams of wet fecal material to milliliters of solvent). Samples were vortexed and allowed to mix by rocking for one hour at room temperature. It is important to note that ethyl acetate reacts with plastic so all extractions were carried out in glass screw-top tubes. All reagents used for hormone extractions and in making buffers and standards for hormone assays were purchased from Sigma Chemical Company.

Because boreal toad fecal samples were small, the whole sample was used for hormone extraction. Boreal toad samples were extracted at 1:10 ratio of grams of wet fecal material to milliliters of ethyl acetate and allowed to mix by rocking one hour at room temperature.

After mixing, samples for both species were centrifuged for ten minutes at 500g. Three milliliters of solvent was removed from each tube, evaporated to dryness, reconstituted in 1.0ml of assay buffer (0.2M NaH_2PO_4, 0.2M Na_2HPO_4, NaCl, and 1%BSA pH 7.0), and stored at -80°C until run in hormone assays. Known amounts of authentic testosterone, progesterone, and estradiol were added to several fecal samples prior to extraction to determine the extraction efficiency. Extraction efficiency for American toad fecal material using this method was 135%, 82%, and 56% for testosterone, estradiol, and progesterone respectively. For boreal toad samples the extraction efficiency was 126% for testosterone, 119% for estradiol, and 49% for

progesterone. Because of variation in measurements both between samples and within the assay it was possible to measure higher amounts of hormone than was originally added to the sample.

Hormone Assays

Enzyme Immunoassay (EIA) was used to quantify hormones in fecal extracts. In this type of EIA an enzyme-conjugated hormone competes with unknown hormones present in the sample for binding positions on an antibody to the authentic hormone. After binding, excess hormone (both labeled and unlabeled forms) is rinsed away and a substrate that reacts with the enzyme is added. The enzyme causes the substrate to cleave, which increases the absorbance of the solution and allows the relative numbers of labeled and unlabeled hormone forms occupying antibody-binding spots to be determined. Higher optical densities (OD) correspond to lower concentrations of hormone measured in the sample. The OD (λ-405nm) of hormone assay plates for antibodies used in this study was determined using a Dynex MRX automatic microplate reader (Dynex Technologies). EIA procedures published by Munro and Stabenfelt (1984), Walker et al. (2002), and Gudermuth et al. (1998) were used to quantify progestagens, estrogens and androgens. C. Munro in the Clinical Endocrinology Lab at UC Davis provided all antibodies and conjugated hormones. Briefly, assay procedures were as follows.

Progesterone- To coat Nalgene 96-well assay plates, 50µl progesterone antibody (CL425) diluted 1:6000 with coating buffer (Na_2CO_3, $NaHCO_3$, NaN_3 pH9.6) was added to wells and allowed to incubate overnight at 4°C. Assay plates were washed 5x with wash solution (NaCl, 5% Tween 20) and 50 µl of standards (0, 7.8, 15.6, 31.25, 62.5, 125, 250,

and 500pg/well of native progesterone), samples (diluted 1:5 with assay buffer), or control standards of assay buffer without extract were added to the wells along with 50 µl of progesterone-3carboxymethyloxime (CMO)-horse-radish peroxidase (HRP). Assay plates were incubated for 2 hours at room temperature. Assay plates were then washed 5x and developed for up to 60 minutes using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (ABTS-$(NH_4)_2$) diluted in citrate buffer (Citric acid-anhydrous, pH 4.0). The measured absorbance was an indicator of the amount of steroid labeled with HRP that was present in each well of the assay plate. Amounts of hormone present in the fecal extract was determined by comparison to the standard curve prepared using known hormone concentrations.

Estradiol- Nalgene 96-well assay plates were coated with 50 µl of estradiol antibody (R4972) diluted to 1:10000 with EIA coating buffer and allowed to incubate overnight at 4°C. The next day, assay plates were removed from 4°C, washed 5x with wash solution and blocked with assay buffer for ~2 hours at room temperature. After blocking, plates were again washed 5x and 20 µl of standards (3.9, 7.8, 15.6, 31.25, 62.5, 125, and 250pg/well of native estradiol), samples (diluted 1:10 with assay buffer), and controls were added to wells. Fifty microliters of estradiol-HRP conjugate was added to wells after samples and assay plates were allowed to incubate for 2 hours at room temperature. Assay plates were washed 5x with assay buffer after incubation and the same ABTS-$(NH_4)_2$ indicator described above was used to develop the assay plates.

Testosterone- Nalgene 96-well assay plates were coated with 100 µl of testosterone antibody (R156/7) diluted to 1:10000 with coating buffer and incubated overnight at 4°C. Day 2, plates were washed 5x with wash solution and 100ul of standards (3.125, 6.25,

12.5, 25, 50, 100, and 200pg/well of native testosterone), samples (diluted 1:30 with assay buffer), and controls were added. Plates were again allowed to incubate overnight at 4°C. Day 3, 50 µl of Testosterone-HRP conjugate was added to wells already containing standards, samples, and controls and incubated at 4°C for 2 hours. Assay plates were washed 5x and developed as described above.

Each assay was validated for use by serially diluting pooled extracts with assay buffer and comparing the OD values with those of a standard curve made from known amounts of the native hormone for that assay. Parallelisms between graphs of the diluted standards and the diluted pooled extracts were used to verify the specificity of the antibody for hormone metabolites in toad feces. Recognition of the hormones in the extract by the antibody was successful by evidence that the added hormone was competing with the conjugated form of the native hormone for binding to the antibody. Serial dilution of pooled samples from both American and boreal toads showed binding parallelism with the testosterone, estradiol, and progesterone antibodies. From these comparisons the best dilution for extracting fecal samples and the best dilution to be run on the assay plates was determined. Extracts of crickets with and without vitamin supplements, obtained using the same methods described previously, were tested for parallelism as well in order to determine if the source of the hormones in the toads' feces were from their diet. Cricket extracts showed no parallelism for any of the three antibodies tested.

Identification of Hormone Metabolites

High Pressure Liquid Chromatography (HPLC) was used in an effort to identify hormone metabolites in feces excreted by both American and boreal toads. The HPLC

apparatus consisted of a Rheodyne Model 7125 Syringe Loading Sample Injector fitted with a 20ul loop, a Whatman Partisphere RTF C18 5u 4.6 x 250mm analytical column, a Ranin Rabbit HP/HPX with a 10ml pump, and a Gilson 112 fixed wavelength UV detector (λ-254 nm). A Dynamax HPLC Method Manager Version 1.2 program run on a MacIntosh SE was used to collect data and control the HPLC. A solvent consisting of 45% methanol, 10% acetonitrile, and 45% distilled, deionized water filtered to 0.45 microns and deaerated under a vacuum was run isocratic at a 1.0ml/min. flow rate. All solvents used in the HPLC analysis were HPLC grade and were purchased from Fisher Scientific.

The hormone standard included 2μg/ml of authentic testosterone and progesterone, and 2mg/ml of estradiol, estriol, and estrone dissolved in 100% ethanol and filtered. Twenty microlitters of this mixed hormone standard was injected into the HPLC apparatus and allowed to run for 60 minutes. Hormone standards produced absorbance peaks at 10, 21, 31, 40, and 44 minutes after injection which corresponded to estriol, testosterone, estrone, progesterone, and estradiol respectively. Collections of timed fractions of HPLC solvent with a Gilson Model 203 Fraction Collector were made at 1-minute intervals during the 60 minutes the standard was running. These fractions were evaporated to dryness, reconstituted with 500ml of assay buffer, and each fraction run in the three hormone assays to confirm the identities of the peaks.

Fecal extracts from American and boreal toads were prepared for HPLC analysis by evaporating 3 ml of ethyl acetate extract to dryness. Extract was reconstituted in 1 ml of 100% methanol and filtered through 0.45μ Whatman syringe filters. A 20μl aliquot of reconstituted fecal extract was injected into the HPLC and allowed to run for 60 minutes.

No peaks corresponding to the hormone standards were observed for the samples using HPLC. Fractions were collected at minute intervals, evaporated, reconstituted, and run in hormone immunoassays to determine if immunoreactivity corresponded with any of the hormone standards. Results are presented in Appendix 1.

Statistical Analysis

All statistical analyses were performed using SYSTAT version 10 software licensed to the University of Cincinnati. In order to confirm that the number of toads available for this study was adequate to reliably report differences between male and female toads, a power analysis was performed. The results of this analysis confirmed that both American and boreal toad sample sizes were sufficient for determining statistical significance in the results.

The amounts of each hormone measured in American toad feces was analyzed by an analysis of variance (ANOVA) with month and sex as factors for all tests and each hormone analyzed as a separate dependent variable. Ratios of testosterone/estradiol, progesterone/testosterone, and progesterone/estradiol were also analyzed as separate dependent variables.

For boreal toad samples a student's t-test was performed to determine if there were differences between samples collected from individuals and those pooled as group samples. No significant difference was found between these two collection techniques so both types of samples were included in the analysis to increase the power of the tests. Boreal toad hormone measurements were also analyzed by ANOVA using the same variables. However because of the breeding study, the analyses were broken into two data sets. One data set consisted of hormone measurements of samples collected only during

the month of May when injections were given to animals in the study. A second data set consisted of hormone measurements of samples for June through November when toads were not participating in the breeding study.

RESULTS

Validation of Hormone Assays-

In order to insure that measurements of hormone metabolite concentrations are reliable for determining gender, the methods had to be validated. Extracts from American and boreal toads were serially diluted with assay buffer and run in EIA hormone assays to quantify estradiol, progesterone, and testosterone metabolites. The optical density (OD, λ-405) values for these extracts were compared with the OD provided by known amounts of authentic estradiol 17β, progesterone, and testosterone that had been serially diluted and run in the EIA together with the pooled extracts to determine if the metabolites in toad feces could be recognized using antibodies to the native hormone. For each toad species three ratios of extraction solvent were used to remove hormone metabolites from feces in order to determine the most effective extraction dilution. In all cases, the amount of immunoreactivity from the toad extracts binding to the antibody changed in a dose dependent manner as the extracts became more dilute. Parallelism between the diluted fecal extracts and estradiol, progesterone, and testosterone standards for the American toads were comparable to those displayed for the boreal toad shown in Figures 1 – 3. For American toads, the 1:20 extraction ratio was chosen to use for extracting further samples because the line created by the ODs of the diluted fecal extracts were not significantly different from the line created by the binding of the standards to the antibodies ($p>0.05$). For boreal toads a 1:10 extraction ratio was the most appropriate for use with further samples because it consistently showed the parallelism for the three hormone standards

($p>0.05$) and provided enough extract solvent to be collected that it could be used in several hormone assays.

The parallelism curves were also important for determining the dilution to run the extracts in the assays in order to be at 50% binding for that antibody, which is the most linear part of the standard curve and therefore gives the most precise measurement. For both the American and boreal toads half of the maximum OD of the standard was considered to be 50% binding for the antibody. Fecal extracts for both toad species were diluted 1:10 with assay buffer for the estradiol assay, 1:5 with assay buffer for the progesterone assay, and 1:30 with assay buffer for the testosterone assay.

Both boreal and American toads were fed a diet consisting almost exclusively of crickets sprinkled with a vitamin supplement. Extracts of crickets were diluted and compared to the hormone standards for parallelism as well to determine if the source of fecal immunoreactivity was from the toads' diets. The cricket extracts did not show dose dependent binding to any of the three antibodies. The dilutions of extracts with and without vitamin supplements were significantly different from the standards in all cases, binding to the estradiol antibody illustrates this relationship (Figure 4).

American toad-

Tanks of individual American toads were checked daily for 12 months for fecal samples. Mean estradiol, progesterone, and testosterone immunoreactivity from feces extracted over this time are expressed in ng/g feces (Table 1). The amount of fecal extract binding to the testosterone antibody was significantly different between the two sexes. Females excreted on average 80.7ng/g feces of testosterone metabolites whereas male values were almost three times that amount (224.3ng/g feces). Plots of the 95%

confidence intervals (CI) about the means for the American toad illustrate the differences between the sexes for the three hormones (Figure 5). Female toads excreted an average of 107.7ng/g feces of estradiol metabolites and 8.3ng/g feces of progesterone metabolites. Male concentrations of these metabolites were slightly lower than female concentrations at 89.4ng/g feces for estradiol metabolites and 6.6ng/g feces for progesterone metabolites. Concentrations did not differ significantly between males and females for these two hormones.

In addition to overall differences in hormone levels between males and females, changes in hormone levels throughout the year were investigated to determine if excreted hormone levels fluctuate. Though American toads were kept at constant daylight and temperature conditions, there were significant differences in estradiol and testosterone levels between months (Figures 6 and 7). Males showed a significant drop in testosterone levels for June and July. There also was an increase during this time in the concentration of testosterone excreted by females. Estradiol concentrations fluctuate throughout the year for both males and females, but do not show any seasonal pattern.

Boreal toad-

Boreal toad feces were collected as group samples, and, when possible, as individual samples. No significant differences were found between hormone levels measured from group samples or individual samples, therefore measurements of both were included in the data analysis for statistical purposes. Measurements from feces collected between June and November (Table 2) were analyzed separately from those taken in the month of May (Table 3) because during May toads were given hormone treatments as part of a breeding study being performed at the same time.

Unlike the American toads, boreal toad fecal extracts showed no significant difference between males and females for binding to the testosterone antibody. From June to November male average testosterone concentrations were 45.2ng/g feces and female values were lower at 29.9ng/g feces. Measurements for both sexes during the month of May were about twice that of the means from June to November. There were no significant differences for males and females in the concentrations of progesterone excreted in the feces for either time period ($p>0.05$). Only estradiol immunoreactivity was significantly different for boreal toad males and females ($p<0.05$). From June to November females excreted a mean estradiol concentration of 48.3ng/g feces. Males excreted 18.9ng/g feces. Figure 8 shows the 95% confidence intervals for concentrations of hormone metabolites excreted by the boreal toads from June to November.

In May, female boreal toads received either one injection of hCG followed by one injection of LH-RH or two injections of LH-RH, while males received either zero, one, or two injections of LH-RH as part of a separate breeding study. Binding to the estradiol antibody in May gave values of almost twice that seen from June to November. Differences in levels of estradiol metabolites for males and females during May were significant ($p<0.05$) with males excreting 33.5ng/g feces and females excreting 107.2ng/g feces (Table 3). The decrease in estradiol metabolite concentrations after May was also significant for both males and females. In addition to the decreases in estradiol, concentrations of testosterone and progesterone metabolites decreased significantly after May as well.

Like the American toads, boreal toads showed seasonal differences in the concentrations of hormone metabolites they excreted. Concentrations of testosterone

metabolites decreased significantly for both males and females from July to October (Figure 9). Males excreted relatively constant amounts of estradiol metabolites, whereas female concentrations were high immediately following hibernation and dropped significantly from June to October. Estradiol immunoreactivity then increases in November in females (Figure 10).

TABLE 1

Mean fecal steroid immunoreactivity for the American toad[a]

		Antibody		
	N	Estradiol	Progesterone	Testosterone
Female	6	107.70 ± 25.60[b]	8.30 ± 1.50	80.70 ± 10.60[c]
Male	7	89.40 ± 12.30	6.60 ± 1.50	224.30 ± 15.50

a) feces collected during a 12-month period from August 2002 to July 2003.

b) All hormone concentrations are given in ng/g feces as the mean ± Standard Error (SE).

c) Significant difference between hormone concentrations for males and females. ($p<0.05$, student's t-test)

TABLE 2

Mean fecal steroid immunoreactivity for the boreal toad (June to November)[a]

		Antibody		
	N	Estradiol	Progesterone	Testosterone
Female	32	48.30 ± 6.30 [bc]	3.60 ± 0.30	29.90 ± 3.60
Male	45	19.00 ± 1.80	3.00 ± 0.30	45.20 ± 7.60

a) Feces collected from August – November of 2002 and June – August of 2003. Missing months due to toads being placed in a breeding study, which prevented fecal collections. Immunoreactivity for May shown in Table 3.

b) All hormone concentrations are given in ng/g feces as the mean ± Standard Error (SE).

c) Significant difference between hormone concentrations for males and females. ($p<0.05$, student's t-test)

TABLE 3

Mean fecal steroid immunoreactivity for the boreal toad (May)[a]

		Antibody		
	N	Estradiol	Progesterone	Testosterone
Female	8	107.20 ± 18.90[bc]	6.40 ± 1.00	69.34 ± 13.30
Male	8	33.50 ± 7.50	5.80 ± 0.50	91.86 ± 20.60

a) Immunoreactivity of feces collected during May of 2003. Toads were administered intraperitoniel one injection of 300IU of human corionic gonadotropin and one injection of 6μg of leutinizing hormone releasing hormone (LH-RH) or one injection of 6μg of LH-RH for females, and males received either one or two injections of LHRH to encourage breeding during May.

b) All hormone concentrations are given in ng/g feces as the mean ± Standard Error (SE).

c) Significant difference between hormone concentrations for males and females. ($p<0.05$, student's t-test)

Figure 1: Boreal toad validation for the estradiol antibody.
Boreal toad feces extracted with ethyl acetate at three different dilutions were serially diluted and compared to a standard curve of serially diluted estradiol 17β. Curves represent ratios of solvent to feces (1:5, 1:10, or 1:20) for fecal extracts compared to a standard. The 1:10 extraction ratio was used for the boreal toad fecal extractions because the curve it produced did not differ significantly from the standard, and allowed sufficient extract to be collected for all hormone assays.

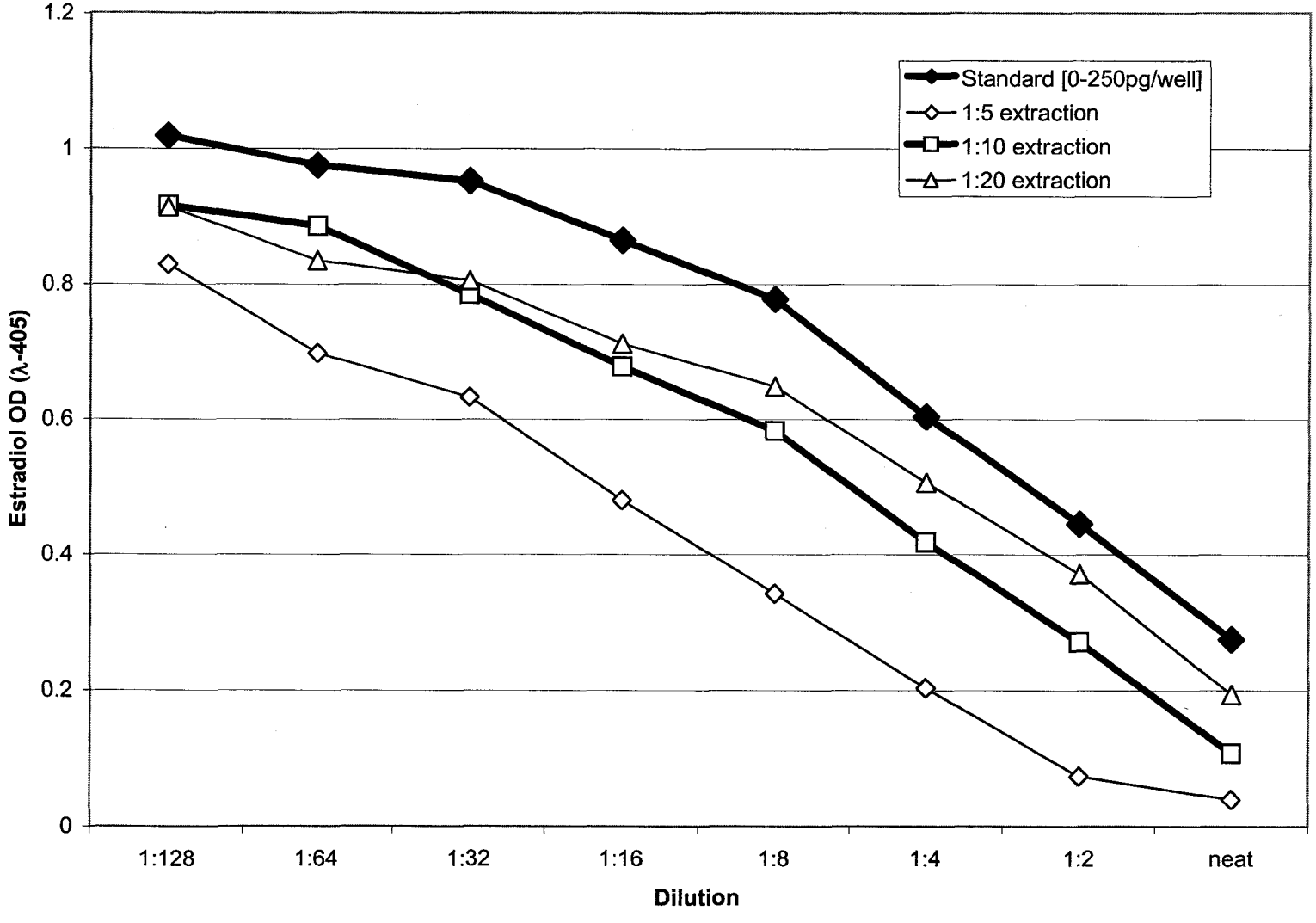


Figure 2: Boreal toad validation for the progesterone antibody
Boreal toad feces extracted with ethyl acetate at three different dilutions were serially diluted and compared to a standard curve of serially diluted native progesterone. Curves represent ratios of solvent to feces (1:5, 1:10, or 1:20) for fecal extracts compared to a standard. The 1:10 extraction ratio was used for the boreal toad fecal extractions because the curve it produced did not differ significantly from the standard, and allowed sufficient extract to be collected for all hormone assays.

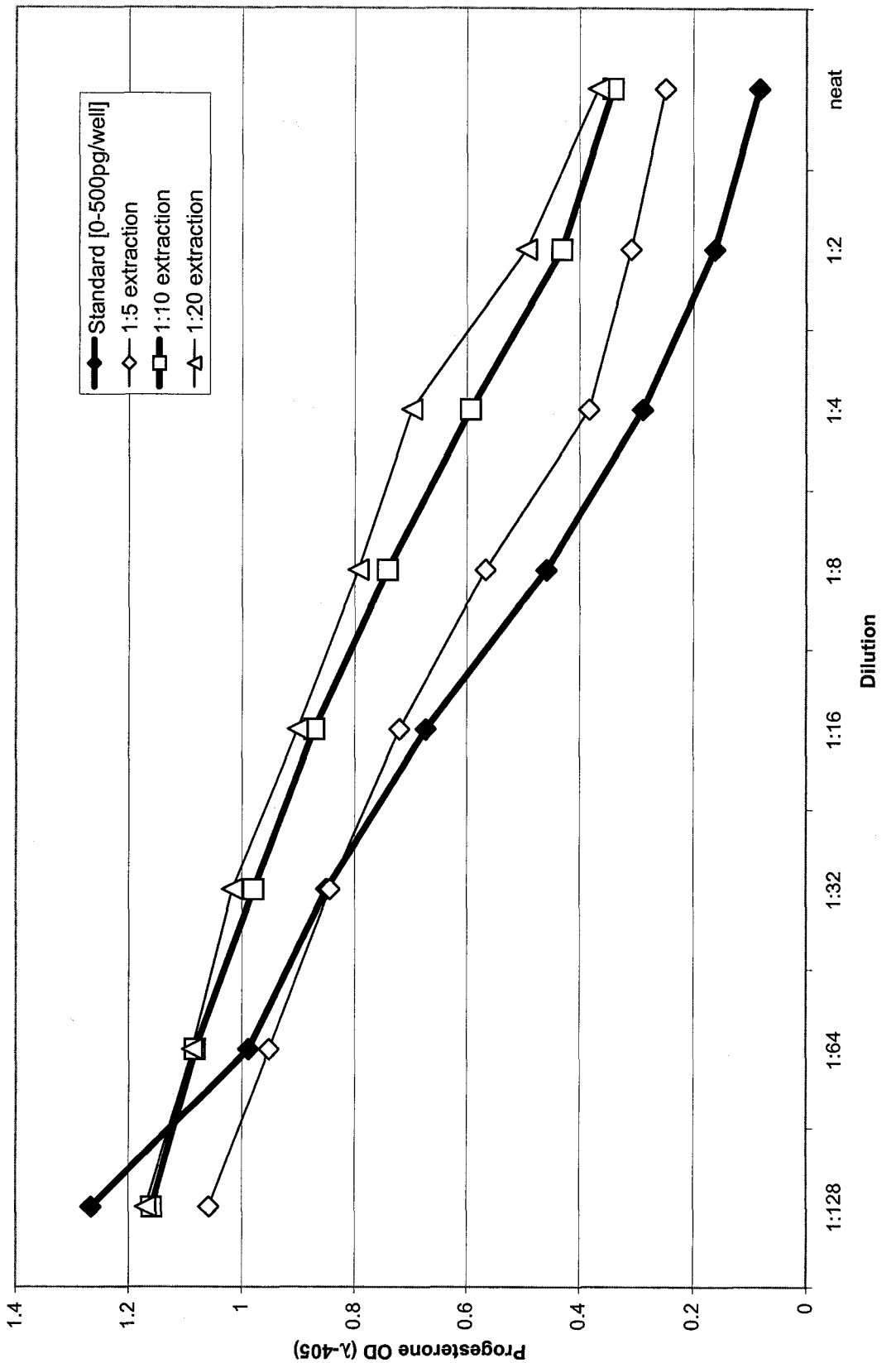


Figure 3: Boreal toad validation for the testosterone antibody
Boreal toad feces extracted with ethyl acetate at three different dilutions were serially diluted and compared to a standard curve of serially diluted native testosterone. Curves represent ratios of solvent to feces (1:5, 1:10, or 1:20) for fecal extracts compared to a standard. The 1:10 extraction ratio was used for the boreal toad fecal extractions because the curve it produced did not differ significantly from the standard, and allowed sufficient extract to be collected for all hormone assays.

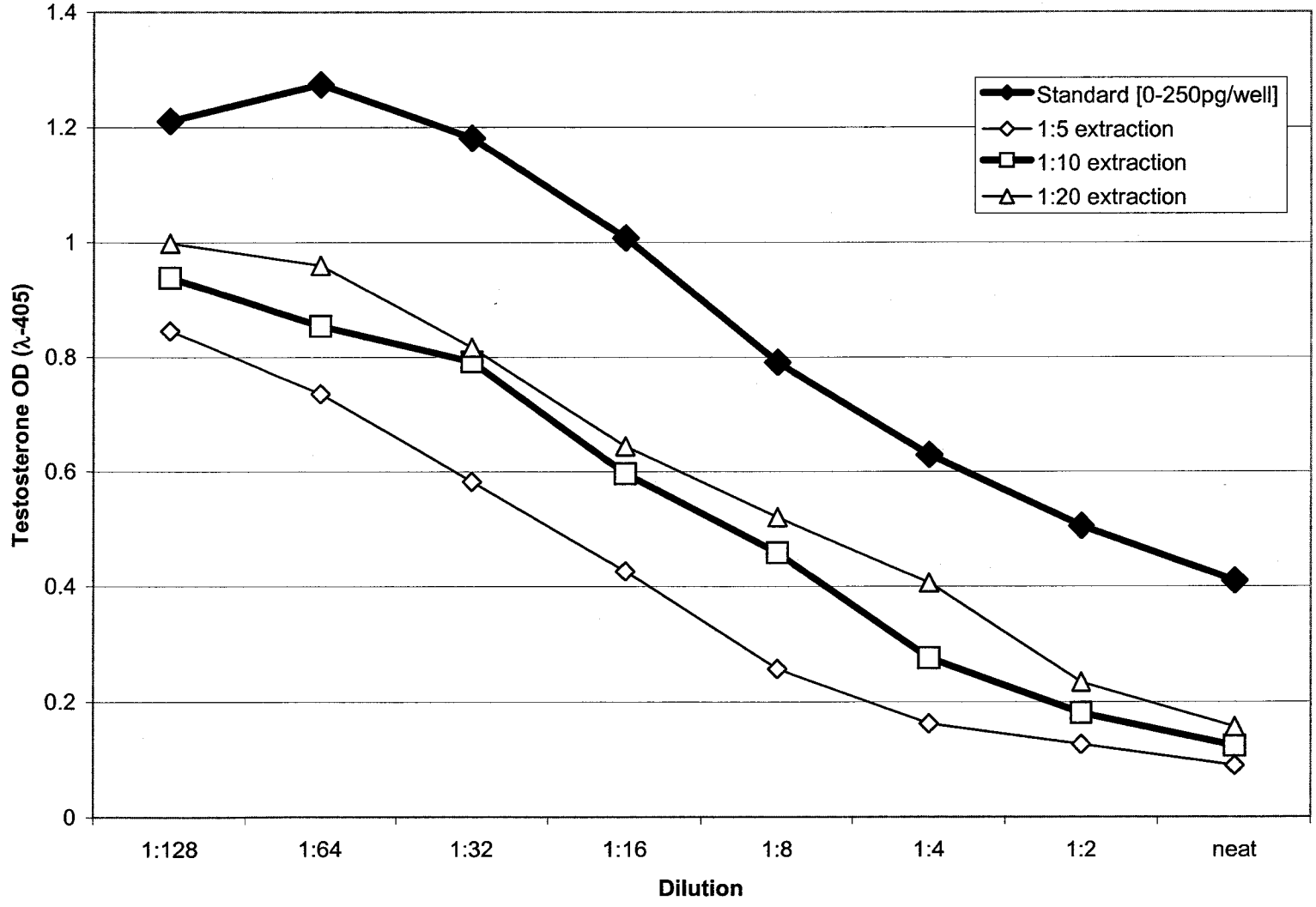


Figure 4: Cricket extract validation for the estradiol antibody
Crickets and crickets sprinkled with vitamin supplement were extracted with ethyl acetate, serially diluted, and compared to a standard curve of serially diluted native estradiol in order to determine whether the antibody was able to bind to hormones extracted from this toad food source. The standard curve differs significantly from the curves for crickets extracted both with and without supplement indicating that the cricket diet is not contributing to the estrogen measured in toad feces.

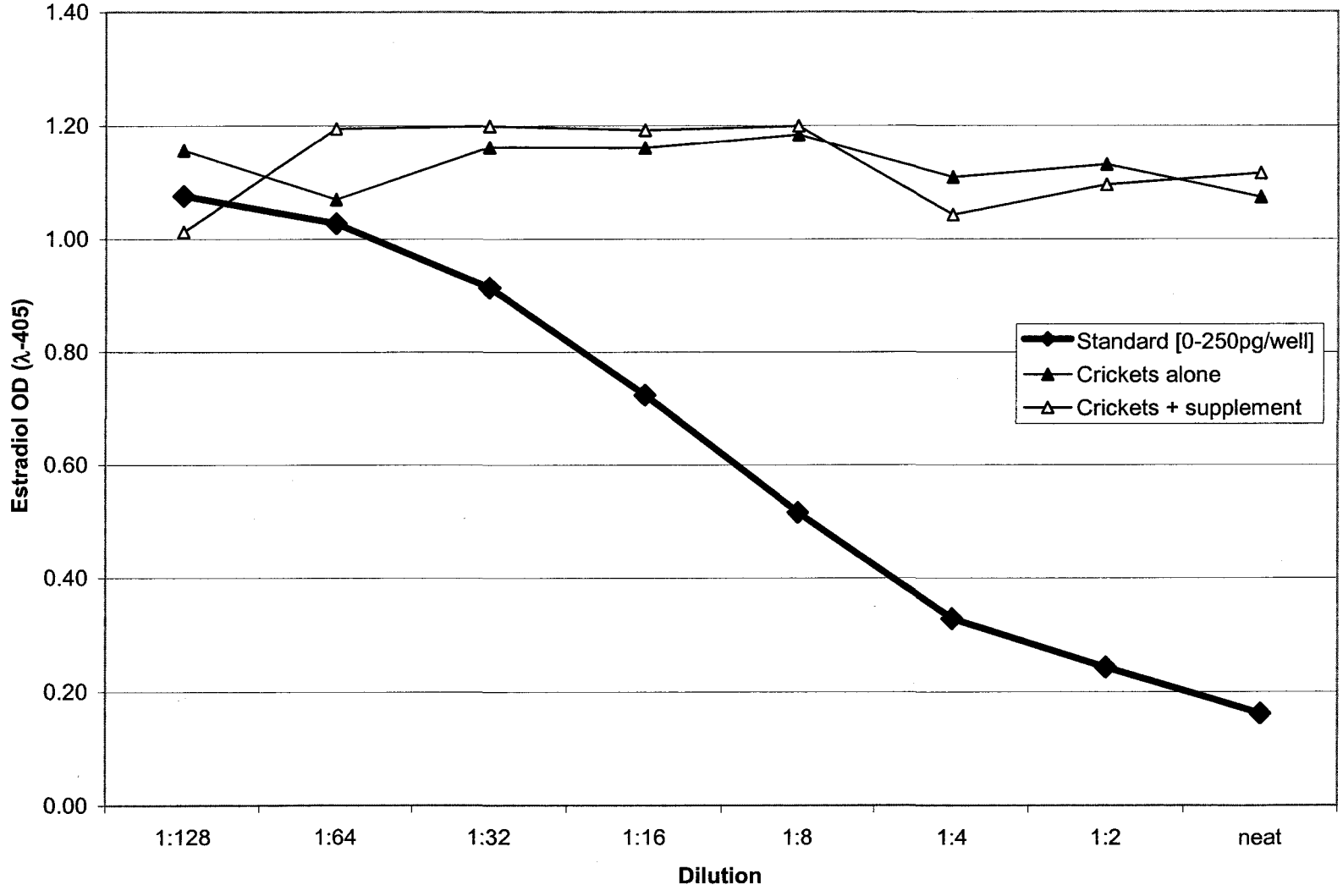


Figure 5: 95% confidence intervals for concentrations of fecal steroids using antibodies to estradiol (E), progesterone (P), and testosterone (T).
Figure is based on fecal samples from 13 American toads (7 male and 6 female) sampled from August 2002 to July 2003. Center bars represent mean hormone concentrations for each sex, boxes represent one standard deviation from the mean, and the horizontal line spans the entire length of the 95% confidence interval for that sex. Asterisks denote outliers.

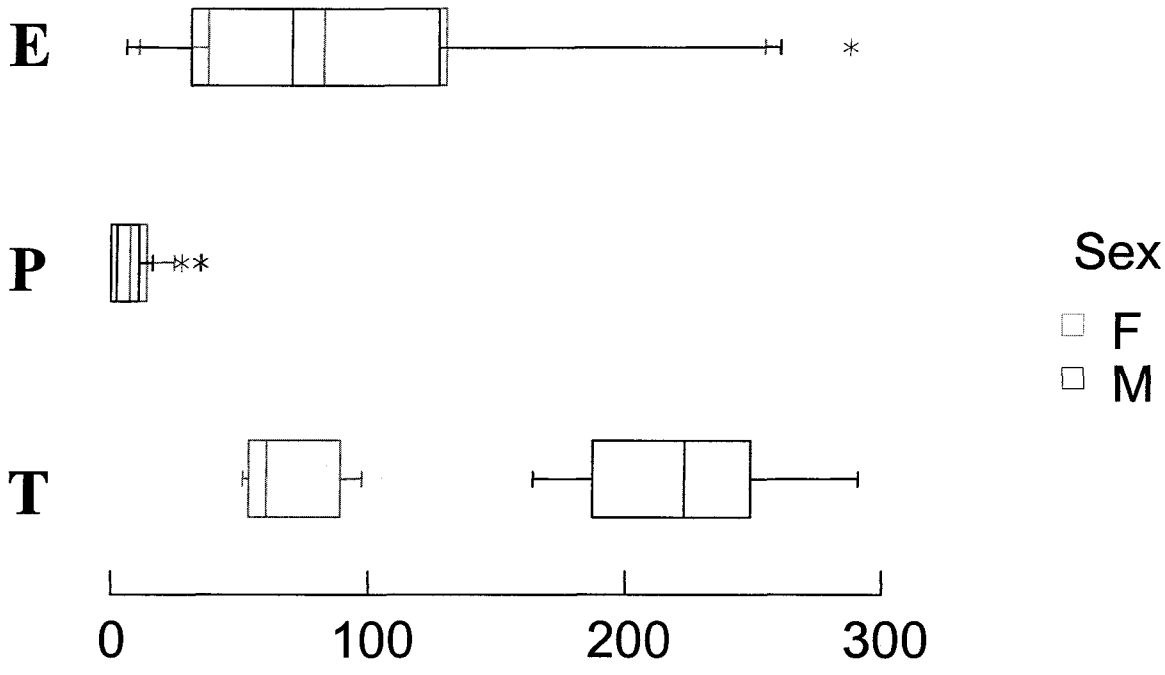


Figure 6: Average estradiol immunoreactivity for male and female American toads by month.

Mean estradiol concentrations $\pm$ SEM for male and female American toads measured by month. Samples were not available from female toads for the months of January and June and points from February, July, and August are based on samples from only 1 female. All other months n=3 female toads. For males, n=5-6, except for the months of June and July when points represent n=1.

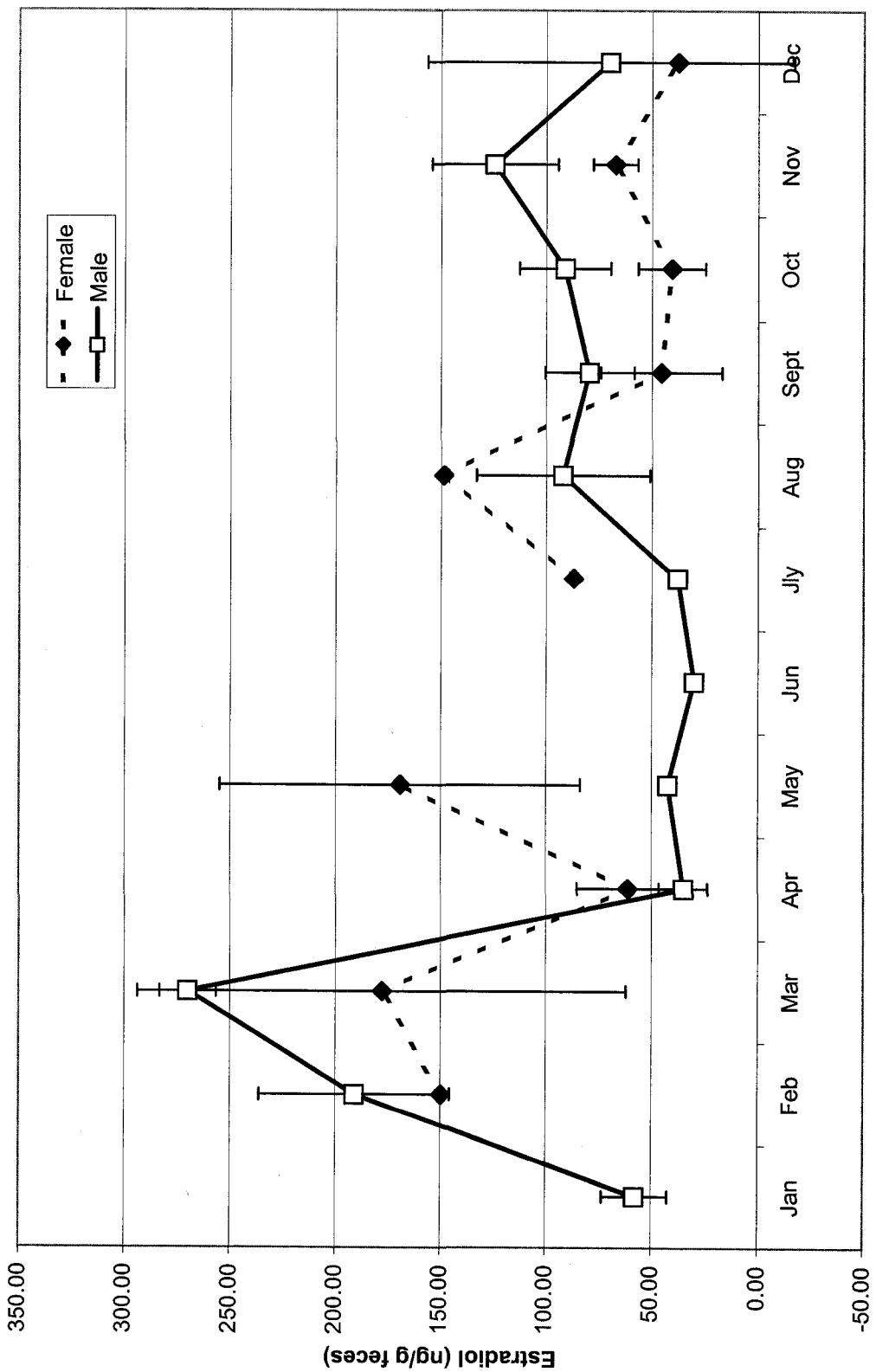


Figure 7: Average testosterone immunoreactivity for male and female American toads by month

Mean testosterone concentrations $\pm$ SEM for male and female American toads measured by month. Samples were not available from female toads for the months of January and June and points from February, July, and August are based on samples from only 1 female. n=3 all other months for female toads. For male toads, n=5-6, except for the months of June and July when points represent n=1.

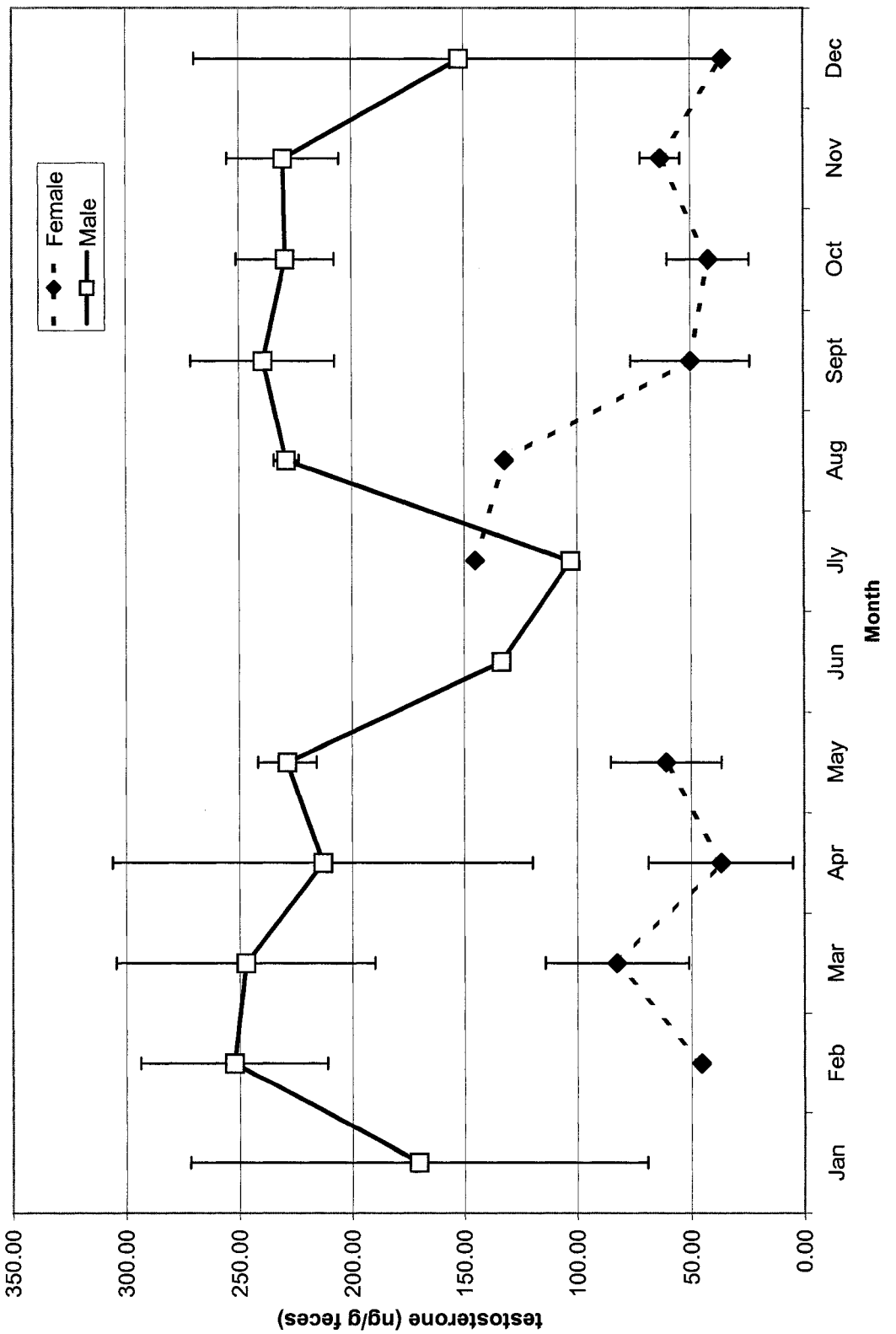


Figure 8: Boreal toad male and female 95% confidence intervals for concentrations of fecal steroids using antibodies to estradiol (E), progesterone (P), and testosterone (T).
Figure is based on fecal samples from 77 boreal toads (45 male and 32 female) sampled from August 2002 to November 2002 and June 2003 to July 2003. Animals were unavailable for sampling due to participation in a breeding study from December 2002 to April 2003, and May values were left out of CI calculation because animals were injected with gonadotropin hormones during this month that may have caused changes in hormone levels. Center bars represent mean hormone concentrations for each sex and the horizontal line spans the length of the 95% confidence interval for that sex. Asterisks and circles denote outliers.

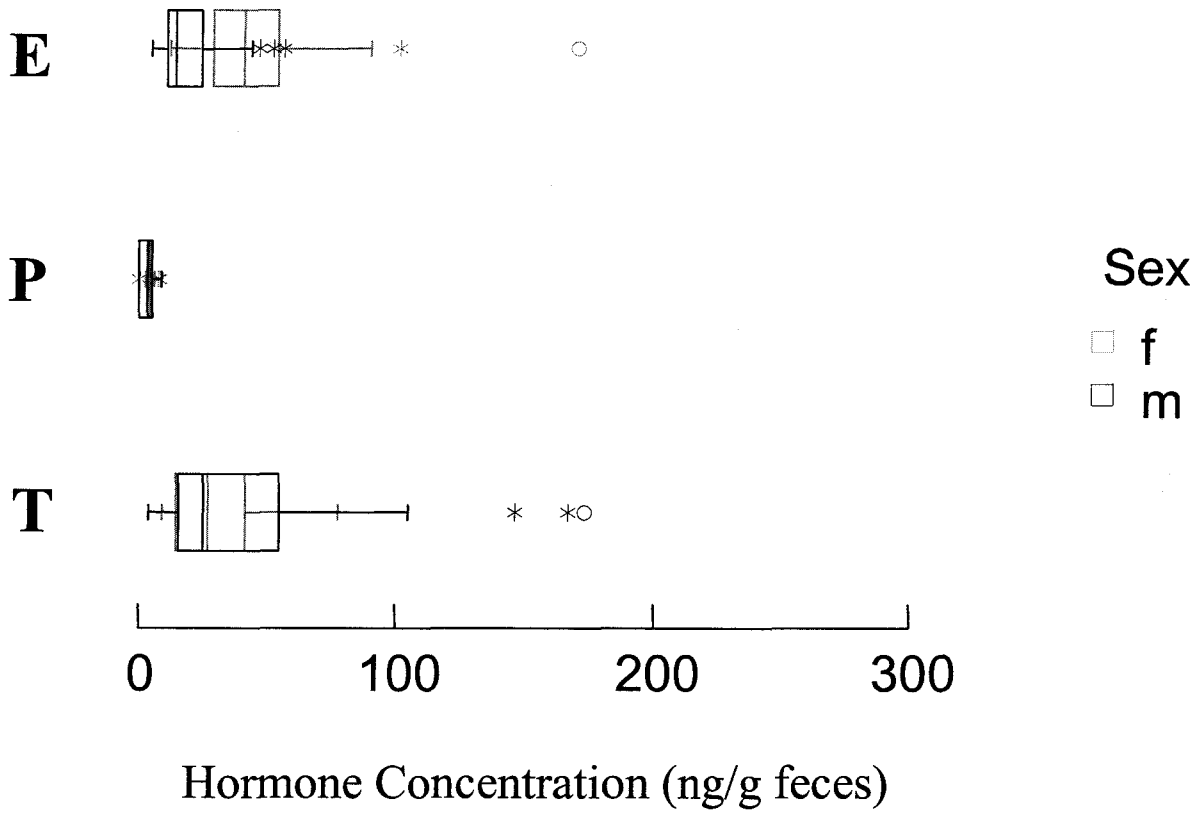


Figure 9: Average testosterone immunoreactivity for male and female boreal toads by month

Points represent mean testosterone concentrations $\pm$ SEM from both pooled and individual samples of male and female boreal toads measured by month. Samples were not available from female toads from December through April because of participation in a breeding study. For all points, both male and female, $n \geq 8$ except for November when the mean for females is based on only 5 individuals.

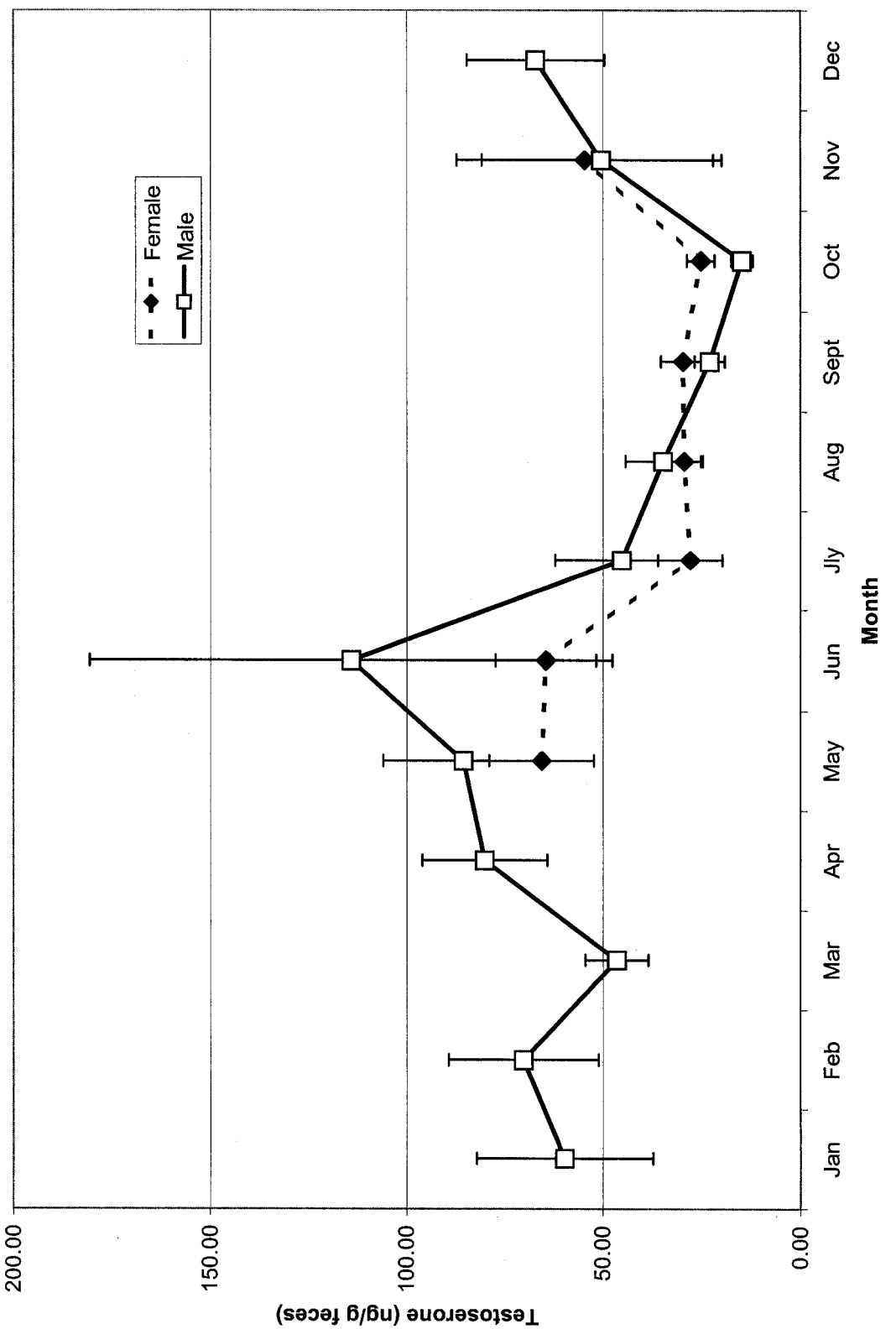
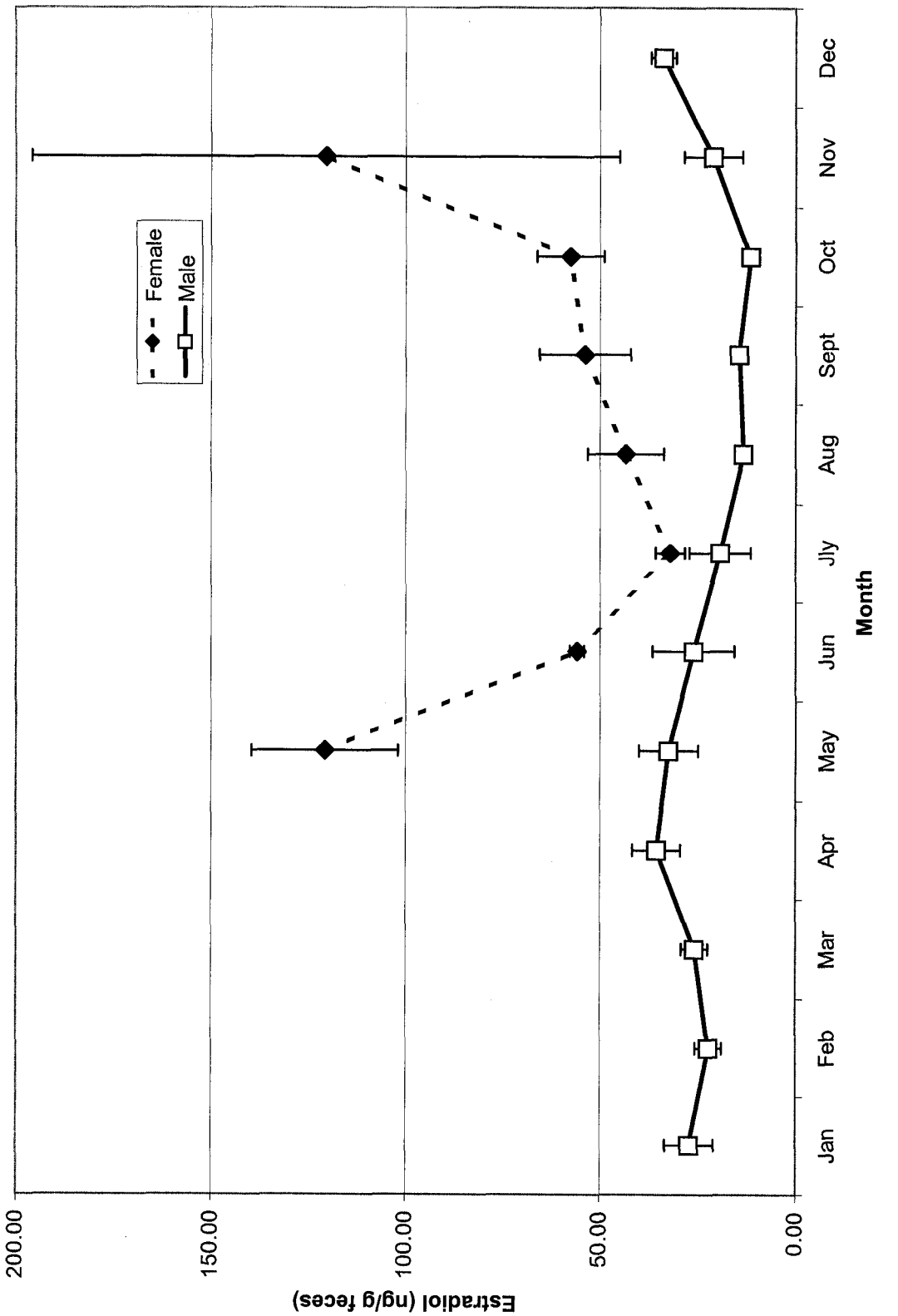


Figure 10: Average estradiol immunoreactivity for male and female boreal toads by month

Points represent mean estradiol concentrations $\pm$ SEM from both pooled and individual samples of male and female boreal toads measured by month. Samples were not available from female toads from December through April because of participation in a breeding study. For all points, both male and female, $n \geq 8$ except for November when the mean for females is based on only 5 individuals.



DISCUSSION

Because sex in many amphibians is difficult to distinguish and currently must be determined in invasive, stressful ways (Roth and Obringer, 2003), this study intended to determine if hormone metabolite measurements from feces could be used as a non-invasive method to distinguish sex in amphibians. The specific aims of this study were to measure the three major classes of steroid hormone metabolites- estrogens, progestagens, and androgens- excreted in the feces of two species of toad, *Bufo americanus* and *Bufo boreas boreas*, in order to determine if qualitative and/or quantitative differences exist in the amounts excreted by males and females. Two species were used to find out if the same pattern of hormone differences occurs across this genus, with the thought that if similarities do exist this method might be applied to other amphibian species.

Before these determinations could be made, the antibodies used in the assays to quantify hormone metabolites had to be validated for use with toads, because antibodies that give good results for some species may not bind to hormones for all species due to subtle differences in structure (Goymann et al., 1999). Hormone assays were validated by determining parallelism between diluted fecal extracts and hormone standards for each antibody. The enzyme immunoassays using antibodies to estradiol, progesterone, and testosterone all showed changes in optical density (OD) measurements as fecal extracts became more dilute. These changes demonstrated that the antibodies to native steroid hormones recognized the hormone metabolites being excreted by the toads. The recognition of the hormone metabolites by the antibodies is specific because the extracted

hormones are able to successfully compete with the labeled forms of the native hormones for binding to the antibodies.

Previous studies in wild baboons have found that diet does not significantly affect hormone measurements in feces (Wasser et al., 1994). To confirm that the toads' diets, which consisted almost exclusively of crickets, did not affect fecal hormone measurements, crickets were extracted both with and without vitamin supplement, diluted in the same manner as fecal extracts, and compared to the hormone standards for parallelism. No parallelism was generated between the cricket extracts and the hormone standards indicating that either the crickets did not form hormones that are recognized by the antibodies used in this study or that the levels of hormones the crickets produced were below the sensitivities of the assays. In either case, the crickets are not the source of the hormones measured from the toads' feces.

The specific hormone metabolites excreted by the toads were not identified in this study, and there are no published reports of steroid metabolites excreted in the feces of amphibians. Hormone metabolites derived from feces in other species include estrone (both conjugated and free forms), pregnanediol-3-glucuronide, and DHT (Shille et al., 1990, Lasley and Kirkpatrick, 1991, and Goymann et al, 2002). Measurements of hormone metabolites cannot be assumed to reflect the total amounts of steroid metabolites present in fecal extracts because only a percentage of the actual metabolite may be binding to the antibodies. Therefore, it is not known whether the immunoreactivity detected in the toad feces was derived from multiple metabolites or a single primary metabolite. Antibodies to the native hormones used in this study do cross-react with metabolites of the native hormones. For example, the testosterone antibody

cross-reacts 100% with testosterone, but it also cross-reacts 57% with the testosterone metabolite DHT and 0.3% with androstenedione. The estradiol antibody cross-reacts 100% with 17β estradiol and 3% with estrone. The progesterone antibody cross-reacts 100% with progesterone as well as with several metabolites, over 100% with 4-pregnen-3α and β-ol-20-one and 4-pregnen-11α-ol-3,20-dione, and between 50% and 95% for the 5α-pregnans (C. Munro, personal communication). The cross-reactivity of these antibodies to conjugated hormone metabolite forms and additional hormone metabolites are not known. Nevertheless hormone metabolites that have not been specifically identified can still show differences between males and females in immunoreactivity, and can, therefore, be used to differentiate between the sexes (Schwartzenberger et al., 1996).

For the American toad, males excreted significantly higher concentrations of testosterone metabolites than females. Although the mean concentrations of estradiol and progesterone metabolites excreted by females were higher than those excreted by males, these differences were not significant. There was a high amount of variability in raw measurements of each hormone group within each sex. Hirschenhauser et al. (2000) found that in domestic geese, fecal metabolites are much more variable than plasma measurements of hormones. Because of this, sources of variability were considered and precautions taken to minimize their effects on analyses of metabolite concentrations. Bamberg et al. (1991) suggested that differences in fecal production could account for variability in metabolite measurements from different individuals; however in the American toad much of the variability appeared to be between concentrations of hormones from the same individual. For example, one individual excreted 260, 23, and 400ng/g feces of testosterone metabolites in one month and 60, 150, and 400ng/g feces

the next; however, in the months before and after these two the immunoreactivity for testosterone was over 200ng/g feces for every sample. Such differences may be due to variation in excretion rates and/or temporal differences in the circulating levels of these hormones. 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. Because there were irregular intervals between defecation, in the present study individual samples were averaged by month to reduce sample-to-sample and sample frequency differences for each toad. In order to reduce variability within an individual's samples, feces were mixed before extraction according to the guidelines recommended by Millspaugh and Nashburn (2003). An additional source of variation both within and between individual hormone metabolite levels may have come from analyzing feces that were stored for varying lengths of time before extraction. Storage has been found to increase scatter among hormone measurements in the baboon (Lynch et al., 2003). In this study the amount of storage time at –20°C was not controlled and, therefore, may have caused immunoreactivity to vary. Future extensions of this work should take these problems into consideration when designing an experiment. In addition, several samples from each individual should be analyzed to avoid misleading results. Despite a high degree of variability between hormone values for the American toad, the differences in testosterone immunoreactivity were distinct for males and females with no overlap of the confidence intervals between the sexes.

To support these findings and confirm that distinguishing sex using differences in hormone metabolite immunoreactivity is applicable to more species than just the American toad, metabolites from boreal toad feces were also assayed to determine

hormone concentrations for males and females. The same extraction solvent and antibodies used for the American toad were used to make these determinations for the boreal toad.

In the boreal toad only estradiol immunoreactivity showed significant differences between males and females. Females excreted higher concentrations of estradiol metabolites than males. While significant, differences in the 95% confidence intervals for estradiol were not as marked for the boreal toad as testosterone was for the American toad and some degree of overlap occurred between the sexes. For the boreal toad, male concentrations of testosterone metabolites were generally higher than concentrations excreted by females, but these differences were not significant. Again, there were large amounts of variation in individual hormone immunoreactivity measurements. These results suggest that differences in hormone metabolite immunoreactivity can be used to distinguish sex in more species than just the American toad, but the differentiating hormone may not be the same from species to species.

In order for the hormone assay to be practical for identifying sex in amphibians, it must be able to work year-round. Therefore, seasonal differences in hormone levels for the American and boreal toads were investigated to determine if the relationships between males and females change throughout the year. For the boreal toads the relationship between males and females for each hormone remained unchanged during May; however, the levels of immunoreactivity for each hormone were significantly higher in May for both sexes than in the other months of the study. The higher hormone metabolite concentrations in May could be due to the hormone injections the toads received or it could be due to seasonal fluctuations in hormone production and excretion.

Because May is part of the boreal toad's normal breeding season, some females were injected with human chorionic gonadotropin (hCG) and all the toads (except a small subset of males not included in the breeding study) were injected with leutinizing hormone releasing hormone (LH-RH), a type of gonadotropin releasing hormone (GnRH), in order to encourage gamete production and stimulate breeding activity. Studies of birds have detected significantly increased levels of androgens excreted in feces after GnRH administration (Hirschenhauser et al., 2000 and Goymann et al., 2002). In amphibians, GnRH caused a significant increase in testicular testosterone in the frog *Rana esculanta* (Pierantoni et al., 1984) and in *Bufo woodhousii* gonadotropins significantly increased plasma testosterone, progesterone, and estradiol levels (Pancak-Roessler and Norris, 1991). However Pozzi and Ceballos (2000) found that spermiation induced by gonadotropins is not dependent on steroid biosynthesis for the toad species, *Bufo arenarum*. Because of conflicting results in these studies on the effects of GnRH on hormone secretions in amphibians, it was difficult to determine if LH-RH caused the increases in hormone concentrations seen in the boreal toad. Therefore, in August of 2003 some males were injected with the same amounts of LH-RH they had been injected with in May in order to determine if LH-RH caused hormone metabolite concentrations in the boreal toad to increase. If LH-RH caused a rise in plasma hormone concentrations, that rise would be reflected in the feces. Lack of defecation by the animals resulted in only five samples being collected before injections and three being collected after injections. While there were increased levels of hormone metabolites in the toads' feces after LH-RH injections, the sample size was insufficient to demonstrate significance.

Alternatively, natural fluctuations in reproductive hormones may also have caused hormone metabolite levels increase significantly in May-especially because the hormone treatments did not augment breeding for the toads. Studies of amphibian plasma androgens have shown that concentrations of testosterone vary throughout the year (Delgado et al., 1989, Ko et al., 1998, and Canosa and Ceballos, 2001b). Boreal toads in the present study showed seasonal differences in the levels of hormone metabolites they excrete. Male boreal toads had a drop in testosterone metabolite excretion shortly after the breeding season in July. The changes in testosterone metabolite levels for females are similar to those for males. Male values of estradiol metabolites remained constant throughout the year despite the LH-RH injections. Female estradiol metabolite levels in the boreal toads are incomplete because fecal samples were unavailable from December through April due to the breeding study, which prevented fecal samples from being collected during this time. Female estradiol metabolites dropped after breeding in May, and increased in November. Again, it is unclear whether these differences were natural seasonal fluctuations or were caused by the hormones the animals were injected with in May to stimulate breeding. It is important to note that although hormone levels fluctuate throughout the year, the relationship between average hormone metabolite concentrations for males and females persist, even through the interval (May) of exogenous hormone administration.

American toads did not receive hormone injections, but also showed changes in the amounts of hormone metabolites excreted throughout the year. Despite the fluctuations in concentrations of fecal testosterone metabolites, average male and female differences persist. The concentration of testosterone metabolites excreted by male

American toads dropped during the breeding season from March to June and reached its lowest levels during July, immediately after the breeding season. Plasma testosterone measurements have shown similar trends for other amphibian species. In *Bufo arenarum,* male plasma androgen levels were lower during the breeding season than during the rest of the year (Canosa and Ceballos, 2002), and plasma testosterone levels in several ranid species were lowest shortly after the breeding season (Delgado et al., 1989 and Ko et al., 1998). Female American toads had higher levels of fecal testosterone metabolites at the same time males have lower levels. Licht et al. (1983) found a peak in plasma testosterone around April for female bullfrogs, however the authors did not report the male values of testosterone in order to compare relative amounts of testosterone between the sexes. In other species of amphibians there are no seasonal elevations of androgens for females (Kang et al., 1995). The increase in fecal testosterone for the female American toads, therefore, may be an artifact of small sample size. These hormone measurements were based on samples from only one individual because three of the females died during the course of the study and feces from the surviving females were, many times, mixed in their water preventing the pellets from being collected. Because of this, there were insufficient samples to establish a year round pattern of hormone metabolite excretion for female American toads. The overlap in male and female testosterone concentrations during May and June indicate that care must be taken when determining sex in individuals from feces. Despite annual changes in hormone metabolite levels, however, male and female differences persist.

Previous studies that have used fecal hormone metabolite concentrations to distinguish sex have compared differences between 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, differences between the sexes for both toad species were analyzed for testosterone/estradiol, progesterone/testosterone, and progesterone/estradiol and all ratios were found to be significantly different for males and females. The ratio of testosterone/estradiol had the most distinct confidence intervals, but was not as distinct in the American toad as testosterone alone. One benefit of this analysis, is that the ratio for males and females stay the same year round eliminating potential problems caused by seasonal hormone changes, which may become important when a complete annual fecal hormone metabolite profile for amphibians is available. For the present study, however, ratios were not better at distinguishing between males and females than absolute hormone concentrations.

This study is the first to use hormones derived from feces to distinguish sex in amphibians. Applications of fecal hormone concentrations may include investigating aspects of amphibian endocrinology in addition to determining sex. Hormone metabolite concentrations from feces could be used for determining seasonal fluctuations in steroids and correlating those levels to behavior. This noninvasive technique would be especially useful in studies of corticosteroid levels to reduce possible fluctuations in measurements caused by handling stress and blood sampling. Fecal metabolite concentrations may also be useful for studies of exposure to exogenous hormones for which blood and tissue

samples are currently used; feces would permit repeated samples to be taken without stress to the animal and long-term studies could be done with the same animal allowing consistency in measurements. Also, because fecal steroid metabolites represent pooled fractions of plasma hormones they may be more effective for determining hormone changes in species with diurnal cycles (Goymann et al., 1999). However, the use of fecal hormones in studies such as these must await identification of hormone metabolites and determinations of lag times between changes in plasma and fecal hormone concentrations as recommended by Wasser et al. (1994).

In conclusion, feces offer a useful source for obtaining and quantifying steroid hormone metabolites in amphibians, however their use in distinguishing sex in amphibians must be determined on a species by species basis. Differences in the concentrations of hormone metabolites were useful for distinguishing between males and females for the American toad because testosterone metabolites showed distinct quantitative differences in immunoreactivity between the sexes. In the boreal toad only estradiol showed significant differences between the sexes, and the confidence interval for each sex overlap despite these differences. Unfortunately this indicates that differences are not distinct enough for accurate gender identification in the boreal toad. Using fecal hormones to determine sex may still be useful for certain species, however because the hormones that show significant differences for the American and boreal toad vary, the assays will have to be validated and concentrations of fecal metabolites will have to be determined for each species individually. In addition, based on the variability in measurements of hormone metabolites in this study, future applications of this technique should rely on more than one sample in order to determine sex in an individual.

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APPENDIX

A mixed hormone standard was injected into high performance liquid chromatography (HPLC) apparatus and allowed to run for 60 minutes. Hormone standards produced absorbance peaks at 10, 21, 31, 40, and 44 minutes after injection which corresponded to estriol (E_3), testosterone (T), estrone (Es), progesterone (P_4), and estradiol (E_2) respectively (Figure 1A). Concentrated extracts of toad feces were run in the HPLC as well. There were a few peaks that showed up in the data printout immediately after injection, however there were no further peaks during the 60 minutes and none of the early peaks corresponded to peaks from the hormone standard.

In order to identify the hormone class the peaks belonged to fractions were collected, dried, resuspended in assay buffer, and run in each of the hormone assays to detect reactivity. Detection on the assay plates of each hormone standard corresponded to the time of the peak on the HPLC printout for that type of hormone. Samples of extracts from toad feces were collected and run on assay plates in the same manner as standards. Binding to the testosterone antibody was highest for fractions collected at 25 minutes, estradiol and progesterone did not show any immunoreactive peaks for binding to toad fecal extracts. To aid the identification of the hormone metabolites excreted by the toads, additional standards were run in the HPLC. The androgen metabolites 5α-androstan3α,17β diol, 5α-androstan 3β, 17α diol, 5α-androstan 3α-ol, 17-one, and 5α-androstan 3β-ol, 17- one were run through the HPLC. However, these compounds gave inconsistent results for both peak formation and for time of peak binding to the testosterone antibody. Because of these inconsistencies there was no further progress made toward identifying the hormone metabolites the toads excreted.

Figure 1A: Curve represents absorbance values for 5 hormone standards separated by high-pressure liquid chromatography (HPLC). Hormone standards produced absorbance peaks at 10, 21, 31, 40, and 44 minutes after injection which corresponded to estriol (E_3), testosterone (T), estrone (Es), progesterone (P_4), and estradiol (E_2) respectively. Arrow denotes the time of fraction from toad fecal extracts that had high immunoreactivity with the testosterone antibody.