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Gonadal sex differentiation, development up to sexual maturity and steroidogenesis in the skipper frog, *Euphlyctis cyanophlyctis*

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ABSTRACT

Gonadal sex differentiation, development up to sexual maturity and steroidogenesis were studied in the Indian skipper frog, *Euphlyctis cyanophlyctis*. In stage 25 tadpoles, gonads contained a few yolk laden germ cells and somatic cells. Ovarian differentiation occurred at stage 27 with the initiation of meiosis. Interestingly, meiosis preceded the formation of a central lumen that was discernible at stage 28. Folliculogenesis in the developing ovary was observed at stage 29. Vitellogenesis was observed in the 3 months old frogs and the females attained sexual maturity around 4 months. Testicular differentiation occurred indirectly through an ovarian phase. In some animals, from stage 37 onwards, oocyte degeneration was observed that was completed around metamorphic climax. Concurrently, large numbers of mesonephric cells were invading the gonads. Around metamorphosis, reorganization of the germ and somatic cells into testicular cords was observed. Following metamorphosis, the formation of seminiferous tubules was observed in the 2 weeks old males. Meiosis in the developing testes was observed in 1.5 months old males. In 3 months old males, the testes contained all stages spermatogenesis including spermatozoa. Steroidogenesis in the developing gonads was studied by immunohistochemical localization of 3β-HSD enzyme. At stage 26, a few immunoreactive cells were seen in the kidneys (interrenal cells). However, during and after differentiation, gonads failed to show positive immunoreaction. In the developing ovary at stage 37, follicular cells surrounding the oocytes were positive for 3β-HSD immunoreactivity. In the ovaries of 3 months old females, follicular cells surrounding the vitellogenic oocytes and stromal cells were positive for 3β-HSD immunoreaction. *E. cyanophlyctis* exhibits undifferentiated type of gonadal differentiation, in which gonadal differentiation precedes steroidogenesis.

1. Introduction

Sexual differentiation is a very complex process involving, at least in some animals, the interaction between extrinsic and intrinsic factors. The process begins with the establishment of genetic sex at fertilization by the fusion of male and female gametes, progresses through the establishment of gonadal sex and culminates in the formation of sexual phenotypes. In vertebrates, two mechanisms determine the sex of individuals; genetic or chromosomal sex determination (GSD or CSD) in which the genes present on the chromosomes determine the sex and environmental sex determination (ESD) in which sex can be determined entirely by the environmental factors [7,9,23,39]. Since temperature is the most common environmental factor known to affect sex determination in vertebrates, temperature dependant sex determination (TSD) is often used synonymously with ESD [7,12,23]. In fishes, however, other factors like pH of the water, density and other social cues are also known to influence sex determination [3,27]. Generally, it is believed that temperature can determine the sex in those species that lack heteromorphic sex chromosomes and reptiles are a major group of vertebrates to exhibit TSD [6,9,17]. Amphibians are unique in that only ~4% of the species studied have heteromorphic sex chromosomes but, TSD is rarely reported [2,7,12]. In amphibians that use GSD, some are male heterogametic (XX/XY) while others are female heterogametic (ZZ/ZW). Interestingly, within a single species, population variation also exists in the male or female heterogamety [7,22,23]. Although in most amphibians, sex chromosomes have not evolved to an extent that they are morphologically distinguishable, sex determination is believed to follow the GSD pattern [7,23].

Once the primary sex is determined, gonadal differentiation follows. Amphibians offer unique model systems for gonadal development studies as sex is determined by more than one mechanism, epigenetic factors can influence sex determination and gonadal differentiation does not follow a pattern; some species exhibit differentiated type in which the bipotential gonads differentiate directly into either testes or ovaries, while many others exhibit undifferentiated type, where indifferent gonads initially differentiate into ovaries in all the individuals followed by a testic-

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ular differentiation in genetic males through an ovarian phase [7,12,23]. A third intermediate type is reported in some species (semi-differentiated), in which testes differentiate through an intersexual stage [12,23]. However, the distinction between undifferentiated and semi-differentiated types is not clear. Large numbers of studies have reported the pattern of gonadal differentiation in anuran amphibians but these studies are limited to the development of the gonads up to metamorphosis. Only a few studies have described complete sequence of events from gonadal differentiation to sexual maturity [10,13,28].

Though sex is determined genetically, in many amphibians, gonadal differentiation can be influenced by the epigenetic factors such as temperature and steroid hormones [7,12,23,29]. In many frog species, administration of sex steroids results in sex reversal raising a possibility of involvement of sex steroids in gonadal differentiation [7,12,23]. However, only few studies have examined the potential of gonads to synthesize sex steroids during differentiation [1,10,15,20,21,31]. Recent studies have shown the involvement of aromatase (aromatase cytochrome P450, the product of CYP 19 gene), an enzyme that catalyzes the irreversible conversion of androgens into estrogens, in gonadal differentiation [1,20,23].

The Indian skipper frog, *Euphlyctis cyanophlyctis* is widely distributed throughout the Indian subcontinent [5]. It inhabits all types of habitats including streams and rivers. The frogs are seen in extremely ephemeral to permanent water bodies where temperature fluctuation is considerable. Our preliminary observations have shown that the sex ratio of *E. cyanophlyctis* at metamorphosis varies greatly depending on the habitat and month of the year. Sex ratio at metamorphosis could be as extreme as 90% of males and 10% females or vice versa. Since, *E. cyanophlyctis* experiences a wide range of temperatures in its geographic range, environmental temperature can possibly influence sex determination and control sex ratios at metamorphosis. Therefore, as a first step in understanding the role of temperature in sex determination and gonadal differentiation, we studied the pattern of gonadal differentiation and development up to sexual maturity and further, the ontogeny of steroidogenesis in the developing/differentiating gonads of *E. cyanophlyctis*.

2. Materials and methods

2.1. Collection and rearing

We collected two amplexed pairs of *E. cyanophlyctis* on 17th September, 2009 night from a temporary pond on the Pune University Campus (18°31′N and 73°51′E) in two separate buckets and immediately transported them to the laboratory where they were maintained in the same buckets overnight. Next morning, one of the females had spawned and the eggs collected from this spawn were maintained in a separate glass aquarium until hatching ($0.6 \times 0.45 \times 0.15$ m^3). Tadpoles hatched from these eggs ($n = \sim 200$) were reared in large glass aquaria at room temperature (22–24 °C) with natural photoperiod. The tadpoles were staged as per Gosner [8] and fed boiled spinach throughout larval development. Following metamorphosis, juvenile frogs were reared in out-door terrarium ($1 \times 1 \times 0.35$ m^3) for approximately 4 months (until sexual maturity). Juvenile ($n = 36$) and adults ($n = 12$) were fed on different insects and their larvae collected from nature. The tadpoles and juvenile frogs from these set-ups were used for histomorphological and immunohistochemical studies.

2.2. Histology

Tadpoles (6–8) at each stage of larval development from stage 25 to 46 (metamorphosis) and juveniles ($n = 3$♀ and 3♂) at specific intervals (weekly in the 1st month and 15 day intervals afterwards) were collected from the stock and dissected out to expose the gonads. The tadpoles were anesthetized using MS 222 before dissection while juveniles (SVL – 26.73 mm ± 0.94 and body mass – 2.26 g ± 0.41) and adults (males; SVL – 33.72 mm ± 1.22 and body mass – 5.2 g ± 1.07, females; SVL – 37.34 mm ± 0.59 and body mass – 6.73 g ± 1.28) were anesthetized using benzocaine gel. Gonads along with the kidneys (torso was fixed during early stages) were fixed in aqueous Bouin's fluid for 24 h. The fixed tissues were dehydrated in alcohol grades, cleared in benzene and embedded in paraffin. Serial sections cut at 5 μm, stained with haematoxylin and eosin were observed and photographed under bright field using a research microscope (Carl Zeiss–Axioscope A1).

2.3. Immunohistochemistry

Occurrence of 3β-hydroxysteroid dehydrogenase (3β-HSD), a key enzyme in the steroidogenic pathway was studied immunohistochemically to ascertain steroidogenic potential of the gonads during development and to determine whether gonadal steroidogenesis precedes sex differentiation. Representative gonads along with the kidneys at specific stages of larval and post-metamorphic development were fixed in aqueous Bouin's for 24 h and then treated with graded solutions of sucrose (10%, 20% and 30%) before suspending in the tissue freezing medium (Cryomatrix-Thermo Scientific, USA) for cryosectioning. Sections cut at 7 μm were preincubated with 0.3% H_2O_2 in methanol for 45 min to block endogenous peroxidise activity, washed in 0.01 M phosphate buffered saline (PBS), treated with BSA gelatine to prevent nonspecific binding, washed in PBS and then treated with normal goat serum. The sections were then incubated overnight at 4 °C with human type 3β-HSD antiserum raised in rabbit diluted 1:500 in PBS at pH 7.4. Next day, the sections were washed and incubated for 1 h at room temperature with biotinylated goat antirabbit IgG diluted 1:200. Tissue sections were then incubated with avidin biotin complex (ABC) for 1 h, washed thoroughly in PBS and developed in the dark using diaminobenzidine (DAB) as a chromogen [19]. Rat adrenal and ovaries served as positive controls while the tissue sections incubated without primary antibody served as negative control. All the chemicals used in immunohistochemistry were procured from Sigma-Aldrich, USA while primary and secondary antibodies were procured from Santa Cruz Biotechnologies Inc. USA and KPL, USA respectively.

3. Results

3.1. Ovary differentiation and development: Stage 25–26 of larval development

In tadpoles at stage 25, indifferent gonads lie ventrally on either side of the dorsal mesentery at the anterior region of the kidneys (Fig. 1A) and contained a few large germ cells and darkly stained somatic cells. The primordial germ cells (PGCs) were still heavily laden with yolk platelets (Fig. 1A). As the development progressed through stage 26, size of the gonads increased with the increasing number of germ cells; gonads were packed with germ cells and somatic cells. Majority of the germ cells with a prominent nucleus and clear cytoplasm were devoid of the yolk platelets and were distributed in the cortical region while darkly stained somatic cells were seen in the medullary region (Fig. 1B). However, the corticomedullary structure was not clear at this stage.

3.1.1. Stage 27–28

In tadpoles at stage 27, size of the gonads further increased with proliferation of germ cells and somatic cells. The germ cells were pushed to the periphery by the invading mesonephric cells. At this