Spermatogenic cycle of a plethodontid salamander, *Eurycea longicauda* (Amphibia, Urodela)

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Previous investigators have described the spermatogenic cycles of numerous species of plethodontid salamanders. Most studies describe a fairly stereotypical cycle with meiotic divisions of spermatogenesis commencing in the spring/summer. However, many studies lack details obtainable from histological examination and/or testicular squashes and, instead, provide only mensural data from the testes. Studies that lacked microscopic evaluation often revealed spermatogenic cycles that varied greatly from that of the stereotypical cycle with meiotic divisions commencing in the fall/winter. Those studies hamper comparisons between the spermatogenic cycles of different species and their environments, as they do not provide a correlation between testicular size and any aspect of the spermatogenic cycle. In the following manuscript, we elucidate the spermatogenic cycle of *Eurycea longicauda longicauda* in an effort to outline an appropriate protocol for analyzing spermatogenesis in salamanders that will facilitate future comparative studies. Like many Nearctic plethodontids, *E. l. longicauda* exhibits a meiotic wave that travels through the testes during the summer; this process is followed by spermiogenesis, spermiation, and recrudescence in the fall, winter, and spring.

Introduction

The Plethodontidae contains 443 of the currently known 665 species of extant salamanders and thus is by far the largest of the 9 families of salamanders.¹ The majority of species occur in the Americas, with centers of diversity in North America and nuclear Central America. Some species of *Hydromantes* also are present in Mediterranean Europe as well as the western United States, and one species, *Karsenia koreana*, occurs on the Korean peninsula. The Plethodontidae is undoubtedly monophyletic with morphological synapomorphies including loss of lungs in metamorphosed individuals and presence of nasolabial grooves among 5 other unique anatomical characters plus extensive molecular evidence.² Traditional phylogenies based on morphology recognized 2 subfamilies, Desmognathinae and Plethodontinae, with 3 tribes in the latter.^{3,4} Recent molecular studies have completely revolutionized our understanding of plethodontid phylogeny, e.g.,⁵⁻⁷ The most recent data suggest a division of plethodontids into a northern clade of *Plethodon*, *Desmognathus*, *Ensatina*, *Aneides*, *Hydromantes*, and *Karsenia*, an eastern/southern clade of *Hemidactylium*, *Batrachoseps*, *Bolitoglossa* (and other neotropical genera), and the Spelerpinae.⁸ The spelerpines are the eastern and middle North American brook salamanders and consist of *Stereochilus* (1 species), *Pseudotriton* (2), *Gyrinophilus* (4), and 28 species of *Eurycea*, many of which are paedomorphic.

Most Nearctic plethodontid salamanders exhibit a spermatogenic cycle with meiosis commencing in the spring or summer⁹⁻²³ with a caudal to cephalic meiotic wave traveling through the testes.^{9,10,14-20,22,24} Meiosis culminates in the summer or fall followed by spermiogenesis, and recrudescence tracking the meiotic wave with the proliferation of spermatogonia and primary spermatocytes (spermatocytogenesis) until the next wave of meiosis in the following spring or summer.^{10,12,14-17,19,21} Increase in temperature and day length has been shown to experimentally accelerate the spermatogenic cycle.¹² Only *Eurycea longicauda melanopleura*,²⁵ *E. multiplicata*,²⁶ and *Pseudotriton ruber*¹⁰ veer greatly from this stereotypical cycle with meiosis delayed until November and culmination occurring at the end of winter.

Neotropical plethodontids appear to possess acyclical spermatogenesis²⁷⁻²⁹ with no indication of discrete seasonal cycles. The histological details gametogenesis within *Bolitoglossa* are vague^{27,29} except for a study on *Bolitoglossa subpalmata*,²⁸ which clearly states that, “active meiotic division and the presence of sperm in testicular lobes and vas deferens can be seen in microscope slides of gonadal tissue from samples taken at all months of the year.” Although multiple taxa of Palearctic plethodontids and one Oriental species (i.e., *Karsenia koreana*³⁰) exist, details of their spermatogenic cycles are lacking.

While previous investigators have examined the spermatogenic cycle of numerous species from Plethodontidae (see above),

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type of examination differs greatly between studies, which makes comparison between species, geographic range, and/or ecological niche difficult. Whereas the most informative studies for comparison describe the spermatogenic cycle of a species throughout the year (at least during the active season) using histology^{9,11,12,14-21,28} or testicular squashes¹⁴⁻¹⁷ to provide cell types found throughout the active season, less revealing studies provide only the initiation of meiosis.¹⁰ Others provide only vague histological elements that may or may not be associated with measurements of the testes,^{25-27,29} presence or absence of mature sperm in the testes,^{22,31} description of only the gross morphology of the testes,¹³ or only measurement data.³²⁻³⁴ The latter studies provide little comparative power, as statements akin to, “an increase in testicular weight reflected increased spermatogenic activity,”²⁵ provide no detail on the transformation and development of germ cells within the testes; likewise, measurement data alone provide no correlation to testicular stage.

Due to accelerated global climate change,³⁵ it is imperative to document variation in aspects of the reproductive cycle (including spermatogenic cycle) across geographical gradients to better understand the effects of climate change on the reproductive biology of species. For example, it was recently predicted that *Acris crepitans* populations are heading toward catastrophe based on a decrease in reproductive investment in concordance with climate change models.³⁶

In the following paper we examine the spermatogenic cycle of *Eurycea longicauda longicauda*. Through this assessment, we provide methodology and detailed analysis that will facilitate future comparison of spermatogenic cycles between plethodontid salamanders. We feel that all future studies should at least analyze spermatogenesis to the same degree that is provided herein. Although our examination focuses solely on a plethodontid salamander, this same analysis can be utilized for any vertebrate with a distinct annual spermatogenic cycle.

Materials and Methods

Specimen collection

Adult male *Eurycea longicauda longicauda* (snout vent length [SVL] range: 42.5 mm – 59.0 mm) were collected from Little Grand Canyon in the Shawnee National Forest, Jackson County, Illinois (37.680517° N, –89.394861° W) from March 2012 to February 2013. With permission from the Illinois Department of Natural Resources (permit numbers NH12.5564 and NH13.5564) and the United States Department of Agriculture (file code 2710), 2 male *E. l. longicauda* were sought for examination each month. No males, females, or juveniles could be found in the months of December, January and February, and we can only assume that *E. l. longicauda* over-winters deep underground or within deep cracks of the canyon walls of Little Grand Canyon.

Tissue preparation

Post collection, *Eurycea longicauda longicauda* individuals were euthanized with a lethal dose of MS-222 and their snout-vent length was recorded. Right testes were excised, measured

(point of greatest length and width), and fixed in 10% buffered formalin for at least 48 h for general histological examination.

After fixation, testes fixed in 10% buffered formalin were rinsed with deionized water, dehydrated through a graded series of ethanol (70%, 95%, and 100%), cleared in toluene, immersed in melted paraplast for at least 12 h, and embedded in paraplast for longitudinal sectioning. Sections were obtained with a rotary microtome at 9 µm and affixed to albumenized slides. Slides were stained with hematoxylin and eosin for general histological examination following Kiernan.³⁷

Qualification of sperm production and transport

The testicular structure and germ cell descriptions from Uribe³⁸ were used to assess the presence or absence of primary and secondary spermatogonia, primary and secondary spermatocytes, spermatids in different stages of elongation, and testicular sperm of male *Eurycea longicauda longicauda* from every month. The presence or absence of Wolffian duct sperm was also recorded.

Results

The testes of *Eurycea longicauda longicauda* were longitudinally-elongated structures suspended into the body cavity via dorsal mesentery. The testes possessed a fibrous tunica albuginea encompassing numerous intratesticular lobules (Fig. 1). Lobules possessed Sertoli cells and cysts of developing sperm depending on the month examined (see below; Fig. 1). The lobules were separated via a thin layer of connective tissue and are aggregated around central testicular ducts that traveled centrally through the testes along their longitudinal axes (Fig. 1). Lobules opened into

Figure 1. Overview of testicular organization from a mid-transverse section through the testis of *Eurycea longicauda longicauda* from November (hematoxylin and eosin; scale bar = 500 µm). Ct, cyst; Ctd, central testicular duct; Fb, fat body; Lb, lobule; Ta, tunica albuginea; Td, testicular ducts; Wd, Wolffian duct.

the central testicular ducts along their entire lengths. Thus, in transverse sections, the testes of *E. l. longicauda* resembled a sliced grapefruit, with lobules surrounding and emptying into central testicular ducts (Fig. 1). Leydig cells were found positioned between lobules. The central testicular ducts communicated with the Wolffian duct via a fine network of sperm transport ducts (for review see Williams et al., 1984; Siegel et al., 2013).

Primary spermatogonia (Fig. 2A) were found in the testes in specimens from July to April, although primary spermatogonia were only found in one specimen in July and April (Fig. 3). Considering these germ cells are precursors to all other germ cell stages, it is assumed that they are present throughout the year, but in such low numbers compared to the other germ cell stages that they become difficult to observe. Secondary spermatogonia (Fig. 2A) were present during every month of the year in every specimen except one from July (Fig. 3). Primary spermatocytes (Fig. 2B) were found in one specimen from March and April, and abundant in every specimen from May to August (Fig. 3). Primary spermatocytes were observed in meiosis I in June through August (Fig. 2B insert b1). During these same months, secondary spermatocytes were observed in meiosis II (Fig. 2B insert b2), indicating meiotic events of germ cell development (Fig. 2B inserts) were restricted to June through August (Fig. 3). Spermatids were found in different stages of elongation (compare Fig. 2C and D) from June to August (Fig. 3). Testicular sperm (Fig. 2E) were observed in the lobules of the testes from July to November, although only one specimen possessed testicular sperm in November (Fig. 3). Spermiation occurred between September and November, which was confirmed by sperm passage from lobules to the testicular ducts (Fig. 2F), lobules with hypertrophied Sertoli cells and sperm rests (Fig. 2G), and sperm in the Wolffian ducts (Fig. 2H; Fig. 3). Sperm persisted in the Wolffian ducts until sometime in the spring based on the

Figure 2. Histology of the spermatogenic cycle of *Eurycea longicauda* (hematoxylin and eosin; scale bar = 50 μ m). (A). High magnification of the testis from a specimen captured in November highlighting the presence of primary and secondary spermatogonia. (B). High magnification of the testis from a specimen captured in June highlighting primary spermatocytes. Insets B1 and B2 show meiosis I (occurring in primary spermatocytes) and meiosis II (occurring in secondary spermatocytes), respectively, during this month. (C). High magnification of the testis from a specimen captured in June highlighting round spermatids and spermatids in early elongation stages of spermiogenesis. (D). High magnification of the testis from a specimen captured in August highlighting spermatids in late elongation stages of spermiogenesis. (E). High magnification of the testis from a specimen captured in September highlighting testicular sperm. (F). High magnification of the testis from a specimen captured in August highlighting spermiation. (G). High magnification of the testis from a specimen captured in October highlighting a lobule post spermiation. (H). High magnification of the testis from a specimen captured in November highlighting sperm in the Wolffian duct. Est, elongating sperm; Lbs, lobule sperm; Lnk, lobule neck; Mpt, mid-principle piece of the sperm tail; Ppt, principle piece of the sperm tail; Rst, round spermatid; Sc, Sertoli cell; Sg1, primary spermatogonia; Sg2, secondary spermatogonia; Sn, sperm nucleus; Sr, sperm rests; Wde, Wolffian duct epithelium.

Figure 3. Spermatogenic cycle of *Eurycea longicauda longicauda* (top) and its correlation with testicular size (average testicular length and width per month; bottom). Broken bars (top) indicate months where cell types were found in only one specimen. Yellow shading (top and bottom) is representative of the major events of meiotic division. Error bars represent standard deviation.

presence in November and in one specimen from April. More caudal regions of the Wolffian ducts where sperm are typically stored in male salamanders were not examined, which most likely resulted in the lack of Wolffian duct sperm presence in the spring. Considering sperm were present in the cranial Wolffian ducts in the fall and spring, it is assumed that they are present in the Wolffian ducts throughout winter. Thus, considering only sperm as a criterion for mating capability, it is possible that *Eurycea longicauda longicauda* males are capable of mating in the fall through the spring.

Both testicular length and width were good indicators of the latter stages of spermatogenesis (meiosis I, meiosis II, and spermiogenesis), as the peak of testicular length/width was correlated with the presence of secondary spermatocytes and spermatids in the testicular lobules (Fig. 3; using testicular size as a percentage of SVL resulted in the same size pattern). Like other plethodontid salamanders, spermatogenesis occurred as a caudo-cephalic wave along the longitudinal axis of the testes. This wave resulted in variation in the width of testes along their length, as portions of the testes with greater meiotic activity and testicular sperm were greater in diameter than those in earlier stages of

spermatogenesis, as described previously by Humphrey.²⁴ Post spermatogenic lobules increased in number after August. Portions of the testes with lobules devoid of sperm were narrower in diameter when compared to those that still possessed maturing spermatozoa.

Discussion

Our analysis of seasonal testicular histology of *Eurycea longicauda longicauda* demonstrates that this species of salamander undergoes testicular recrudescence in the fall and commences meiotic divisions of spermatogenesis in the summer, followed by spermiation in the late summer to fall. Increase in testicular size is correlated with proliferation of primary spermatocytes in the spring and filling of lobules with mature spermatozoa through meiotic divisions in the summer. Decreases in testicular size are due to emptying of lobules through spermiation in the late summer and fall where sperm pass through the testicular ducts and genital kidney nephrons^{39,40} to fill the Wolffian ducts for mating, which probably occurs through the fall and spring. Only 2 observations of mating behavior exist and confirm mating in the fall during October (field observation of potential courtship behavior⁴¹) and November (laboratory observation⁴²).

Abundance of sperm in the Wolffian ducts indicates mating occurs from at least November to February in a different subspecies of *Eurycea longicauda*, *E. l. melanopleura*²⁵; however, meiotic divisions of the spermatogenic cycle in this subspecies occur during the late fall and through winter,²⁵ a strikingly different cycle than that observed for *E. l. longicauda*. This previous study provides only measurement data with no indication of the correlation of the measurement data with germ cell stages found at different times of the year. If measurement data from this previous study correlate with germ cell stages found in *E. l. longicauda*, *E. l. melanopleura* possesses a drastically different spermatogenic cycle (in terms of timing) than *E. l. longicauda*, and the majority of other Nearctic plethodontid salamanders that were examined using testicular histology or squashes;^{9,11,12} i.e., meiotic divisions of spermatogenesis commencing in the spring or summer. Whether this difference is due to local niche variation, inter-seasonal variation, or a unique ancestry is unknown; however, considering the majority of Nearctic plethodontid salamanders commence meiotic divisions of spermatogenesis during the warmer months (spring and summer), besides *Eurycea multiplicata*²⁶ and *Pseudotriton ruber* from one population,¹⁰ which also

lack sufficient seasonal delimitation of germ cell stages, we caution future investigators from making too broad of conclusions from the previous literature without histological data also present for the spermatogenic cycle.

Data from the spermatogenic cycle of male *Eurycea longicauda longicauda* meld well with previous data on their reproductive cycle. After spermiation in the fall, mating occurs in the fall,^{41,42} potentially over winter, and the spring (based on sperm presence in the Wolffian ducts). The mating season appears to be concordant with oviposition, as eggs have been observed at early stages of development from the late fall to winter.^{25,43,44} Furthermore, recently hatched larvae have been observed from late fall to spring,^{45,46} indicating the lack of a precise timing for oviposition.

The main purpose of this study was to demonstrate what we propose is the minimum standard for examining spermatogenic cycles in salamanders. Providing both morphometric data and stages of the testicular cysts allows for qualitative correlations between testicular growth and testicular stage. If testicular size and germ cell stages are identical between different species, easy statistical comparisons can be made to test the relationships of spermatogenic cycles (using the measurement data) between species. Unfortunately, few previous investigations¹² have used this simple protocol, making comparisons between sperm

development and valuable historical data difficult, at least through quantifiable approaches. To test hypotheses on how geographic variation, climate variation, and phylogenetic position influence reproduction in salamanders, it is paramount that future investigators use a common means of histological analysis to examine reproductive cycles.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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