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Sex hormones in the Axolotl, *Ambystoma mexicanum*: potential method for sex determination

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***Abstract.*—Axolotls, *Ambystoma mexicanum* (Shaw and Nodder 1798), are a Critically Endangered, aquatic salamander species. Despite being imperiled in the wild, laboratory colonies are bred extensively for developmental and embryological research. Verifying the sex of young-of-year would benefit researchers and culturists especially when organisms take a long time to develop or become sexually mature. In this study, we investigated whether steroid hormone metabolites (i.e., testosterone, 17β-estradiol) could be measured in three age classes of axolotls (classified as juvenile, sub-adults, and adults). Our objectives were firstly to validate whether significant levels of steroid metabolites could be detected in axolotls at various developmental stages using a previously unexplored method of extraction. Secondly, if significant levels of hormones were detected, could we differentiate between the sexes of adults by examining differences in the concentrations of steroid hormone metabolites? Steroid hormone analysis of tissue samples determined both testosterone and 17β-estradiol were present in detectable concentrations in all age classes. There was no significant difference in estradiol between females and males (t [11] = 0.89, p = 0.3881), however testosterone concentrations were approaching significance (t [10] = 1.81, p = 0.0569) with females having over twice as much testosterone (x = 0.36 ± 0.07 ng/mL, n = 7) compared to males (x = 0.17 ± 0.04 ng/mL, n = 5). There was not a significant difference (t [10] = 1.81, p = 0.1112) in the ratio of testosterone to estradiol between sexes. The presence of these hormones in earlier developmental stages was also confirmed providing the prospect that hormone changes can be tracked over the course of sexual development.**

Keywords. Estradiol, testosterone, salamanders, conservation, enzyme immunoassays, Mexico

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Introduction

Ambystoma mexicanum (Shaw and Nodder 1798), the axolotl, is native to Lake Xochimilco, a high-altitude lake in the Central Valley of Mexico. The expansion of agriculture and human development, compounded with the effects of deteriorating water quality and the introduction of non-native fish, has decimated *A. mexicanum* populations (Recuero et al. 2010; Alcaraz et al. 2015; Contreras et al. 2009), such that the species is listed as Critically Endangered (Shaffer et al. 2004; Zambrano et al. 2010; Eisthen and Krause 2012). Few individuals exist outside of captivity with many axolotls kept as exotic pets or maintained in laboratory colonies where they are used as model organisms for biological research (Contreras et al. 2009).

Captive breeding and reintroduction programs are being used to address dwindling wild populations and for axolotls and other salamanders, knowing the sex of individuals early in their development can play an essential role in conservation planning. Determining sex at a younger age benefits researchers and culturists, especially when individuals take a long time to develop and become sexually mature. Additionally, verifying the sex of young-of-year can be crucial in understanding demographics in field studies. Having to wait until maturity to ascertain the impacts of environmental conditions is not conducive to developing feasible and relevant conservation methods. By decreasing the time it takes to determine sex, management efforts can be greatly expedited and both captive reproductive efforts and field assessments can be improved..

One method of identifying sex of anuran amphibians is through hormone monitoring using urinary and fecal steroid hormonal immunoassays (Germano et al. 2009, 2012; Hogan et al. 2013; Narayan 2013; Graham et al.

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2016). These studies infer that these endocrinological methods could be implemented for identifying sex in salamanders and caecilians. To that end, our objectives in this study are to determine whether hormone measurements are reliable methods for identifying sex in salamanders. We investigated steroid hormone metabolites in three age classes of axolotls, *A. mexicanum* (Shaw and Nodder 1798), (classified as juvenile, sub-adults, and adults) with the goal of developing protocols for quantifying sexual hormones. Previous methods for quantifying steroid metabolites in axolotls required highly invasive procedures that resulted in termination of the experimental subjects (Eisthen and Krause 2012). Our goals were to develop a procedure which posed minimal risk for the subjects that could reliably quantify steroid hormone concentrations in the three age classes, as defined above. Prior to this study, we attempted to develop methods for extracting urinary and fecal samples from axolotls. However, we were unable to consistently collect adequate volumes of urine and fecal matter which could reliably quantify steroid metabolites so this study focuses on tissue samples. Second, we explored the possibility of differentiating between females and males by examining differences in the concentrations of steroid hormone metabolites (testosterone and 17β-estradiol [hereafter estradiol]) and calculating differences in the ratio of the concentrations of testosterone to estradiol. Both objectives are an attempt to develop a foundation for verifying sex of axolotls at an earlier age class. Verifying the sex of juvenile axolotls, a surrogate for other salamanders (especially *Ambystoma* species), may aid in the management of various species of concern.

Materials and Methods

Animals

We used 13 reproductively mature adult *A. mexicanum* that were maintained at the Warm Springs Fish Technology Center in Warm Springs, Georgia, USA. Of these, five were verified male subjects (snout-vent length [SVL] = 145.7 mm ± 10.0 mm), and eight were verified female subjects (snout-vent length = 138.0 mm ± 9.9 mm). We obtained 16 *A. mexicanum* of unknown sex from the *Ambystoma* Genetic Stock Center (AGSC) at the University of Kentucky and classified these either as sub adults (SVL = 115.0 mm ± 3.9 mm; approximately 8 to 10 months old) or juveniles (SVL = 99.9 mm ± 2.2 mm; approximately 5 to 6 months old). From these individuals, we chose six sub-adults and six juveniles for this study. The adult females, adult males, and sub-adults were housed in individual tanks, while juveniles were housed in tanks with other individuals which were of similar size and age. All tanks were supplied with a steady flow of fresh spring water at a rate of ~0.5 L/min. The axolotls were fed a standard pellet diet three to four days per week (three pellets twice a day when fed). Food pellets, "soft-moist" pellets (5 mm in size) were made by Rangen, Inc. and were purchased from the AGSC.

Tissue Collection and Steroid Tissue Extraction

Toe clips provided tissue samples from eight adult female subjects, five adult male subjects, and six sub-adults of unconfirmed sex and six juveniles of unconfirmed sex. Prior to sample collection, the animals were transferred to buckets containing an anesthetic bath prepared with the standard protocol for anamniote vertebrate sedation using tricaine mesylate (MS-222). Each bath was prepared at room temperature with 200 mg/L MS-222 in fresh tap water. The pH of each bath was neutralized using NaOH, which has previously been found to increase anesthetic efficacy and reduce stress in amphibians (Robinson and Scadding 1983). The bath was aerated with a solitary pump while organisms occupied the bath. Toe clips are a standard marking method for amphibian subjects in ecological studies and have not been shown to have a significant detrimental effect on other *Ambystoma* species (Otto and Scott 1999). Based on the extensive research on limb regeneration in *A. mexicanum*, it was assumed that when executed with extreme care, toe clips would not have a damaging effect on this species. Steroid hormones are more likely to be stored in the bone than the skin, and to maximize the amount of steroid hormone that would be found in each sample, the middle digit of the right forelimb was clipped on every subject. This is the longest digit with the most bone, so it is assumed this is the most efficient method of obtaining bone samples while minimizing risk to the organism.

Samples were processed using the protocol developed by Arbor Assays for steroid tissue extraction but modified for small sample volumes. Samples were dried at 70 °C for four hours. Samples were weighed periodically until constant weight was reached, and they were completely dry. Samples were homogenized by mortar and pestle and 1.0 g of each sample was transferred to new microcentrifuge tubes. To each tube, 10 ml of acetonitrile was added, and then the tube was sealed, and vortexed thoroughly. To determine extraction efficiency, one randomly chosen sample was split into two tubes and 10 µL of either 100,000 pg/mL estradiol or 200,000 pg/mL testosterone was added in addition to acetonitrile. All samples were centrifuged at 4,700 rpm at 4 °C for 15 minutes The supernatants of the centrifuged samples were transferred to clean 15 mL conical tubes and frozen at 4 °C overnight. The samples were gradually thawed over two hours. Once thawed, solvents were extracted using organic phase separations. To each sample, 30 ml of hexane was added and the sample was vortexed for two minutes. The solution was transferred to a separatory funnel, and the supernatant was transferred to a clean tube while the top layer of hexane was discarded. The supernatant which still contained acetonitrile was evaporated to dryness in SpeedVac over the course of two hours. The dried, extracted samples were reconstituted using 100 µL of ethanol and then diluted with 450 µL of Assay Buffer provided in the enzyme immunoassay kit from Arbor Assays (Ann Arbor, Michigan, USA).

Hormone Enzyme Immunoassays and Analysis

The reconstituted tissue samples were immediately run on DetectX Testosterone ELISA kit (Cat. # K032-H1, Arbor Assays, Ann Arbor, Michigan, USA) and 17β Estradiol ELISA kit (Cat. # K030-H1, Arbor Assays, Ann Arbor, Michigan, USA) based on instructions provided in the kit. Serially diluted samples were run alongside samples on each plate. All samples and standards were run in duplicate. Plates were read at an absorbance of 450 nm on a microplate reader. Concentrations were calculated from a standard curve using 4PLC fitting software provided by the free online software MyAssays, Ltd. For both enzyme immunoassays, one sample was extracted twice with a standard concentration of steroid hormone added to one portion of the sample to test extraction efficiency. Blank wells were run on each plate as a control. Concentrations that fell outside the range of the standard curve were excluded from the analysis.

Data Analysis

Statistical analyses were conducted using Minitab® Statistical Software. Testosterone and estradiol concentrations were compared between adult males and adult females using Student's t-tests. The ratios of testosterone:estradiol across categories were also compared using Student's t-tests. Extraction efficiency was calculated according to each steroid metabolite kit.

Results

Two of the 27 samples were not processed due to low sample weight after drying. The extraction efficiencies were 46.3% for estradiol extraction and 6.7% for testosterone extraction. The limit of detection for the testosterone kit was 0.031 ng/mL and the limit of detection for the estradiol kit was 0.027 ng/mL. All samples read were above these limits. The regression slopes were parallel to the standard curve for both estradiol (R^2 = 0.998) and testosterone (R^2 = 0.997). The intraassay coefficients of variation for estradiol and testosterone were 11.4% and 34.9%, respectively. To minimize variation in testosterone, it is suggested that extraction efficiency be maximized and an increase in serial dilutions be implemented.

Estradiol concentrations for females varied from 0.69 to 1.47 ng/mL, with a mean (± S.D.) of 1.56 ± 0.23 ng/mL, while estradiol concentrations for males ranged between 0.50 and 1.31 ng/mL, with a mean of 0.88 ± 0.38 ng/mL. Estradiol for subadults was between 0.49 and 1.27 ng/mL, with a mean of 0.78 ± 1.12 ng/mL, whereas for juveniles it was from 0.66 to 1.68 ng/mL, with a mean of 1.05 ± 1.57 ng/mL. Estradiol concentrations were higher than testosterone concentrations (Table 1), however there was no significant difference in estradiol between females and males (t [11] = 0.89, p = 0.3881).

Testosterone concentrations for females were between 0.15 and 0.63 ng/mL, with a mean of 0.359 ± 0.18 ng/mL, while testosterone concentrations for males ranged 0.07 to 0.28 ng/mL, with a mean of 0.17 ± 0.09 ng/mL, and differences in testosterone concentrations between the sexes were significantly different at the p = 0.0569 level (t [10] = 1.81). The range of testosterone concentrations for subadults was 0.17 with a mean of 0.66 ng/mL, 0.30 ± 0.75 ng/mL, and 0.15 with a mean of 0.45 ng/mL, 0.24 ± .05 ng/mL for juveniles. The ratio of testosterone:estradiol concentrations did not differ significantly between adult females and adult males (t [10] = 1.81, p = 0.1112).

Discussion

This study establishes a viable protocol for collecting tissue samples that can be analyzed for steroid hormone metabolite content. In addition, our findings validate the modified steroid extraction protocol developed for this study as both testosterone and estradiol were present in detectable concentrations in the collected tissue samples. The presence of these hormones in earlier developmental stages was also confirmed, providing the prospect that hormone changes can be tracked over the course of sexual development. All estradiol concentrations for females fell within previously reported ranges (0.2–2.8 ng/mL) (Eisthen and Krause 2012), while testosterone levels in males were lower than previously reported (0.2 to 0.8 ng/mL, Eisthen and Krause (2012)) potentially resulting from the lower testosterone extraction efficiency (6.7%) (Jacobs and Kühn 1988).

It may be possible to determine sex based solely on steroid hormone concentrations. Female axolotls had over twice as much testosterone than males (on average), however ranges of this hormone overlapped between the sexes, so these results must be viewed with caution. Future studies with a larger sample size and with methods that optimize extraction efficiency would potentially yield discernable differences in concentrations.

Previous studies on axolotls required dissection of the organism or invasive techniques which resulted in the termination of the subject to measure hormone levels (e.g., Eisthen and Krause 2012). In this study, all subjects survived the toe clip procedure, and no significant detriment occurred in any animals from the loss of the digit. This suggests that this procedure might be both a reliable and feasible method for steroid hormone measurement.

This study can serve as a basis for research into the environmental sex determination interests of the species, and consequently the concern for alterations of sex ratios in amphibian populations that might result from rearing/growth in different water temperatures. Axolotls are known to have a genetic sex determination system, referred to as the ZZ/ZW system, with females being the heterogametic sex (Armstrong and Malacinski 1989). Research by Bodney (1982) determined that increased levels of estradiol benzoate in water would induce sexual role reversal in male axolotls. Humphrey (1948) examined the functional role reversal of adult female axolotls due to the implementation of male sex structures. Further, Eisthen and Krause (2012) found that several environmental factors influenced estradiol levels in axolotl females but not in males. These studies show that axolotls have a flexible sexual system,

Table 1. Table of raw data for the estradiol (E) and testosterone (T) concentrations (ng/mL) of all tissue culture samples that were processed and analyzed via enzyme immunoassay as well as the ratios of the concentrations (T:E). Concentration marked with double asterisk (**) indicates the sample was out of range of the standard curve.

Subject	Female			Male			Subadult			Juvenile		
	E	**T**	**T:E**	**E**	**T**	**T:E**	**E**	**T**	**T:E**	**E**	**T**	**T:E**
1	0.857	0.373	0.435	1.242	0.285	0.229	0.586	0.315	0.537	0.738	0.225	0.304
2	1.466	**	n/a	1.310	0.086	0.066	0.781	0.167	0.214	0.168	0.189	0.113
3	0.966	0.633	0.655	0.557	0.206	0.370	0.690	0.259	0.375	1.326	0.163	0.123
4	1.012	0.559	0.552	0.501	0.071	0.143	0.491	0.206	0.418	0.102	0.149	0.145
5	1.039	0.151	0.145	0.803	0.208	0.259	1.272	0.659	0.518	0.905	0.453	0.501
6	1.143	0.251	0.241	-	-	-	0.862	0.205	0.237	0.659	0.264	0.400
7	1.176	0.337	0.287	-	-	-	-	-	-	-	-	-
8	0.693	0.215	0.310	-	-	-	-	-	-	-	-	-

suggesting the determination of sex can be influenced by environmental conditions. Hopefully, our research can provide a foundation for the understanding of hormones and sexual development and assist future investigations of how climate changes influence sexual determination and sex role reversal in *A. mexicanum*.

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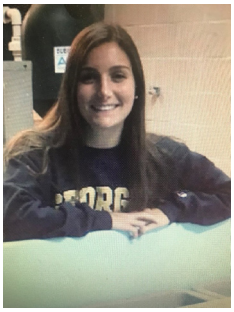

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