

Climatic correlates of female and male reproductive cycles and plasma steroid hormone levels in the many-lined sun skink *Eutropis multifasciata*

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ABSTRACT

Gonadal activity and plasma steroid hormone (testosterone and 17 β -estradiol) levels in males and females of a viviparous skink (*Eutropis multifasciata*) were investigated. Changes in the hormone profiles were then correlated to changes in environmental factors such as temperature and rainfall and were found to vary seasonally in both sexes. Gonadal activity, calculated using testicular mass and volume, seminiferous tubule diameter and epithelial height in males and, in females, ovary mass and largest follicular volume also varied seasonally. Peak spermiogenesis was in synchrony with maximal vitellogenic activity, but reproductive synchronicity among females was low. Ovary mass and largest follicular volumes were negatively related to air temperature and rainfall. Testicular mass and volume were not related to air temperature, but both were negatively related to rainfall. Rainfall explained a greater proportion of variation in vitellogenic activity than temperature. As for the climatic correlates of seasonal variation in plasma steroid hormones, we found only in males that the plasma level of testosterone was negatively related to rainfall. Taken together, our data show that male and female reproductive activities are more tightly correlated with rainfall than temperature in *E. multifasciata*.

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1. Introduction

Seasonal timing and other characteristics of the reproductive cycles are linked to exogenous (environmental) and endogenous (mainly hormonal) factors for a wide range of vertebrate taxa [9,6,8,46,38]. Studies of reptile reproduction have mostly been anatomical and limited to the examination of reproductive organs for evidence of spermatogenesis and vitellogenesis [46]. These studies generally show that temperature and rainfall influence reproductive cycles in reptiles, and that species and populations may not necessarily respond to environmental factors in the same way. For example, for many temperate reptiles, lower temperatures associated with autumn and winter limit activity and physiological processes and, consequently, they reproduce in spring and summer, with male and female cycles well synchronized [13,14,23]. Reproductive activity in tropical reptiles is also correlated with environmental factors. However, in contrast to temperate zones, annual climatic variation is low in the tropics and, correspondingly, tropical reptiles are either seasonal breeders, dependent on rainfall and food availability, or aseasonal breeders, reproducing throughout the year [13,14,23,10]. Hormonally regulated reproduction has been documented in several lizards where seasonal gonadal

recrudescence and elevation in plasma sex steroids are correlated [2,3,11,27,48,34], but how changes in hormone levels are associated with environmental factors remains a sparsely studied area.

The pantropical scincid genus *Mabuya* was recently split into four genera, and only the American forms retain that generic designation [28]. The other three genera are *Chioninia* endemic to Cape Verde Islands, *Euprepis* in Africa, and *Eutropis* in Asia [28]. Although more than 100 species of “*Mabuya*” skinks have been described worldwide, male and/or female reproductive cycles have been described in detail for six species only [33,15,50–52,37]. Males and females are seasonally reproductive in *Euprepis capensis* [15], *Mabuya bistrata* [51], *Mabuya frenata* [52] and *Mabuya heathi* [50], but are continuously reproductive in *Mabuya mabouya* [37]. In two subspecies of *Euprepis striata*, females of the low altitude subspecies (*E. s. striata*) are reproductive throughout the year apart from a brief period at the start of the rainy season, whereas females of the high altitude subspecies (*E. s. punctatissima*) are seasonally reproductive and give birth in the late dry season only [33]. Previous studies of “*Mabuya*” skinks generally show that environmental factors such as temperature and moisture influence reproductive activity in both sexes.

The many-lined sun skink (*Eutropis multifasciata*) is one of four “*Mabuya*” species in China, occurring in five subtropical provinces of the country [21]. This species is an ideal model to explore the proximate and ultimate causes of variation in reptile reproductive

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patterns because of its tropical–subtropical distribution and the fact that the flow of herpetological research out of China remains small. This study focuses on climatic correlates of the reproductive cycles and plasma steroid hormone (testosterone and 17 β -estradiol) levels in both males and females of a subtropical population of *E. multifasciata*. We first document annual changes in gonadal activities and plasma steroid hormone levels in males and females, and then investigate whether these changes are associated with environmental factors such as temperature and rainfall.

2. Materials and methods

2.1. Study area and climate

The study population is located between Lingshui (18°48'N, 110°02'E) and Ledong (18°73'N, 109°17'E), Hainan. Data from Hainan Meteorological Administration show that the climate of the study area is seasonal. Mean annual temperature is 26 °C and total annual rainfall is 1475 mm. Monthly mean temperatures in the warm season (April to October) are higher than the cool season (November to February) by an average of 4 °C. Rainfall is 1010 mm in the wet season (July to October, monthly mean rainfall >210 mm) and 310 mm in the dry season (November to April, monthly mean rainfall <70 mm). From September to October, rainfall is at its highest (~290 mm) and at its lowest (~5 mm) in January–February (Fig. 1).

2.2. Annual changes in gonadal activities and plasma steroid hormone levels

A total of 199 females (>96 mm SVL) and 188 males (>99 mm SVL) were collected in 2008 to study the reproductive cycles. The smallest reproductive female of *E. multifasciata* is 90 mm SVL, and adult males are larger than adult females by an average of 4 mm SVL [25]. Thus, all individuals collected were adults. Between 14 and 26 adults of each sex were sampled each month. These individuals were decapitated to collect blood samples into heparinised tubes. Blood samples were immediately centrifuged at 4000 rpm for 20 min to separate plasma which was stored at –80 °C until analysis. The bodies were dissected for gonadal analyses. The following measurements were taken for each individual: SVL, gonadal mass, length and width of left and right testes in males, and length and width of the largest ovarian follicle in

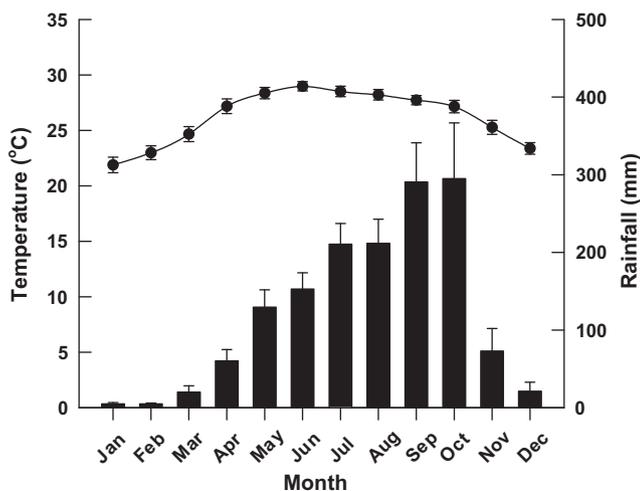


Fig. 1. Means (\pm SE) monthly air temperature (solid dots) and rainfall (solid bars) over the 6-year period (January 2006 through December 2011) in the study area. Data were obtained from Hainan Meteorological Administration.

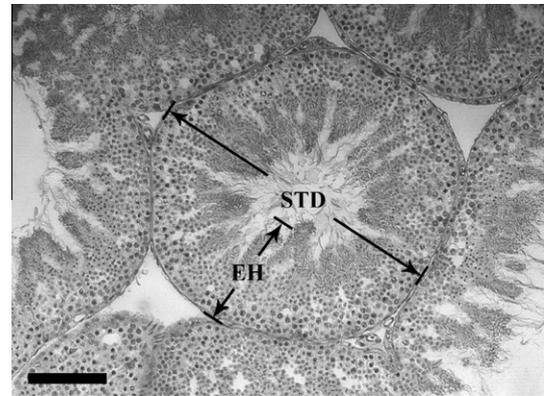


Fig. 2. A cross-section of seminiferous tubules. STD: seminiferous tubule diameter; EH: epithelial height; Scale bar = 100 μ m.

females. The number of vitellogenic and nonvitellogenic follicles, and/or embryos in each oviduct was also noted. We calculated testicular and follicular volumes with the formula for the volume of an ellipsoid: $V = 4/3 \pi ab^2$, where $a = 1/2$ the longest diameter and $b = 1/2$ the shortest diameter. Testicular and follicular volumes were used as indicators of reproductive activity of both sexes [52].

Testes were preserved in Bouin fixer solution for 24 h, and dehydrated using 75% ethanol solution. Tissue segments were infiltrated with and embedded in paraffin wax, and 3 μ m sections were cut from each block with an ultramicrotome (LKB Produkter AB, Sweden). Slides were stained with haematoxylin and eosin and examined by light microscopy. Cross-sections of testes were taken serially and inspected histologically to assess male gonadal activity using a light microscope (Nikon E600, Japan). We randomly inspected ten cross-sections of seminiferous tubules for each testis (Fig. 2), and measured seminiferous tubule diameter and epithelial height using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, USA) to obtain mean values for the two variables.

Concentrations of two plasma steroid hormones, testosterone (T) and 17 β -estradiol (E2), were determined by enzyme-linked immunosorbent assay via EIA kits (T product No. 582701, and E2 product No. 582251; Cayman Chemical Inc). The interassay coefficient of variation was 6.6% for T and 12.3% for E2, and the intrassay coefficient of variation was 7.5% for T and 5.5% for E2. Before the EIA assay, steroids were extracted with 5 \times the sample volume of diethyl ether for three times and dried under a nitrogen stream in a dry bath at 30 °C. Recovery rates averaged 71.8% for T and 85.3% for E2.

2.3. Statistical analyses

Statistica 6.0 (StatSoft, Tulsa, USA) was used to analyze data. Data were tested for normality using Kolmogorov–Smirnov test, and for homogeneity of variances using Bartlett's test. Log_e transformations were performed when necessary to satisfy the assumptions for parametric tests. Linear regression analysis was used to examine whether a trait was dependent on body size, and whether reproductive activity of both sexes was dependent on environmental factors (air temperature and precipitation). Spearman correlation analysis was done to examine whether there was a correlation between the plasma level of a given steroid hormone and gonadal activity in each sex. To describe male and female reproductive cycles, we firstly tested the relationship between the log_e-transformed gonadal size (mass and volume) and the log_e-transformed body size (SVL). Body size influenced gonadal size in males but not in females. We therefore calculated residuals of log_e-transformed gonadal size against log_e-transformed SVL in

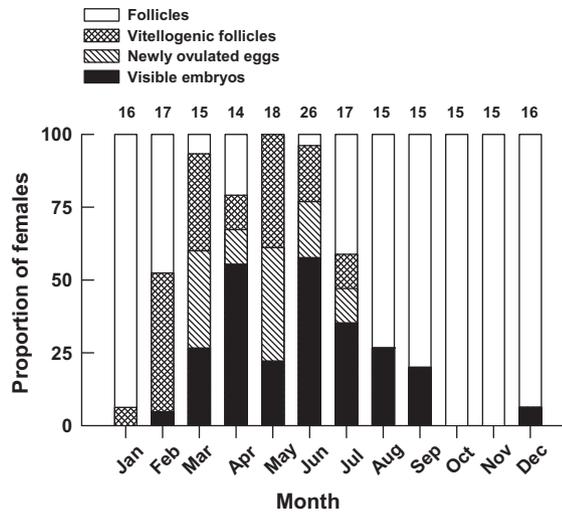


Fig. 3. Proportion of females represented in each of four reproductive states in monthly samples. Numbers in the figure indicate sample size for each month.

males. The female reproductive cycle was best represented by actual gonadal data. One-way analysis of variance (ANOVA) with month as the factor was used to examine whether gonadal size and plasma steroid hormones examined varied seasonally. Multiple comparisons were performed when necessary using Tukey's *post hoc* test. Values were presented as mean \pm SE, and the significance level was set at $\alpha = 0.05$.

3. Results

3.1. Female reproductive cycle

Females sampled in October and November did not have vitellogenic follicles and embryos; females with vitellogenic follicles were observed in January to July; females with newly ovulated eggs were observed in March to July; females with visible embryos were observed in February to September, and in December (Fig. 3).

There was no relationship between \log_e -ovary mass and \log_e -SVL ($r^2 = 0.004$, $F_{1, 197} = 0.84$, $p = 0.362$), nor was the relationship between \log_e -largest follicular volume and \log_e -SVL ($r^2 = 0.004$, $F_{1, 197} = 0.83$, $p = 0.363$). Ovary mass varied among months throughout the year (ANOVAs on \log_e transformed data; $F_{11, 187} = 8.28$, $P < 0.0001$; Fig. 4A), so did the largest follicular volume (ANOVA on \log_e transformed data; $F_{11, 187} = 8.42$, $P < 0.0001$; Fig. 4B). Ovaries began to increase in size in July and reached maximum size in January.

3.2. Male reproductive cycle

The regression of \log_e -testis mass against \log_e -SVL was significant ($r^2 = 0.18$, $F_{1, 186} = 40.23$, $P < 0.0001$), so was the regression of \log_e -testicular volume against \log_e -SVL ($r^2 = 0.27$, $F_{1, 186} = 69.22$, $p < 0.0001$). Testis mass varied significantly among months throughout the year (ANOVAs on regression residuals; $F_{11, 176} = 16.50$, $p < 0.0001$; Fig. 5A), so did the testicular volume (ANOVAs on regression residuals; $F_{11, 176} = 10.45$, $p < 0.0001$; Fig. 5B). Testes began to increase in size in August, remained enlarged in November–May, after which they decreased in size. Monthly changes in seminiferous tubule diameter (ANOVA on \log_e transformed data; $F_{11, 176} = 9.47$, $p < 0.0001$; Fig. 6A) and epithelial height (ANOVA on \log_e transformed data; $F_{11, 176} = 8.40$, $p < 0.0001$; Fig. 6B) were significant. Seminiferous tubule diameter and epithelial height were positively related to testicular mass and volume ($r^2 > 0.39$ and $p < 0.03$ in all cases; Fig. 7).

3.3. Annual variation in plasma steroid hormones

Plasma T and E2 levels varied monthly in both sexes (ANOVAs on \log_e transformed data; $p < 0.0001$ in all cases) (Fig. 8). The annual mean T level was higher in males than in females ($F_{1, 186} = 921.46$, $p < 0.0001$), so was the annual mean E2 level ($F_{1, 197} = 23.54$, $p < 0.0001$). Plasma T and E2 levels were not correlated with ovary mass or largest follicular volume (Spearman correlation analysis; all $r < 0.27$ and all $p > 0.191$). Plasma T levels were positively correlated with testis mass ($r = 0.72$, $p < 0.008$) and testicular volume ($r = 0.66$, $p = 0.020$). Plasma E2 levels were

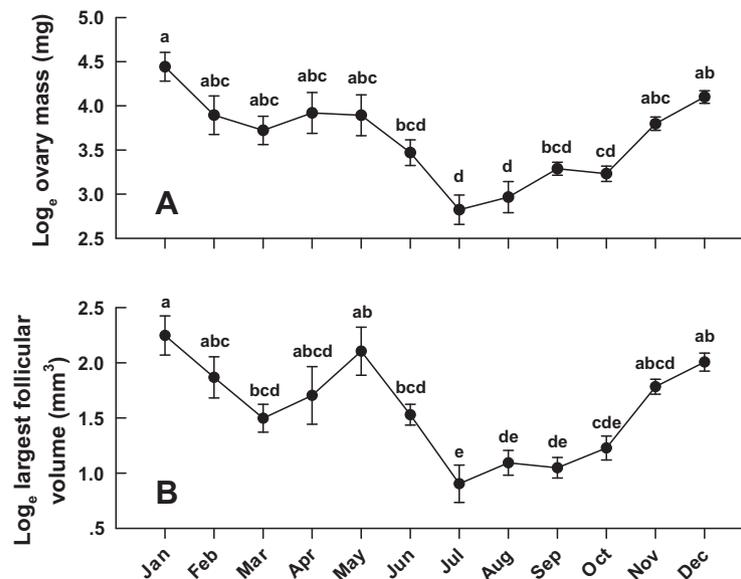


Fig. 4. Monthly changes in ovary mass (A) and largest follicular volume (B). Data are expressed as mean \pm SE. See Fig. 3 for sample size for each month. Means with different letters differ significantly (Tukey's *post hoc* test, $\alpha = 0.05$).

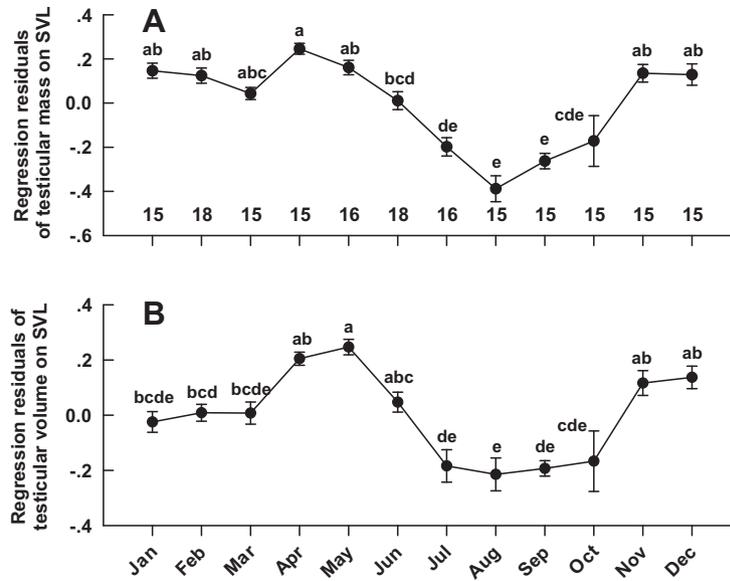


Fig. 5. Monthly changes in testicular mass (A) and volume (B). Data are mean (\pm SE) residuals from regressions of \log_e -testicular mass (mg) and volume (mm^3) against \log_e -SVL (mm). Numbers in the figure indicate sample size for each month. Means with different letters differ significantly (Tukey's *post hoc* test, $\alpha = 0.05$).

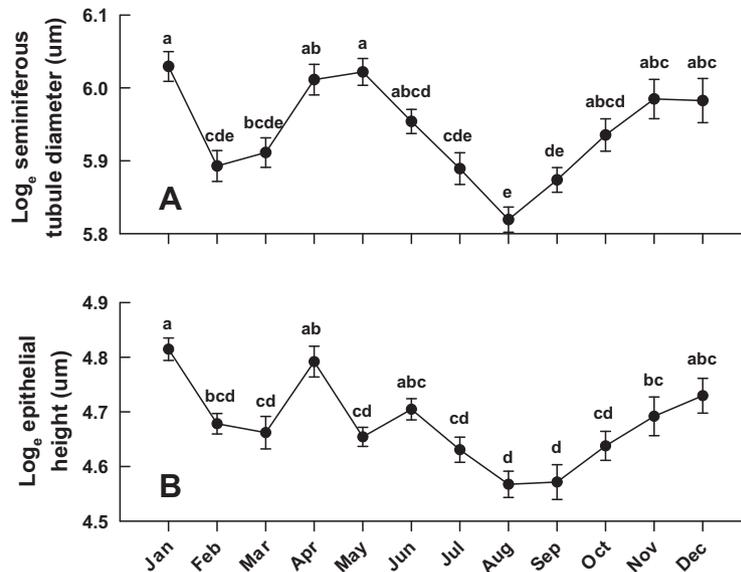


Fig. 6. Monthly changes in seminiferous tubule diameter (A) and epithelial height (B). Data are expressed as mean \pm SE. See Fig. 5 for sample size for each month. Means with different letters differ significantly (Tukey's *post hoc* test, $\alpha = 0.05$).

not correlated with testis mass ($r = 0.18$, $p = 0.587$) or testicular volume ($r = 0.30$, $p = 0.342$).

3.4. Climatic correlates

Ovary mass and largest follicular volumes were negatively related to air temperature and rainfall (linear regression analysis; $p < 0.03$ in all cases; Fig. 9). Testicular mass and volume were not related to air temperature (linear regression analysis; $p > 0.091$ in both cases), but both were negatively related to rainfall (linear regression analysis; $p < 0.022$ in both cases; Fig. 10). As for the climatic correlates of seasonal variation in plasma steroid hormones, we found only in males that the T level was negatively related to rainfall ($F_{1, 10} = 23.93$, $p < 0.001$; Fig. 11).

4. Discussion

4.1. Female and male reproductive cycle

Female *E. multifasciata* with visible embryos were observed in February to September and in December (Fig. 3). This pattern is similar to that reported for tropical lizards, for example for *Anolis opalinus* [24], *Japalura swinhonis* [22], *M. heathi* [51], *M. mabouya* [37] and *E. s. striata* [33] where reproductive synchronicity among females is low. Females with newly ovulated eggs were observed in March to July (Fig. 3). This observation together with the findings that embryonic development in wild sampled individuals often take about two months to complete and most females give birth between March–August [25,44] suggest that female *E. multifasciata*

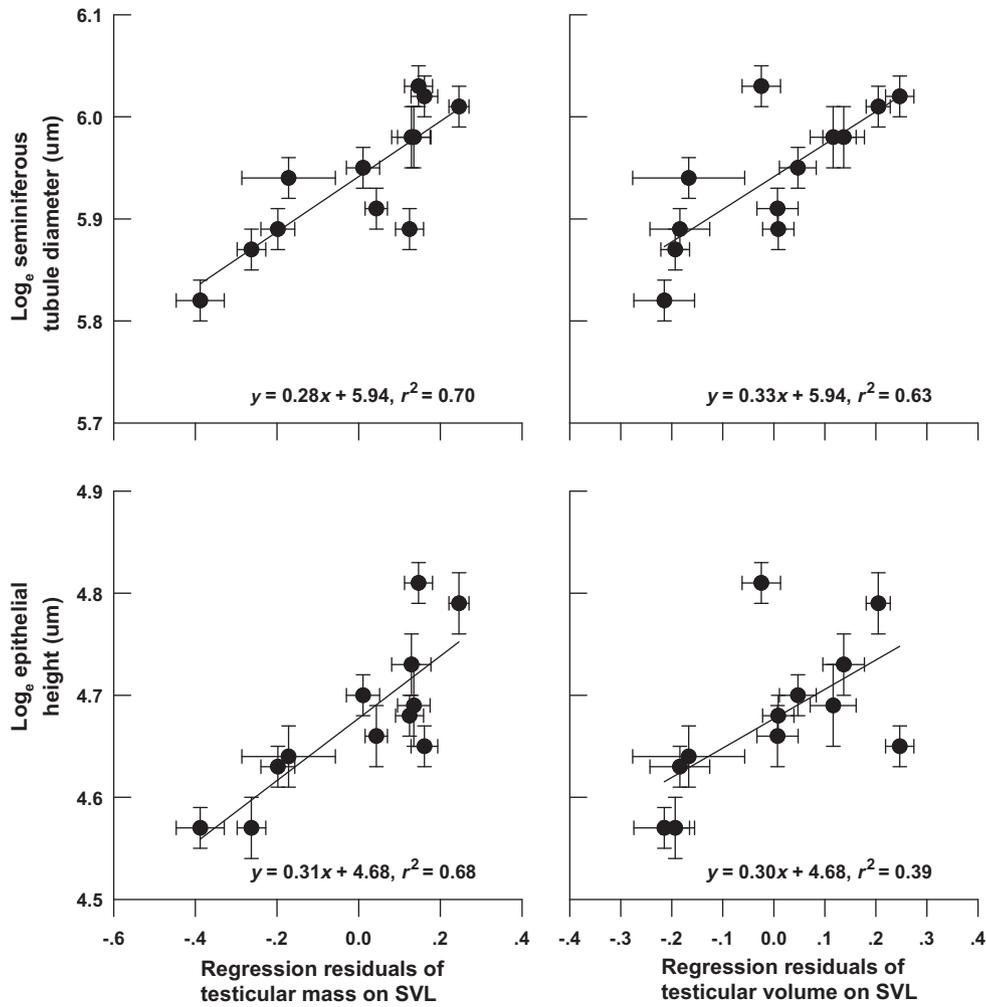


Fig. 7. Seminiferous tubule diameter and epithelial height in relation to testicular mass and volume. Data are expressed as mean \pm SE. Regression equations and coefficients are given in the figure.

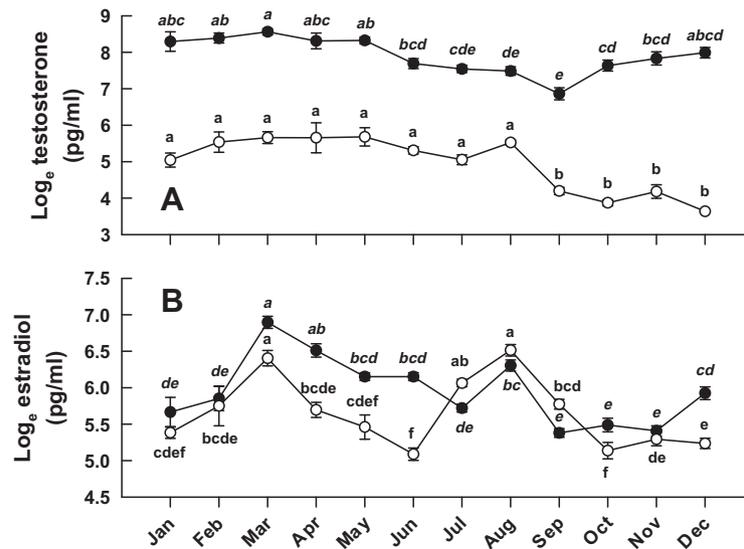


Fig. 8. Monthly changes in plasma testosterone (A) and 17 β -estradiol (B) in male (solid dots) and female (open dots) adults. Data are expressed as mean \pm SE. See Fig. 3 (females) and Fig. 5 (males) for sample size for each month. Means with different letters differ significantly (Tukey's post hoc test, $\alpha = 0.05$).

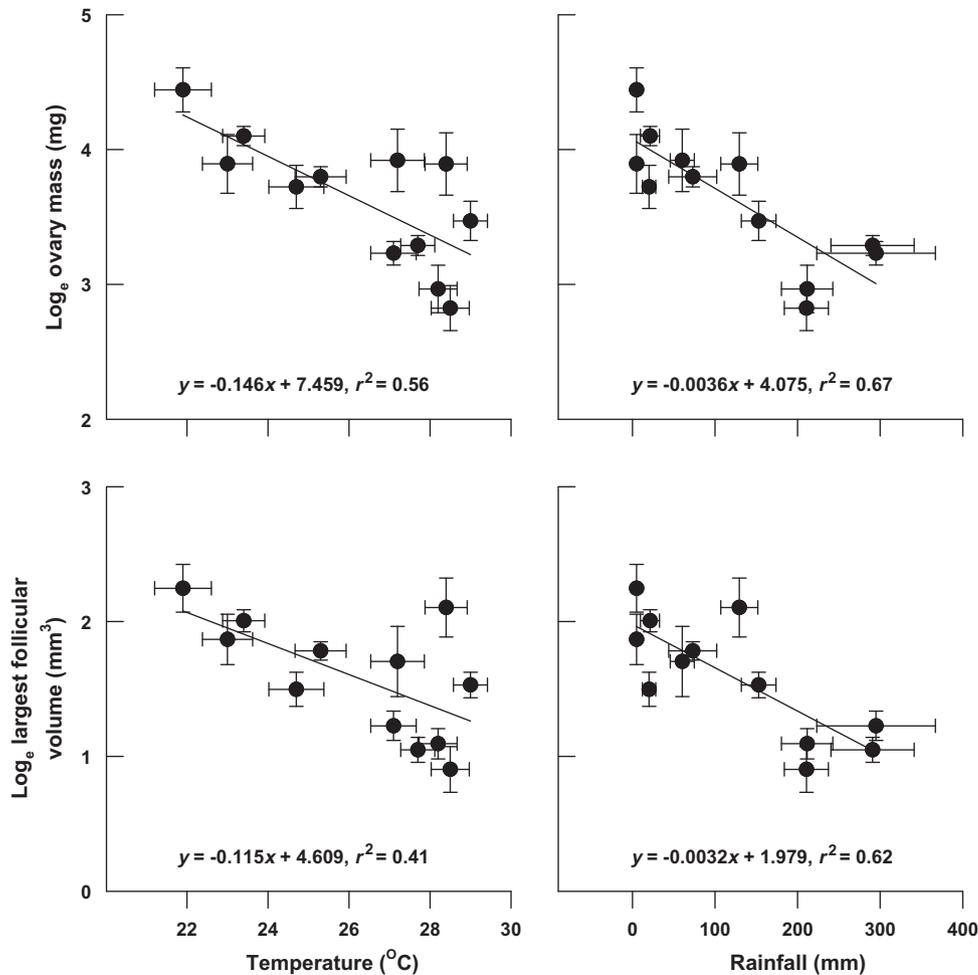


Fig. 9. Ovary mass and largest follicular volume in relation to air temperature and rainfall. Data are expressed as mean \pm SE. Regression equations and coefficients are given in the figure.

have the potential to produce two litters per year. Among the “Mabuya” species, only *E. striata* [42,33] and *M. mabouya* [37] have been reported to be multi-clutched.

There has been evidence that in lizards seminiferous tubule diameter and epithelial height become enlarged in the season when testes are spermatogenically active [17,39]. Our finding that seminiferous tubule diameter and epithelial height were positively related to testicular mass and volume adds evidence that spermatogenic activity can be better estimated by the size of testes in lizards [42,12,51,36,37,25,22]. Testicular mass and volume, seminiferous tubule diameter, epithelial height, ovary mass and largest follicular volume varied seasonally, and this indicates that, as in other “Mabuya” lizards such as *E. capensis* [15], *E. s. punctatissima* [33], *M. bistrata* [51], *M. frenata* [52] and *M. heathi* [50], males and females of *E. multifasciata* are seasonally reproductive. It is not surprising that the aforementioned species are seasonally reproductive because they inhabit seasonal tropical zones. Interestingly, however, *E. s. striata* [33] and *M. mabouya* [37] also inhabit seasonal tropical zones but are either continuously reproductive (*M. mabouya*) or reproductive throughout most of the year (*E. s. striata*). Why is reproductive seasonality evident in some seasonal tropical lizards but not in others? The answer presumably is that the rainfall regime and temperature may affect reproductive activity in tropical lizards. For example, the local rainfall regime is bimodal for *M. mabouya* [37], but is monomodal for *E. capensis* [15], *E. striata* [33], *E. multifasciata* (Fig. 1) and *M. frenata* [52]. Reproductive seasonality is evident in *E. s. punctatissima* but not

in *E. s. striata*, although the two subspecies both inhabit regions with a monomodal rainfall regime [33]. It is noteworthy that *E. s. striata* uses warmer habitats than *E. s. punctatissima* [33]. That reproduction is constrained by relatively lower temperature in *E. s. punctatissima* provides evidence that temperature may influence reproductive activity in tropical lizards.

4.2. Climatic correlates of reproduction

Temperature is the most important environmental factor controlling and regulating reproductive cycles of temperate and cold-climate reptiles, either by direct action, or due to the existence of a temperature threshold to facilitate a photoperiod response [12,37,32,20]. In tropical zones seasonal variation is greater for rainfall than temperature. This is also the case for Hainan Island (Fig. 1), and so we hypothesize that seasonality in rainfall would play a more important role than temperature in influencing reproductive activity in *E. multifasciata*. This hypothesis was supported because (1) the relationship between testicular size and rainfall was significant, whereas the relationship between testicular size and temperature was not, and (2) rainfall explained a greater proportion of variation in vitellogenic activity (as estimated by ovary mass and largest follicular volume) than temperature. These results suggest that vitellogenic and spermatogenic activities are more tightly correlated with rainfall than temperature in *E. multifasciata*. Spermatogenic and vitellogenic activities are lower in the wet season in *E. multifasciata*, as revealed by the

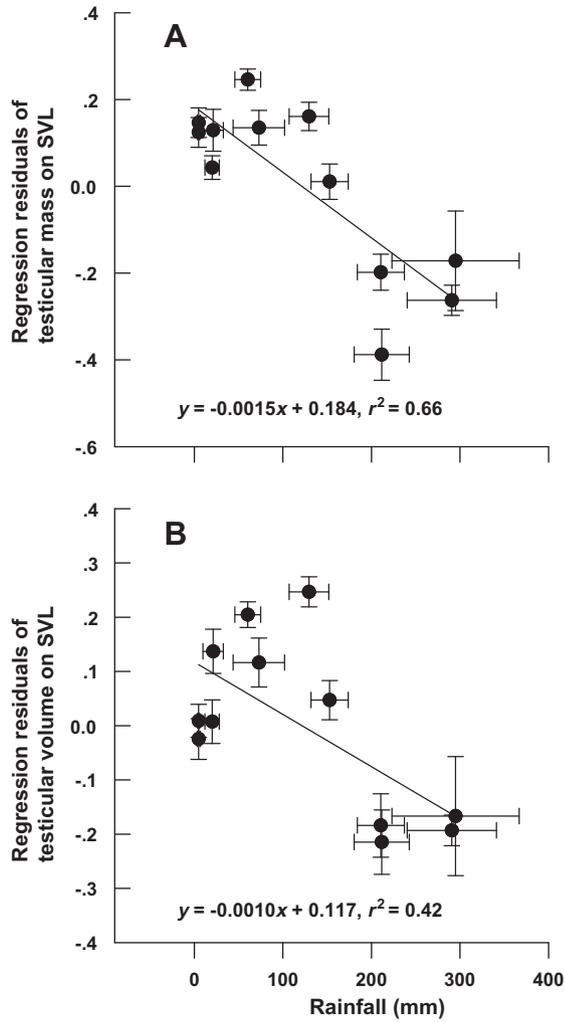


Fig. 10. Testicular mass (A) and volume (B) in relation to rainfall. Data are expressed as mean ± SE. Regression equations and coefficients are given in the figure.

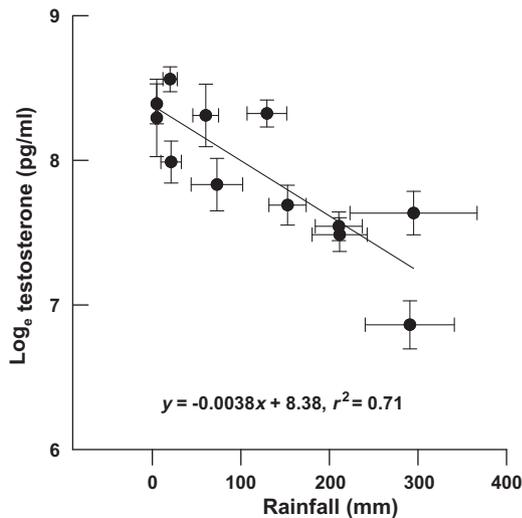


Fig. 11. The level of plasma testosterone in relation to rainfall in adult males. Data are expressed as mean ± SE. Regression equation and coefficient are given in the figure.

fact that testicular mass and volume, ovary mass and largest follicular volume were negatively correlated with rainfall. Peak spermiogenesis overall was in synchrony with maximal vitellogenic activity, suggesting the existence of a prenuptial spermatogenic cycle in male *E. multifasciata*.

Female *E. multifasciata* never give birth in the driest (January–February) and wettest (September–October) seasons [44]. Thus, it is possible that some degree of synchronization of the reproductive activity exists in female *E. multifasciata* and is related to rainfall, such that most neonates are found in months (April–August) with moderate rainfall. The finding that parturition date begins at the end of the driest season through the onset of the wettest season is consistent with results for other “*Mabuya*” lizards, for example for *E. capensis* [15], *E. striata* [42,33], *M. frenata* [52] and *M. mabouya* [37]. This consistency suggests that availability of moisture is an important factor affecting reproduction due to the effect of rainfall on food availability for newly emerging young. In turn, optimal time of birth can correlate with synchronization of reproductive activity in lizards [33,37,35,29,32].

4.3. Hormonal regulation of reproduction

Estrogens such as 17β-estradiol (E2) stimulate reproductive behavior and vitellogenesis in female reptiles, and are expected to be elevated during these events [19,7,4]. Androgens such as testosterone (T) stimulate reproductive behavior and spermatogenesis in male reptiles, and are expected to be elevated during periods of spermatogenesis and mating [31,47,41,16]. The role of androgens in female reproduction is thought to include sensitization of the follicles to preovulatory gonadotropin stimulation, influencing courtship and mating behavior, stimulation of oviductal hypertrophy, and the mobilization of lipids, carbohydrates and proteins for vitellogenesis [26,54,18,43,53]. Different species vary in the relative concentration of T and E2 and the exact relationship of these hormones to follicular development and mating activity. Here, plasma levels of T and E2 varied seasonally in both sexes, suggesting that in *E. multifasciata*, as in other reptiles [18,40], T and E2 may have physiological roles in influencing male and female reproductive cycles. However, plasma T levels were low in females and did not rise alongside vitellogenic activity, but were high in males and exhibited an elevation with spermatogenic activity. These results suggest that the role of testosterone in regulating reproduction is less important in females compared to males. A correlation between the annual cycle of testicular activity and plasma T levels has been also observed in other warm- and cold-climate lizards, for example in *Cordylus giganteus* [49], *Niveoscincus metallicus* [45], *Pogona barbata* [1], *Sceloporus jarrovi* [30] and *Tiliqua rugosa* [5]. Plasma E2 levels are correlated with vitellogenic activity in lizards where reproductive synchronicity among females is high [2,3,11,27,48,34]. Such a correlation was not found in this study, presumably because reproductive synchronicity among female *E. multifasciata* is low. Plasma T levels were correlated with spermatogenic activity, whereas plasma E2 levels were not. These results suggest that T rather than E2 is involved in the regulation of male reproduction in *E. multifasciata*. We found only in males that the gonadal size (mass and volume) and T level were negatively correlated with rainfall. These findings reinforce our conclusion that the male reproductive cycle is more tightly correlated with rainfall than temperature in *E. multifasciata*.

4.4. Conclusions

Our data show that gonadal activities and plasma levels of testosterone and 17β-estradiol vary seasonally in both sexes of *E. multifasciata*. Peak spermiogenesis is in synchrony with maximal vitellogenic activity, but reproductive synchronicity among

females is low. Annual changes in gonadal activities are associated with rainfall in both sexes, and are associated with temperature only in females. As for the climatic correlates of seasonal variation in plasma steroid hormones, we can find only in males that the plasma level of testosterone is negatively correlated with rainfall. Taken together, our data show that male and female reproductive activities are more tightly correlated with rainfall than temperature in *E. multifasciata*.

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