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Induction of ovarian follicular development in the subadult frog *Rana tigrina* using luteinizing hormone releasing hormone-acetate

B HOQUE*, S K SAIDAPUR† and D R NAIK**
Department of Zoology, Karnatak University, Dharwad 580 003, India
*Present address: Department of Zoology, University of Chittagong, Chittagong, Bangladesh
**Department of Zoology, Utkal University, Bhubaneswar 751 004, India

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Abstract. In the subadult *Rana tigrina* administration of 2 µg luteinizing hormone releasing hormone-acetate/frog six days a week for 4 weeks in April resulted in the formation of medium (in all 8 frogs) and large sized (in 4 out of 8 frogs) yolky oocytes and, concomitant increases in the oviductal mass. The ovarian and oviductal masses showed a 10-fold increase over the control frogs. In untreated frogs the ovaries were transparent and contained first growth phase oocytes only. The oviducts were also infantile.

The pituitary sections were stained using antisera raised in rabbit against the β-subunit of human luteinizing hormone and human follicle stimulating hormone. Immunoreactivity, staining intensity, cytoplasmic granulation and, cell, nuclear and cytoplasmic areas of gonadotrophs (B_2 cells) increased significantly in luteinizing hormone releasing hormone treated frogs.

The above findings suggest that pituitary-ovarian axis in the subadult *Rana tigrina* is responsive to luteinizing hormone releasing hormone and that long-term treatment with the hormone induces cytomorphological changes in the gonadotrophs which result in the conversion of inactive cells into secretory cells. This is accompanied by precocious vitellogenic growth of oocytes in the subadult frogs.

Keywords. LHRH-acetate; ovary, gonadotrophs; subadult *Rana tigrina.*

1. Introduction

Immunocytochemical and/or biochemical studies have shown that luteinizing hormone releasing hormone (LHRH) like substances are present in the brain of diverse nonmammalian vertebrates and mammalian LHRH or its agonists induce hypophyseal secretion of gonadotropin in them. Among the amphibians intracerbral immunoreactive LHRH containing neurons have been demonstrated in *Bufo vulgaris, Rana esculenta, Xenopus laevis, Rana pipiens, Rana catesbeiana, Cynops pyrrhogaster ensicauda, Rana brevipoda, Rana japonica, Bufo marinus, Bufo bufo japonicus, Gastrophryne carolinenis* and *Rana ridibunda* and in the salamander *Taricha granulosa gracile* (Alpert *et al* 1976; Doerr-Schott and Dubois 1976; Goos *et al* 1976; Nozaki and Kobayashi 1979; Crim 1985; Jokura and Urano 1986; Sherwood *et al* 1986; Andersen

†Corresponding author.
Abbreviations used: LHRH, Luteinizing hormone releasing hormone; GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone; FGP, first growth phase; MSGP, medium sized second growth phase; LSGP, large sized second growth phase; AF, atretic folicles; TSH, thyroid stimulating hormone.

et al 1988). Further, several of the above species are known to respond *in vivo* and *in vitro* to gonadotropin releasing hormone (GnRH) and release both luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Daniels and Licht 1980; McCreery *et αl* 1982; McCreery and Licht 1983a,b; Porter and Licht 1985; Gracia-Navarro *et al* 1990; Stamper and Licht 1993). Furthermore, *in vivo* and *in vitro* studies have shown that LHRH and its agonists have direct effect on testicular steroidogenesis in *R. pipiens* (Segal and Adjuwon 1979) and *R. esculenta* (Pierantoni *et al* 1984; Zerani and Gobbetti 1990). Interestingly, in *R. pipiens* and in *R. catesbeiana* GnRH failed to show any direct effect on ovarian steroidogenesis and germinal vesicle breakdown (Hubbard and Licht 1985) but recent studies on *R. esculenta* suggested a direct action of GnRH in androgen and estradiol synthesis in the ovary (Gobbetti and Zerani 1990, 1992; Zerani *et al* 1991).

Studies on the effect of LHRH on ovarian follicular development in nonmammalian vertebrates are scarce and among anurans limited to two species viz., *Rana cyanophlyctis* and *Rana tigrina* (Saidapur and Kupwade 1989; Saidapur and Pramoda 1993). The present work was therefore undertaken to elucidate the long-term effect of LHRH-acetate on ovarian follicular development and also on hypophyseal gonadotrophs in the subadult *R. tigrina.* Such studies will help in precocious induction of vitellogenic growth of oocytes in subadult frogs.

2. Materials and methods

Female subadult *R. tigrina* (70–99 g) were collected from the surrounding areas of Karwar city (latitude 14° 50" N, longitude 74° 15"E) in the first week of April, 1992. Six frogs were killed on the day of their arrival to laboratory to serve as initial controls. The body, ovary, and oviduct weights were recorded. The ovaries were fixed in Bouin's fluid for histological study. The remaining 16 frogs were divided into two groups and acclimated for 4 days before use. One group was treated with 2 µg/frog/day LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)-acetate (Sigma Lot 68F-7134) dissolved in 0·05 ml 1% acetic acid. The control group received the vehicle (0·25 ml/day). All injections were given (ip) between 1100–1130 h 6 days a week for 4 weeks. The frogs were maintained under natural photoperiod (LD : 12,12) and ambient temperature (27·5°C–30·3°C). They were fed live guppies (*Gambusia affinis*) 6 days a week *ad libitum.* The frogs were autopsied on day 31 of treatment.

At autopsy, weights of the body, ovaries and oviducts were recorded. The excised ovaries of each frog were fixed in Bouin's fluid for analysis. An ovarian sample weighing 10% of the total Bouin's fixed ovary was used for counting oocytes under a dissecting microscope. The oocytes were classified into first growth phase (FGP), medium sized second growth phase (MSGP), large sized second growth phase (LSGP) oocytes and atretic follicles (AF) as in an earlier study (Hoque and Saidapur 1994). A piece of ovary was processed for histology. The diameter of 50 largest oocytes/frog was measured using an ocular micrometer to determine the maximum size attained by the oocytes.

For histological and immunocytological analysis pituitaries plus surrounding brain tissue from frogs of the treated and control groups were fixed *in situ* in Bouin's Hollande sublimate. After fixation for 24 h the pituitaries were processed for paraffin embedding. Sagittal sections were cut at 4 µm thickness.

For immunostaining of the pituitary sections, the unlabelled antibody enzyme method was followed (Sternberger *et al* 1970; Kar and Naik 1987). In brief, the sections were deparaffinized and hydrated, rinsed in phosphate saline buffer (PBS, 0·01 M, pH 7·5) and treated with 3% H_2O_2 and 10% egg albumin in order to eliminate non-specific staining (Zehr 1978). After washing in PBS the sections were incubated with suitable dilutions (1 : 500; 1 : 1000) of primary antisera, anti-human LH β (AFP-54372; NIH, Bethesda, USA), anti-human FSH ß (AFP-3710194; NIH, Bethesda, USA), and anti-human TSH β (AFP-62423473; NIH, Bethesda, USA), raised in rabbit against β-subunit of human LH, FSH and thyroid stimulating hormone (TSH) respectively in a closed moist chamber at 4°C for 24 h. The sections were then incubated with a second (link) antibody, anti-rabbit γ-globulin (ARGG, antibodies Inc., Davis, California, USA) at a dilution of 1 : 100 at 24°C. After washing in PBS they were incubated with peroxidase anti-peroxidase complex (PAP, Dakopatts, Denmark) at a dilution of 1 : 100 at 24°C. The immunoreactive sites in the tissue were visualized as brown reaction products using 0·075 % solution of 3,3-diaminobenzidine tetrahydrochloride (DAB, Fluka Ag. Buchs SG) in PBS (0·01 M, pH 7·4) containing 0·03% H_2O_2. After washing in PBS and rinsing in distilled water the sections were counterstained with light green, dehydrated in alcohol series, cleared in xylene and mounted with DPX.

Specificity of the immunoreaction was tested by omission of one of the steps in the immunostaining procedure and/or replacement of the primary antiserum with normal goat serum. In both the cases immunostaining was abolished indicating that immunoreactivity was specific.

The outlines of 20 randomly selected gonadotrophs (B_2 cells) from each pituitary were drawn using camera lucida at × 1000. The cell and its nuclear areas were measured by a planimeter. The cytoplasmic area of each cell was then calculated by subtracting the nuclear area from the cell area of the corresponding cell (Masood-Hussain and Saidapur 1983).

Statistical comparison between paired groups as carried out by the Student's *t* test and the nonparametric Kruskal-Wallis test followed by a multiple comparison method (Conover 1980) to compare multiple groups. Since LSGP oocytes were formed in 4 out of 8 LHRH treated frogs the coefficient of correlation (*r*) was determined by using Pearson's formula to assess the relationship, if any, between the body mass and production of SGP oocytes. The value of *r* was also checked for statistical significance at 5% level of significance.

Results

3.1 *Effect of LHRH-acetate on ovary and oviduct*

The ovaries of initial control frogs were small (table 1) and transparent. They contained mainly, healthy FGP and a few atretic oocytes (table 2). There were neither pigment scars nor SGP oocytes in the ovary (figure 1). The largest oocytes were 3186 ± 14 µm in diameter (table 2). Oviducts were infantile. The ovaries and oviducts of vehicle injected control frogs resembled those of the initial controls. However, FGP oocytes were slightly larger (tables 1–2) and some of them had yolk vesicles at the periphery (figure 2).

Table 1. Effect of LHRH-acetate (2 μg/frog) on ovary and oviduct of subadult *R. tigrina.*

Group	Body weight (g) (mean ± SE)	Weight (g)/100 g body weight (mean ± SE)	
		Ovary	Oviduct
Initial control (6)	85·00 ± 4·00	0·64 ± 0·07	0·20 ± 0·03
Control (8)	87·25 ± 3·08	0·74 ± 0·11	0·21 ± 0·03
LHRH treated (8)	97·87 ± 5·46	7·60 ± 1·24*	1·91 ± 0·35*
T value (Kruskal-Wallis test)	3·21	15·81**	14·60**

Values in parenthesis represent number of frogs
* $P < 0·05$ compared to controls.
Tabulated $T = 5·991$.
** Significant at 5% level of significance.

Table 2. Effect of LHRH-acetate (2 μg/frog) on oocyte growth in subadult *R. tigrina.*

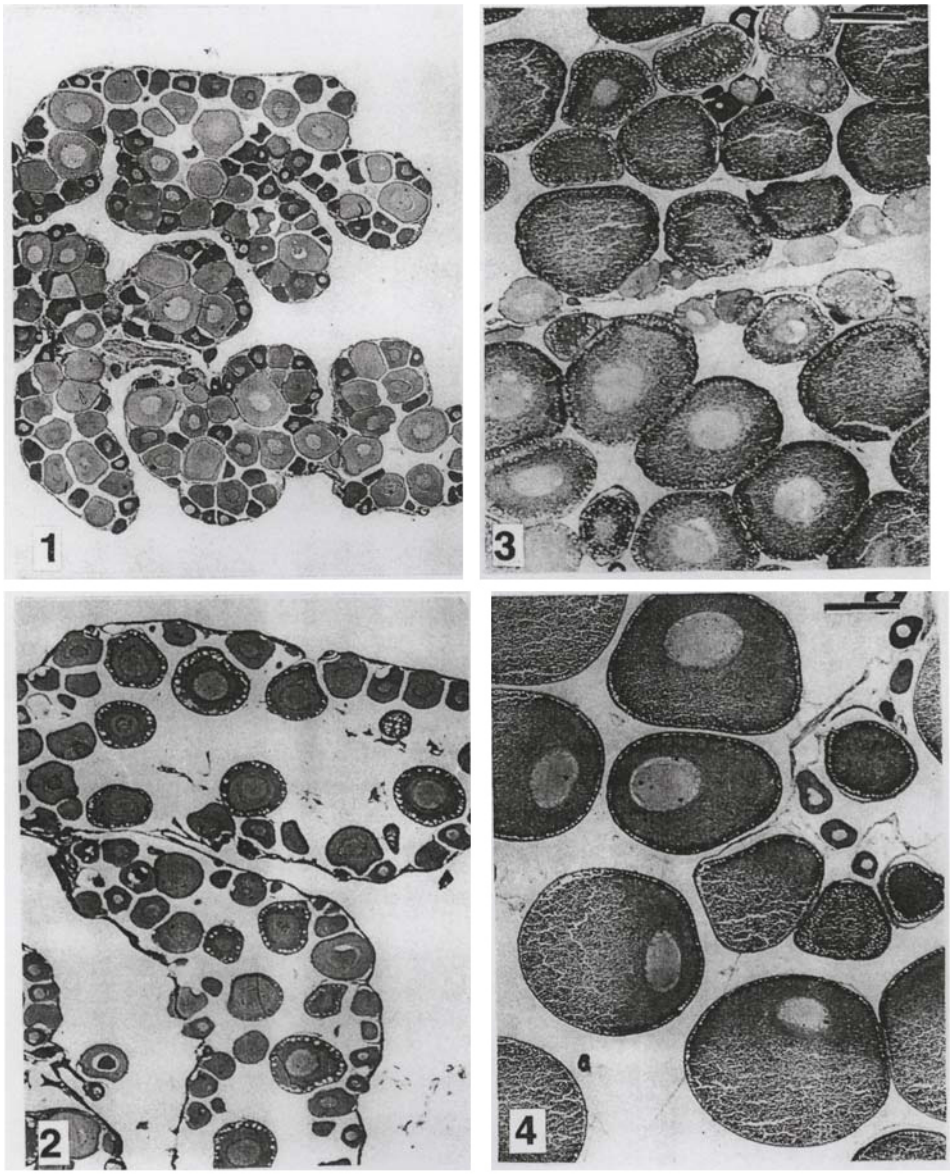
Group	Number of oocytes (mean ± SE)/frog				Largest oocyte diameter (μm) (mean ± SE)
	FGP	MSGP	LSGP	AF	
Initial control (6)	39623 ± 1388	—	—	498 ± 74	318 ± 14
Control (8)	40298 ± 2791	—	—	535 ± 109	351 ± 10
LHRH treated (8)	40460 ± 4127	4795 ± 489*	1792 ± 810*	777 ± 123	1061 ± 72*
T value (Kruskal-Wallis test)	0·16	—	—	3·53	16**

Values in parenthesis represent number of frogs.
* $P < 0·05$ compared to controls.
Tabulated $T = 5·991$.
** Significant at 5% level of significance.

Administration of 2 μg LHRH-acetate six days a week for 4 weeks caused significant increase in ovarian and oviductal weights and diameter of the largest oocytes (tables 1 and 2). Ovarian mass exhibited a 10-fold increase over that of the control frogs (table 1) due to the recruitment of MSGP and LSGP oocytes (table 2, figures 3 and 4). There was no significant change in the number of AF, and FGP oocytes (tables 2). The diameter of the largest oocytes in 4 out of 8 frogs of this group exceeded 1200 μm (figure 4) while in the remaining 4 frogs it was 800–900 μm (figure 3). Further, the number of SGP (MSGP + LSGP) oocytes increased in frogs with larger body mass ($r = 0·74$; $P < 0·05$). There was a concomitant increase (9-fold) in the oviductal weight in treated frogs (table 1).

3.2 *Effect of LHRH-acetate on gonadotrophs*

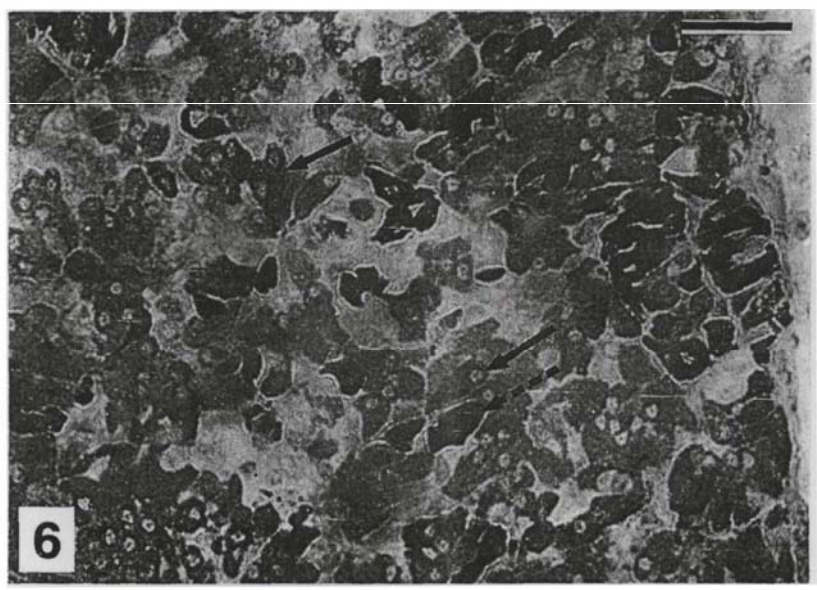
The gonadotrophs (B_2 cells) of *R. tigrina* were recognized by their immunoreactivity to both hLH β and hFSH β antisera and the thyrotrophs (B_1 cells) by their specific immunoreactivity to hTSH β antiserum. Further, the anti-hLH β and anti-hFSH β sera also showed intense cross reactivity with the TSH cells (figures 5 and 6). However, no cross-reaction was seen in the gonadotrophs with the hTSH β antiserum.



Figures 1–4. Cross sections of subadult *R. tigrina* ovary (H × E). (**1,2**) Show the presence of only FGP oocytes in the ovaries of initial (**1**) and final control frogs (**2**). (**3,4**) LHRH-acetate injected frog ovaries showing the formation of medium (**3**) and large sized (**4**) second growth phase oocytes. Scale lines: 500 µm.

The gonadotrophs are oval or elongated in shape with a distinct central or eccentric nucleus surrounded by immunoreactive cytoplasm.

The B_2 cells of the control frogs were small (table 3) and they lacked secretory granules. Their staining intensity/immunoreactivity was very low (figure 5). In



Figures 5 and 6. Sagittal sections of pituitary pars distalis showing immunoreactive B_1 and B_2 cells. Note that B_2 cells are less immunoreactive and faintly stained in controls (**5**) while LHRH treated frogs are distinctly immunoreactive and hence markedly stained (**6**). Scale line: 30μm.

LHRH treated frogs there was a significant ($P < 0{\cdot}001$) increase in B_2 cell, nuclear and cytoplasmic areas over the controls (figure 6, table 3). The secretory granules were clearly more in the B_2 cells of LHRH treated frogs and the cells were more immunoreactive in comparison to those of the controls (figure 6).

Table 3. Effect of LHRH-acetate (2 µg/frog) on B_2 cells of pituitary pars distalis in subadult *R. tigrina.*

Group	B_2 cells		
	Cell area (μm^2)	Nuclear area (μm^2)	Cytoplasmic area (μm^2)
Control	84·0 ± 0·63	31·2 ± 0·37	52·8 ± 0·58
LHRH treated	133·8 ± 1·15*	45·0 ± 0·31*	88·4 ± 0·92*

Values are mean ± SE of 5 frogs in each group.
* $P < 0{\cdot}001$ compared to controls.

Discussion

The effect of LHRH and its agonists on the pituitary and gonads have been extensively studied in mammals (Fink 1988). In a variety of mammals including men and other primates GnRH agonists are known to inhibit a wide range of reproductive functions (Rastogi and lela 1994). In contrast, other studies indicate that GnRH also has the stimulatory actions on the mammalian ovary (Fink 1988). Comparative studies on nonmammalian vertebrates are scanty. Among the female anurans previous studies in juvenile *R. catesbeiana* have shown that there was virtually no response to doses of GnRH up to l000 ng/ml (Porter and Licht 1985). The frogs in their early follicular stages were also relatively unresponsive to infusion of 1 to 10 µg/h GnRH. In juvenile *R. catesbeiana* while the postmetamorphic males showed progressive increase in the magnitude of pituitary response with age, the females remained insensitive until maturation (McCreery and Licht 1983a,b). In contrast, in juvenile *R. cyanophlyctis* (7–8 g), daily injection of LHRH -ethylamide (0·01 or 0·01 µg/frog/day) for 30 days induced significant increases in ovarian mass and vitellogenic oocytes (Saidapur and Kupwade 1989). In the adult *R. tigrina,* injection of LHRH in the postbreeding phase when hypophyseal gonadotrophs and ovaries were highly regressed, resulted in the production of vitellogenic follicles (Saidapur and Pramoda 1993). These studies on juvenile *R. cyanophlyctis* and adult *R. tigrina* (in the postbreeding regression phase) thus showed that LHRH induces hypertrophy in B_2 cells of pituitary pars distalis and production of secretory granules (AB-PAS-OG positive) and also ovarian growth. The limitation of these studies was that the gonadotrophs were stained using AB-PAS-OG and not by immunocytological techniques and, gonadotropin levels were not measured. In the present study, use of immunocytological technique demonstrates that B_2 cells of the pituitary pars distalis of *R. tigrina* are indeed gonadotrophs. The intense cross reaction of the hLH β and hFSH β antisera with the B_1 (in addition to B_2) cells of *R. tigrina* was also seen in the case of *R. hexadactyla* (unpublished results). This is somewhat intriguing, but may be due to certain homology between the β-subunit of hLH and hFSH with that of the frog TSH. Similar cross reaction between different adenohypophysial cell types was reported earlier in *X. laevis* (Van Kemenade *et al* 1984) and *R. limnocharis* (Kar and Naik 1987).

LHRH induced production of SGP oocytes in subadult *R. tigrina* is similar to that reported earlier for juvenile *R. cyanophlyctis* (Saidapur and Kupwade 1989).

In agreement with the findings in *R. cyanophlyctis* is the fact that in the present study also production of SGP oocytes was enhanced in frogs with higher body mass ($r = 0{\cdot}74$; $P < 0{\cdot}05$). Further, corresponding increase in the oviductal mass due to LHRH indicates enhanced ovarian secretion of steroid hormones.

The mechanism of LHRH action on ovaries of amphibians is not clear. It was shown that injection of GnRH in adult (Daniels and Licht 1980) and subadult (McCreery *et al* 1982) *R. catesbeiana* causes several hundred-fold increase in the levels of plasma FSH and LH. Likewise, stimulation of hypophyseal gonadotrophs following LHRH treatment was observed in *R. cyanophlyctis* (Saidapur and Kupwade 1989), *R. pipiens* (Gracia-Navarro *et al* 1990) and *R. tigrina* (Saidapur and Pramoda 1993). Whereas, an *in vitro* study in *R. pipiens* and *R. catesbeiana* failed to demonstrate any action of GnRH at the level of ovary. Similarly, there was no effect of GnRH on basal or LH-stimulated *in vitro* steroid secretion or oocyte maturation in the ovaries of *R. pipiens* (Hubbard and Licht 1985). Interestingly, GnRH analogue (HOE 766) stimulated both *in vivo* and *in vitro* spermatogonial multiplication in *R. esculenta* (Minucci *et al* 1986). Likewise, in *R. esculenta* GnRH seems to directly stimulate ovarian steroidogenesis (Gobbetti and Zerani 1990, 1992; Zerani *et al* 1991). In view of the species differences more studies are needed before drawing conclusions with regard to the mode of action of LHRH in the frog ovary in bringing about follicular growth.

In summary, the present study demonstrates that LHRH can induce inactive gonadotrophs to undergo transformation into secretory cells following long term treatment in subadult *R. tigrina.* Present findings lead to the hypothesis that LHRH in anurans not only causes gonadotropin release but also acts as a trophic hormone. The present findings also show that subadult *R. tigrina* responds positively to LHRH by exhibiting precocious ovarian growth.

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Corresponding editor: SAMIR BHATTACHARYA