Temporal Difference Between Testis and Ovary Determinations With Possible Involvement of Testosterone and Aromatase in Gonadal Differentiation in TSD Lacking Lizard, Calotes versicolor

SUBRAMANIAM GANESH, BIBHA CHOUDHARY, AND RAJIVA RAMAN*
Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

ABSTRACT

In the garden lizard, Calotes versicolor, which lacks identifiable sex chromosomes, incubation temperature also does not have a deterministic effect on the gender. However, the embryos reared at high temperature (33–35°C) have a shorter duration of incubation as well as gonadal differentiation. In contrast, exogenous application of the male hormone testosterone to embryos at ambient temperature (28°C) results in almost all individuals with only testis. Thus the testosterone treatment reverts genic females to males and accelerates the differentiation of testis, a feature similar to the high-temperature treatment. Treatment of eggs with estradiol shows no difference from that seen in the untreated eggs. The present series of experiments was done to establish the “window” of testosterone sensitivity and to understand the interaction between sex hormones and high temperature on gonadal differentiation. The period between day 5 and 15 of embryonic development was the window period of testosterone sensitivity for sex reversal. This period coincided with the formation of the genital ridge and its differentiation into cortex and medulla. Treatment of the 33°C-reared embryos with testosterone resulted in hatchlings of both the sexes, in contrast to only males at the ambient temperature. In contrast, at the same temperature (33°C), all the dihydrotestosterone (nonaromatisable testosterone)–treated embryos hatched into males. However, those given estradiol showed no sex bias regardless of the day of application and the concentration of drug. Eggs were also treated with aromatase inhibitor, CGS 16949 A, at ambient temperature and at 33°C. All the 33°C eggs to which the drug was given on day 25 hatched into males. These results suggest that though high temperature has no direct effect on sex determination in this species, it may have a stimulatory effect on aromatase activity, leading to the conversion of the exogenously applied testosterone into estradiol and permitting ovarian differentiation in the genic females. It also follows from the present report that the pathway of testis formation in Calotes versicolor is triggered much earlier, and irreversibly, than that for the ovary. J. Exp. Zool. 283:600–607, 1999. © 1999 Wiley-Liss, Inc.

Recent studies on the effects of exogenously applied sex hormones, their agonists, and their antagonists on reptilian embryos have demonstrated that in a number of species hormones influence the differentiation of developing gonads (reviewed by Crews et al., ’94; Pieau, ’96). As a general rule, estrogen induces ovary in all the embryos, turning them into female. Though seen predominantly in the TSD (temperature-dependent sex determination) species, this feature has also been observed in a few of those endowed with the sex chromosomes (CSD) (Bull, ’83). In the TSD species, even testosterone treatment at the male-producing temperature results in the female (Crews, ’94; Pieau, ’96). However, administration of the nonaromatisable androgen dihydrotestosterone (DHT) to Chelydra serpentina at an intermediate temperature induces development of testis in all the individuals, resulting in all male hatchlings (Wibbels et al., ’92). It has therefore been inferred that the feminizing effect of testosterone in this species is due to its aromatisation into estrogen by the enzyme aromatase. This suggestion is fortified by the observation that coadministration of

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S. Ganesh’s present address: Lab for Neurogenetics, Brain Science Institute (RIKEN), 2-1 Hirosawa, Wako-Shi, Saitama, 351-0198, Japan.

*Correspondence to: Rajiva Raman, Lab for Neurogenetics, Brain Science Institute (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan. E-mail: raman@banaras.ernet.in

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testosterone and an aromatase inhibitor results
in the development of a male even at the female-
producing temperature (Crews, '94; Jeyasuria et al., '94). These observations lead to the sugges-
tion that in TSD reptiles (1) estradiol is essential for
the determination of female sex, (2) aromatase
is the key enzyme in gonadal differentiation, and
(3) its activity is regulated by the incubation tem-
perature of eggs (Crews et al., '94; Pieau, '96).

In the Indian garden lizard, Calotes versicolor,
which lacks the identifiable sex chromosomes, the
incubation temperature also does not have a de-
terministic effect on the gonadal phenotype (Ga-
ness and Raman, '95). Most of the naturally born
hatchlings have an indifferent gonad until about
7 to 10 days after birth. Nevertheless, when reared
at a higher temperature, the incubation period is
markedly shortened and the gonads in the major-
ity of hatchlings are fully differentiated in both
the sexes within a day or two of birth. In con-
trast, the application of testosterone to embryos
in the first half of development at ambient tem-
perature induces testis development in almost all
the embryos, though the duration of incubation
is not shortened. Estrogen treatment to the em-
bryos, however, has no effect either on the gonadal
differentiation or on the duration of incubation
(Ganesh and Raman, '95). The present series of
experiments has been done to define the window
of testosterone sensitivity and to under-
stand the interactive effects of temperature and
sex hormones on gonadal differentiation by ap-
plying hormones to the eggs reared at high tem-
perature.

MATERIALS AND METHODS

The procedure of collection and maintenance of
eggs was essentially the same as reported previ-
ously for this species (Ganesh and Raman, '95).
The eggs were recovered from gravid females
cought during the breeding season (July–August).
For all the experiments, eggs from different fe-
males were mixed together and reallocated into dif-
f erent groups for various experiments. The eggs
were maintained in moist sand at a constant in-
cubation temperature (28°C—ambient; 33°C,
35°C—high) in BOD incubators. All the hormones,
testosterone (17β-hydroxy 3-oxo-4-androstene;
Sigma, St. Louis, MO), dihydrotestosterone (DHT:
5α-androstan-17β-ol-3-one; Sigma), β-estradiol
(Sigma), and the aromatase inhibitor CGS 16949
A (Ciba-Geigy, Summit, NS), were dissolved in
90% alcohol at the required concentration, and 5
µl of the solutions was applied topically to the eggs
at different stages of embryonic development. Un-
treated eggs at 33°C and those treated with test-
ostosterone at the ambient temperature (28°C)
served as two different controls.

RESULTS

Window of testosterone sensitivity in embryo

In the previously reported experiments on the
effect of testosterone on gonadal differentiation in
C. versicolor, the hormone was applied on days 5,
10, and 15 of embryonic development, and the
majority of hatchlings were born as male (Ganesh
and Raman, '95). To check up to which stage of
development was testosterone effective in sex re-
versal, the treatment of the hormone was ex-
tended to the 20-, 25-, and 30-day embryos (at
ambient temperature; 10 embryos each). The sex
of the individual was established by examining
histological sections of the gonads of 8- to 10-day-
old hatchlings; only about 50% of the hatchlings
were male on day 20 and 25, and a few of them
had clear ovary. Most of the eggs treated on day
30 did not survive till hatching. This was in con-
trast to the 5- to 15-day embryos, in which more
than 80% hatchlings were males and there were
no females. Thus the testosterone-sensitive period
of C. versicolor, the “window,” appears to be be-
tween day 5 and 15 of embryonic development (Fig. 1). Histological sections of the mesonephro-
gonal complex (MGC) of the “window” were
studied in 5-, 10-, 15-, and 20-day embryos. Al-
though MGC was seen from day 5 itself, the geni-
tal ridge was first seen in the day 10 embryo as a
thickening on the ventromedian surface of the
mesonephros, which further enlarged and differ-
entiated into cortex and medulla in the day 15
samples. In the day 20 MGC, sex cords (primor-
dial seminiferous tubule of testis) were formed in
the medulla, but the cortical region did not dif-
f erentiate any further at this stage (Fig. 2). Thus
the testosterone-sensitive period of the embryo co-
incided with the formation and further differen-
tiation of the genital ridge. Also, the formation of
sex cords in the medulla indicated a step toward
testis formation much in advance of cortical de-
velopment.

The effect of testosterone, dihydrotestosterone, and aromatase inhibitor on gonadal differentiation
at high temperature

The untreated embryos at ambient temperature
as well as at 33°C showed different degrees of go-
nal differentiation; many had either testis or ovary, but a few had an indifferent gonad, retaining both testicular as well as ovarian anlage (Ganesh and Raman, '95). However, in the testosterone-treated (100 µg/egg; applied on day 10 of incubation) ambient temperature group of eggs, the majority of hatchlings had only testis (11 out of 14; Table 1). Both these results were in agreement with those previously reported for this species (Ganesh and Raman, '95). But unlike the previous group of experiments (Ganesh and Raman, '95), in which more than 80% of the 35°C eggs had survived, administration of testosterone (100 µg/egg) to the 35°C eggs led to very high mortality (>90%; data not given). Hence the present set of experiments was done on the eggs reared at 33°C, and the one-time application of testosterone was given in two concentrations (50 µg/egg and 100 µg/egg) at three different stages of embryonic development (on day 10, 15, and 20 of embryonic development; see Table 1). Although even in these groups the survival rate was only about 50%, it was better than that with the 35°C group. The surviving eggs hatched on day 41 ± 1 irrespective of the concentration of the hormone and the stage of treatment. The results summarized in Table 1 show two significant departures from the controls (testosterone at ambient temperature; 33°C untreated); individuals of both the sexes were produced (sex ratio slightly skewed toward female) and the ovary in certain females had large follicles surrounded by numerous supporting cells (Fig. 3b). Such advanced ovarian differentiation was never seen in the 7- to 10-day-old hatchlings. Instead, they were comparable to the ovary of about 2-month-old hatchlings (see Fig. 3c). When the eggs were treated with the nonaromatisable DHT (20 µg/egg and 30 µg/egg; on day 15), all the surviving hatchlings born to the 20 µg DHT /33°C) were male. All the 30 µg eggs died at different stages of development (see Table 1).

The difference between the results of testosterone and DHT hinted that the aromatisation of testosterone to estrogen could have a role in the testosterone-induced feminization. Therefore, another set of experiments was done with the Cytochrome P450 aromatase inhibitor, CGS 16949 A, which was applied (5 µg/egg) to two groups of eggs incubated at 28°C and 33°C, either on day 15 or day 25 of incubation. All the hatchlings of the 28°C
Fig. 2. Transverse sections of 10-, 15-, and 20-day embryos through their MGC. Note the extension of the genital ridge in the day 10 embryo (a), the differentiation of cortex and medulla in the genital ridge of the day 15 embryo (b), and the formation of sex cords in 20-day-old embryos. gc, germ cells; c, cortex; m, medulla; sc, sex cords. Magnification ×900.
eggs, regardless of the day of application, were unaffected by the drug. The same was true of the day 15, 33°C samples. In contrast, all the hatchlings from the day 25, 33°C eggs turned out to be males (Table 2).

**Effect of estradiol at ambient and high temperature in gonadal differentiation**

The above results indicated that the testosterone-induced feminization at high temperature could be due to precocious availability of estradiol. Therefore, fresh experiments were done with estradiol at ambient and high temperature. Since in the previously reported series of experiments the 5-, 10-, and 15-µg estradiol, administered to 5, 10, and 15 days ambient temperature-reared embryos, did not show any effect on sex determination (Ganesh and Raman, '95), we applied the hormone (16 µg/egg) on day 30 at ambient temperature. The results were identical to those pre-

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**TABLE 1. Gonads in the hatchlings of the eggs treated with androgens reared at ambient and 33°C temperature**

<table>
<thead>
<tr>
<th>Group</th>
<th>Incubation temperature</th>
<th>No. of hatchlings</th>
<th>Gonadal differentiation</th>
<th>Mean incubation period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Survived</td>
<td>Testis</td>
</tr>
<tr>
<td>No treatment</td>
<td>33°C</td>
<td>20</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Testosterone treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On day 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg/egg</td>
<td>28 ± 2°C</td>
<td>15</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>50 µg/egg</td>
<td>33°C</td>
<td>15</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>100 µg/egg</td>
<td>33°C</td>
<td>15</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>On day 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µg/egg</td>
<td>33°C</td>
<td>15</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>100 µg/egg</td>
<td>33°C</td>
<td>15</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>On day 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µg/egg</td>
<td>33°C</td>
<td>15</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>100 µg/egg</td>
<td>33°C</td>
<td>15</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Dihydrotestosterone treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On day 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µg/egg</td>
<td>33°C</td>
<td>50</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>30 µg/egg</td>
<td>33°C</td>
<td>30</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

128°C = ambient temperature.

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**Fig. 3.** Histological sections of ovary from 10-day-old hatchlings after (a) high temperature (33°C; control) and (b) high temperature (33°C) + testosterone (100 µg/egg) treatment, and (c) of around a 2-month-old normally grown hatchling. Note the large-sized ova in (b), which are comparable to those in (c). of, ovarian follicle. Bar = 50 µm.
TABLE 2. Gonads in the hatchlings of the eggs treated with CGS 16949 A (aromatase inhibitor) reared at ambient and 33°C temperature

<table>
<thead>
<tr>
<th>Group</th>
<th>Incubation temperature</th>
<th>No. of hatchlings</th>
<th>Gonadal differentiation</th>
<th>Mean incubation period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Survived</td>
<td>Testis</td>
</tr>
<tr>
<td>On day 15</td>
<td>28°C</td>
<td>5 µg/egg</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>On day 25</td>
<td>33°C</td>
<td>5 µg/egg</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

Previously obtained except that in the ovaries the thickness of the germ cell layers of the cortex was much greater than in the controls. However, when 25 µg estradiol was given to the 20-day embryo at 33°C, only 2 out of the 30 embryos survived. Nearly the same was true with the embryos given 16 µg of the drug on day 20 at 33°C (7 survived out of 25). Gonadal sections from these 7 revealed the presence of both male and female individuals. Whereas in the females the ovary was hyper- 

trophied, in the 7-day-old males the testis still retained a rudimentary layer of cortical epithelium. In another series of experiments, 10 µg and 5 µg/egg estradiol was given on day 25 to the 33°C-reared eggs. As seen in Table 3, there was no striking difference between the control (ambient) and the experimentals. Thus estradiol in any combination did not have any effect on the differentiation of testis, but it could have an effect on the maturation of ovary.

**DISCUSSION**

The present study on the effect of testosterone on the day 20, 25, and 30 eggs at ambient temperature reveals that, unlike the 5-, 10-, and 15-day embryos, the sex-reverting effect of testosterone is reduced or absent. Thus the period between days 5 to 15 appears to be the “window” for androgen sensitivity and for determination of testis in *Calotes*. The histological examination of the MGC shows that the window coincides with the evagination of the genital ridge followed by its regionalization into the medulla (primordium of testis) and cortex (primordial ovary) and finally the formation of sex cords in the medulla with not much differentiative activity in the cortex. It appears that the pathway of testis differentiation is initiated in the indifferent gonad during the testosterone-sensitive window.

The most intriguing result in the present set of experiments is the differentiation of ovary in the window period–testosterone–treated eggs when they were incubated at high temperature. Even more, in a number of females, the ovary was endowed with enlarged follicles, as large as those of approximately 2-month-old naturally growing females. Though in reptilian embryos the feminizing effect of testosterone is not unusual (Pieau, ’96), in *C. versicolor* it is, because in this species the same hormone induces only testis at ambient temperature, and the untreated eggs at high temperature show no preference for ovarian differentiation (Ganesh and Raman, ’95; see control groups in Table 1 of this study). In the present set of experiments, where a substantial amount of mortality was encountered, on the whole more females were obtained. However, with the presence of a fair proportion of males it is unlikely that the relative abundance of females is because of sex reversal of genic males to females. At the same time, the apparent decline in the frequency

**TABLE 3. Gonads in the hatchlings of the eggs treated with estradiol reared at ambient and 33°C temperature**

<table>
<thead>
<tr>
<th>Group</th>
<th>Incubation temperature</th>
<th>No. of hatchlings</th>
<th>Gonadal differentiation</th>
<th>Mean incubation period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Survived</td>
<td>Testis</td>
</tr>
<tr>
<td>On day 15</td>
<td>28°C</td>
<td>10 µg/egg</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>On day 20</td>
<td>28°C</td>
<td>10 µg/egg</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>On day 25</td>
<td>33°C</td>
<td>5 µg/egg</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>33°C</td>
<td>10 µg/egg</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
of males, when viewed along with the high rate of mortality, could be due to preferential loss of the male embryo. Contrary to the testosterone experiments, treatments with its nonaromatisable derivative, DHT, and the aromatase inhibitor, CGS 16949 A (applied on day 25 at 33°C), produced only males despite high temperature. Both these experiments provide evidence that differentiation of ovary into the treated eggs was due to aromatisation of the exogenously given testosterone, resulting into estradiol. It is also conceivable that on day 25 both testosterone and the enzyme aromatase were available only in the high-temperature eggs and not in those reared at ambient temperature. It is to be noted that in *C. versicolor* histochemical analysis failed to detect sex steroid synthesis till hatching (Gaintonde and Gouder, '84). That is, in *C. versicolor*, at high temperature the synthesis or activity of the enzyme cytochrome P-450 aromatase is induced earlier than at the lower, ambient temperature. The same is true for a number of other reptiles. This would not only lead to ovary formation but also advance its differentiation and accelerate the subsequent steps in ovarian maturation, leading to more mature eggs in these females than in those naturally developed. However, since a fair proportion of hatchlings were males, either the precocious synthesis or activity of aromatase occurred only in the “genic” female or else the male embryos were refractory to the enzyme at this stage of development.

Temperature-induced aromatase activity has been shown to mediate gonadal differentiation in a number of TSD reptiles (Desvages and Pieau, '92; Desvages et al., '93). Even in the chromosomally sex determined (CSD) amphibian, *Pleurodeles wallii* (ZZ/ZW sex chromosomes), the aromatase level showed a temperature-specific change (Chardard et al., '95). In these, the incubation temperature does not increase the rate of aromatase activity per se; instead it may induce fresh synthesis of the enzyme. *C. versicolor* appears to be among the first non-TSD/CSD reptiles to show aromatase-mediated testosterone feminization under the influence of temperature. Even though under indirect and artificial (temperature) conditions, the evidence is good enough to conclude that aromatase has a crucial role in the determination of ovary in *C. versicolor*.

The existence of the “window” period of testosterone receptivity and the results with DHT, aromatase inhibitor, and estradiol collectively provide some insight into the time and stage of development when the pathways toward testis and ovary are fixed in *C. versicolor*. It is important to note that, for both the male hormones, testosterone (at ambient temperature) and DHT (even at high temperature), the window period of their effectiveness in inducing testis was between day 10 to 15, the first quarter of embryonic development. On the other hand, since the aromatase inhibitor became effective much later (day 25) and only at elevated temperature (33°C), it can be safely assumed that the effective aromatase activity at the ambient temperature would occur even later than day 25. The same conclusion may be derived from all the estradiol experiments; estradiol, unlike testosterone, does not revert the indifferent gonad toward ovary regardless of the stage (window or afterwards) or temperature at which it is applied. That is, in *C. versicolor*, estradiol does not seem to modulate gonadal differentiation; instead aromatase may be the more critical molecule in ovary differentiation. These results imply that in this species the initial cue for sex determination is genic rather than hormonal, and that male is the heterogametic sex. These results also show that during development the pathway toward testis differentiation is fixed much earlier than that of ovary; and the default female embryos remain in a flexible state by the time the pathway to testis differentiation is irreversibly fixed in genic males. Therefore, whereas the “female” embryos can be reverted, the male embryos cannot.

These experiments add to our knowledge of sex determination and differentiation in the lizard, *C. versicolor*, which lacks both CSD and TSD, in the following two ways: (1) the deterministic cues for testis and ovary are temporally distinct, testis preceding ovary by about 10 days, and (2) the enzyme aromatase plays an important role in the differentiation of ovary. Whereas the first feature looks very similar to that seen in mammals, the other is common to the TSD reptiles. Since there are indications of the male-specific distribution of the SRY/ZFY family of genes in *C. versicolor* (Ganesh et al., '97), could it be that this species is one among those on the crossroads of the CSD/GSD and TSD? Our present efforts are directed toward cloning and characterizing the sex-related genes of *Calotes versicolor* and deriving their sequence of expression in gonadal differentiation.

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LITERATURE CITED


