Seasonality, microhabitat and cryptic variation in tropical salamander reproductive cycles

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In many salamanders, most aspects of reproduction coincide with seasonal changes in abiotic factors such as temperature and humidity. However, while these reproductive patterns have been well documented for temperate salamanders, detailed data for tropical species are relatively sparse. I used histological techniques to examine temporal variation in the spermatogenetic cycles of four species of Guatemalan bolitoglossine salamanders (Bolitoglossa occidentalis, B. rostrata, Dendrotriton bromeliacia and Pseudoerycea goebeli) from different microhabitats along an elevational gradient. All four species have mature sperm present in the testis throughout the year, irrespective of patterns of courtship and egg-laying. Additionally, the two species from more seasonal habitats (B. occidentalis and B. rostrata) exhibit significant but cryptic levels of variation in the amount of spermatozoa present in the testis not detectable by external appearance or the presence of secondary sexual characteristics. For the two species from less seasonal cloud forests (D. bromeliacia and P. goebeli), there were no detectable patterns to variation within the testis. Regional variation in climate undoubtedly influences reproductive cycles, however, microhabitat and the immediate environment are also important determinants of reproductive strategy. © 2003 The Linnean Society of London. Biological Journal of the Linnean Society, 2003, 78, 489–496.


INTRODUCTION

Timing of courtship activity, egg-laying, gonad activity and other temporal aspects of amphibian reproduction are presumably under selection and constrained in part by particular ecological and environmental parameters (Stebbins, 1954; Salthe & Mecham, 1974; Harvey et al., 1997). Ephemeral pools for egg-laying, moisture to prevent desiccation of adults, and adequate heat to meet the energetic costs of development, oogenesis and spermatogenesis are examples of specific environmental requirements (Ifft, 1942; Werner, 1969). One apparent consequence of such relationships is that reproductive cycles generally coincide with seasonal climatic trends (Salthe & Mecham, 1974). For example, in temperate regions, reduced humidity, temperature and water levels associated with autumn and winter limit activity patterns and physiological processes (Rastogi, 1976). Correspondingly, many temperate amphibians are active for only a short period in the spring during which all reproduction occurs (Miller & Robbins, 1954; Stebbins, 1954). Similar correlations between the environment and reproductive traits are found among tropical amphibians (Houck, 1977a). However, in contrast to temperate amphibians, annual climatic variation may be reduced in the tropics and, correspondingly, many tropical anurans and salamanders have prolonged breeding seasons with egg-laying and courtship occurring throughout the year (Valdivieso & Tamsitt, 1965; Inger & Bacon, 1968; Vial, 1968; Crump, 1974; Kurian & Saidapur, 1983; Saidapur, 1983).

Interestingly, both seasonal and aseasonal oviposition occurs among tropical salamanders while other aspects of reproduction such as mating activity and spermatogonic cycle occur continually (Bille, 2000;
Valdivieso & Tamsitt, 1965; Vial, 1968; Houck, 1977b). While broad-scale patterns of annual climatic variation may account for some differences in reproduction between temperate and tropical salamanders, the variation among reproductive patterns within each region may reflect the importance of microhabitat and associated local selective pressures on the evolution of reproductive cycles. Thus, we can gain a better understanding of how reproductive cycles evolve by comparing variation in life history and reproductive traits across gradients of environmental conditions and selective regimes.

Neotropical salamanders are an ideal system for investigating the interplay between reproductive traits and the environment because they share a close evolutionary history, but exhibit ecological and reproductive diversity (Wake, 1966; Wake & Lynch, 1976; Wake, 1987). All neotropical salamanders belong to the tribe Bolitoglossini (Family Plethodontidae); these lungless salamanders have direct development of young within terrestrially laid eggs (i.e. no aquatic, larval stage; Wake, 1966). Microhabitat specializations and preferences contribute to high species diversity of bolitoglossine salamanders in the tropics and distinct species found in geographical proximity may experience different microclimatic regimes throughout the year (Wake & Lynch, 1976; Wake, 1987). The fine-scale heterogeneity of abiotic conditions and the ecological affinities of many species provide a unique opportunity to examine how life history traits and patterns of reproduction vary in light of these selective pressures. Previous studies investigating reproduction in tropical salamanders have not quantitatively assessed changes within the testis; male cycles of gametogenesis have been deduced from the presence of sperm in the testis, the development of secondary sexual characteristics, and the volume and pigmentation of the testis (e.g. Valdivieso & Tamsitt, 1965; Houck, 1977b; Bille, 2000). Through more detailed examination of the testis we can identify subtle levels of variation and detect cryptic temporal patterns that may not be obvious or apparent using techniques that focus on external characteristics.

Here, I examine the testicular cycles of four species of tropical bolitoglossine salamanders that differ in altitudinal distribution, microhabitat preferences and seasonality of egg-laying (Table 1). Because annual cycles of reproduction depend in part on the environmental variation individuals encounter, both regional climatic and local ecological conditions play a role in the evolution of reproductive strategies. Rainfall patterns, temperature levels and air humidity levels depend on the climate of the area while the degree to which an individual is exposed to fluctuations in abiotic conditions is dependent on microhabitat use. For example, arboreal specialists residing in bromeliads and plant leaf axils will be well-buffered against changes in temperature and humidity, while terrestrial, ground dwelling species will not necessarily be protected against such fluctuations (Feder, 1982; Wake, 1987). By examining species from different climates and with different microhabitat preferences we can determine the combined effects that regional variability and microhabitat buffering have on patterns of reproduction.

### MATERIAL AND METHODS

#### THE STUDY SPECIES AND SYSTEM

The four focal species of salamander used in this study were collected in the Departamento de San Marcos, Guatemala by D. B. Wake, J. F. Lynch and L. D. Houck from 1969 to 1979 and deposited in the Museum of Vertebrate Zoology (MVZ), University of California,

<table>
<thead>
<tr>
<th>Species</th>
<th>Elevation (m)</th>
<th>Habitat type (Seasonality)</th>
<th>Microhabitat preference</th>
<th>Activity</th>
<th>Egg-laying</th>
<th>Sperm in testis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bolitoglossa occidentalis</em></td>
<td>0–1600</td>
<td>Lowland wet forest (high)</td>
<td>Arboreal (bromeliads and banana plants)</td>
<td>Year-round</td>
<td>November/December</td>
<td>All year</td>
</tr>
<tr>
<td><em>Bolitoglossa rostrata</em></td>
<td>2700–3200</td>
<td>Pine-cypress/ Pine-bunchgrass (high)</td>
<td>Terrestrial</td>
<td>Year-round</td>
<td>November/December</td>
<td>All year</td>
</tr>
<tr>
<td><em>Dendrotriton bromeliacia</em></td>
<td>1900–2700</td>
<td>Cloud forest (low)</td>
<td>Arboreal (bromeliads)</td>
<td>Year-round</td>
<td>Year-round</td>
<td>All year</td>
</tr>
<tr>
<td><em>Pseudoeurycea goebeli</em></td>
<td>2400–2900</td>
<td>Cloud forest (low)</td>
<td>Terrestrial</td>
<td>Year-round</td>
<td>November/December</td>
<td>All year</td>
</tr>
</tbody>
</table>
These species were chosen for this study based on the availability of an adequate sample of preserved individuals and the availability of detailed data on elevational distributions, microhabitat preferences (Wake & Lynch, 1976; Campbell, 1999) and seasonality of oviposition (Houck, 1977a; Houck, 1977b). They occur within the same general geographical region, but experience unique seasonal environments because they occupy microhabitats at different elevations (Table 1).

**EXPERIMENTAL PROCEDURES**

For each species, I chose 11–16 adult male individuals collected and preserved in the field. Whenever possible, the sample included individuals collected in each month of the year as well as multiple individuals from each species for a subset of collection dates. To minimize sampling error, I used specimens with similar collecting dates and localities. Standard measurements such as snout–vent length and tail length were taken for each individual. In plethodontid salamanders, external secondary sexual characteristics are indicative of reproductive activity and condition (e.g. Weichert, 1945; Houck & Sever 1994; Bille, 2000); therefore, I examined each individual for the presence of enlarged naso-labial grooves and developed mental glands.

Previous studies of these four species did not reveal any discernible differences between the right and left testis (Houck, 1977b; Houck, 1977c). Therefore, I removed the entire right testis and a portion of the connected vas deferens for histological procedures (for four individuals, the right testis had previously been removed so I used the left testis instead). I measured the length of each testicular lobe and recorded the external coloration and general appearance of the testis and vas deferens. Pigmentation and the gross appearance of the testis and the vas deferens are often associated with the presence and absence of spermatozoa in the testis and can therefore be used as rough indicators of the reproductive state of the testis (Lofts, 1974). The bolitoglossine testis consists of one or more lobes, each an aggregation of numerous lobules (Humphrey, 1922; Lofts, 1974). A single lobe contains cells at the same stage of maturation. Within a lobe, maturation is completed first in the most posterior lobules and more anterior lobules contain successively more immature cells (Fig. 1). From the posterior lobules, gametes empty centrally into the collection duct and then continue on to the vas deferens. Empty lobules are reabsorbed and Sertoli cells migrate longitudinally to the anterior end of the testicular lobe where after some delay, new lobules are formed and immature cysts begin to develop into spermatozoa (Humphrey, 1922; Vial, 1968; Lofts, 1974). Thus, the spermatogenetic ‘wave’ of male gamete maturation progresses from the posterior end to the anterior end of the testis (described by Humphrey, 1921; Humphrey, 1922). For individuals with more than one testicular lobe, both lobes were sectioned and examined. If the lobes were similar in size, each was examined to confirm that the progression of the spermatogenetic wave was similar between lobes. Because small lobes of the testis are in the process of being either reabsorbed or maturing (Vial, 1968), I considered them to be less representative of spermatogenetic cycles and, therefore, I always used the lobe of greater length for analyses. In most cases, this was the posterior lobe. Individuals with multiple lobes that were very different in appearance were omitted from analyses.

For each individual, the testis and a portion of the vas deferens were dehydrated in a series of ethanol dilutions, cleared with Histoclear, and embedded with paraffin. Serial, longitudinal sections 7 µm thick were mounted on glass microscope slides and stained with Ehrlich’s haematoxylin followed by eosin counterstain. A coverslip was placed over each slide using Permount solution and slides were curated at the MVZ. Because the plethodontid testis is spindle shaped, the

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**Figure 1.** Schematic of sampling method for a typical testicular lobe. Measurements were taken from digital images at the anterior, middle, and posterior points of the testis (respectively, one-quarter, one-half, and three-quarters of the way down the length of the testis). The images on the right are examples of primary spermatocytes (1° Sc), secondary spermatocytes (2° Sc) and spermatozoa bundles (Sz).
central axis of the testis should be the most representative of the overall condition of the gonad. Thus, for each individual I identified the central section of the testis by the presence and completeness of the central collecting duct. This section and two additional sections 21 μm (four sections) to each side of the central section were used for measurements. Every individual examined had all three main spermatogonogenic stages present (primary spermatocytes, secondary spermatocytes, and spermatozoa). Therefore, I standardized measurements by defining the length of each testis as the distance between the most posterior boundary of lobules containing spermatozoa to the anterior boundary of lobules containing primary spermatocytes. To characterize the state of the testis and examine how the composition of the testis varies throughout the year, I took digital photographs through a Leica compound microscope at ×400 magnification of representative stages at one-quarter, one-half, and three-quarters of the way down the length of the testis (regions referred to, respectively, as ‘anterior portion’, ‘middle portion’ and ‘posterior portion’; see Fig. 1) for a total of nine representative images per individual. In addition, an image of a cross section of the vas deferens was taken to address questions regarding sperm evacuation.

I used Object Image 1.62p7 to take quantitative and qualitative data from each digital image. I randomly placed a 0.005 mm² circle within each image, determined the cell stage present within the circle, then counted the number of cells within or touching the circle as primary spermatocytes, secondary spermatocytes or spermatozoa. Because spermatozoa are present in dense clusters, I counted the number of spermatozoa bundles and noted the gross appearance and development, rather than counting individual spermatids. I examined the vas deferens of all individuals, noting the presence of spermatozoa in the tubule and Sertoli cells in the wall of the vas deferens, as well as the relative density of spermatozoa if present.

DATA ANALYSES

Individuals were grouped by species for all analyses. The three measurements for each region of the testis for each individual were considered for all analyses to account for slight sampling biases. To census the overall degree of variation within each species, I counted the number of individuals with each of the three sperm cell stages at the anterior, middle and posterior points in the testis (Fig. 2). For most individuals, all three images for a given region had the same cell stage present. In the few instances when cell stages were variable (e.g. two sections with primary spermatocytes and one section with secondary spermatocytes), I used the stage that was present in two of the three sections.

I also examined temporal variation in spermatogenesis. For each individual, averages for the three sections within each region were used. In instances of stage variation among sections, averages including only the sections with the stage of interest were used. For initial searches of temporal patterns, I plotted

![Figure 2](image_url)  
**Figure 2.** Frequency distribution of spermatogonogenic stages found at the anterior, middle and posterior portions of the testis in four species of tropical salamander.
data using standard calendar days starting with January 1st (not shown). From these preliminary analyses, I identified natural breaking points based on the temporal distribution of cell stages that I later used as begin/end dates of cycles. June 1st was used as the starting point for further investigations of annual patterns and temporal variation for B. occidentalis, D. bromeliacia and P. goebeli; November 1st was used for B. rostrata. June 1st and November 1st correspond, respectively, to the beginning and end of the wet season in Guatemala (Houck, 1977a; Wake, 1987).

The spermatogenetic wave progresses down the length of the testis, thus, variation in the composition of the testis will be more evident in the middle of the testis than at either end. Thus, to examine temporal variation in the presence of each of these stages, I plotted the stage present in the middle portion against day of year. If there is a temporal pattern to the spermatogenetic cycle, individuals with the same stage should have collection dates clustered in the same part of the year.

The density of primary spermatocytes at the anterior portion of the testis is a quantitative measure of the progression of the spermatogenetic wave. Almost all individuals have primary spermatocytes present at the anterior portion of the testis. Initial analyses down the length of testes revealed that spermatocyte densities decrease linearly from anterior to posterior, with the more mature cells in lower densities than the newer cells. Because of the nature of the spermatogenetic wave, directional seasonal variation in primary spermatocyte density at this point in the testis can be taken as an indicator of changes in the overall composition of the testis and the degree to which the spermatogenetic wave has progressed. Thus, for these additional analyses of temporal variation, I used linear regression analyses to examine the relationship between day of the year and spermatocyte density.

**RESULTS**

All three spermatogenetic stages were present in every individual examined but the portion of the testis occupied by each of the three stages varied among species (Fig. 2). For all individuals examined of B. rostrata, D. bromeliacia and P. goebeli, only primary spermatocytes were present in the anterior portion of the testis and only spermatozoa were found in the posterior portion. Additionally, within each of those species, individuals with primary spermatocytes in the middle portion of the testis were found in near equal frequency to those with secondary spermatocytes. *Bolitoglossa occidentalis* had considerably greater variation in testicular composition. Two of 14 individuals had secondary spermatocytes present at the anterior end of the testis instead of primary spermatocytes. In the posterior portion of the testis, each of the three stages was found in at least one individual although most individuals had spermatozoa present. Likewise, for this sample of *B. occidentalis*, individuals with each of the three stages present at the middle section were equally frequent.

There were detectable patterns of temporal variation in the stage present in the middle portion of the testis for both *Bolitoglossa* species, but not for either *D. bromeliacia* or *P. goebeli* (Fig. 3). For *B. rostrata* and *B. occidentalis*, successively more mature stages were found in this portion of the testis over time. For

**Figure 3.** Stage present at the middle portion of the testis (top) and primary spermatocyte density at the anterior portion of the testis (bottom) against collection date in four species of tropical salamander. Based on the presence of natural temporal breaks in the preliminary analysis, the x-axis for *Bolitoglossa rostrata* begins with November while the x-axis for all other species begin with June.

B. rostrata, the middle portion of the testis contained primary spermatocytes from November through March and secondary spermatocytes from April to October. In contrast, for B. occidentalis, the most mature stages found at this point were spermatooza rather than secondary spermatocytes and they were present from March to July.

Anterior densities of primary spermatocytes were the same throughout the year for all species examined except B. occidentalis (Fig. 3). Bolitoglossa occidentalis had directional, temporal variation: primary spermatocyte densities decreased linearly with the maximum occurring in mid-July and the minimum in early February. Two individuals, both collected in early February, were omitted from these analyses because they contained secondary spermatocytes at this point of the testis. However, the middle portion of the testis in these individuals was the boundary region between lobules containing primary spermatocytes and those containing secondary spermatocytes. Densities of the primary spermatocytes in these lobules were less than 10 cells 0.005 mm⁻² supporting this pattern of decreased density (increased maturity) of primary spermatocytes over the course of one year. Every individual examined had Sertoli cells in the wall of the vas deferens and spermatooza were present in the vas deferens for all individuals of B. rostrata, D. bromeliacia and P. goebeli. Three of the 14 individuals of B. occidentalis (collected in early September and early November) did not have spermatooza present in the vas deferens. The temporal distribution of these individuals was not significantly different from that predicted by random, however, that may be due to low detection power as a result of small sample size.

DISCUSSION

The observation of spermatooza in the testis of all individuals examined here corroborates previous studies of male reproductive patterns in tropical bolitoglossine salamanders (Valdivieso & Tamsitt, 1965; Vial, 1968; Houck, 1977a; Bille, 2000). Additionally, I found evidence for constant production and evacuation of spermatooza suggesting that these species are truly continually spermatogenetic and capable of inseminating females at any time of the year. In most species of temperate salamanders, the state of the testis changes greatly in size and composition throughout the year (Humphrey, 1921; Humphrey, 1922; Joly, 1971; Uribe, Gomez-Rios & Brandon, 1994). For some species, males undergo a period of complete testicular regression during which the testis does not contain any mature gametic cells, followed by a period of recrudescence where the testis is occupied only by immature cells (Weichert, 1945; Miller & Robbins, 1954; Stebbins, 1954). In contrast, the testes of all individuals in this study have newly formed cells in addition to spermatooza and all but three individuals have sperm in the vas deferens as well. The presence of developed secondary sexual characters in almost every individual examined further suggests that males are capable of courtship and mating at all times of the year as the hormones responsible for the maintenance of these characteristics are also associated with spermatogenesis. The evacuation of mature sperm from the testis (Houck & Woodley, 1995; Woodley, 1994). Thus, the occurrence of spermatooza in the testis at all times of the year is most likely not the result of a single spermatogenetic event with subsequent storage of that sperm. In many temperate salamanders, the production of spermatooza is restricted during the year because of unfavourable environmental conditions (Ifft, 1942; Werner, 1969). However, even in the most seasonal of tropical habitats conditions may be sufficiently benign to allow for the continual production of sperm. If the cost of maintaining and producing mature sperm is low, then continual spermatogenesis may represent an evolutionarily stable strategy as suggested by Houck (1977c).

ECOLOGICAL CONTEXT OF TEMPORAL PATTERNS

Although all species in this study are spermatogenetic throughout the year, there are cryptic levels of variation in the male reproductive cycle that might not be detectable in studies that focus exclusively on the presence or absence of spermatooza. The presence of temporal variation in spermatogenesis for species from seasonal habitats (low elevation B. occidentalis and high elevation B. rostrata) and the absence of variation in the species from the more aseasonal cloud forests (D. bromeliacia and P. goebeli) correlate with the amount of climatic seasonality in each life zone. However, there are dissimilarities in reproductive cycles that cannot be accounted for by these broad-scale differences. Understanding the significance of temporal variation in spermatogenesis given other aspects of reproductive biology requires consideration of how regional climate and species-specific ecological parameters together determine the immediate environment an individual experiences.

Of the two species from the more seasonal elevations, the bromeliad dweller (B. occidentalis) has more pronounced temporal variation than the ground dweller (B. rostrata) contrary to predictions based on the capacity of arboreal microhabitats to better protect against temperature and humidity extremes (Feder, 1982). In addition, the timing of significant changes in the testicular cycle of each of these species differs despite the fact that both species lay eggs at the end of the wet season (Houck, 1977a; Houck, 1977b).
Abrupt changes in the testis in *B. occidentalis* occur between May and June; the proportion of the testis occupied by spermatozoa decreases and the testis instead has a high proportion of primary spermatocytes (Fig. 3). Measurements of primary spermatocyte densities at the anterior portion of the testis corroborate this pattern, suggesting that the rate of evacuation of sperm increases at the start of the wet season. This may be in response to increased mating opportunities in the ensuing months. Salamander densities within bromeliads are greater during the dry season than the wet season, suggesting that arboreal habitats provide a refuge from unfavourable conditions and that individuals may be confined to bromeliads at particular times of the year (Wake, 1987). The onset of more favourable conditions during the wet season is likely to be accompanied by greater surface activity and, thus, the increase in the rate of sperm evacuation in May and June may correspond to more mating opportunities that result from increased activity. Likewise, the three individuals found in September and November without sperm in the vas deferens may represent the end of the intense mating season at which time the relative disadvantage of having an empty vas deferens is minimal.

Significant variation in the spermatogenetic cycle of the high elevation terrestrial species, *B. rostrata*, is subtler than that of *B. occidentalis* and occurs at the end of the wet season in October and November rather than at the beginning. Although terrestrial habitats are less efficient at buffering against environmental fluctuations, suitable terrestrial microhabitats are more abundant and widely distributed throughout the landscape than typical arboreal microhabitats such as bromeliads. While a greater proportion of matings may take place around the time of egg-laying, accounting for the slight variation observed, the movement of individuals may not be drastically restricted during the dry season resulting in a relatively constant rate of encounters with potential mates throughout the year.

Although neither of the cloud forest species have any detectable temporal cycle of spermatogenesis, the arboreal species (*D. bromeliacta*) lays eggs throughout the year while the terrestrial species (*P. goebeli*) is a seasonal egg-layer. Mating opportunities are likely to be relatively constant throughout the year for both species irrespective of microhabitat preference because of stability in moisture and temperature levels in the cloud forest. The lack of seasonal variation in the testis suggests that rates of maturation and evacuation are similar so that the overall composition of the testis does not change greatly over the course of the year. Differences in the seasonality of oviposition may be best explained by microhabitat preference and life-stage specific abiotic requirements; terrestrial habitats may be inadequate buffers for eggs and young even in a fairly aseasonal environment, despite being sufficient for adult activity. Thus, only the arboREAL cloud forest species is able to achieve completely aseasonal reproduction.

By examining fine-scale variation within the testes of tropical bolitoglossine salamanders, it is possible to detect cryptic temporal patterns that are not obvious from spermatozoa presence/absence data. These slight levels of variation help us to understand better how individual reproductive traits fluctuate with environmental conditions. Ecological parameters at the regional scale certainly influence reproductive seasonality; however, local-scale environmental conditions are also important determinants of overall reproductive strategy. Depending on the environmental background determined by regional patterns, microhabitat preference and the associated local climate may constrain or release certain aspects of reproductive biology. Because plethodontid salamanders are secretive and sedentary, behavioural observations on reproductive cycles in the field are difficult. Furthermore, densities of tropical bolitoglossine salamanders have declined in recent years making field observations even more unlikely (J. Campbell, pers. comm.; Parra-Olea, Garcia-Paris & Wake, 1999). Thus, detailed examination of temporal and spatial series of museum specimens is a useful technique for inferring physiological and behavioural patterns in light of ecological parameters.

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