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Ambiguities in the relationship between gonadal steroids and reproduction in axolotls (*Ambystoma mexicanum*)

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ABSTRACT

Axolotls (*Ambystoma mexicanum*) are aquatic salamanders that are widely used in research. Axolotls have been bred in laboratories for nearly 150 years, yet little is known about the basic biology of reproduction in these animals. We investigated the effects of changing day length, time of year, and food availability on levels of circulating estradiol and androgens in adult female and male axolotls, respectively. In addition, we examined the effects of these variables on the mass of ovaries, oviducts, and eggs in females and on mass of testes in males relative to each individual's body weight, to calculate a form of gonadosomatic index (GSI). In both sexes, GSI was not correlated with levels of circulating steroids. In female axolotls, estradiol levels were influenced by food availability, changes in day length, and season, even when animals were held at a constant temperature and day length was decorrelated with calendar date. In addition, the mass of ovaries, oviducts, and eggs varied seasonally, peaking in the winter months and declining during the summer months, even though our animals were not breeding and shedding eggs. In males, levels of androgens appeared to vary independently of external conditions, but GSI varied dramatically with changes in day length. These results suggest that reproduction in axolotls may vary seasonally, as it does in many other ambystomid species, although both male and female axolotls are capable of reproducing several times each year. The physiological basis of this ability remains enigmatic, given the indications of seasonality contained in our data.

1. Introduction

The axolotl (*Ambystoma mexicanum*) is a paedomorphic salamander: it becomes sexually mature while retaining many morphological characteristics of the larval form. Axolotls were first brought to Europe in 1863, where they generated much interest for their ability to reproduce while resembling aquatic larvae, and then in some cases to metamorphose into an animal resembling a common terrestrial salamander, which was equally capable of reproducing [50]. Since their introduction to the research community, axolotls have become a model organism for biological research, used primarily in studies of embryology and regeneration.

The natural history of axolotls is poorly understood. Axolotls are native to two lakes, Xochimilco and Chalco, which have been subsumed by present-day Mexico City. Human alteration of this habitat for agricultural purposes dates back hundreds of years [7]; their environment is now so badly degraded that axolotls are listed as "critically endangered" on IUCN Red List. Recent studies indicate that few individuals exist in the wild [e.g., [12]], and it seems likely that the ecology and natural history of axolotls will never be known.

Given that axolotls have been bred in laboratories for nearly 150 years, it is perhaps particularly surprising that their reproductive patterns have not been thoroughly documented. Their courtship behaviors [18] and the anatomy of both male and female reproductive systems have been described [38,56], but the physiology of reproduction in axolotls has not been explored. In this study, we measured the gonadosomatic index (GSI) as well as levels of circulating estradiol and androgens in female and male axolotls, respectively. In addition to examining the relationship between GSI and specific gonadal steroids, we examined the effects of food availability, photoperiod, and season on both GSI and circulating levels of steroids.

2. Materials and methods

2.1. Subjects

All subjects were sexually mature, captive-bred axolotls (*A. mexicanum*). Animals were housed in individual bowls, and

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none of our subjects were given breeding opportunities during the course of the study. Data presented here were collected from 31 females and 30 males.

Axolotls were fed commercial salmon chow (Rangen; Buhl, ID) on one of two feeding regimes: "low feed" animals were fed five pellets each twice per week, and then food-deprived for 10 days prior to sacrifice for the study (15 females and 17 males), and "high feed" animals were fed five pellets each three times per week, including the day before sacrifice (16 females and 13 males). Aside from the terminal period of food deprivation, animals in our colony are maintained on the "low feed" schedule; thus, food availability was increased relative to baseline in the high feed group. Animals used as subjects in this study were maintained in the low feed condition for 9–600 days (median = 31 days) prior to the experiment; animals were maintained in the high feed condition for 7–354 days (median = 30 days).

To approximate changing seasonal conditions in the animals' native habitat, the animals were maintained in one of two windowless rooms in which the temperature and light cycle were tightly controlled. In the "short days" room, the temperature was maintained at 18 ± 1 °C, and in the "long days" room, the temperature was maintained at 21 ± 1 °C. The timing of light onset and offset in both rooms was digitally controlled, and adjusted daily to match sunrise and sunset times in Mexico City. The shortest day for any of these animals was 11:05, and the longest was 13:20. The remaining animals were maintained in a room in which the windows were blocked with foil, and the day length was controlled using an analog timer that was adjusted monthly to approximate summer conditions in Mexico City; days for these animals were 13:00 or 13:15.

All procedures were carried out in accordance with US Public Health Service regulations, under the guidance of the Institutional Care and Use Committees of the Marine Biological Laboratory and Michigan State University.

2.2. Data collection

Prior to blood collection and dissection, each animal was deeply anesthetized with pH-corrected 0.1% tricaine methanesulfonate (MS222; Sigma, St. Louis, MO). The chest was surgically opened, and a 25-g needle inserted directly into the aorta to collect 100–1000 μl blood from each animal. Whole blood was stored on ice in a Vacutainer (Becton–Dickinson, Franklin Lakes, NJ) for 5–15 min before being centrifuged at 4 °C for 15 min. The plasma was then removed and stored in microcentrifuge tubes at −80 °C until processing.

Each animal was weighed prior to blood collection and dissection. After blood collection, the animal was decapitated. For males, the testes were dissected out and weighed; for females, the ovaries and oviducts as well as the eggs ovulated into the coelomic cavity were dissected out and weighed. For ease of expression, we refer to the results of these measures as the gonadosomatic index (GSI), even though the mass used for females includes more than simply gonads.

2.3. Hormone assays

Levels of sex steroids in circulating plasma were quantified using testosterone and estradiol enzyme immunoassay (EIA) kits from Cayman Chemical (Ann Arbor, MI). Assays were carried out in accordance with the manufacturer's instructions. Standard curves were generated using serial dilutions of estradiol and testosterone that were supplied with the kits, and were run in duplicate for all samples. According to information supplied with the kits, the anti-17β-estradiol antiserum cross-reacts somewhat with estrone, estradiol-3-glucuronide, and estradiol-17-glucuronide in competitive binding assays. Similarly, the anti-testosterone antiserum cross-reacts with 5α- and 5β-dihydrotestosterone (DHT), and to a lesser extent with androstenedione and 11-ketotestosterone. In both kits, the cross-reactivity of other steroids is less than 1%. For ease of expression, we will generally refer to the measured steroids as "estradiol" and "androgens".

Estradiol was extracted from plasma four times using dichloromethane, and testosterone was extracted three times using diethyl ether. Samples were quickly dried under a vacuum at room temperature and reconstituted using EIA buffer supplied with the kits. Samples from females were assayed for estradiol in triplicate using one or two dilutions ranging from 1:10 to 1:45. Samples from males were assayed for androgens using eight dilutions spanning a range from 1:10 to 1:250. After incubation, all plates were read at an absorbance of 415 nm.

2.4. Data analysis

For both estradiol and androgen assays, controls included assaying plasma from the same animal on separate plates or more than once on a single plate. As an additional control, in some cases plasma from a given tube was extracted twice separately and the samples run on the same plate. Results obtained from repeated samples from the same animal generally fell within 20% of each other, as indicated in the kit specifications.

For all assays, results were excluded if the ratio of the percent of steroid bound to the maximum bound ($\%B/B_0$) fell on the ends of the standard curve, that is, at a value of less than 20% or more than 80%. In addition, in androgen assays in which eight dilutions were used, values falling more than one standard deviation from the mean were excluded. Finally, for all assays, samples for which the coefficient of variation (standard deviation/mean) of the calculated quantity of estradiol or androgens was greater than 20% were excluded from the analysis. The 3–10 remaining values for each individual were averaged to obtain a single value used in the analyses presented here.

Gonadosomatic index was calculated as the ratio mass of the gonads to the mass of the body, with the gonads subtracted from the body mass. In females, oviduct and egg mass was included in the gonad mass. Statistical analyses were carried out using JMP 5.0 (SAS Institute; Cary, NC).

3. Results

3.1. Correlation between levels of sex steroids and GSI

The detection limit for the estradiol EIA kit is 20 pg/ml, and that for the testosterone kit is 6 pg/ml. All levels that we measured were substantially above these limits. Estradiol levels in female subjects ranged from 0.2 to 2.6 ng/ml, with a median level of 0.6 ng/ml; the mean (±SEM) estradiol level was 0.85 ± 0.11 ng/ml. Levels of androgens in males were somewhat higher, ranging from 0.5 to 7.2 ng/ml; the median value was 2.1 ng/ml, and the mean (±SEM) was 2.52 ± 0.29 ng/ml.

We also calculated the GSI for each individual. For females, GSIs ranged from 0.6% to 10%, with a median value of 6.0%. The mean (±SEM) GSI value for females was 5.8 ± 0.4%. GSIs for males were more homogeneous than for females, ranging from 0.9% to 2.1% with a median value of 1.5%. The mean (±SEM) GSI for males was 1.5 ± 0.1%.

The hormone levels that we measured do not correlate well with GSI for either female or male axolotls (Fig. 1). Specifically, $R^2 = 0.005$ for the correlation between estradiol and GSI in females ($p = 0.70$), and $R^2 = 0.002$ for the correlation between androgens and GSI in males ($p = 0.81$). Further, we examined the possibility