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Effects of 17β-estradiol on liver vitellogenin gene expression in immature female frogs, *Hoplobatrachus rugulosus*

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ABSTRACT: We aimed to determine the vitellogenin gene expression in response to 17β-estradiol (E_2) treatment in livers of immature female Chinese edible frogs (*Hoplobatrachus rugulosus*). Frogs reared during rainy or dry seasons were intramuscularly injected daily with E_2 at doses of 0, 50, 500, or 5000 µg/kg body weight for five days. During the rainy season, treatment with E_2 within the dose range studied significantly decreased the vitellogenin gene transcript levels compared to those of the control. In contrast, during the dry season, treatment with E_2 at all doses tended to increase the vitellogenin gene transcript levels. The endogenous plasma E_2 levels in frogs reared during the rainy season were higher than those reared during the dry season. There was no significant difference in liver-somatic index or gonadosomatic index among the treated frogs during the rainy season. However, during the dry season, the 500 µg/kg E_2 treatment significantly increased the frog liver-somatic index. The hepatocytes from the dry season frogs appeared smaller and flatter. The histology of ovaries from rainy season frogs showed that the oocytes were bigger and of several stages. We therefore concluded that the injection of E_2 into immature female *H. rugulosus* may stimulate vitellogenesis if administered during the dry season, when the endogenous E_2 is lower. This knowledge could be used as a basis for inducing precocious maturation or mating of *H. rugulosus* outside the breeding season.

KEYWORDS: liver-somatic index, gonadosomatic index

INTRODUCTION

The Chinese edible frog or rice field frog, *Hoplobatrachus rugulosus* Dubois, 1992 (synonyms: *Rana tigerina rugulosa* Fang and Chang, 1931, *Rana rugulosa* Wiegmann, 1835) is a common amphibian found throughout Southeast Asia. It is one of the major food resources providing protein to local people. During the reproductive cycle of *H. rugulosus*, the oogenesis is completed at the age of 12 months[1]. It is a seasonal breeder under both natural and farmed conditions[2]. The breeding season of these frogs occurs between May and October (the rainy season), when the plasma gonadal steroid levels increase[2,3]. This period is followed by a hibernation period between November and April (the dry season) when the frog activity and food intake decrease.

Control of the reproduction in this species is the same as in other vertebrates. There are two types of oocytes in the frog ovaries: nonvitellogenic oocytes (stages I and II) and vitellogenic oocytes (stages III–VI). It was reported that ovaries of 2–4-month-old *R. tigerina* contain only stage I oocytes, whereas the ovaries of 12-month-old frogs contain oocytes of all stages, which indicate the maturity of the female frogs[1]. Growth and maintenance of vitellogenic oocytes are regulated by the hypothalamus-pituitary-gonadal-liver axis[4]. Vitellogenin (Vtg), a precursor of the yolk proteins lipovitellin and phosvitin, is synthesized in the liver of female oviparous vertebrates[5]. It is well known that estrogen stimulates vitellogenesis in liver cells[6]. Moreover, the expression pattern of 17β-estradiol (E_2) matches that of the plasma vitellogenin protein levels[7,8]. In adult female *R. esculenta*, the ovarian level of E_2 increases during follicle growth and decreases during spawning[8]. Treatment of ovariectomized female frogs with E_2 increases the Vtg levels in the plasma[7]. In *R. temporaria*, exposure of hepatocytes to both estrone and E_2 increases Vtg synthesis in a dose-dependent manner[9].

As mentioned above, E_2 stimulates vitellogenesis as well as *vtg* gene expression in sexually mature

female frogs. It is therefore of interest to know if the administration of exogenous E_2 can stimulate vitellogenesis in immature (5-month-old) female frogs whose oocytes are known to be mainly in the non-vitellinogenic stage. In addition, we also want to know if the expression of the *vtg* gene in immature female frogs differs between the rainy (or breeding) season and the dry (or hibernation) season after estrogen treatment.

MATERIALS AND METHODS

Animals

Colonies of *H. rugulosus* frogs were bred and reared in the frog farm at the Huai Sai Royal Development Study Centre, Petchaburi Province. They were reared in a 2.0 m $\times$ 2.5 m $\times$ 1.0 m concrete tank containing water at a constant depth of 10 cm. The water was changed every 2 days. Frogs were fed with frog chow twice daily (5% of kg body weight) and kept in a natural (outdoor) environment (average temperature: 25–29 °C, RH: 69–81%). The natural daily light and dark cycles were approximately 12 h each.

Four-month-old frogs were randomly sampled from the colonies in October and March. They were transferred from the farm to the animal facility at the Department of Biology, Chulalongkorn University, and housed under essentially the same conditions as in the farm for 1 month prior to the start of the experiments. At the age of 5 months, when the female frogs can be distinguished from male frogs by their larger size and absence of vocal sac, healthy female frogs (28 frogs in each season) were randomly collected.

Treatment schedule

The 28 female frogs were allocated to one of the 4 groups and were given daily intramuscular injections of E_2 (Fluka) at doses of 0, 50, 500, or 5000 µg/kg body weight (hereafter abbreviated as E-0, E-50, E-500, and E-5000, respectively) for 5 days[10]. After the 5-day treatment, the frogs were weighed and humanely sacrificed by quick decapitation using a guillotine. The liver and ovary were dissected out, weighed, and fixed in 10% neutral buffered formalin solution. A portion of the liver was immediately transferred to a tissue stabilizing solution (Ambion) and kept at -20 °C for subsequent RNA extraction for determination of *vtg* mRNA levels.

Isolation of total RNA

The excised liver tissues from 7 frogs in each treatment group were pooled. Then 100 mg of liver tissue was frozen and homogenized under liquid nitrogen in a mortar. Total RNA was isolated by the SV total RNA isolation system (Promega), quantified by a spectrophotometer, electrophoresed through a 1% (w/v) formaldehyde MOPS-agarose denaturing gel, stained with 2 µg/ml of ethidium bromide, and visualized under a UV transilluminator. The purity of the product was determined from the ratio of the absorbance at 260 and 280 nm.

Reverse transcription PCR

The amplification of the *vtg* gene from *H. rugulosus* using the primers previously designed for *Xenopus laevis*[11] gave no PCR product. We then designed new primers based on the consensus sequences derived from the alignment of *vtg* cDNA sequences of *X. laevis* (M18061, Y00354), *Oncorhynchus mykiss* (S82450), and *Gallus gallus* (M18060, X13607) deposited in the GenBank nucleotide database. However, the designed primers were unable to amplify DNA products for *H. rugulosus*. We therefore individually tested the primers designed for *vtg* sequences of *X. laevis*, *O. mykiss*, and *G. gallus*. We obtained PCR product from the primer designed for the *G. gallus vtg* sequence. The forward (vtgF) and reverse (vtgR) primers were 5′-CAAGGTCATTCGAGCAGACA-3′ and 5′-ACAGCTGGGAACCACGTATC-3′, respectively. An amplification product of 230 bp was expected. The gene coding for β-actin (*act*) was used as an internal reference amplification control (a house keeping gene). The primers for the *act* gene were designed from conserved sequences of *Engystomops pustulosus* (AY226144), *Hyla japonica* (AB092520), *R. lessonae* (AY272629, AY272627), and *R. catesbeiana* (AB094353). The sequences of the forward (ActF) and reverse (ActR) primers were 5′-GATCTGGCATCACACTTTCT-3′ and 5′-TGGGTGACACCATCACCAGA-3′, respectively. An amplification product of 212 bp was expected.

The RNA samples were reverse-transcribed and PCR-amplified using an Access Quick RT-PCR system according to the manufacturer's protocol (Promega). Two samples of total RNA (100 ng each) were used in each reaction. The first strand cDNA of *vtg* and *act* were synthesized at 48 °C for 45 min. Amplification was carried out in a PCR machine (Thermocycler: model 9700) using an initial denaturation step of 95 °C for 2 min followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s. The PCR amplification was concluded with an additional extension period at 68 °C for 7 min.

The PCR products were separated by electrophoresis in a 1.2% (w/v) agarose gel, and were stained with ethidium bromide. The DNA bands were visualized under a UV transilluminator and imaged using a gel documentation system (GeneGenius Classic). The intensity of each band from the *vtg* and *act* amplification products was measured by QUANTITY ONE (version 4.6.1). For each sample, the RT-PCR reaction was done twice and the intensity of each band was measured thrice.

Before using the designed primers for the amplification of the *vtg* and *act* genes in the *H. rugulosus* samples, the primers were first tested for contingency in a 12-month-old mature female *H. rugulosus*. To ensure that the PCR product obtained was the *vtg* gene, the PCR product was sequenced, and the sequences were aligned with the reported *vtg* sequences of *G. gallus* and *X. laevis* to determine sequence similarity. The designed primers were also tested to determine if they could amplify the *vtg* gene of *R. catesbeiana*, a species related to *H. rugulosus*[4]. The samples were taken from mature female *H. rugulosus* and *R. catesbeiana* collected during rainy season when many follicles were found in their ovaries.

Liver-somatic index and gonadosomatic index

During *vtg* synthesis, there is an increase in the liver weight of mature female oviparous fish[12,13]. The gonadosomatic index (GSI) could indicate the sexual maturity in *H. rugulosus* and *R. catesbeiana* frogs of both sexes[3]. The liver-somatic index (LSI) is defined as the percentage of the liver mass with respect to the body mass. Similarly, the GSI is defined as the percentage of ovary mass with respect to the body mass.

Plasma E_2 levels determination

Since E_2 plays an important role in vitellogenesis, it is of interest to know if the background levels of plasma E_2 in immature female *H. rugulosus* differ in the rainy and dry seasons. Plasma E_2 levels were measured by the established radioimmunoassay method of the World Health Organization after extraction of samples by diethyl ether[3,14]. The intra-assay and inter-assay coefficients of variation were 7.3% and 13.8%, respectively. The assay was done only in the E-0 treated frogs, because the exogenous estrogen could cross-react with the antibody of the radioimmunoassay system used. Therefore, the plasma E_2 levels measured in E-50, E-500, and E-5000 treated frogs are the levels of endogenous E_2 plus the exogenous E_2 treatment and cannot indicate changes of endogenous E_2 levels.

Data analysis

Differences of plasma E_2 levels, body weight, liver *vtg* gene expression, LSI, and GSI between seasons in E-0 treated frogs were tested by the independent-sample *t*-test. The comparison of the difference between the mean of treatments was analysed by ANOVA. The statistical significance was determined using a post-hoc LSD test.

RESULTS

RNA extraction and contingency of the vtg primers on *vtg* gene amplification

The quality of extracted RNA was found to be satisfactory since the 260 to 280 nm absorbance ratio was 1.79 and the 28S and 18S rRNA bands were visibly intact.

The contingency of the vtgF/vtgR primer pair was tested in mature female *H. rugulosus* and *R. catesbeiana* samples and the RT-PCR products of approximately 230 bp were obtained (Fig. 1). The result shows that the primers could amplify the *vtg* gene in mature female frogs of both species. Additionally, the intensity of the band obtained from the mature female *H. rugulosus* sample was higher than that of *R. catesbeiana*.

The obtained *vtg* nucleotide sequences of *H. rugulosus* were aligned with the reported *vtg* sequences of *G. gallus* and *X. laevis* with similarities of 53.5% and 47.8%, respectively. The *vtg* nucleotide sequence of *H. rugulosus* had only a 85.7% similarity to that of *R. catesbeiana*.

Seasonal variation

Body weight, LSI, GSI, and liver *vtg* gene expression in immature female *H. rugulosus* collected during the rainy season were significantly higher than those of frogs collected during the dry season (Table 1). The plasma E_2 level in the rainy season was somewhat higher than that of the dry season, but the difference

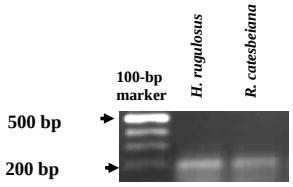

Fig. 1 RT-PCR amplification of *vtg* cDNA of *Hoplobatrachus rugulosus* and *Rana catesbeiana* using the vtgF/vtgR primer pairs.

Table 1 Comparison of various parameters obtained from immature female *H. rugulosus* collected during rainy and dry seasons.

Parameters	Rainy season	Dry season
Body weight (g) ($n = 7$)	195.7 ± 7.2	140 ± 21[a]
LSI ($n = 7$)	4.61 ± 0.36	2.22 ± 0.38[b]
GSI ($n = 7$)	7.2 ± 1.3	2.86 ± 0.99[a]
E_2 level (pg/ml) ($n = 7$)	330 ± 190	87 ± 24
Liver *vtg* gene expression ($n = 6$)	0.85 ± 0.03	0.65 ± 0.04[b]

[a] $p < 0.05$
[b] $p < 0.01$

was not significant ($p = 0.06$).

Effects of E_2 on liver *vtg* gene expression

During the rainy season, E_2 significantly decreased the relative *vtg* gene expression levels (E-50: $p < 0.05$; E-500, E-5000: $p < 0.01$) in immature female frogs (0.65 ± 0.02, 0.60 ± 0.02, and 0.58 ± 0.02 for the E-50, E-500, and E-5000 groups, respectively, compared to 0.85 ± 0.03 for the E-0 group; Fig. 2a). However, there was no significant difference in the relative *vtg* gene expression levels between the E-500 and E-5000 treatments.

During the dry season, E_2 at all doses tended to increase *vtg* gene expression levels in immature female frogs (0.68 ± 0.04, 0.78 ± 0.05, and 0.72 ± 0.04 for E-50, E-500, and E-5000 groups, respectively, compared to 0.65 ± 0.04 for the E-0 group; Fig. 2b). However, the increase in *vtg* levels was independent of the doses of the E_2 treatment. No significant difference was observed ($p > 0.05$) between the E-50, E-500, and E-5000 treatments.

Effects of E_2 on body weight, LSI, and GSI

During the rainy season, the body weight of the immature female frogs treated with different doses of E_2 was not significantly different ($p > 0.05$) among treatment groups, except for the E-500 group which was significantly lower than that of the E-0 group ($p < 0.05$) (Fig. 3). Neither the LSI nor the GSI of the immature female frogs treated with different doses of E_2 was significantly different ($p > 0.05$) among the treatment groups, despite the fact that the LSI increased in a dose-dependent manner (Fig. 3).

During the dry season, the body weight of the immature female frogs treated with 50–5000 µg/kg body weight of E_2 was not significantly different ($p > 0.05$) among the treatment groups. The LSI of the immature female frogs treated with different doses of E_2 tended to increase with dose, but this was not significant ($p > 0.05$), except for the E-500 group which was significantly higher than that of the E-0 group ($p < 0.05$) (Fig. 3).

The GSI of the immature female frogs treated with different doses of E_2 during the dry season showed no significant difference ($p > 0.05$) among the groups, except for the E-50 group which was significantly higher than those of the other groups ($p < 0.05$) (Fig. 3).

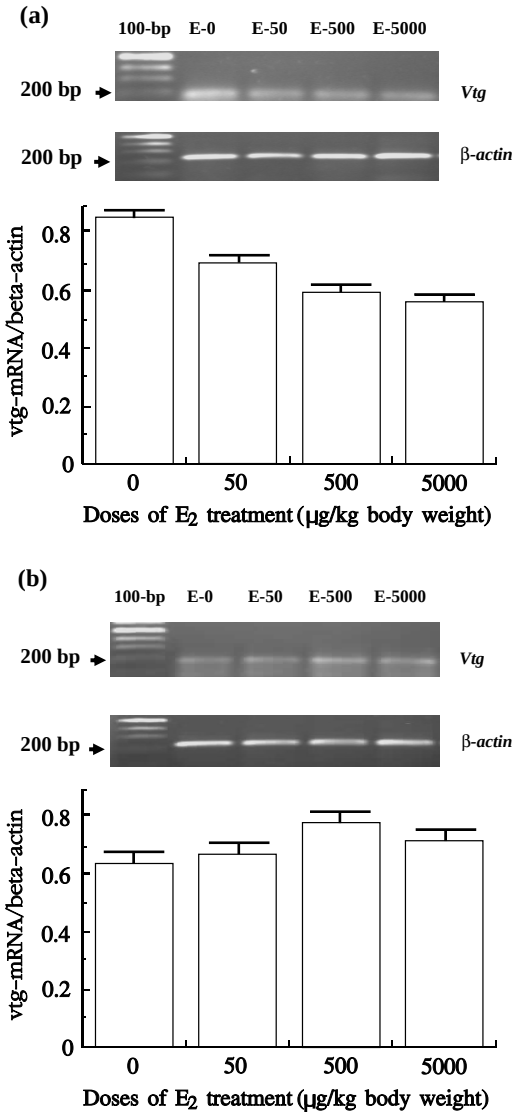

Fig. 2 Vitellogenin (*vtg*) gene expression levels in immature female *H. rugulosus* treated with E_2 during (a) rainy season (b) dry season. Data presented as mean ± SEM.

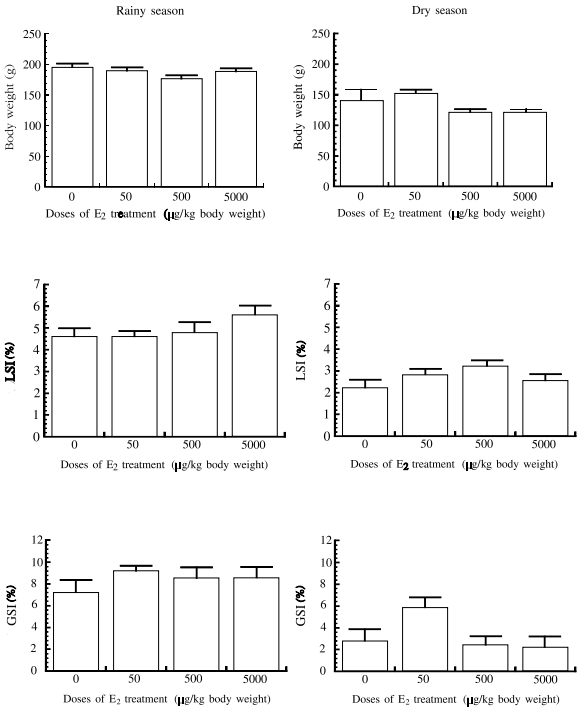

Fig. 3 Body weight, LSI, and GSI in immature female frogs treated with E_2.

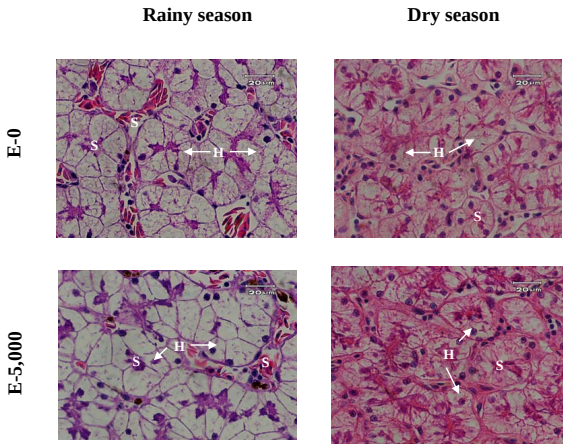

Fig. 4 Effect of season and E_2 dose on histological structure of frog liver (stained with hematoxilin-eosin). H = hepatocytes, S = sinusoid.

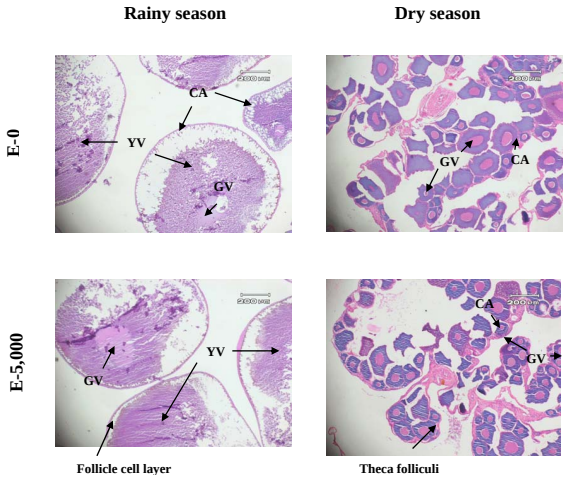

Fig. 5 Effect of season and E_2 dose on histological structure of frog gonad (stained with hematoxilin-eosin). CA = cortical alveoli, GV = germinal vesicle, YV = yolk vesicle.

Effects of E_2 on histology of the liver and ovary

The E-50, E-500, and E-5000 treatments had no effect on the histology of the liver and ovary of immature female frogs during either season. However, comparison of the liver and ovary of E-0 treated frogs between rainy and dry season revealed some structural differences. During the rainy season, the hepatocytes were arranged in strands and vacuolated (Fig. 4). Sinusoids entered the central vein in several places. The central vein was supported by a small amount of connective tissue and lined by endothelial cells. Flattened endothelial cell nuclei appeared in the central vein and sinusoids. During dry season, the hepatocytes were arranged in a similar fashion but were smaller and flatter.

Histological analysis of frog ovaries during the rainy season indicated a late vitellogenesis stage (Fig. 5). The vitellogenic gonad had yolk vesicles. Previtellogenic cells were characterized by a germinal vesicle and cortical alveoli. A thin layer of flat follicle cells surrounded the oocytes. Egg yolk granule filled almost all spaces outside the nucleus, with only a little cytoplasm surrounding the nucleus. The nucleus showed a wavy edge with a few nucleoli inserted in the troughs and most of them located near the centre. During the dry season, frog ovaries consisted of previtellogenic cells (Fig. 5). The theca folliculi was intact. The oocytes showed a heavily basophilic (opaque) cytoplasm, with the germinal vesicle occupying a large portion of its surface, and cortical alveoli in only some of them. Unexpectedly, 3 of the 7 frogs in E-50 group collected during the dry season had small follicles in their ovaries. This might explain why the GSI in this group increased significantly after the E_2 treatment.

DISCUSSION

We have shown that the *vtg*F/*vtg*R primers designed from the *vtg* sequence of domestic chicken could be used to amplify the *vtg* gene of *H. rugulosus* as well as *R. catesbeiana*, despite there being only 53.5% similarity of *vtg* nucleotide sequences between *H. rugulosus* and *G. gallus*. The *vtg* gene of *H. rugulosus* was found to be evolutionarily far (47.8% similarity) from that of *X. laevis* which belongs to a different family, but close (85.7% similarity) to that of *R. catesbeiana* which belongs to a closely related genus[4].

As expected, we found that the plasma E_2 level in immature female *H. rugulosus* collected during the rainy (breeding) season was higher than that of frogs collected during the dry (hibernation) season which is similar to those of mature female *H. rugulosus*[3]. On the other hand, we found that injecting exogenous E_2 significantly decreased the level of hepatic *vtg* gene expression in immature female *H. rugulosus* during the rainy season, in contrast to the finding that E_2 stimulates *vtg* gene expression in mature frogs[15–17]. When different stage animals are used, there are also different responses of E_2 to vitellogenesis[18,19]. Ng et al[18] found that the ontogenic competence of embryonic liver to respond to the first exposure to E_2, in terms of activation of transcription of this multigene family, is acquired late in metamorphosis at around Nieuwkoop-Faber stage 58. Exposure of tadpoles to triiodothyronin hormone (10^{-9} M) at the metamorphic climax (stages 60–64) but not mid-metamorphosis (stages 56–58) enhances and accelerates the precocious activation of the silent vitellogenin genes by E_2 treatment[19]. Mature female liver of *R. esculenta* cultured with 1 nM of E_2 increased *vtg* mRNA expression levels in frogs from both prereproductive and reproductive periods[20]. Exposure of *R. temporaria* hepatocytes to both estrone and E_2 increased *vtg* mRNA synthesis in a dose-dependent manner[9]. However, exogenous xenoestrogens and estrogen-mimic-containing sewage increased *vtg* mRNA expression levels in immature female *X. laevis*[21]. These different responses of *vtg* gene expression after E_2 injections are likely to be due to the different developmental stages of the animals assayed (immature or adult), the different experimental procedures used (in vitro or in vivo assays), or the differences among frog species.

The data shown here indicate that the injection of exogenous E_2 into immature female frogs during the two seasons seemed to have opposite effects, with decreased expression during the rainy season and a tendency for increased expression during the dry season. Thus, immature female frogs reared during the dry season should be more responsive to E_2 for inducing *vtg* mRNA production compared to those reared during the rainy season. The endogenous hormone levels seem to be one of the factors that can cause differences in the sensitivity to exogenous endocrine active substances. When exogenous hormones are administered to animals having different concentrations of the endogenous hormone(s) in the blood, the effects of the administered hormone would be expected to be higher in an animal with low levels of hormone than in the one with high levels due to competition between the exogenous and endogenous hormone for binding to the receptor[22]. The results obtained in this study may therefore be due to the fact that exogenous E_2 might interfere with the endogenous level of E_2 resulting in decreased *vtg* mRNA expression in immature females reared during the rainy season. On the other hand, exogenous E_2 might down-regulate the expression of the estrogen receptor[23–25]. However, the reasons why the *vtg* gene expression in frogs studied during dry season tended to increase after E_2 treatment, whereas those of frogs studied during rainy season were decreased, need further investigation.

In agreement with the increase in E_2 level and the body weight, the LSI and GSI of frogs collected during rainy season (breeding season) were higher than those of frogs collected during the dry season (non-breeding season)[3,7,8]. The data from our experiments showed that the LSI and GSI obtained in E_2 treated frogs reared during the rainy season showed no significant differences, though *vtg* gene expression was decreased. Moreover, during the dry season, only frogs treated with E_2 at a dose of 500 µg/kg body weight showed a significant increase of LSI. These results are consistent with an increase in *vtg* gene expression. Vitellogenin is normally produced in the liver of mature oviparous animals in response to E_2 treatment[26]. Consequently, the liver weight increases during *vtg* synthesis[12,13]. The *vtg* is then transported via the bloodstream to the ovary where it is incorporated into the developing oocyte. As a result, the gonad weight increases. Thus, mature females normally have a higher LSI and GSI than immature females[27]. Immature female squirrelfish (*Holocentrus adscensionis*) intraperitoneally injected with 5 mg/kg body weight of E_2 for 4 days showed an increase in LSI due to increased *vtg* protein production, but no increase in GSI which was attributed to the short duration of the treatment[27]. This interpretation was confirmed by another study. Male leopard frogs, *R. pipiens*, implanted with silastic capsules containing

E_2 for 20 days showed no correlation between GSI and the size of GnRH neurons[28]. It was suggested that change in GSI was only manifested after being exposed to elevated steroid hormone levels for prolonged periods. It is therefore to be expected that no changes in organ levels (LSI and GSI) will be observed when changes in cellular levels (liver and ovarian histology) cannot be detected in our study. Thus, the incongruence between the changes in LSI and GSI and changes in the *vtg* gene expression levels in immature female *H. rugulosus* frog treated with E_2 for 5 days might be due to the short treatment period, the season (or environmental factors), or susceptibility of the animals to E_2 treatment. Because increases in LSI (especially for the E-500 treated frogs) and GSI (especially for 3 of the E-50 treated frogs) are correlated with increases in *vtg* gene expression only during the dry season, the LSI and GSI would not be good indicators for vitellogenesis in immature female *H. rugulosus*. In addition, the increase in liver and gonad size can also be caused by other factors such as hyperplasia or hypertrophy of the cells.

From our study, it may be concluded that injection of exogenous E_2 into immature female *H. rugulosus* may stimulate vitellogenesis when the administration is done during the dry (hibernation) season. This knowledge could be applied to induce precocious maturation or the mating out of the breeding season in *H. rugulosus*. To better understand the mechanisms, however, further studies are required. In particular, studies using greater variation of E_2 doses between 0 and 500 µg/kg body weight (because the increase of GSI and LSI can be seen in E-50 and E-500, respectively) are needed along with studies that increase the duration of treatment and use frogs with different developmental stages (from 5 to 12-month-old) in early, middle, or late dry seasons.

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