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.

Keywords

androgen
estradiol
model organism
salamander
seasonal breeding
testosterone

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 (*Ambystoma mexicanum*). Animals were housed in individual bowls, and 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 5 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 5 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 (Figure 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 that levels of sex steroids might covary with either body or gonad mass, independent of their ratios. They do not. Specifically, the females included in this study ranged in body mass from 40 to 129 g (median = 67 g); the mass of their ovarian tissue and eggs ranged from 0.3 to 9.5 g (median = 3.6 g). Estradiol levels do not correlate with females' body mass ($R^2 = 0.004$; $p = 0.73$) or gonad/oviduct/egg mass ($R^2 = 0.004$; $p = 0.74$). The body mass of males ranged between 46 and 114 g (median = 63 g), and their gonadal mass ranged from 0.6 to 1.4 g (median = 0.9 g). Androgen levels do not correlate with males' body mass ($R^2 = 0.0005$; $p = 0.91$) or gonad mass ($R^2 = 0.002$; $p = 0.80$).

3.2. Analysis of female data

Either estradiol levels or GSI, or both, might be affected by the feeding regimes that our animals were exposed to, by changes in the duration of daylight, by the actual time of year (regardless of photoperiod), or some combination of these factors. In all such analyses reported here, change in day length was calculated over the two weeks prior to blood collection and the date the animal was sampled was represented by the month alone.

Stepwise multiple regression analysis of the effects on estradiol levels of feeding regime, change in photoperiod, and month indicates that feeding regime significantly affects estradiol levels ($R^2 = 0.18$, $p = 0.02$). As illustrated in Figure 2A, estradiol levels were significantly higher in animals in the high-feed condition than in the low-feed condition (Tukey HSD test, $p < 0.05$). Change in photoperiod and month do not, alone, significantly affect estradiol levels: change in photoperiod, $R^2 = 0.08$, $p = 0.13$; month, R^2

= 0.02, p = 0.38. Estradiol levels in relation to changes in day length and to calendar month are illustrated in Figures 2B and C, respectively.

Multiple regression analysis revealed that all potential two-factor models account for between 21 and 27% of the variance. A model that incorporates both feeding regime and change in photoperiod produces results (R^2 = 0.27, p = 0.04) that are indistinguishable from model that includes feeding regime and month (R^2 = 0.26, p = 0.04). On the other hand, a model that includes only change in photoperiod and month does not explain the data quite as well (R^2 = 0.21, p = 0.09). Finally, a model that incorporates all three variables accounts for 43% of the variance (p = 0.04).

In contrast, similar analyses examining the effects of feeding regime, change in photoperiod, and month on GSI yielded no statistically significant results. The strongest single factor was change in photoperiod, but it accounts for little of the variance (R^2 = 0.006, p = 0.68). Two-factor models did not perform well, producing R^2 values that ranged from 0.005 to 0.02, with p values between 0.91 and 0.99. A model that included all three variables also performed poorly (R^2 = 0.06, p = 0.98).

Interestingly, the mass of a female's gonads, oviducts, and eggs varied somewhat across the year (R^2 = 0.17, p = 0.07); this relationship is illustrated in Figure 3A. Nevertheless, gonad/oviduct/egg mass is not correlated with estradiol levels (R^2 = 0.004, p = 0.73), and adding this mass into the multiple regression model along with change in photoperiod, month, and feeding regime, reduced the ability of each model to predict estradiol levels. Thus, it appears that mass of gonads, oviducts, and eggs varies independently of estradiol.

Although we were concerned that feeding regime might directly affect either body mass or gonad/oviduct/egg mass, neither differed significantly between females in the high-feed and low-feed conditions (t(29) = 1.66, p = 0.15, and t(29) = 1.23, p = 0.23, respectively).

3.3. Analysis of male data

Data from male axolotls were analyzed as described above for females. Unlike the results obtained with female data, stepwise multiple regression analysis revealed no statistically significant effects on androgen levels in male axolotls. As with females, feeding regime was the strongest single factor, but did not explain much of the variance (R^2 = 0.04, p = 0.25). None of the three possible two-factor models produced statistically significant results, with R^2 values ranging between 0.08 and 0.11 and p values between 0.40 and 0.56. The three-factor model explained only 23% of the variance in the data (p = 0.49).

In contrast to the results obtained for females, analyses using GSI as the dependent variable produced statistically significant results, illustrated in Figure 4. Notably, change in photoperiod had a dramatic effect on GSI (R^2 = 0.25, p = 0.005), with GSI decreasing as days grew shorter and increasing as days grew longer over the two-week interval prior to blood collection (Fig. 4A). This effect is specific to *change* in photoperiod, as the correlation between the length of daylight and GSI is not statistically significant (R^2 = 0.07, p = 0.29). Neither feeding condition nor month alone significantly affected GSI (R^2 = 0.05, p = 0.24 and R^2 = 0.02, p = 0.44; Figs. 4B and C respectively). Nevertheless, two-factor models that included change in photoperiod produced statistically significant results: a model that combined change in photoperiod with month explained 30% of the variance (p = 0.03) and one that combined change in photoperiod with feeding condition explained 26% of the variance (p = 0.05). A model that combines month and feeding condition did not perform well (R^2 = 0.12, p = 0.34). Finally, a model that combines all three variables does not produce statistically significant results (R^2 = 0.33, p = 0.24).

Among males, gonad mass did not vary across the year (Fig. 3B; R^2 = 0.06, p = 0.45); gonad mass also did not vary with androgen level (R^2 = 0.002, p = 0.80). As we found with females, feeding condition did not affect either males' body mass (t(28) = 0.64, p = 0.53) or gonad mass (t(27) = 0.39, p = 0.70).

4. Discussion

4.1. Levels of sex steroids in axolotls and other salamanders

The levels of circulating sex steroids that we measured in axolotls fall within the range of

values reported for other salamanders. The highest levels of circulating estradiol reported for any female salamander occur in the Japanese black salamander *Hynobius nigrescens*, ranging from 2 to 22 ng/ml [25]. In female mudpuppies, *Necturus maculosus*, estradiol levels range between 0.6 - 6.4 ng/ml [5]. In female Ocoee salamanders, *Desmognathus ocoee*, serum levels of estradiol are approximately 3 – 6 ng/ml [64]. Finally, estradiol levels have been measured in females of two species of salamandrids, and fall in a similar range: 0.8 – 4.7 ng/ml in Pyrenean brook salamanders, *Calotriton* (formerly *Euproctus*) *asper*, [11] and 0.8 – 3.1 ng/ml in the sharp-ribbed salamander, *Pleurodeles waltl* [16].

In our study, we found that estradiol levels in female axolotls ranged between 0.2 and 2.6 ng/ml, with a mean of 0.9 ng/ml. Interestingly, Katsu and colleagues [31] have cloned the ERα and ERβ receptors from axolotls and report that the half-activation for heterologously expressed receptors is about 2.7 pg/ml for ERα and 0.7 ng/ml for ERβ. Thus, the EC50 for ERα is approximately 100-fold lower than the lowest levels of circulating estradiol that we measured, whereas that for ERβ is within the range of circulating estradiol levels.

In male axolotls, we found that circulating androgen levels range from 0.5 to 7.2 ng/ml, with a mean of 2.5 ng/ml. These levels are somewhat higher than those described for axolotls by Jacobs and Kühn [30], who report baseline levels of serum testosterone around 0.2 ng/ml, which were elevated to as much as 0.8 ng/ml following injection of GnRH. The source of the discrepancy is not clear. The specificity of the method used by Jacobs and Kühn is not described, raising the possibility that the levels we measured are higher due to the cross-reactivity of our antibody with DHT, although the cross-reactivity is not high (approximately 27% in competitive binding assays) and serum levels of DHT are generally lower than those of testosterone in salamanders. The animals in the two studies were of similar sizes (Jacobs and Kühn report that the mean body mass of their animals was 64 g; the mean for our animals was 63 g) and were maintained at similar temperatures, but many other variables, including the type of food used and the genetic backgrounds of the animals, may have contributed to differences among the two groups.

Levels of serum androgens have been reported in males of three other species of ambystomid salamanders, and exceed the values we obtained for axolotls. Specifically,

Norris et al. [42] quantified androgens (T + DHT) in neotenic tiger salamanders, *Ambystoma tigrinum*, which range between 0.6 and 39.0 ng/ml. In the spotted salamander, *A. maculatum*, androgen levels range from 2 to 16.7 ng/ml [13], and values in marbled salamanders, *A. opacum*, range from 5.2 to 116.7 ng/ml [27].

Testosterone or androgen levels in other families of salamanders are as high or higher than those reported in ambystomids. In male *Necturus maculosus*, circulating testosterone ranges from 4 to 202 ng/ml [5], and in male plethodontid salamanders range from 5 to 439 ng/ml in redback salamanders, *Plethodon cinereus* [10], to approximately 200 – 350 ng/ml in male *Desmognathus ocoee* [64]. Estradiol levels in female *Hynobius nigrescens* are high, and so are androgen levels in males: 60 - 844 ng/ml [25]. Finally, in salamandrids, testosterone levels in male *Salamandra salamandra* range between 25 and 85 ng/ml [34]; androgen levels in male *Calotriton asper* range from 1.7 to 9.8 ng/ml [11] and in *Pleurodeles waltl* range from 5.2 to 37 ng/ml [17].

Interspecific differences in levels of circulating androgens appear to be greater among male salamanders than are levels of circulating estradiol among female salamanders. The basis of this variability is unknown, but a recent analysis suggests that levels of circulating androgens in male reptiles and amphibians increase with latitude [40], much as levels of androgens in birds increase with distance from the equator [19]. Axolotls generally fit this pattern, with the lower levels of circulating androgens than have been found in ambystomids obtained from more northern locations.

4.2. Androgens, estradiol, and reproduction in salamanders

Our results indicate that food availability, change in day length, and season all influence estradiol levels in female axolotls; in contrast, none of these variables influences androgen levels in male axolotls. Interpreting the significance of these effects is complicated by a lack of available information concerning relationship between gonadal steroids and reproductive behavior or gametogenesis in axolotls. Nevertheless, some data are available for other salamanders, including a few ambystomid species, and we will briefly review the relevant literature here to provide context for our results.

Few studies directly address the role of gonadal steroids in facilitating or inducing reproductive behavior in salamanders by manipulating steroid levels. Nevertheless, data from members of the family Salamandridae suggest that androgens may be generally necessary but not sufficient for induction of courtship behavior in males. For example, in the newt *Taricha granulosa*, male courtship clasping (amplexus) depends on circulating androgens, but hormones and other factors, like vasopressin, corticosterone, and GABA, are involved in releasing or suppressing clasping behavior. Similarly, androgens are required for male courtship displays in two species of newts that do not engage in amplexus, *Cynops pyrrhogaster* and *Triturus carnifex*, but prolactin may serve as the more immediate trigger for courtship behavior [reviewed in 39]. Androgens have also been shown to be necessary for expression of courtship behavior in the plethodontid salamander *Desmognathus ochrophaeus* [4]. Curiously, in the congener *D. ocoee*, handling stress suppresses testosterone but does not alter male courtship behavior, suggesting either that testosterone is not necessary for courtship in this species or that the stressor did not alter testosterone levels enough to produce a behavioral effect [64].

Additional studies provide correlational data concerning the role of androgens in male courtship behavior by indicating whether the timing of peaks in androgen levels coincides with peaks in breeding activity. Given the data described above, it is reassuring that androgen levels have been shown to peak during the breeding season in the salamandrids *Cynops pyrrhogaster* and *Triturus carnifex* [53, 65]. Similar results have been obtained for the salamandrids *Calotriton asper*, *Taricha granulosa*, and *Salamandra salamandra*, which is unusual in having a fall breeding season, and *Pleurodeles waltl*, which has two breeding seasons [8, 11, 16, 34, 51]. Within the family Plethodontidae, similar relationships are seen: plasma androgen or testosterone levels are highest during the breeding season in male redback salamanders, *Plethodon cinereus* [10], as well as in two species with uncommon breeding patterns, *Desmognathus ochrophaeus*, which breeds twice year, and *Plethodon jordani*, which breeds in the fall [62]. Circulating levels of androgens also peak during the spring breeding season in *Hynobius nigrescens* [25].

The closest relatives of axolotls are tiger salamanders, *A. tigrinum* [48, 49]. In a neotenic population of tiger salamanders in Colorado, androgen levels are moderate during the spring breeding season (4 ng/ml) and decline in June (0.6 ng/ml) before peaking in the

fall (38 ng/ml) [42]. Although the highest levels of androgen do not occur during the breeding season, the levels measured during the spring are similar to those that we measured in axolotls and may be sufficient to support reproductive behavior. Houck [27] found that androgen levels do not differ between male marbled salamanders (*A. opacum*) that were given the opportunity to court females and those that were not. Nevertheless, given that all individuals were examined during the breeding season and were collected during the spring migration to breeding ponds, this study does not rule out the possibility that androgens are required for courtship behavior in male *A. opacum*. Bolaffi and Callard [5] report no seasonal variation in plasma steroid levels in male mudpuppies, *Necturus maculosus*, although this result is difficult to interpret because the animals used in the study were kept in the laboratory for up to five weeks and some at temperatures much lower than they would normally experience during the breeding season. In summary, although the data concerning the relationship between circulating androgens and reproductive behavior are ambiguous for ambystomatid and proteid salamanders, androgens peak during the breeding season, and may be necessary for expression of courtship behavior, in salamandrids, plethodontids, and hynobiids.

The role of hormones in courtship and mating behavior in female salamanders has been the subject of very few studies [63]. In the salamandrid *Taricha granulosa*, courtship behavior leads to elevated circulating estradiol levels [44]. In two other salamandrids, *Pleurodeles waltl* and *Triturus carnifex*, both estradiol and androgen levels peak in females during the annual breeding period [8, 17, 65], but estradiol levels peak somewhat after the breeding season in *Calotriton asper* [11]. In the plethodontid salamander *Desmognathus ocoee*, handling stress elevates plasma estradiol, but does not alter courtship behavior in females [64]. Circulating steroid levels in female *Necturus maculosus* have been reported to lack seasonality [5], but, as mentioned above, the conditions in which the animals were kept weakens this conclusion. On the other hand, in female *Hynobius nigrescens* estradiol levels are low throughout the spring breeding season [25]. Thus, estradiol may be related to courtship behavior in some salamander taxa, but not in others. Overall, the direction of causality in the relationship between elevated estradiol levels and courtship behavior is not clear, and no study has addressed the possibility that moderate levels of estradiol may be necessary for courtship behaviors to occur.

In addition to courtship and mating behavior, androgens and estradiol may also be involved in gametogenesis. In salamanders with a spring breeding season, males often use sperm that were produced during the previous fall and stored in the vas deferens over the winter [35]. The process of spermatogenesis in salamanders has recently been reviewed by Propper [43]. Briefly, spermatogenesis occurs sequentially in different zones of the testis, such that posterior regions contain more mature sperm and the least-developed sperm are found most anteriorly. Development of Leydig cells follows the posterior-anterior gradient of spermatogenesis, and tends to lag behind sperm development; thus, androgens are not secreted from local cells during at least the early stages of spermatogenesis. The period of spermatogenesis can be lengthy, but in most species ends at spermiation, when the mature sperm move into the vas deferens. The emptied testicular lobules then differentiate into glandular tissue that secretes androgens and estradiol into the circulatory system. This basic pattern holds in the salamandrid *Paramesotriton hongkongensis* as well as in the proteid *Necturus maculosus* [35, 45].

The timing of major stages of spermatogenesis and their relationship to circulating androgen levels have been studied in a number of species. In salamandrids, androgen levels are lowest at the beginning of spermatogenesis, increase during the period of spermatogenesis, and peak around the time of spermiation *[Calitriton asper*, 11, *Pleurodeles waltl*, 16, *Taricha granulosa*, 51]. A similar pattern has been described in the *Plethodon jordani* [62]. In *Hynobius negrescens*, spermatogenesis is synchronized throughout the testis and spermiation occurs in the spring when males enter breeding ponds, rather than in the fall as in many other Northern temperate salamanders. Nevertheless, androgen levels are still low when spermatogenesis begins and peak at the time of spermiation [24, 25]. In the neotenic ambystomids *A. tigrinum* and *A. dumerilii*, as well as in the plethodontid *Desmognathus ochrophaeus*, spermatogenesis begins at the end of the breeding season, when androgen levels are moderate; androgen levels then fall before rising to a peak at the time of spermiation [42, 55, 62]. Thus, it seems that in salamanders androgens may not be required for the initiation of spermatogenesis, and androgen levels tend to peak at spermiation. Lazard [33] studied the activity of two enzymes involved in androgen production in the testes of axolotls, and found that, as expected, their activity in Leydig cells peaked around the time of spermiation; however, their activity in Sertoli cells peaked while spermatogonia were

dividing, suggesting that androgen levels may not be minimal at the beginning of spermatogenesis in axolotls.

The role of gonadal steroids in oogenesis has not been the subject of detailed study in ovariparous salamanders, but is presumed to follow the pattern established in frogs in which estradiol levels are generally low at the beginning of oogenesis and gradually increase until the time of ovulation [54]. Estradiol causes the release of vitellogenin, and is therefore necessary for deposition of yolk in eggs [54]. Estradiol is also necessary, but not sufficient, to stimulate production of the jelly coat in oviducts of female *Cynops* [29]. Oviducts elongate and their walls become thicker during the breeding season in *A. tigrinum*, and both estradiol and testosterone are required for these changes to occur; the two hormones are also required for sensitizing oviducts to hypophyseal hormones that stimulate the smooth muscle contractions involved in oviposition [21]. Thus, the involvement of estradiol in female gametogenesis seems to begin with yolk deposition and continue through egg laying.

Correlational studies of reproductive anatomy and circulating estradiol levels support this general scenario. In the salamandrids *Calitriton asper* and *Pleurodeles waltl*, estradiol levels are low in the summer, after the breeding season, and increase through the fall until the time of vitellogenesis [11, 17]. In *Pleurodeles waltl* and *Triturus carnifex*, the ovaries and oviducts increase in mass during the fall, as circulating estradiol levels are rising [17, 65]. In *Hynobius nigrescens*, which is unusual in having external fertilization, estradiol levels are low during the spring breeding season and rise in the fall [25]. This fall increase in estradiol levels coincides with growth of the ovaries, oviducts, ovisacs, and cloacal glands [23].

4.3. External factors influencing reproduction in female axolotls

Estradiol levels in female axolotls are more clearly related to food availability than to any other variable (Fig. 2A). Why should estradiol levels increase in well-fed females? As described above, estradiol levels are frequently elevated during the breeding season, but reproductive behavior does not appear to require dramatic expenditures of energy. On the other hand, estradiol levels are also frequently associated with an increase in the mass of ovaries and oviducts and the production of vitellogenin, which are energetically

expensive events [54]. In *A. opacum*, females with greater access to food are more likely to be reproductive, have larger clutch sizes, produce larger eggs, and invest their eggs with more lipids than do females given moderate or low access to food [47]. Studies of female plethodontids (*Hemidactylium scutatum*) indicate that those that skip a breeding season gain more body mass than those that reproduce, but that females provisioned with extra food are less likely to skip a breeding season [22]. Reproduction is expensive for female axolotls, which typically lay 175 - 200 eggs in a single clutch, and can lay up to 400 eggs at a time [20]. Thus, we surmise that estradiol levels increase in well-fed females because food availability is a key factor limiting reproduction in female salamanders.

Our data also suggest that estradiol levels in female axolotls may increase with increasing day length (Fig. 2B). Given day length increases through the winter and spring in the Northern hemisphere, this result may indicate that estradiol levels increase during a spring breeding period, perhaps in association with reproductive behavior, in axolotls. We also find peaks in estradiol levels in January and October, regardless of day length (Fig. 2C). Given that photoperiod was decorrelated with calendar date and all animals were maintained at constant temperatures we are at a loss to explain this apparent seasonality in estradiol levels, but seasonal differences in breeding success have been repeatedly reported in axolotl colonies. Despite keeping the animals at a constant temperature and unchanging day length, the probability of obtaining successful spawns peaked from January through May and decreased dramatically during the summer months in axolotl colonies at Indiana University [37], the University of Kentucky (Laura Muzinic, pers. comm.), and the University of Ottawa [3]. In addition, one source asserts that although absolute day length does not affect reproductive success in axolotls, an increase in day length over a 2-3 week period enhances the probability of obtaining spawns [3], suggesting that changing day length may alter hormonal activity in axolotls. Some species of salamanders show two peaks in estradiol levels, one during the spring breeding season and a second in the fall, associated with vitellogenesis and ovulation [Pleurodeles waltl, 17, Hynobius nigrescens, 23, 25], and the same may occur in axolotls.

We find that the mass of the females' ovaries, oviducts, and eggs declines in the summer and peaks in the winter (Fig. 3A), corroborating a previous anecdotal report that

the gonads in axolotls tend to regress during the summer [37]. A similar decline has been described in salamandrids and hynobiids [17, 23, 65]. Nevertheless, we found no correlation between circulating estradiol levels and the combined mass of ovaries, oviducts, and eggs (Fig. 1A), a result unlike those described in the salamandrid *Triturus carnifex* [65] and in *Hynobius nigrescens* [23, 25]. Either the relationship is different in axolotls or ambystomids than in other salamanders, or perhaps a more detailed analysis that separated the masses of the ovaries, oviducts, and eggs would reveal a relationship that was obscured by our methods.

4.4. External factors influencing reproduction in male axolotls

In contrast to our results with females, we found no relationship between androgen levels and food availability, change in photoperiod, or time of year in male axolotls. Although severely malnourished male salamanders may not reproduce in the wild, all our animals were sufficiently well fed that a lack of effect of feeding regime is not surprising. However, the absence of changes in androgen levels with either time of year or change in photoperiod is surprising given that seasonal differences in androgen levels have been reported in many male salamanders, including *A. maculatum* and neotenic *A. tigrinum* [13, 42]. It seems possible that decorrelating change in day length from time of year confounded the two variables, obscuring differences, although both variables contributed to differences in estradiol levels in females.

On the other hand, we found that GSI in males varied dramatically with change in day length (Fig. 4A); this effect was not due to changes in the absolute size of the testes or the body. These observations suggest that spermatogenesis in axolotls increases with day length, that is in the spring. The data illustrated in Figure 4C further suggest that spermatogenesis continues through the summer and that spermiation, which causes declines in testis mass, begins in the fall or winter. This result fits with an estimate of the life cycle of sperm in axolotls, which indicates that sperm may be stored in vasa for only days or at most weeks, rather than the months seen in salamanders in which spermiation occurs in the fall and breeding in the spring [38]. If so, the pattern in axolotls differs from that of other ambystomids. In neotenic *A. tigrinum*, the GSI is moderate in March and declines throughout the breeding season, reaching a nadir in June; it then increases rapidly, peaking in August before beginning a gradual decline through the

following spring [42]. A similar pattern has been described for *A. macrodactylum* in Washington State [58], as well as for a salamandrid, *Triturus carnifex* [65]. Species differences, ecological differences, and differences between laboratory and wild populations may all contribute to the differences between axolotls and the other species described here.

Previous studies of the effect of photoperiod on spermatogenesis in plethodontids and salamandrids have produced mixed results, with some researchers concluding that long days can alter the rate of spermatogenesis and others concluding that day length is irrelevant [e.g., 15, 28, 61]. We are unaware of any study, however, that has systematically examined the effect of *changing* photoperiod on reproductive activity in salamanders. Given the strong correlation we found between GSI and change in day length, our results suggest that previous conclusions about the effects of long days or short days should be reconsidered.

We found no correlation between plasma androgen levels and GSI in axolotls (Fig. 1B). In the salamandrid *Triturus carnifex*, androgens also do not correlate with GSI in males [65]. On the other hand, we have analyzed the data for neotenic *A. tigrinum* presented by Norris et al. [42], and find a slight association between androgen levels and GSI (R^2 = 0.43, p = 0.07). Interestingly, levels of DHT are strongly correlated with GSI in these animals (R^2 = 0.60, p = 0.02). It is not clear whether this result represents another difference between axolotls and tiger salamanders, or if we would obtain the same result if we were to measure DHT levels in axolotls.

4.5. Are axolotls seasonal breeders?

The reproductive activity of axolotls has not been studied in their native habitat; thus, we do not know whether they breed seasonally in the wild. Most ambystomids that have been examined have been reported to breed seasonally, including *A. annulatum* [52], *A. californiense* [36], *A. maculatum* and *texanum* [32], *A. macrodactylum* [58, 59], *A. opacum* [27], and *A. talpoideum* [46]. Seasonal breeding has also been described in neotenic populations and species, including *A. dumerilii* [6, 55], *A. gracile* [14], and *A. tigrinum* [41]. Nevertheless, not all ambystomids are seasonal breeders. *A. ordinarium* in the Michoacan district of Mexico breed either biannually or continuously: Anderson and

Worthington [2] describe finding eggs and larvae in both summer and winter. *A. rosaceum*, which is found in Western Mexico, generally breeds in during the summer rainy season but may breed biannually or year-round when permanent ponds are available [1]. Furthermore, studies of spermatogenesis in neotropical bolitoglossine salamanders indicate that males produce new sperm year-round, even in species that live in habitats with some seasonal variation in ecological factors, strongly suggesting that these animals are not seasonal breeders [9, 26, 57, 60]. The lack of seasonality in bolitoglossines is thought to be due to relative constancy of their environment; given that axolotls were originally in found in permanent lakes in the tropics, it seems possible that they also breed aseasonally.

Publications that describe conditions for breeding axolotls in captivity indicate that males can spawn once a month or more and females every four to five months, although the success rate declines in the summer, as noted above [3, 20]. In addition, spermatogenesis in male axolotls occurs asynchronously among individuals in a colony, suggesting a lack of seasonality when animals are kept under constant conditions [38]. Given that axolotls have been bred in laboratories for nearly 150 years, it seems possible that they have been subjected to strong artificial selection for year-round breeding, and have perhaps lost their annual cycle. Nevertheless, the first published description of mating behavior in axolotls [18] mentions that Duméril obtained six clutches of eggs from a single female in 1865-66. Given that axolotls were brought to Duméril's laboratory in 1863 and axolotls require at least a year to reach sexual maturity, this female cannot have been more than two generations removed from the wild, indicating that the ability of axolotls to breed year-round is not a product of intensive laboratory inbreeding. On the other hand, Gasco also describes his attempts to observe mating in axolotls that were kept on at near-ambient temperatures and a natural photoperiod in Genoa, Italy; he write that males started courting and laying spermatophores in February and that he obtained his first successful spawn in March, suggesting a role for environmental cues in controlling reproduction in axolotls. Our data indicate that changing day length and time of year both contribute to estradiol levels in females and that GSI in males can be dramatically altered by changing photoperiod over just two weeks, suggesting that seasonal factors play a role in reproduction in axolotls. In the laboratory, and perhaps in the wild, axolotls seem to be able to take advantage of

optimal conditions to breed more than once a year, or even continuously. The physiological basis of this ability is curious and worthy of examination.

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References

[1] J.D. Anderson, R.G. Webb, Life history aspects of the Mexican salamander *Ambystoma rosaceum* (Amphibia, Urodela, Ambystomatidae). J. Herpetol. 12 (1978) 89-93.

[2] J.D. Anderson, R.D. Worthington, The life history of the Mexican salamander *Ambystoma ordinarium* Taylor. Herpetologica. 27 (1971) 165-176.

[3] J.B. Armstrong, S.T. Duhon, G.M. Malacinski, Raising the axolotl in captivity. In: J.B. Armstrong, G.M. Malacinski, (Eds.), Developmental biology of the axolotl, Oxford University Press, New York, 1989, pp. 220-227.

[4] S.L. Benner, S.K. Woodley, The reproductive pattern of male dusky salamanders (genus *Desmognathus*) is neither associated nor dissociated. Horm. Behav. 51 (2007) 542-547.

[5] J.L. Bolaffi, I.P. Callard, Plasma steroid profiles in male and female mudpuppies, *Necturus maculosus* (Rafinesque). Gen. Comp. Endocrinol. 37 (1979) 443-450.

[6] R.A. Brandon, Size range, size at maturity, and reproduction of *Ambystoma* (*Bathysiredon*) *dumerilii* (Dugès), a paedogenetic Mexican salamander endemic to lake Pátzcuaro, Michoacán. Copeia (1970) 385-388.

[7] R.A. Brandon, Natural history of the axolotl and its relationship to other ambystomatid salamanders. In: J.B. Armstrong, G.M. Malacinski, (Eds.), Developmental biology of the axolotl, Oxford University Press, New York, 1989, pp. 13-21.

[8] C. Cayrol, D.H. Garnier, P. Deparis, Comparative plasma levels of androgens and 17 beta-estradiol in the diploid and triploid newt, *Pleurodeles waltl*. Gen. Comp. Endocrinol. 58 (1985) 342-346.

[9] L. Chan, Seasonality, microhabitat and cryptic variation in tropical salamander reproductive cycles. Biol. J. Linn. Soc. 78 (2003) 489-496.

[10] D.R. Church, R.K. Okazaki, Seasonal variation of plasma testosterone titres in male redback salamanders, *Plethodon cinereus*. Amphib. Reptil. 23 (2002) 93-97.

[11] M. Clergue, J.P. Moatti, P. Milhet, Androgènes et 17 beta-Estradiol plasmatiques de l'Urodèle pyrénéen *Euproctus asper* durant la période de reproduction. Gen. Comp. Endocrinol. 58 (1985) 337-341.

[12] -Meyer, E. Valiente, L. Zambrano, Recent decline and potential distribution in the last remnant area of the microendemic Mexican axolotl (*Ambystoma mexicanum*). Biol. Cons. 142 (2009) 2881-2885.

[13] M.D. Cooperman, J.M. Reed, L.M. Romero, The effects of terrestrial and breeding densities on corticosterone and testosterone levels in spotted salamanders, *Ambystoma maculatum*. Can. J. Zool.-Rev. Can. Zool. 82 (2004) 1795-1803.

[14] G.W. Eagleson, A comparison of the life histories and growth patterns of populations of the salamander Ambystoma gracile (Baird) from permanent low-altitude and montane lakes. Can. J. Zool. 54 (1976) 2098-2111.

[15] B. Fraile, R. Paniagua, M.C. Rodriguez, F.J. Saez, Effects of photoperiod and temperature on spermiogenesis in marbled newts (*Triturus marmoratus marmoratus*). Copeia (1989) 357-363.

[16] D.H. Garnier, Androgen and estrogen levels in the plasma of *Pleurodeles waltl*, Michah., during the annual cycle. I. Male cycle. Gen. Comp. Endocrinol. 58 (1985) 376-385.

[17] D.H. Garnier, Androgen and estrogen levels in the plasma of *Pleurodeles waltl*, Michah., during the annual cycle. II. Female cycle. Gen. Comp. Endocrinol. 60 (1985) 414-418.

[18] F. Gasco, Les amours des axolotls. Bull. Soc. Zool. France. 6 (1881) 151-162.

[19] W. Goymann, I.T. Moore, A. Scheuerlein, K. Hirschenhauser, A. Grafen, J.C. Wingfield, Testosterone in tropical birds: effects of environmental and social factors. Am. Nat. 164 (2004) 327-334.

[20] J. Gresens, An introduction to the Mexican axolotl (*Ambystoma mexicanum*). Lab. Anim. (NY). 33 (2004) 41-47.

[21] L.J. Guillette, D.O. Norris, M.F. Norman, Response of amphibian (*Ambystoma tigrinum*) oviduct to arginine vasotocin and acetylcholine in vitro: Influence of steroid hormone pretreatment *in vivo*. Comp. Biochem. Physiol. C-Pharmacol. Toxicol. Endocrinol. 80 (1985) 151-154.

[22] R.N. Harris, P.M. Ludwig, Resource level and reproductive frequency in female four-toed salamanders, *Hemidactylium scutatum*. Ecology. 85 (2004) 1585-1590.

[23] M. Hasumi, Seasonal fluctuations of female reproductive organs in the salamander *Hynobius nigrescens*. Herpetologica. 52 (1996) 598-605.

[24] M. Hasumi, H. Iwasawa, Y. Nagahama, Seasonal dynamics of reproductive organs in male salamanders of the species *Hynobius nigrescens*. Copeia. 1990 (1990) 367-377.

[25] M. Hasumi, H. Iwasawa, Y. Nagahama, Seasonal changes in plasma concentrations of sex steroids in the salamander *Hynobius nigrescens*. Gen. Comp. Endocrinol. 90 (1993) 51-57.

[26] L.D. Houck, Reproductive biology of a neotropical salamander, *Bolitoglossa rostrata*. Copeia (1977) 70–83.

[27] L.D. Houck, M.T. Mendonca, T.K. Lynch, D.E. Scott, Courtship behavior and plasma levels of androgens and corticosterone in male marbled salamanders, *Ambystoma opacum* (Ambystomatidae). Gen. Comp. Endocrinol. 104 (1996) 243-252.

[28] J.D. Ifft, The effect of environmental factors on the sperm cycle of *Triturus viridescens*. Biol. Bull. 83 (1942) 111-128.

[29] T. Iwata, F. Toyoda, K. Yamamoto, S. Kikuyama, Hormonal control of urodele reproductive behavior. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 126 (2000) 221-229.

[30] G.F. Jacobs, E.R. Kühn, Luteinizing hormone-releasing hormone induces thyroxine release together with testosterone in the neotenic axolotl *Ambystoma mexicanum*. Gen. Comp. Endocrinol. 71 (1988) 502-505.

[31] Y. Katsu, E. Taniguchi, H. Urushitani, S. Miyagawa, M. Takase, K. Kubokawa, et al., Molecular cloning and characterization of ligand- and species-specificity of amphibian estrogen receptors. Gen. Comp. Endocrinol. 168 (2010) 220-230.

[32] W.H. Keen, Breeding and larval development of three species of *Ambystoma* in central Kentucky (Amphibia: Urodela). Herpetologica. 31 (1975) 18-21.

[33] L. Lazard, Steroidogenesis in axolotl testis. Histochemistry of two major enzymes related to cell type, spermatogenesis, and substrate. Gen. Comp. Endocrinol. 39 (1979) 381-387.

[34] A. Lecouteux, D.H. Garnier, T. Bassez, J. Joly, Seasonal variations of androgens, estrogens, and progesterone in the different lobules of the testis and in the plasma of *Salamandra salamandra*. Gen. Comp. Endocrinol. 58 (1985) 211-221.

[35] B. Lofts, Reproduction. In: B. Lofts, (Ed.), Physiology of the Amphibia, Vol. 11., Academic Press, New York, 1974, pp. 107-218.

[36] I. Loredo, D. VanVuren, Reproductive ecology of a population of the California tiger salamander. Copeia (1996) 895-901.

[37] G.M. Malacinski, The Mexican axolotl, *Ambystoma mexicanum*: Its biology and developmental genetics, and its autonomous cell-lethal genes. Am. Zool. 18 (1978) 195-206.

[38] M.J. Miltner, J.B. Armstrong, Spermatogenesis in the Mexican axolotl, *Ambystoma mexicanum*. J. Exp. Zool. 227 (1983) 255-263.

[39] F.L. Moore, S.K. Boyd, D.B. Kelley, Historical perspective: Hormonal regulation of behaviors in amphibians. Horm. Behav. 48 (2005) 373-383.

[40] I. Moore, C. Escallon, J. Husak, C. Eikenaar, Testosterone and corticosterone increase with latitude in reptiles and amphibians. Front. Endocrin. Conference Abstract: ISAREN 2011: 7th International Symposium on Amphibian and Reptilian Endocrinology and Neurobiology (2011).

[41] D.O. Norris, Hormones and reproductive patterns in urodele and gymnophionid amphibians. In: D.O. Norris, K.H. Lopez, (Eds.), Hormones and Reproduction of Vertebrates. Volume 2: Amphibians, Elsevier Science, Burlington, 2011, pp. 187-202.

[42] D.O. Norris, M.F. Norman, M.K. Pancak, D. Duvall, Seasonal variations in spermatogenesis, testicular weights, vasa deferentia, and androgen levels in neotenic male tiger salamanders, *Ambystoma tigrinum*. Gen. Comp. Endocrinol. 60 (1985) 51-57.

[43] C.R. Propper, Testicular structure and control of sperm development in amphibians. In: D.O. Norris, K.H. Lopez, (Eds.), Hormones and Reproduction of Vertebrates. Volume 2: Amphibians, Elsevier Science, Burlington, 2011, pp. 39-53.

[44] C.R. Propper, F.L. Moore, Effects of courtship on brain gonadotropin hormone-releasing hormone and plasma steroid concentrations in a female amphibian (*Taricha granulosa*). Gen. Comp. Endocrinol. 81 (1991) 304-312.

[45] J. Pudney, J.A. Canick, P. Mak, G.V. Callard, The differentiation of Leydig cells, steroidogenesis, and the spermatogenetic wave in the testis of *Necturus maculosus*. Gen. Comp. Endocrinol. 50 (1983) 43-66.

[46] D.E. Scott, Timing of reproduction of paedomorphic and metamorphic *Ambystoma talpoideum*. Am. Midl. Nat. 129 (1993) 397-402.

[47] D.E. Scott, M.R. Fore, The effect of food limitation on lipid levels, growth, and reproduction in the marbled salamander, *Ambystoma opacum*. Herpetologica. 51 (1995) 462-471.

[48] H.B. Shaffer, Phylogenetics of model organisms: the laboratory axolotl, *Ambystoma mexicanum*. Syst. Biol. 42 (1993) 508-522.

[49] H.B. Shaffer, M.L. McKnight, The polytypic species revisited: Genetic differentiation and molecular phylogenetics of the tiger salamander *Ambystoma tigrinum* (Amphibia: Caudata) complex. Evolution. 50 (1996) 417-433.

[50] H.M. Smith, Discovery of the axolotl and its early history in biological research. In: J.B. Armstrong, G.M. Malacinski, (Eds.), Developmental biology of the axolotl, Oxford University Press, New York, 1989, pp. 3-12.

[51] J.L. Specker, F.L. Moore, Annual cycle of plasma androgens and testicular composition in the rough-skinned newt, *Taricha granulosa*. Gen. Comp. Endocrinol. 42 (1980) 297-303.

[52] J.R. Spotila, R.J. Beumer, The breeding habits of the ringed salamander, *Ambystoma annulatum* (Cope), in northwestern Arkansas. Am. Midl. Nat. 84 (1970) 77-89.

[53] S. Tanaka, H. Takikawa, Seasonal changes in plasma testosterone and 5alpha-dihydrotestosterone levels in the adult male newts, *Cynops pyrrhogaster*. Endocrinol. Jpn. 21 (1983) 1–6.

[54] M.C. Uribe Aranzábal, Hormones and the female reproductive system of amphibians. In: D.O. Norris, K.H. Lopez, (Eds.), Hormones and Reproduction of Vertebrates. Volume 2: Amphibians, Elsevier Science, Burlington, 2011, pp. 55-81.

[55] M.C.A. Uribe, G. Gomez Rios, R.A. Brandon, Spermatogenesis in the urodele *Ambystoma dumerilii*. J. Morphol. 222 (1994) 287-299.

[56] M.C.A. Uribe, L.F. Mena, M.R.C. Carrubba, E.F. Estrada, B.D. Palmer, L. Guillette Jr., Oviductal histology of the urodele, *Ambystoma mexicanum*. J. Herpetol. 23 (1989) 230-237.

[57] D. Valdivieso, J.R. Tamsitt, Reproduction in the neotropical salamander, *Bolitoglossa adspersa* (Peters). Herpetologica. 21 (1965) 228–236.

[58] P.A. Verrell, The male reproductive cycle of the North American salamander *Ambystoma macrodactylum columbianum*. Amphib. Reptil. 25 (2004) 349-356.

[59] P.A. Verrell, The female reproductive cycle of the North American salamander *Ambystoma macrodactylum columbianum*. Amphib. Reptil. 27 (2006) 274-277.

[60] J.A. Vial, The ecology of the tropical salamander, *Bolitoglossa subpalmata*, in Costa Rica. Rev. Biol. Trop. 15 (1968) 13–115.

[61] J.K. Werner, Temperature-photoperiod effects on spermatogenesis in the salamander *Plethodon cinereus*. Copeia. 1969 (1969) 592-602.

[62] S.K. Woodley, Plasma androgen levels, spermatogenesis, and secondary sexual characteristics in two species of plethodontid salamanders with dissociated reproductive patterns. Gen. Comp. Endocrinol. 96 (1994) 206-214.

[63] S.K. Woodley, Hormones and reproductive behavior in amphibians. In: D.O. Norris, K.H. Lopez, (Eds.), Hormones and Reproduction of Vertebrates. Volume 2: Amphibians, Elsevier Science, Burlington, 2011, pp. 143-169.

[64] S.K. Woodley, E.L. Lacy, An acute stressor alters steroid hormone levels and activity but not sexual behavior in male and female Ocoee salamanders (*Desmognathus ocoee*). Horm. Behav. 58 (2010) 427-432.

[65] M. Zerani, C. Vellano, F. Amabili, O. Carnevali, G.E. Andreoletti, A. Polzonetti-Magni, Sex steroid profile and plasma vitellogenin during the annual reproductive cycle of the crested newt (*Triturus carnifex* Laur.). Gen. Comp. Endocrinol. 82 (1991) 337-344.

Figure Legends

Figure 1. Relationship between gonadosomatic index (GSI) and levels of sex steroids in adult female (**A**) and male (**B**) axolotls. Each point represents the mean of 3-10 measurements for a single individual. Levels of both estradiol and androgens varied greatly among individuals, and in neither case is the correlation between hormone levels and GSI statistically significant.

Figure 2. Relationships between levels of estradiol and external factors in adult female axolotls. (**A**) Animals were fed twice weekly and then food deprived for 10 days prior to blood collection (*low* feed condition) or were fed three times weekly, including the day before blood collection (*high* feed condition). Levels of estradiol in animals in the high feed condition were significantly higher than in those in the low feed condition. Bar height indicates mean of all individuals; error bar indicates SEM. Estradiol was also influenced by both change in day length (**B**) and calendar month (**C**), which were decorrelated in our experimental setup. When feeding condition was taken into account, both variables were revealed to have significant effects on estradiol levels.

Figure 3. Changes in mass of ovaries, oviducts, and eggs in females (**A**) and testes in males (**B**) over the course of the calendar year, regardless of day length. In females, mass of reproductive tissue tends to be lower in the summer months and higher in the fall and winter ($p = 0.07$), but is not correlated with estradiol levels; in males, no seasonal effect is seen, and testis mass does not correlate with androgen levels.

Figure 4. Relationships between GSI and external factors adult male axolotls. (**A**) Change in day length significantly altered GSI. In contrast, feeding condition (**B**) and month (**C**) did not by themselves have statistically significant effects on GSI; however, if change in photoperiod is taken into account both variables significantly alter GSI.

Figure 1

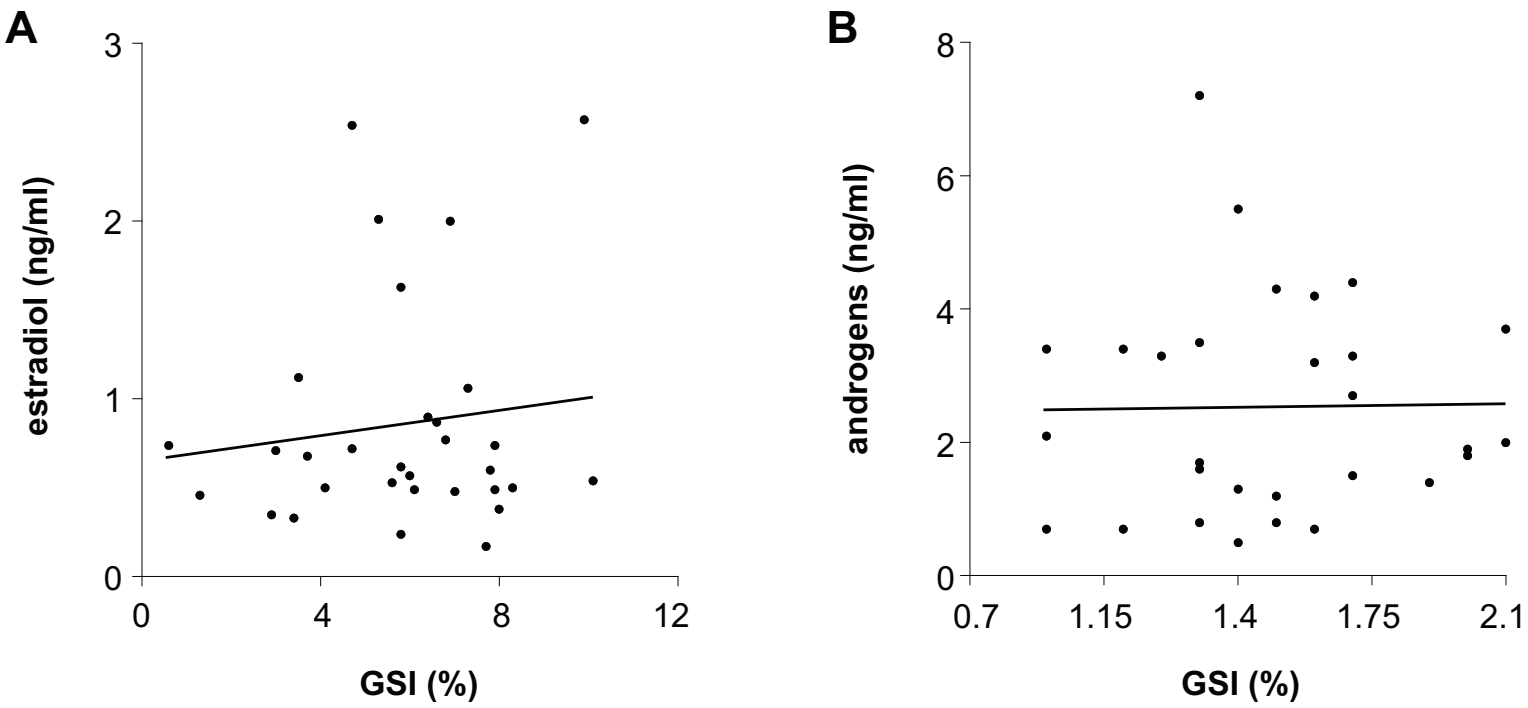
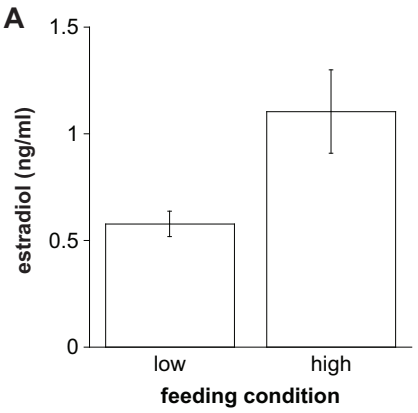
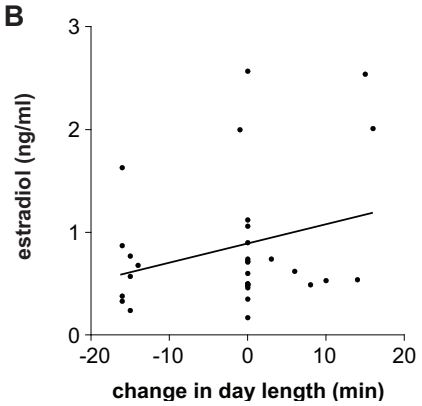

A
estradiol (ng/ml)
0
1
2
3
0
4
8
12
GSI (%)
B
androgens (ng/ml)
0
2
4
6
8
0.7
1.15
1.4
1.75
2.1
GSI (%)

Figure 2


A
1.5
1
0.5
0
estradiol (ng/ml)
low
high
feeding condition


B
3
2
1
0
estradiol (ng/ml)
-20
-10
0
10
20
change in day length (min)

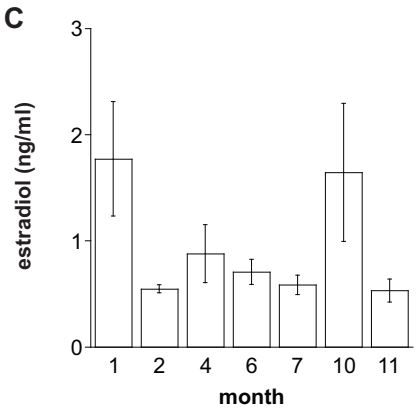
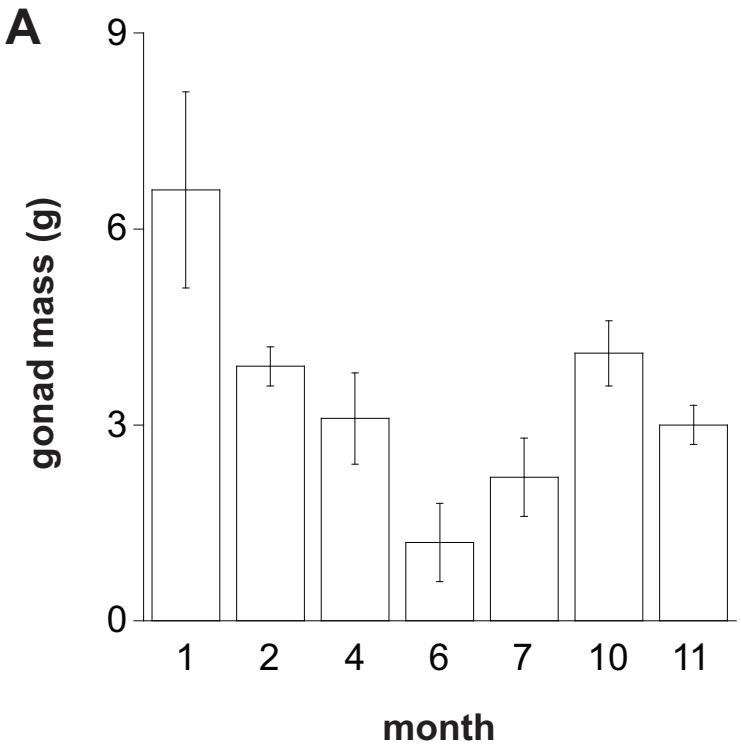

C
3
2
1
0
estradiol (ng/ml)
1
2
4
6
7
10
11
month

Figure 3


A
gonad mass (g)
0
3
6
9
1
2
4
6
7
10
11
month

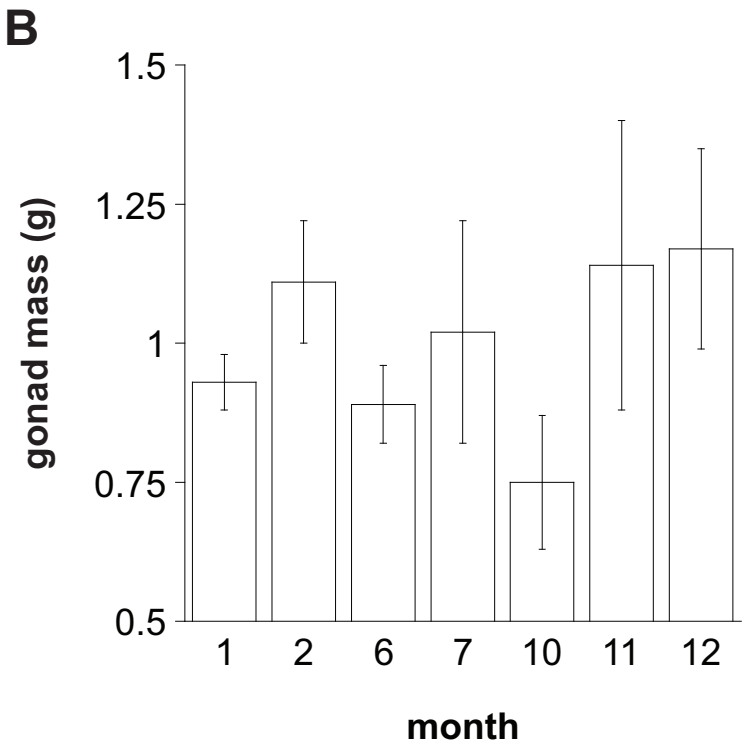
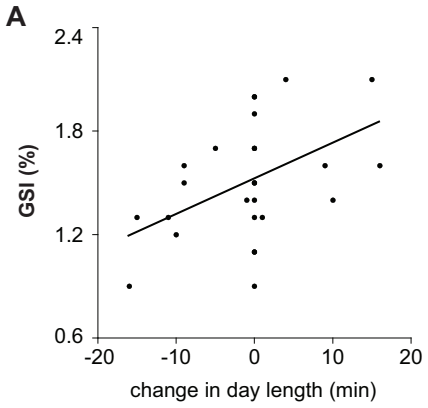
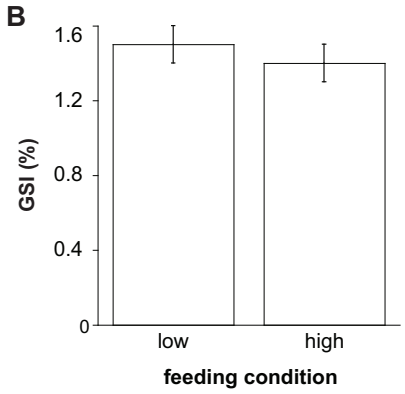
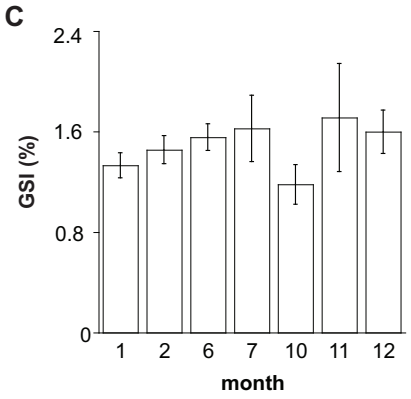

B
gonad mass (g)
0.5
0.75
1
1.25
1.5
1
2
6
7
10
11
12
month

Figure 4


A
2.4
1.8
1.2
0.6
GSI (%)
-20
-10
0
10
20
change in day length (min)


B
1.6
1.2
0.8
0.4
0
GSI (%)
low
high
feeding condition


C
2.4
1.6
0.8
0
GSI (%)
1
2
6
7
10
11
12
month