Reproductive pattern and sex hormones of *Calotes emma* Gray 1845 and *Calotes versicolor* Daudin 1802 (Squamata; Agamidae)

Worawitoo MEESOOK1, Taksin ARTCHAWAKOM2, Anchalee AOWPHOL1, Panas TUMKIRATIWONG1,*

1Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand
2Sakaerat Environmental Research Station, Thailand Institute of Scientific and Technological Research, Ministry of Science and Technology, Nakhon Ratchasima, Thailand

**Abstract:** We monitored testicular and ovarian morphologies, seminiferous tubules, the sexual segments of the kidneys (SSK), follicular histologies, and male testosterone and female estradiol to define the reproductive pattern of *Calotes emma* and *Calotes versicolor*. Samples were collected monthly at Sakaerat Environmental Research Station in Thailand for 1 year. Testicular hypertrophies occurred at a time characteristic for each species, with their time course corresponding well to both active spermatogenesis and the hypertrophied SSK. Gravid females were also found at a time characteristic for each species. In active reproductive females, oviductal eggs were concomitantly encountered with ovarian vitellogenic follicles. The previtellogenic and vitellogenic follicles corresponded well to granulosa layer alterations. The distinct large pyriform cells were present in the granulosa layer of previtellogenic follicles but disappeared from the vitellogenic follicles. Male testosterone levels rose during testicular and SSK hypertrophies, and female estradiol levels increased during active reproductive stages of late vitellogenic follicles and gestation. We suggest that the reproductive patterns of *C. emma* and *C. versicolor* fall into the same reproductive pattern of annual continual reproduction, but that the time courses of such events are different in the 2 *Calotes*, and even in individuals of the same *Calotes* population.

**Key words:** Estradiol, sexual segment kidney, spermatogenesis, testosterone, vitellogenesis

1. Introduction

Lizards (including snakes) are the most speciose living clade of reptiles, with almost 7200 species. Even excluding snakes, lizards are still the most speciose extant reptiles, with approximately 4450 species. Lizards, which belong to the family Agamidae, are classified into 52 genera with approximately 400 species (Vitt and Caldwell, 2009), 31 species of which are found in Thailand (Laohachinda, 2009). The genus *Calotes* Cuvier 1817, a genus of Agamidae, currently contains 23 species (Hartmann et al., 2013). There are 7 species of *Calotes* known to occur on the Southeast Asian mainland: *C. chincollium*, Vindum 2003; *C. htnwnwi*, Zug and Vindum 2006; *C. irawadi*, Zug et al. 2006; *C. jerdoni*, Günther 1870; *C. emma*, Gray 1845; *C. mystaceus*, Duméril and Bibron 1837; and *C. versicolor*, Daudin 1802. The latter 3 species are well known to Indochina, including Thailand (Zug et al., 2006; Hartmann et al., 2013).

Male reproductive displays are directly correlated to testicular hypertrophy in the majority of seasonally breeding vertebrates, and are accompanied by an elevated level of circulating sex steroids. This is termed an associated reproductive pattern (Crews, 1984, 1999; Crews et al., 1984; Licht, 1984; Norris, 2013). In contrast, in a small number of vertebrate species, such as some turtles, snakes, and bats, males mate at a time when their gonads are quiescent (QU) and the levels of circulating sex steroids are low (Garstka et al., 1982). This pattern of reproduction is referred to as a dissociated reproductive pattern (Volsøe, 1944; Crews, 1976, 1984, 1999; Lofts, 1977; Licht, 1984; Norris, 2013). In snakes, especially in the family Colubridae, various terms have been used to describe the male reproductive patterns observed in different species. Volsøe (1944) introduced the term prenatal spermatogenesis to describe sperm production that occurs immediately prior to mating, and postnatal spermatogenesis to describe sperm production that occurs after mating. Aldridge et al. (2009) introduced the term preovulatory spermatogenesis to describe sperm production immediately prior to ovulation, and postovulatory spermatogenesis as sperm production that occurs after ovulation. They assumed that androgenesis was associated with the development of secondary sex characters and mating behavior.
Vitt and Caldwell (2009) added that the currently known diversity of seasonal patterns of tropical squamate reproduction suggests that no single explanation is sufficient. Snakes are included in the same order (Squamata), but demonstrate a more diverse reproductive pattern in contrast to lizards. Tumkiratiwong et al. (2012) studied the reproductive patterns of captive male and female *Naja kaouthia*, monocled cobra, Lesson 1831, suggesting that its reproductive pattern exhibited either postnuptial spermatogenesis or a dissociated reproductive pattern.

There is no detailed information available on testicular and ovarian cycles, plasma sex hormonal profiles during gonadal cycles, or male reproduction-associated sexual segment of the kidney (SSK), especially in *Calotes emma*. This paper, therefore, monitored annual alterations of reproductive organs of males and females of 2 *Calotes* species, i.e., *C. emma* and *C. versicolor*, and investigated male and female sex hormones, male testosterone, and female estradiol to define the reproductive patterns of 2 such *Calotes* species. We expected that annual male and female reproductive alterations of the testes, male SSK, and the ovaries would require underlying morphological and histological investigations, and also that annual sex hormonal levels would reveal the reproductive patterns of 2 such *Calotes* species.

2. Materials and methods

2.1. Animals

We caught the adult lizards monthly over 1 year by hand or with a noose in the 3 forest types: a dry evergreen forest, a deciduous dipterocarp forest, and an ecotone forest at Sakaerat Biosphere Reserves of the Sakaerat Environmental Research Station (14°26′33″–14°32′50″N, 101°50′43″–101°57′21″E; 720–770 m above sea level), located at Nakhon Ratchasima Province, northeastern Thailand. We classified adults of *C. emma* and *C. versicolor* based on external morphological differences (Figure 1). The adult stages of the 2 species were diagnosed based on the internal morphology of active male and female gonads (Figure 1) and expressed in terms of the snout–vent length (SVL) as: (1) male, *C. emma*, >5.78 cm; and *C. versicolor*, >5.40 cm; (2) female, *C. emma*, >6.57 cm; and *C. versicolor*, >5.99 cm. We attempted to collect 10 adult males and females, but in some months the samples were smaller than expected and sometimes we could not collect any samples.

Figure 1. External morphologies of the representatives of 2 *Calotes* species. Top, *C. versicolor*: A, no patch of granular scales in front of forelimb insertion; bottom left, *C. emma*: B, crescent-shaped patch of small granular scales in front of forelimb insertion, and C, large postorbital spine present. Bottom middle, dissections of urogenital morphology of male *Calotes*: T, testis; Vd, vas deferens; K, kidney; bottom right, female *Calotes*: OvaF, ovarian follicles; OviE, oviductal eggs. Lines were drawn from a total preparation (in ventral view).
The months that lacked samples in each species are shown in parentheses as follows: males of *C. emma* (May, July, and August) and *C. versicolor* (December); females of *C. emma* (February to March, May, and December) and *C. versicolor* (March to May, July, October, and December). We anesthetized the samples with diethyl ether and collected blood samples by puncturing the cardiac chamber; however, it was not possible to acquire samples from those lizards with very small cardiac chambers. Blood samples were kept at 4 °C in heparinized vials and centrifuged within 2–3 h following blood collection. The blood was centrifuged at 1600 × g for 10 min. Plasma was then aspirated off and frozen at −79 °C for later analysis of plasma levels in male testosterone and in female estradiol.

### 2.2. External and internal reproductive morphologies

We measured SVL, which ranges from the snout tip to the anterior margin of the vent. We sacrificed male and female lizards to investigate the general reproductive morphologies (Figure 1, bottom middle, male; bottom right, female) and measured follicular size and testicular mass. Each specimen was weighed to the nearest 0.01 g; its SVL was measured to the nearest 0.05 mm.

#### 2.3. Testicular, male SSK, and ovarian histologies

Testes, male SSK, and ovaries were excised and fixed in a 10% v/v buffered neutral formalin solution, processed by the paraffin technique (Avwioro, 2011). The tissue was cut in a cross-section to 6 µm in thickness using a LEICA RM2145 (Nussloch, Germany). Sections were stained with hematoxylin and eosin. The reproductive stage of adult females was determined on the basis of the presence or absence of types of pyriform cells in the granulosa layer. Follicles with the pyriform cells, which appeared in the granulosa layer, were considered to be previtellogenic follicles, and follicles not containing the pyriform cells were considered to be vitellogenic follicles (Tumkiriatiwong et al., 2012). Females having vitellogenic follicles and/or oviductal eggs were considered to be at the active reproductive stage, while females having only previtellogenic follicles were considered to be at the QU reproductive stage. Females with regressed follicles were considered to be at the postparturient stage. Males with the appearance of sperm bundles and/or free sperm in the seminiferous tubules (ST) were assessed as being at the active reproductive stage, while males without those attributes were assessed as being at the inactive reproductive stage (Tumkiriatiwong et al., 2012). Males with SSK that had strongly eosinophilic-stained granules were regarded as being at the active reproductive stage (Sever et al., 2002).

#### 2.4. Categorization of female individuals based on reproductive status

Similar-sized follicles were organized into distinct groups. The total number and diameter of follicles belonging to the group with the largest-sized follicles were recorded from both ovaries. The follicular size was measured with a Vernier caliper (0–150 × 0.02 mm). Follicles greater than or equal to 2.5 mm in diameter represented vitellogenic status (Shanbhag and Prasad, 1993).

Since ovarian follicular development, ovulation, and gestation occur asynchronously in a population, individuals with differing reproductive statuses are encountered. Therefore, the data from the reproductive phase were classified based on the reproductive status of individuals rather than on a monthly classification as follows: (1) the QU stage: previtellogenic follicles sized <2.5 mm in diameter; (2) the early vitellogenic (EV) stage: initiation of vitellogenesis with follicles sized 2.5–5.0 mm in diameter and without oviductal eggs; (3) the late vitellogenic (LV) stage: late period of vitellogenesis with follicles sized >5.0 mm in diameter; (4) the early gestation (EG) stage: oviductal eggs with previtellogenic follicles sized <2.5 mm in diameter; (5) the midgestation (MG) stage: oviductal eggs with vitellogenic follicles sized 2.5–5.0 mm in diameter; and (6) late gestation (LG) stage: oviductal eggs with vitellogenic follicles sized >5.0 mm in diameter (modified from Radder et al., 2001).

#### 2.5. Measurement of testosterone

The plasma level of testosterone was measured by a 125I radioimmunoassay (RIA). We added a 500-µL sample of plasma to 5.0 mL of dichloromethane in a screw-top glass extraction tube. We then capped the mixture and mixed it for 60 min by gentle inversion with an end-over-end rotator, and then centrifuged the sample for 5 min at 1600 × g to separate the layers. The upper phase was aspirated without disturbing the interface; 2.0 mL of the lower phase was then transferred to a clean 12 × 75 mm glass tube and evaporated to dryness under a gentle stream of nitrogen at 37 °C. Finally, we reconstituted the extract with 200 µL of testosterone buffer. The testosterone extraction procedure was performed using a Coat A Count Testosterone RIA Kit (Diagnostic Products, Los Angeles, CA, USA). The intra- and interassay variations expressed as coefficients of variation (CVs) were 8.4% and 7.9%, respectively. The approximate sensitivity of this assay was 40 pg/mL. The cross-reactivity with androstenedione was 0.5%. The spiking recovery values averaged 98.3 ± 0.6%. Dilutions of 50%, 25%, and 12.5% of the undiluted concentration of 7300 pg/mL were 3490 pg/mL, 1700 pg/mL, and 780 pg/mL, respectively.

#### 2.6. Measurement of estradiol

The plasma level of estradiol was measured by a 125I RIA. We added 250 µL of plasma to 2.0 mL of diethyl ether in a screw-top glass extraction tube, and then capped and mixed it by gentle inversion with an end-over-end rotator for 30 min. It was centrifuged for 5 min at 1600 × g to separate the layers. The lower (aqueous) phase was...
frozen using dry ice; the organic phase was then decanted into another vial and evaporated to dryness under a gentle stream of nitrogen at 37 °C. Finally, the extract was reconstituted with 250 µL of estradiol buffer. The estradiol extraction procedure was performed using a Coat A Count Estradiol (TKE2) RIA kit (Diagnostic Products). The intra- and interassay variations calculated as CVs were 5.3% and 6.4%, respectively. The approximate sensitivity of this assay was 10 pg/mL. The specificity of cross-reactivity with estradiol was 0.32%. The spiking recovery values averaged 96.8 ± 3.3%. Dilutions of 50%, 25%, 12.5%, and 6.25% of the undiluted concentration of 230.9 pg/mL were 114.4 pg/mL, 60.0 pg/mL, 27.9 pg/mL, and 14.8 pg/mL, respectively.

2.7. Statistical analysis

Testicular masses, testosterone levels, ovarian weights, estradiol levels, and diameters of the largest follicle were expressed as the mean ± standard error of the mean (SEM). The Kolmogorov–Smirnov test and Levene's test were used to determine if the data were normally distributed and the homogeneity of variance, respectively. Nonparametric tests were used, as all data mentioned above were nonnormal and heteroscedastic. Therefore, the Kruskal–Wallis H test was used to test for differences in the ovarian weights, the estradiol levels, and the diameters of the largest follicle among the reproductive stages of nongestation periods. The Mann–Whitney U test was then used to compare differences in the ovarian weights, the estradiol levels, and the diameters of the largest follicle between each reproductive stage of the various follicular growths of C. emma only because of the insufficient sample size. There was no statistical analysis of differences in male testosterone levels or testicular masses based on months due to the small amount of data available. Spearman's correlation coefficients were used to determine relationships between the testicular mass and testosterone, and the diameter of the largest follicles and estradiol levels. The level of significance was set to P < 0.05.

2.8. Ethical aspects

This study was approved by the Ethics Committee of the Department of National Parks, Wildlife, and Plant Conservation, Ministry of Natural Resources and Environment, Thailand (License No. 0909.302/18344).

3. Results

3.1. Annual alterations in male and female reproductive morphologies of Calotes

Annual changes in testicular morphological events between C. emma and C. versicolor are depicted in Figure 2. The representative C. emma testes were recrudesced in December, continued to hypertrophy from January to April and in June, and completely regressed from September to November. In the representative C. versicolor, the testes became hypertrophied from January to September and regressed from October to November.
Annual changes in ovarian morphological events (Figure 3) and annual changes in the number of follicular types and egg types (Table 1) of the representative 2 *Calotes* species are shown. In the representative *C. emma*, we found that in January, April, from June to July, and from August to November, follicles were in the EV, EG, LV, and QU stages, respectively (Figure 3, top; Table 1). In the representative *C. versicolor*, we found that, in January and February, the ovaries contained only QU follicles; in June and August, QU and EV follicles and oviductal eggs; in September, QU follicles and oviductal eggs; in November, only QU follicles (Figure 3, bottom; Table 1).

### 3.2. Annual histological alterations in ST, SSK, and ovaries of *Calotes*

In the male representative *C. emma*, the ST and SSK were hypertrophied from January to April and in June, then regressed from September to November, becoming active again in December (Figure 4, top left and top right, respectively). Spermatozoal masses were contained inside the hypertrophied ST. Spermatogonia initially appeared in November; additionally, a few types of germ cells occurred, but there were still no active spermatozoa in December (Figure 4, top left). In the representative *C. versicolor*, both ST and SSK were active from January to September but were inactive from November to December (Figure 5, top left and top right).

We found that in the female representative *C. emma*, active ovaries contained follicles of vitellogenic stages in January and June, but inactive ovaries contained previtellogenic and atretic follicles in August (Figure 4, bottom left and bottom right). In the representative *C. versicolor*, ovaries contained previtellogenic and atretic follicles in January and both previtellogenic and EV follicles in June (Figure 5, bottom left and bottom right). Additionally, we found corpus luteum in the ovaries of 1 representative in September (Figure 5, bottom left and bottom right). We also observed cellular alterations in the granulosa layer of both *C. emma* and *C. versicolor*. It was demonstrated that, inside the granulosa layer of the previtellogenic follicles, both types of small and pyriform cells appeared. However, both such cell types disappeared in the granulosa layer of the vitellogenic follicles (Figures 4 and 5, bottom right).

### 3.3. Annual plasma testosterone levels and testicular masses in *Calotes*

Annual variations in plasma testosterone levels and testicular masses between the 2 *Calotes* species are depicted (Figures 6a and 6b). In the representative *C. emma*, plasma testosterone levels and testicular masses initially increased in December, peaked in March, and thereafter gradually reduced from April to November (Figure 6a). In the representative *C. versicolor*, plasma testosterone
levels and testicular masses initially increased in January, peaked in April, and thereafter gradually reduced from May to November (Figure 6b). The changes in testicular masses tended to correspond well to the changes in plasma testosterone levels between the 2 species in the genus *Calotes* \( r = 0.789, P = 0.001; \) and \( r = 0.732, P = 0.001 \) for *C. emma* and *C. versicolor*, respectively).

3.4. Annual plasma estradiol and ovarian cycles in *Calotes*

Variations in ovarian masses and the diameter of the largest follicle were observed between the 2 *Calotes* species during their reproductive stages (Table 2). The ovarian weights and the diameters of the largest follicles differed significantly from the QU stage to the LV stage regarding the growth of ovarian follicles \( P = 0.001 \) in *C. emma*. It was also observed that the growth of ovarian follicles concomitantly occurred with the growth of oviductal eggs during the gestation period of the 2 species.

Variations in plasma E₂ levels in relation to the diameters of the largest follicles between the 2 *Calotes* species during reproductive stages are graphically depicted (Figures 7a and 7b). Regarding the groups of follicular sizes.

---

**Table 1.** Annual changes in numbers of follicular and egg types according to *Calotes* species.

<table>
<thead>
<tr>
<th>Follicular size and eggs</th>
<th>Jan</th>
<th>Feb</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. emma</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.5 mm</td>
<td>21</td>
<td>-</td>
<td>18</td>
<td>-</td>
<td>18</td>
<td>22</td>
<td>41</td>
<td>33</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>2.5–5.0 mm</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&gt;5.0 mm</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oviductal eggs</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. versicolor</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.5 mm</td>
<td>16</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>28</td>
<td>19</td>
<td>16</td>
<td>-</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2.5–5.0 mm</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&gt;5.0 mm</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oviductal eggs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>6</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: Jan–Nov denotes January to November, respectively.
of *C. emma*, plasma E2 levels were the difference between the QU and the EV stages (U = 9.00, P = 0.090), the QU and the LV stages (U = 2.00, P = 0.009), and the EV and the LV stages (U = 2.00, P = 0.083). Additionally, the diameters of the largest follicles were the difference between the QU and the EV stages (U = 0.00, P = 0.004), the QU and the LV stages (U = 0.00, P = 0.004), and the EV and the LV stages (U = 0.00, P = 0.021). The correlation coefficient between plasma E2 and the diameter of the largest follicle was 0.82 (P = 0.001) and 0.32 (P = 0.365) of *C. emma* and *C. versicolor*, respectively.

4. Discussion
Annual variations in the timing of male and female reproductive stages were encountered among populations of *C. emma* and *C. versicolor*, even in individuals of the same population. Annual changes in testicular sizes were categorized into 2 phases in both species: (1) an active hypertrophied testicular phase; and (2) an inactive regressed testicular phase. However, the timing of those 2 events appeared asynchronously in the 2 *Calotes* species, even in individuals of the same population (data not shown here). The testes were hypertrophied with active spermatozoal production from December to June and January to September in the representative *C. emma* and *C. versicolor*, respectively. A study by Gouder and Nadkarni (1979) showed that males of *C. versicolor*, widely distributed in India, were spermatogenetically active from April to September. Active vitellogenic follicles and oviductal eggs were concomitantly encountered in *C. emma* and *C. versicolor*. Therefore, these 2 *Calotes* species exhibited polyautochrony and multiclutches. Radder et al. (2001) reported that Indian garden lizards, *C. versicolor*, showed polyautochrony and multiclutches. The representative gravid lizards were found in April in *C. emma*, and in June and from August to September in *C. versicolor*. Gravid *C. versicolor*, whose habitat is in India, was encountered from May to October (Shanbhag and Prasad, 1993). We
MEESOOK et al. / Turk J Zool

Table 2. Changes in ovarian weight and diameter of the largest follicle in *Calotes* during the reproduction cycle.

<table>
<thead>
<tr>
<th>Reproductive stages</th>
<th>N</th>
<th>Ovarian weights</th>
<th>Diameter of the largest follicle</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. emma</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QU</td>
<td>11</td>
<td>0.02 ± 0.00</td>
<td>1.78 ± 0.06</td>
</tr>
<tr>
<td>EV</td>
<td>4</td>
<td>0.11 ± 0.09</td>
<td>3.50 ± 0.33</td>
</tr>
<tr>
<td>LV</td>
<td>4</td>
<td>1.71 ± 0.49</td>
<td>8.02 ± 0.85</td>
</tr>
<tr>
<td>EG</td>
<td>1</td>
<td>0.02</td>
<td>2.40</td>
</tr>
<tr>
<td>MG</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LG</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>C. versicolor</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QU</td>
<td>4</td>
<td>0.02 ± 0.00</td>
<td>1.52 ± 1.99</td>
</tr>
<tr>
<td>EV</td>
<td>1</td>
<td>0.2</td>
<td>4.28</td>
</tr>
<tr>
<td>LV</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EG</td>
<td>3</td>
<td>0.02 ± 0.00</td>
<td>2.22 ± 0.69</td>
</tr>
<tr>
<td>MG</td>
<td>2</td>
<td>0.11 ± 0.09</td>
<td>4.36 ± 0.36</td>
</tr>
<tr>
<td>LG</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. The differences in superscript alphabets indicate the significant differences among the reproductive stages of nongestation at P < 0.01 (N, sample sizes).
Notes: QU, quiescent; EV, early vitellogenic; LV, late vitellogenic; EG, early gestation; MG, mid-gestation; and LG, late gestation.

Figure 7. Changes in the plasma levels of estradiol and the diameter of the largest follicle: (a) *C. emma*; (b) *C. versicolor*.
Notes: QU, quiescent; EV, early vitellogenic; LV, late vitellogenic; EG, early gestation; MG, mid-gestation; LG, late gestation.
Data are presented as mean ± SEM. The differences in superscript alphabets (estradiol levels) and in the numbers of asterisks (diameters of the largest follicles) indicate the significant differences between the various follicular sizes at P < 0.01. The number (in parentheses) represents the analyzed samples in each month.

are likely to suggest after studying the annual alterations in the male and female reproductive morphologies of the 2 *Calotes* species that the active reproductive events of both males and females of the *Calotes* species lasted nearly 1 year with only a few months of reproductive arrest, which is especially seen in *C. versicolor*.

According to our investigations on annual histological alterations in ST and male SSK timing of the 2 *Calotes* species, both ST and SSK were in active spermatogenic and hypertrophied stages, respectively, which corresponded well with the timing of the testicular hypertrophied stage mentioned above. In other words, the timing of arrested spermatogenesis and regressed SSK was in accordance with that of the regressed testes. Likewise, we confirmed that the 2 *Calotes* species have an active reproductive stage that is much longer than the inactive reproductive stage.

SSK is present in a variety of male snakes and lizards, but is absent in both turtles (Regaud and Policard, 1903).
and crocodilians (Fox, 1952). Bishop (1959) found that the testes of the male garter snake, *Thamnophis sirtalis*, were spermatogenically active during the same time as the hypertrophied SSK; during the active reproductive period, the diameter of the SSK tubule was 5 times greater than that of the SSK tubule during the inactive reproductive period. However, SSK development in female lizards has been reported in the genus *Cnemidophorus* (Del Conte, 1972; Del Conte and Tamayo, 1973) and *Scincella laterale* (Sever and Hopkins, 2005). They suggested that the females had a low level of natural androgens, which caused the SSK development (Del Conte and Tamayo, 1973; Sever and Hopkins, 2005). In the present study, we did not monitor the annual seasonal alterations in female SSK. The hypertrophy of the SSK is synchronous with androgen secretion and spermatogenic activity (Sever and Hopkins, 2005). Norris (2013) also stated that the SSK of sexually active squamates undergoes hypertrophy and is under the influence of androgens. In this study, we did not investigate any alterations in annual SSK with annual androgen secretion, but we did demonstrate that the hypertrophy and the regression of SSK changed seasonally and synchronously with the active spermatogenic event and the spermatogenic arrest, respectively. In the Iberian rock lizard, *Lacerta monticola*, SSK secretions form a copulatory plug that adheres to the female's cloaca following copulation to occlude oviductal openings; however, such a plug does not prevent subsequent mating nor does it reduce the female's attractiveness (Moreira and Birkhead, 2003).

With our investigations on annual alterations in female ovarian morphologies between 2 *Calotes* species, we found that individuals in the same *Calotes* species showed different timing of reproductive events throughout a 1-year period (the data are not shown here). Additionally, there was quite clear evidence that QU, EV, and LV follicles and oviductal eggs overlapped among individuals within the same populations of both *Calotes* species. Female reproductive status is definitely distinguishable between the 2 *Calotes* species. Gravid lizards were encountered in 1 individual of *C. emma* in April, and in 3 individuals of *C. versicolor* in June, August, and September. Shanbhag et al. (2000) reported that female *C. versicolor* showed inactive reproduction from December to April, and gravidity was encountered from May to October.

We found that in the previtellogenic follicles (QU and EV) of females of both *Calotes* species the granulosa layer contained 2 types of cells: pyriform and small cells. Uribe et al. (1996) stated that in squamates the follicular epithelium or granulosa initially consists of small cuboidal cells, but differentiates during the previtellogenic phase and becomes multilayered and polymorphic by the presence of unique flask-shaped pyriform cells, intermediate cells, and small cells. These pyriform cells differentiate from small somatic follicular cells early in follicular development via the intermediate-cell stage to become nurse cells, in direct contact with the developing oocytes (Maurizii et al., 2004). Differentiation of the small cells into pyriform cells appears to be linked to the progressive appearance of glycoproteins with terminal α-N-acetylgalactosamine residues on the cell surface, which may be involved in fusion between the oocyte and the follicle cell membranes as well as maintenance of the differentiated pyriform cells. The pyriform cells are connected to the oocyte via intercellular bridges containing a cytoskeleton of α-tubulin and cytokeratin microtubules (Maurizii et al., 2004). Tumkiratiwong et al. (2012) also demonstrated that the previtellogenic follicles of the captive monocled cobra, *Naja kaouthia*, had many pyriform cells in the granulosa layer, but fewer in the vitellogenic follicles. In this study, the pyriform cells disappeared when the follicles entered the vitellogenic stage. Andreuccetti (1992) studied the differentiation of pyriform cells and their contribution to oocyte growth in 3 lizards, namely *Tarentola mauritanica*, *Cordylus wittifer*, and *Platysaurus intermedius*, and a colubrid snake, *Coluber viridiflavus*, and revealed that pyriform cells differentiate from small follicle cells via intermediate cells after establishing an intercellular bridge with the oocyte. Once pyriform cells are differentiated, they display ultrastructural features indicative of synthetic activity, including abundant ribosomes, Golgi membranes, vacuoles, mitochondria, and lipid droplets. These cellular components extend to the apex of the cell at the level of the intercellular bridge, suggesting that constituents of pyriform cells may be transferred to the oocyte. Pyriform cells and the oocytes may fulfill similar vitellogenic functions. The establishment of an intercellular bridge may represent a crucial event in the development of an integrated system in which pyriform cells and oocytes cooperate. Norris (2013) reported that the squamate granulosa contains the pyriform cells, which are in direct contact with the developing oocyte and are apparently involved with early steps in oocyte development soon after the onset of vitellogenesis. As ovulation approaches, the granulosa cells, as well as some thecal cells, accumulate cholesterol-positive lipids, and proliferate and luteinize to form corpora lutea following ovulation. Follicular atresia is a common occurrence in reptilian ovaries as in other vertebrates (Norris, 2013), as we found the corpora lutea in the follicle in accordance to oviductal egg appearances of a representative *C. versicolor* collected in September. Additionally, as shown in Table 1, several atretic follicles <2.5 mm in diameter were commonly encountered in *C. emma* and *C. versicolor*.

Based on both morphological and histological investigations, we found that testes, ST, and SSK were
concomitantly active and were associated with high levels of plasma testosterone. We also demonstrated that there were high correlations between levels of plasma testosterone (T) and testicular mass where annual changes occurred in the same direction as the testicular size and time of spermatogenetic events among the males of the Calotes species. Radder et al. (2001) reported that in the male tropical or oriental garden lizard, Calotes versicolor, plasma T is highest during the breeding season, which correlated with testis mass and reproductive behavior. Changes in T levels are associated with high spermatogenetic activity. Radder et al. (2001) also stated that the changes in plasma T levels during different phases of the male reproductive cycle in C. versicolor follow a reproductive pattern of a prepubertal type of spermatogenesis that is similar to that of some other species of lizards: the spiny-tailed lizard, Uromastix hardwickii (Arslan et al., 1978a); the viviparous lizard, Lacerta vivipara (Courty and Dufaure, 1982); the western shingleback lizard, Tiliqua rugosa (Bourne et al., 1986); the male lizard Podarcs s. sicula (Ando et al., 1990); Podarcs s. sicula Raf. (Ando et al., 1992); the white-throated savanna monitor, Varanus albigularis (Phillips and Millar, 1998); and the male brown anoles, Anolis sagrei (Tokarz et al., 1998).

We found that estradiol (E2) levels increased in vitellogenic females; its high levels were associated with the presence of the largest vitellogenic follicles in the 2 Calotes species. Radder et al. (2001) reported that in female C. versicolor with overlapping reproductive events, such as vitellogenesis and gestation, E2 was at low levels when the ovaries were regressed and at high levels at vitellogenic follicular recruitment, reaching peak level at the time of preovulatory follicles. The same patterns of E2 secretion were found again when the second set of follicles underwent vitellogenesis (Radder et al., 2001). Surprisingly, Amey and Whittier (2000) reported that in female bearded dragons, Pagona barbata, plasma E2 was low or nondetectable across all reproductive states. In C. versicolor, E2 levels were low in nonreproductive females with small previtellogenic follicles and those in the EG phase (Radder et al., 2001). We do not discuss the level of the plasma progesterone (P) during the gestation period, as its level was not detectable in this study. The gravid lizards in EG exhibited low plasma E2, but high P levels, and the highest P levels coincided with eggshell production. P levels declined after eggshell formation as reported in other gravid individuals in several species of lizards that do not possess vitellogenic follicles of the subsequent clutch, including C. versicolor (Radder et al., 2001); Uromastix hardwicki (Arslan et al., 1978b); Agama atra (Van Wyk, 1984); Euemeces obsoletus, Sceloporus undulatus, and Crotaphytus collaris (Masson and Guillette, 1987); and Psammodromus algirus (Diaz et al., 1994). However, a decline in P levels in MG with vitellogenic follicles did not seem to facilitate recruitment or growth of the subsequent set of vitellogenic follicles in gravid Sceloporus jarrovi (Guillette et al., 1981).

In the present study, there were variations in the timing of breeding between the 2 Calotes species and even within populations of the same species. We could not relate the copulation timing of 2 such Calotes species to gonadal activity or sex hormonal surges, as the timing of natural mating could not be observed during the times we collected data. Lizard species that inhabit temperate zones have mostly exhibited seasonal reproduction (Fitch, 1970; Licht, 1984; Pianka and Vitt, 2003). The 10 lizard species that have been studied widely to date exhibit an associated reproductive pattern (Lovern, 2011); that is, green anoles, Anolis carolinensis (Crews, 1980; Lovern et al., 2004); brown anoles, Anolis sagrei (Lee et al., 1989; Tokarz, 1998); eastern fence lizards, Sceloporus undulatus (Cox et al., 2005); mountain spiny lizards, Sceloporus jarrovi (Woodley and Moore, 1999); tree lizards, Urosaurus ornatus (French and Moore, 2008); wall lizards, Podarcs sicula (Putti et al., 2009); common lizards, Lacerta vivipara (Vercken and Clobert, 2008); little striped whiptail lizards, Cnemidophorus inornatus (Crews, 2005); garden lizards, Calotes versicolor (Shanbhag, 2003; Lovern, 2011); and leopard geckos, Eublepharis macularius (Rhen et al., 2005). The temperate Florida populations of the brown anole, Anolis sagrei, show a strong seasonality in reproduction (Lee et al., 1989), while the tropical Caribbean (Licht and Gorman, 1970; Sexton and Brown, 1977) and Hawaiian populations of this species (Goldberg et al., 2002) show a less-pronounced seasonality, in which reproductively active individuals can be found throughout the year. Although individuals within a population of many tropical lizard species can breed at any time, no individuals within the population breed year-round (Lovern, 2011). Additionally, Vitt and Caldwell (2009) stated that the belief used to be that tropical squamates had continuous reproduction in aseasonal tropical environments, or reproduced during the wet season in a wet–dry seasonal tropical environment. Many tropical lizard species, namely the anoles Anolis acutus (Ruibal et al., 1972), Anolis limifrons (Sexton et al., 1971), and Anolis opalinus (Jenssen and Nunez, 1994), as well as the gecko Cyrtodactylus malyanus, the flying lizard Draco melanopogon (Inger and Greenberg, 1966), and the parthenogenetic, oviparous whiptail lizard Cnemidophorus nativo (Menezes et al., 2004), showed slightly more frequent breeding during the wet season than during the dry season (Jenssen and Nunez, 1994).

Reproductive patterns can be described in a variety of ways, but not all species fit neatly into such categorizations. However, 2 general types of reproductive patterns are
recognized in terms of prenuptial and postnuptial reproductive patterns (Lance, 1998). Prenuptial reproductive pattern terms, such as gonadal recrudescence, sex steroid production, and gametogenesis occur in advance of mating, whereas postnuptial reproductive patterns occur following mating. In other words, in a high-elevation population of Sceloporus grammicus in Parque Nacional de Zoquíapan in central Mexico, an active reproductive event occurring in the early fall is described as dissociated from testicular recrudescence in males but is associated with the initiation of ovarian recrudescence in females (Guillette and Casas-Andreu, 1980, 1981; Zuniga-Vega et al., 2008). This is in contrast to S. grammicus from Teotihuacan, Mexico, in which testicular recrudescence and breeding occur in the summer and fall, at the onset of female ovarian recrudescence (Jimenez-Cruz et al., 2005). In S. mucronatus from Valle de la Cantimplora, Mexico, peak testicular recrudescence and mating occur during the summer, prior to ovarian recrudescence, which does not occur until several months later (Ortega-Leon et al., 2009). This is distinct from many fall-breeding populations elsewhere (Mendez-De La Cruz et al., 1994; Villagran-Santa Cruz et al., 1994). The examples above demonstrate that gonadal activity and mating behavior are clearly variable, but hormone analyses have not been performed in these species and so endocrine relationships cannot be assessed at this point.

In conclusion, we suggest that the males and females of the 2 Calotes species have much more prolonged, active reproductive phases than inactive reproductive phases. The reproductive patterns of C. emma and C. versicolor were classified into the same reproductive pattern of continual reproduction.

Acknowledgments
We thank the Department of Zoology of Kasetsart University for financial support. We also thank the staff of Sakaerat Environmental Research Station, Nakhon Ratchasima Province, for devoting time for research collaboration. We also thank Mrs Sureerat Sangkrut for drawing all illustrations. In addition, we wish to thank the anonymous referees for many helpful suggestions.

References


