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Source: Zoological Science, 14(2) : 363-367

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.363

ZOOLOGICAL SCIENCE **14**: 363–367 (1997)

Ontogeny of the Expression of Messenger Ribonucleic Acid Encoding Lutropin Receptor in Chicken Embryo

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ABSTRACT—Ontogeny and cellular localization of the lutropin receptor (LH-R) mRNA were studied in fowl gonads between day 5 of incubation and day 1 of posthatch. *In situ* hybridization using an antisense cRNA probe demonstrated that LH-R gene expression began at embryonic day 7 in females and day 14 in males. In female embryos, the specific hybridization was detected in not only developing left ovaries but also regressing right ovaries. The LH-R mRNA expression in the right ovary was detectable until hatch. LH-R mRNA was localized in the medullary cords in ovaries and interstitial tissues in testes. These results are consistent with previous reports on ontogenic analysis of LH-dependent steroidogenesis in chicken embryo gonads.

INTRODUCTION

Onset of the expression of lutropin receptor (LH-R) in embryo gonads plays a pivotal role in the commencement of steroidogenesis, resulting in the expression of sexual dimorphism. Sokka *et al.* (1992) and Zhang *et al.* (1994) have determined the day of onset of LH-R gene expression in rat fetal ovary and testis, respectively. Namely, full-length LH-R mRNA appears in testis in 15.5-day embryo and in ovary in 7-day neonate. These facts indicate that in rat a developing testis is more active in the LH-dependent steroidogenesis than developing ovary. Since avian embryo develops much more rapidly than mammalian fetus, sexual manifestation appears at an earlier stage of development (for review, Johnson, 1990). Tanabe *et al.* (1986) have revealed that in chicken embryo the ovary is more active than the testis in the production and secretion of estrogen, while production of androgen dose not differ significantly between the two. They also showed that LH is detectable in 10-day embryo pituitaries of both sexes, as well as in 10-day female embryo plasma and in 12-day male embryo plasma. On the other hand, equine LH administration increases in plasma 17β-estradiol in 7.5-day embryo (Woods *et al.*, 1981), and in vitro treatment with human chorionic gonadotropin stimulates testosterone and estradiol production in embryo ovary by day 8 of incubation (Teng and Teng, 1977). These facts suggest that embryo gonads are responsive to LH before day 10, though circulating endogenous LH remains undetectable level. Tanabe *et al.* (1986) also investigated the plasma and gonadal levels of sex hormones (testosterone, 17β-estradiol and estrone) from day 10 of incubation to day 14 after hatching, and demonstrated that plasma and gonadal sex hormones are detectable in these stages. Woods and Erton (1978) demonstrated that estrone- and 17β-estradiol-immunoreactive cells appear on day 3.5 of incubation in both testis and ovary. Recently, Yoshida *et al.* (1996) have revealed that P450 aromatase (which converts testosterone into 17β-estradiol) mRNA first appears in 6.5-day embryo ovary, which suggests that ovarian LH sensitivity is acquired before day 10 of incubation.

We are greatly interested in how the appearance of LH-R in chicken embryo gonads relates to the establishment of pituitary-gonadal axis in avian species. Information of sequnces of avian LH-R cDNAs (quail, Akazome *et al.*, 1994; chicken, Johnson *et al.*, 1996) enables the evaluation of LH-R gene expression in avian species. In the present study we used *in situ* hybridization techniques to determine the day of onset and cellular localization of the expression of mRNA encoding LH-R in chicken embryo gonads.

MATERIALS AND METHODS

Animals

Fertilized chicken eggs of the White Leghorn were purchased from a commercial source. Eggs were incubated in an incubator with agitation (one stroke per 2 hr) at 37.6-37.7°C and 58-69% humidity. A White Leghorn cock was also purchased from a commercial source.

In situ *hybridization*

To determine the day of onset of LH-R mRNA expression precisely, we used an *in situ* hybridization technique. Embryos were removed at various incubation days; day 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 21 (hatching day) and 1-day posthatch, and fixed overnight in freshly prepared Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered

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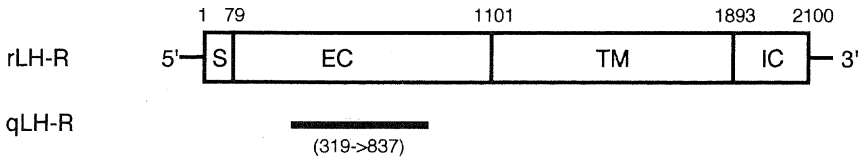

Fig. 1. Schematic presentation of position of quail LH-R (qLH-R) cRNA fragment for *in situ* hybridization with rat (r) LH-R cDNA. Because full-length chicken and quail LH-R cDNAs are not available, the fragment is aligned to rLH-R cDNA. Localizations indicated by numbers of base pairs are based on original references (McFarland *et al.*, 1989). Abbreviations used are as follows, S, signal peptide; EC, extracellular domain; TM, transmembrane spanning domain; IC, intracellular domain.

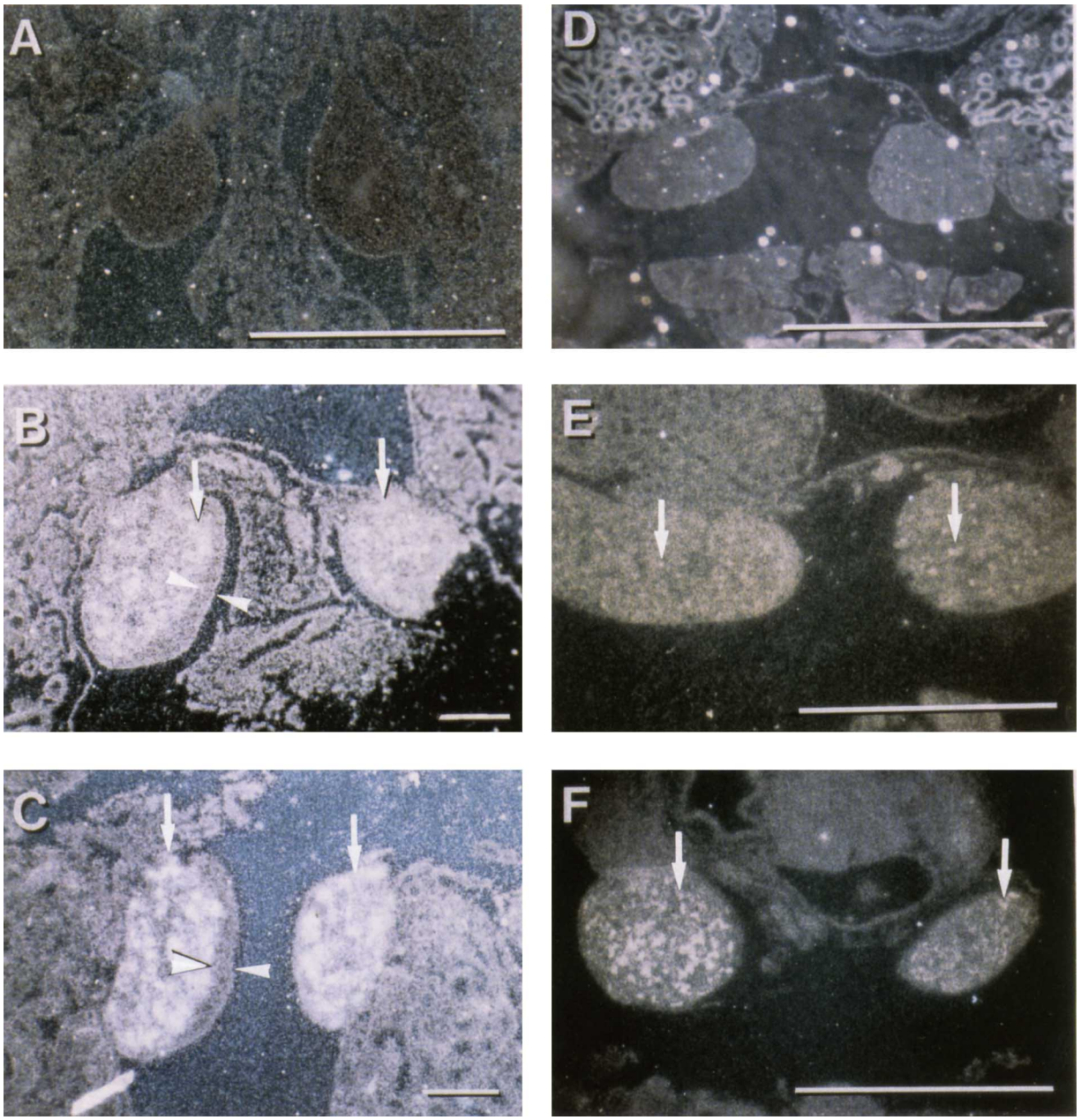

Fig. 2. *In situ* hybridization of LH-R mRNA in chicken embryo gonads. Darkfield photomicrographs of transverse sections of 6-day embryo undifferentiated gonads (**A**), 7- (**B**), 8-day embryo ovary (**C**), 12- (**D**), 14- (**E**) and 18-day embryo testis (**F**) hybridized with antisense cRNA probe. Arrows indicate silver grains, and opposite arrowheads indicate the cortex. Bars represent 1 mm (**A**, **D**, **E**, **F**) or 100 μm (**B**, **C**).

saline (PBS) containing 4% paraformaldehyde. Fixed embryos were dehydrated with ethanol, embedded in paraplast, dissected into 5 μm thick sections and mounted upon gelatin-coated slideglasses. Before hybridiztion, sections were dewaxed in xylene and rehydrated through an ethanol series. After rehydration, the sections were treated with 0.3% Triton X-100 in PBS to extract cell membrane, immersed in 0.2 M HCl to remove basic substances, digested with 2 μg/ml of proteinase K in P buffer (50 mM Tris-Cl pH 7.5, 2 mM $CaCl_2$) for 15 min, and fixed in 4% paraformaldehyde at room temperature for 15 min. After these treatments, the sections were dehydrated through an ethanol series, immersed in xylene, and airdried at room temperature for an hour.

As a template for the cRNA probe, a 519 base pair DNA fragment of the extracellular domain of quail LH-R (Akazome *et al.*, 1994) was used. The corresponding region of the probe is shown in Fig. 1. This fragment has 96.0% identity with chicken LH-R at the level of DNA sequence (Johnson *et al.*, 1996). This fragment was inserted into pAM18 and pAM19 plasmid vectors containing the promotor for SP6 RNA polymerase. Sense and antisense cRNA probes were generated in the mixture containing SP6 RNA polymerase (Amersham, UK) and [α-^{35}S]CTP (>1000 Ci/mmol; Amersham). Before hybridization, cRNA probes were partialy digested with alkali solution (80 mM $NaHCO_3$, 120 mM Na_2CO_3, 10mM DTT) to make fragments of about 200 bases which can easily soak into tissue sections.

Hybridization mixture (50% formamide, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 600 mM NaCl, 10 mM DTT, 1 × Denhardt's reagent, 0.25% SDS, 10% PEG6000, 200 μg/ml yeast tRNA) containing 1.5×10^4 cpm/μl of probe was applied to the dried sections. Hybridization was performed in a moist chamber at 50°C for 16 hr. After hybridization, the sections were rinsed in 2 × SSC containing 50% formaldehyde at 42°C for 30 min twice. Non-hybridized cRNA was digested with RNase A (Sigma Chemical, Co., USA). Furthermore, sections were washed in 2 × SSC at 42°C for 20 min twice and in 0.5 × SSC at 42°C for 20 min twice, dehydrated in an ethanol series containing 0.3 M ammonium acetate (pH 5.2), and dried in air. The slides were coated with autoradiographic emulsion (NR-M2; Konica Medical, Japan). Exposure was performed at 4°C in a light-tight box for one week. After developing, the sections were counterstained with Mayer's hematoxylin.

Histological analysis was performed following the references (Willier, 1927; Romanoff, 1960).

RESULTS

As a preliminary, we performed RT-PCR to detect LH-R mRNA of total RNA from 16-day embryo gonads (at this stage embryos have gonads which are easily sexed by shape), using oligonucleotide primers as described previously (Akazome *et al.*, 1994). The DNA fragment of LH-R was successfully amplified in both ovarian and testicular cDNAs in 16-day embryo.

To determine both the day of onset and the cellular localization of the expression of LH-R mRNA, *in situ* hybridization with a ^{35}S labelled cRNA probe corresponding to a part of the extracellular domain was performed. No visible silver grains were detected in either sex until embryonic day 6 (Fig. 2A). Silver grains were visible in medulla of 7-day embryo ovary (Fig. 2B, arrows) while the grain distribution of cortex is almost similar to those of extragonadal tissues (Fig. 2B, between arrowheads). In 8-day embryo ovary, the grain distribution is almost the same as that in 7-day embryo ovary but more intensive (Fig. 2C). The grains were localized in the medullary cord (Fig. 3A, B). In 10-day embryo, the regression of right ovary begins. Regressing right ovary also exhibited grains, which were visible at least until the day of hatch (not shown). In contrast, no signals was detectable in testis until embryonic day 12 (Fig. 2D). In 14-day embryo, slight signals were detectable in the testis (Fig. 2E, arrows), and in 18-day embryo, clear signals were detected (Fig. 2F, arrows), but not in all samples. The signals were localized in the interstitial tissue which would differentiate into Leydig cells (Fig. 3C, D). The results of positive reaction to LH-R mRNA in developing gonads are summarized in Table 1.

DISCUSSION

Using RT-PCR, the expression of LH-R mRNA was detected in 16-day embryo gonads of both sexes. We also applied a pair of primers for amplifying the FSH-R (Akazome *et al.*, 1996). However, FSH-R mRNA was detected neither in ovary nor testis on day 16, though FSH immunoreactive cells are detectable in Rathke's Pouch of 4-day embryo (Woods *et al.*, 1985). At present, there are much fewer reports on avian embryonic FSH than on LH. This might be due to a very low level of FSH and FSH-R expression during embryogenesis.

In the present study, the day of onset and cellular localization of the expression of LH-R mRNA in chicken embryo gonads were elucidated using a cRNA probe. In ovary, the expression of LH-R mRNA was detectable from embryonic day 7. Woods *et al.* (1988) showed that plasma LH is detectable in 10.5-day embryos of both sexes, and Tanabe *et al.* (1986) detected plasma LH in 10-day female embryo. LH-binding cells, as identified by immunocytochemistry, are detectable in the medullary interstitial cells in 6.5-day embryo (Woods *et al.*, 1988). According to Woods and Erton (1978), estrogen immunoreactive cells appear in both rudimentary ovary and testis in 3.5-day embryo. However, the expression of P450 aromatase gene was first detected in 6.5-day embryo (Yoshida *et al.*, 1996). Therefore, a small amount of LH is likely to be secreted in 7- and 8-day embryo.

During ovarian development, the follicular cells are differentiated from the cortical cord. Woods *et al.* (1989) showed that the LH-binding cells are distributed both in the cortical cord and the cortical interstitial cells in 13.5- to 19.5-day embryos. In the present study, however, the expression of LH-R mRNA was clearly localized in medullary cord until hatch. Despite the inconsistency between the distribution of LH-R mRNA and the presence of LH binding sites, the localization in the present experiment is consistent with that of P450 aromatase expressing sites (Yoshida *et al.*, 1996), at least in 7-, 8- and 9-day embryos.

In the present study, the expression of LH-R mRNA was visible in regressing right ovary. Recently, the expression of aromatase and P450α17 mRNA (they are responsible for estrogen synthesis) in both left and right ovaries from day 6.5 to day 9 of embryo development was reported (Yoshida *et al.*, 1996). Indeed, Woods and Erton (1978) revealed that estrogen immunoreactive cells exist in both left and right ovaries from day 3.5. According to them, in the right ovary, levels of

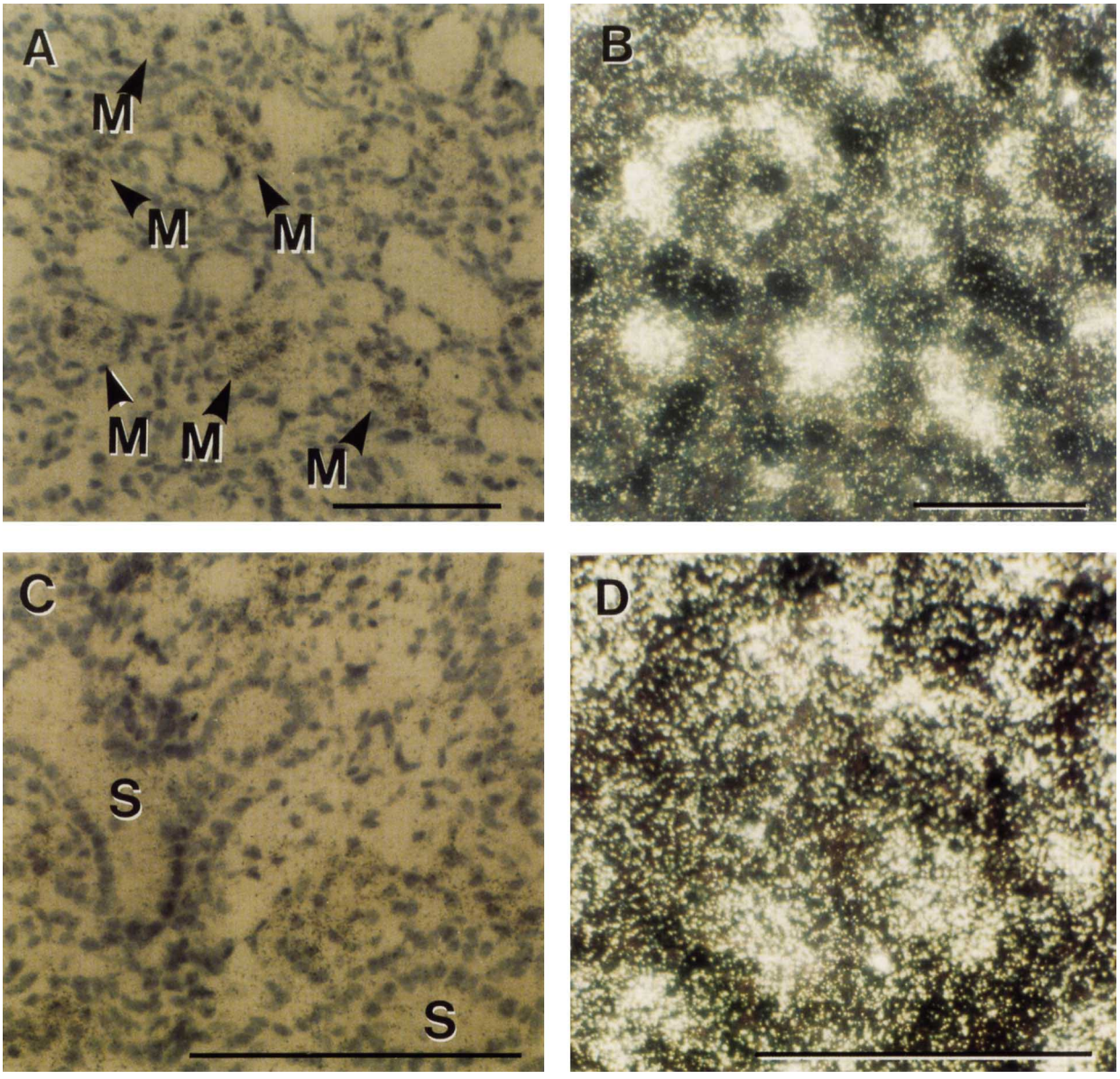

Fig. 3. Magnified photomicrographs of *in situ* hybridization. (**A**) Lightfield photomicrograph of left ovary at day 19 of incubation. (**B**) Darkfield photomicrograph of the same section in **A**. (**C**) Lightfield photomicrograph of testis at day 19 of incubation. (**D**) Darkfield photomicrograph of the same section in **C**. M, medullary cord; S, seminiferous tubule. Bars represent 100 μm.

immunoreactivity maximize on day 5.5 (estrone) or day 6.5 (17β-estradiol), then decrease gradually (estrone) or rapidly (17β-estradiol). Furthermore, they showed that on day 11.5, the immunoreactivity of both estrogens reached very low levels. However, in the present experiment, LH-R mRNA expression was detected until the day of hatch. Why did gonadotropin fail to save the right ovary? Both the left and right ovaries are able to generate estrogen by LH. On day 6 of incubation, the estrogen target cell distribution in germinal epithelium is limited in the left gonads in both sexes while left and right medulla have estrogen target cells on the same day. However, on day 10 of incubation, gonadal estrogen target cells are localized only in the germinal epithelium of the left testis (Gasc, 1980). Treatment of embryo gonads collected from 6- and 8- day males with 17β-estradiol or aromatizable androgen, androstendione, induces the sex reversal of gonads (Willier *et al.*, 1935; Narbaitz and Teitelman, 1965). Taken together, a high dose of estrogen in female embryo may promote the protrusion of the cortical cord in the left ovary by day 10 of incubation. Therefore, it is possible that left-right asymmetry in female chicken embryo gonads is due to the asymmetric expression of estrogen receptor in the germinal epithelium.

In the testis, a slight LH-R mRNA signals were visible in

Table 1. Summary of *in situ* hybridization analysis of LH-R mRNA in chicken embryonic gonads

Age in days	Female	Male
Embryo		
5	0/8	
6	0/11	
7	2/5	0/5
8	5/6	0/4
9	6/6	0/3
12	7/7	0/7
14	3/3	1/3
16	3/3	0/1
18	3/3	2/3
19	7/7	2/2
21	3/3	2/3
Chick		
1	3/3	1/1

Data are presented as number of samples with LH-R mRNA signals relative to total number of samples examined. Five- and 6-day embryos could not be sexed by histological observation.

14-day embryo. This finding is in accord with a previous report that the testicular testosterone concentration increases gradually from embryonic day 16 to adulthood (Tanabe *et al.*, 1986). The silver grains were localized in the interstitial tissue, and the rudimentary Leydig cells. This result is consistent with those of studies on adult quail testis (Akazome *et al.*, 1994) and mammalian testis (Zhang *et al.*, 1994).

The onset of expression of LH-R mRNA, as detected by in situ hybridization, was 7 days earlier in ovary than in testis. This is reasonable because avian embryo ovary synthesizes a relatively large amount of estrogen than the testis while no significant difference exists in testosterone production between the ovary and testis (Tanabe *et al.*, 1986).

ACKNOWLEDGMENTS

This study was supported by a JSPS Research Fellowship for Young Scientists awarded to Y. Akazome, and by Grants-in-aid for Fundamental Scientific Research and Developmental Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan to T. Mori.

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(Received November 20, 1996 / Accepted December 25, 1996)