ผลของ 17 เบตา-เอสตราไดออล ต่อการเหนี่ยวนำให้เกิดการสร้างไวเทลโลเจนิน ในกบนาเพศเมียและเพศผู้ตัวไม่เต็มวัย

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECT OF 17 β - ESTRADIOL ON INDUCTION OF VITELLOGENESIS IN IMMATURE FEMALE AND MALE FROGS, *Hoplobatrachus rugulosus*

Miss Phunee Ratanasaeng

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Thesis Title	EFFECT OF 17β- ESTRADIOL ON INDUCTION OF VITELLOGENESIS IN IMMATURE FEMALE AND MALE FROGS, *Hoplobatrachus rugulosus*
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วัตถุประสงค์ของงานวิจัยคือ ศึกษาผลของ 17เบตา - เอสตราไดออล (E_2) ต่อการสร้างไวเทลโลเจนินในกบนา *Hoplobatrachus rugulosus* ไม่เต็มวัยอายุ 5 เดือน ซึ่งเพาะเลี้ยงในฤดูฝน (มิถุนายน-ตุลาคม) และฤดูแล้ง (พฤศจิกายน-มีนาคม) โดยฉีด E_2 ที่ขนาด E50, E500 หรือ E5,000 ไมโครกรัมต่อน้ำหนักตัว เข้ากล้ามเนื้อเป็นเวลา 5 วัน แล้วเก็บตัวอย่างเพื่อตรวจหาการแสดงออกของยีนไวเทลโลเจนิน ปริมาณฟอสโฟโปรตีน (ตัวเริ่มต้นของไวเทลโลเจนิน) ในพลาสมา จำนวนและขนาดของไข่ เปอร์เซ็นต์น้ำหนักตับต่อน้ำหนักตัว เปอร์เซ็นต์น้ำหนักอวัยวะสืบพันธุ์ต่อน้ำหนักตัว และลักษณะทางจุลกายวิภาคของตับและรังไข่ ทำการสกัด RNA จากตับแล้วเพิ่มปริมาณของยีนไวเทลโลเจนินด้วยปฏิกิริยา อาร์ที-ลูกโซ่พอลิเมอเรส โดยใช้ไพรเมอร์ที่ออกแบบจากส่วน cDNA ของไก่ *Gallus gallus* ภายใต้ภาวะที่เหมาะสม

ผลการทดลองพบว่า E_2 ทุกขนาดมีผลทำให้การแสดงออกของยีนไวเทลโลเจนิน ของกบนาเพศเมียที่เพาะเลี้ยงในฤดูฝน ลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) (0.65 ± 0.02, 0.60 ± 0.02 และ 0.58 ± 0.02 ตามลำดับ) เมื่อเปรียบเทียบกับกลุ่มควบคุม (0.85 ± 0.03) และ E500 เท่านั้นที่มีผลทำให้การแสดงออกของยีนไวเทลโลเจนิน ของกบนาเพศผู้เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเทียบกับกลุ่มควบคุม (0.80 ± 0.06 เทียบกับ 0.61 ± 0.04) เมื่อฉีด E_2 ให้แก่กบนาไม่เต็มวัยที่เพาะเลี้ยงในฤดูแล้ง พบว่า E_2 ทุกขนาดที่ให้มีแนวโน้มทำให้การแสดงออกของยีนไวเทลโลเจนินเพิ่มขึ้นทั้งในเพศเมีย (0.68 ± 0.04, 0.78 ± 0.05, 0.72 ± 0.04 เทียบกับกลุ่มควบคุม 0.65 ± 0.04) และเพศผู้ (0.70 ± 0.06, 0.72 ± 0.05, 0.73 ± 0.03 เทียบกับกลุ่มควบคุม 0.68 ± 0.04) จากเทคนิควิธีการย้อมฟอสโฟโปรตีน พบว่า E_2 สามารถเพิ่มปริมาณพลาสมาฟอสโฟโปรตีนทั้งในเพศเมียและเพศผู้ ที่ถูกเพาะเลี้ยงในทั้งสองฤดูกาล E_2 ทุกขนาดที่ให้ ไม่มีผลต่อจำนวนและขนาดของไข่ เปอร์เซ็นต์น้ำหนักตับต่อน้ำหนักตัว เปอร์เซ็นต์น้ำหนักอวัยวะสืบพันธุ์ต่อน้ำหนักตัว และการเปลี่ยนแปลงลักษณะทางจุลกายวิภาคของตับและไข่ และพบว่าระดับ E_2 ในพลาสมาของกบนาไม่เต็มวัยเพศเมียที่เพาะเลี้ยงในฤดูฝน (327.24 ± 188.61 พิโคกรัมต่อมิลลิลิตร) สูงกว่า ระดับ E_2 เพศเมียที่เพาะเลี้ยงในฤดูแล้ง (87.33 ± 24.18 พิโคกรัมต่อมิลลิลิตร) และเพศผู้ไม่เต็มวัยที่เพาะเลี้ยงในทั้งสองฤดู (17.59 ± 4.47 และ 68.03 ± 23.81 พิโคกรัมต่อมิลลิลิตร)

จากผลการทดลองสรุปได้ว่า ไพรเมอร์ที่ออกแบบจากส่วน cDNA ของ *Gallus gallus* สามารถนำมาเพิ่มปริมาณของยีนไวเทลโลเจนินในกบนาได้ ซึ่งแสดงว่าส่วนของยีนนี้เป็นส่วนอนุรักษ์ในสัตว์มีกระดูกสันหลัง และการที่ E_2 ยับยั้งการแสดงออกของยีนไวเทลโลเจนิน ของกบในฤดูฝนที่มีระดับ E_2 สูง จึงอาจเป็นไปได้ว่าเมื่อกบเหล่านี้ได้รับ E_2 เพิ่มจากสิ่งแวดล้อมข้างนอก อาจทำให้ไม่สามารถสืบพันธุ์ตามปกติได้

สาขาวิชา สรีรวิทยา (สหสาขาวิชา)
ปีการศึกษา 2550

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4589700020: MAJOR PHYSIOLOGY
KEY WORD: Estrogens / Vitellogenin / Vitellogenesis / Frog / *Hoplobatrachus rugulosus*
PHUNEE RATANASAENG: EFFECT OF 17ß- ESTRADIOL ON INDUCTION OF VITELLOGENESIS IN IMMATURE FEMALE AND MALE FROGS, *Hoplobatrachus rugulosus*. THESIS ADVISOR: ASSOC. PROF. PRAKONG TANGPRAPRUTGUL, Ph. D., THESIS COADVISOR : ASSIST. PROF. CHANPEN CHANCHAO, Ph. D. AND ASSOC. PROF. PUTSATEE PARIYANONTH, M.Sc., 102 pp.

The objectives of the study were to elucidate the effect of 17ß-estradiol (E_2) on vitellogenesis in immature frogs, *Hoplobatrachus rugulosus (H. rugulosus)* which reared in the rainy season (June-October) and the dry season (November-March). Female and male frogs at the age of 5 months old of each season were daily intramuscularly injected with E_2 at a dose of 0, 50, 500 or 5,000 µg/kg body weight for five days. Liver vitellogenin (*Vtg*) gene expression, plasma phosphoprotein (precursor of Vtg), the number and size of oocytes, liver-somatic index (LSI), gonadal-somatic index (GIS) as well as histology of the liver and gonad were determined.

Results obtained showed that primers of *Vtg* gene sequence from the *Vtg* gene sequence of the chicken, *Gallus gallus* were used for RT-PCR amplification of *H. rugulosus* liver total RNA. Sequence of the derived RT-PCR amplicons and sequence similarity suggested that it was a *Vtg* gene of *H. rugulosus*. The expression level of liver *Vtg* gene was determined by semi-quantitative RT-PCR. Treatment with E_2 in frogs which reared during rainy season within the dose range studied significantly decreased the *Vtg* gene expression levels in immature female frogs (0.65 ± 0.02, 0.60 ± 0.02 and 0.58 ± 0.02 vs 0.85 ± 0.03, respectively). E_2 at dose of 500 µg/kg body weight significantly increased the level of *Vtg* gene expression (0.80 ± 0.06) in immature male frog compared to control group (0.61 ± 0.04). In contrast, female frogs reared during dry season, treated with E_2 at all doses tended to increase the *Vtg* gene expression levels (0.68 ± 0.04, 0.78 ± 0.05 and 0.72 ± 0.04 vs 0.65 ± 0.04). Similar results were obtained from the immature male frog (0.70 ± 0.06, 0.72 ± 0.05 and 0.73 ± 0.03 vs 0.68 ± 0.04). Phosphoprotein staining showed that E_2 could stimulate plasma phosphoprotein levels in both immature female and male frogs which reared in both seasons. There were no changes in the number and size of oocytes, LSI and GSI as well as in the histology of the liver and oocytes in the immature female frogs which reared during rainy season were observed. Results obtained might be due to the short period of treatment (5 days). When plasma E_2 levels were measured, it was found that the levels of E_2 in immature female reared in the rainy season (327.24 ± 188.61 pg/ml) were higher than the female frog reared in the dry season (87.33 ± 24.18 pg/ml) and than the E_2 levels found in immature male in both seasons (17.59 ± 4.47 and 68.03 ± 23.81 pg/ml).

It is therefore concluded that the primers designed from part of cDNA sequence of vitellogenin gene of *Gallus gallus* was able to amplify a fragment of at least one of the *Vtg* genes in the frogs, *H. rugulosus* which confirmed that this part of the gene is conserved among vertebrates. Since E_2 inhibited *Vtg* gene expression in frogs reared in the rainy season when the endogenous E_2 was high, hence it is possible that frogs with high levels of endogenous E_2 may be interfered with exogenous E_2 such as xenoestrogens in the environment resulted in impairment of reproduction in the frog.

Department: Inter-department
Field of study: Physiology
Academic year 2007

Student's signature.....
Advisor's signature.....
Co-advisor's signature.....
Co-advisor's signature.....

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LIST OF ABBREVIATIONS

ACN	Acetronitrile
APS	Ammonium persulfate
Bis	*N, N'*-methylenebisacrylamide
Bp	Base pair
^{o}C	Degree celcius
CA	Cortical alveoli
cDNA	Chromosomal DNA
DNA	Deoxyribonucleic Acid
E_1	Estrone
E_2	17-β estradiol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERE	Estrogen responsive elements
FSH	Follicle stimulating hormone
g	Gram
GnRH	Gonadotropin releasing hormone
GSI	Gonadal-somatic index
GtHs	Pituitary gonadotropins
GV	Germinal vesicle
h	Hour
H	Hepatocytes
HPGL	Hypothalamus-pituitary gonadal-liver axis
kDa	Kilodalton
kg	Kilogram
LH	Luteinizing hormone
LSI	Liver-somatic index
µl	Microlitre
µg	Microgram
MALDI	Matrix Assisted Laser Desorption Ionization
min	Minute
M	Molar

mA	Milliampere
mg	Milligram
µM	Micromolar
ml	Millilitre
mm	Millimetre
mM	Millimolar
MOPS	3-(N -morpholino)-propanesulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
m/z	Mass per charge
nm	Nanometre
O.D.	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
pH	hydrogen potential
PMSF	Phenylmethylsulfonyl fluoride
RER	Rough endoplasmic reticulum
rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	Sinusoids
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide-gel electrophoresis
SEM	Standard error of mean
SHBGs	Sex hormone binding globulins
TBE	Tris-Borate-EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
Tof	Time of flight
Tris	Tris(hydroxymethyl)-aminoethane
U	Unit (s)
UV	Ultra violet spectroscopy
V	Volt
Vtg	Vitellogenin

VtgF	Forward primer of Vtg
VtgR	Reverse primer of Vtg
VTGRs	Vitellogenin receptors
v/v	Volume by volume
w/v	Weight by volume
YV	Yolk vesicle
%	percentage

CHAPTER 1

INTRODUCTION

Reproductive behavior in most vertebrates is widely understood to be affected by the hypothalamo-pituitary gonadal axis. The change in environment, such as photoperiod and water temperature, provides signals that can be received by the central nervous system. These signals lead to oocyte growth and maturation that are regulated by pituitary gonadotropins and ovarian sex steroids. Vitellogenin (Vtg) is a precursor of the yolk proteins lipovitelline, phosvitin and β-component. Estrogens stimulate vitellogenesis in the liver cells (Denslow *et al*., 1999) of female oviparous vertebrates. Vtg level can serve as an indicator of female maturation because serum Vtg concentration rises markedly during oocyte growth (Matsubura *et al*., 1994).

Vitellogenin is generally being low in immature female. In males, the *Vtg* gene, although present, is normally not expressed. Detection of mRNA-levels of *Vtg* using semiquantitative RT-PCR showed a marked increase of *Vtg*-mRNA in juvenile *Xenopus laevis* (*X. laevis*), with increasing portions of estrogenic substance (sewage) (Bögi *et al*., 2003). One report has shown that zebrafish *zvtg1* and medaka *mvtg1* mRNAs were induced either by intramuscular injection of E_2 or immersion of fish in E_2 water (Tong *et al.*, 2004). In adult female green frogs, *Rana esculenta*, the levels of E_2 progressively increase during recovery period when many follicles in the ovary grow. The sex pattern matches that of plasma vitellogenin level (Di Fiore *et al*., 1998; Polzonetti-Magni *et al.*, 1998). The E_2 treatment of males, *X. laevis* resulted in increased synthesis of Vtg protein in the liver (van Wyk *et al*., 2003).

Rice field frogs, *Hoplobatrachus rugulosus*, are economic as one of main food sources in Thailand. They can be found throughout the country. It is a very good source of protein. Pariyanonth *et al*. (1985) have shown that it exhibits seasonal breeding, both under natural and farmed conditions. In 1996, Tangpraprutgul *et al*. reported that plasma gonadal steroids markedly increased during breeding season (May to October) in adult rice field frogs both females and males reared under farmed conditions. The data implied that sexual maturity is in the breeding season. They

reach sexual maturity at the age of 6 and 12 months for males and females, respectively (Sretarugsa *et al*., 1997; 2001).

As mentioned above, E_2 stimulates vitellogenesis as well as *Vtg* gene expression in the adults of a diverse array of species including sexually mature frogs. At present, vitellogenesis in the rice field frog has not been reported and it is unclear whether E_2 can induce vitellogenesis in the immature female and male frogs. Moreover, it is of interest to investigate of exogenous E_2 interfere the *Vtg* gene expression in those immature frogs.

The objectives were to:

1. Investigate *Vtg* gene expression in immature and mature frog
2. Determine effect of E_2 on the following parameters in immature female and male frogs which were reared in different seasons, rainy season and dry season:
    2.1 *Vtg* gene expression
    2.2 plasma phosphoprotein levels
    2.3 number and size of oocytes
    2.4 liver-somatic index (LSI) and gonadal-somatic index (GSI)
    2.5 histology of liver and gonad
3. Investigate partial sequence of *Vtg* gene in *H. rugulosus*

CHAPTER 2

LITERATURE REVIEWS

2.1 Rice field frogs (*Hoplobatrachus rugulosus*)

Rice field frogs are amphibians classified in

Kingdom Animalia

Phylum Chordata

Subphylum Vertebrata

Class Amphibian

Order Anura

Family Ranidae

Genus *Hoplobatrachus*

Species *rugulosus*

Rice field frogs, *Hoplobatrachus rugulosus* Dubois, 1992 are a common amphibians found throughout Southeast Asia such as Thailand, Cambodia, Laos, Malaysia, Myanmar, Philippines, etc. These animals play very important role as predator in an ecosystem. Moreover, they are one of main food sources providing protein. The frogs spend most of their lives in aquatic habitats but can also hunt for food and shelter on dry ground. Life maturity is about 12 months in the females and 6 months in the males. Their characteristic were characterized by olive brown in color with numerous small black spots, nostril much nearer tip to snout than to eyes, lips with dark spots separated by black band. The mature female and male have body size about 85-125 mm and 70-100 mm, respectively. (http://www.amphibiaweb.org/aw/amphibian/facts.html).

2.2 Frog reproduction

In *H. rugulosus*, the oogenesis of female is completed at the age of 12 months whereas the spermatogenesis in male is completed at the age of 6 months. It has been observed that breeding season of these frogs is during a rainy season (between May and October) (Pariyanonth *et al*., 1985). This period is followed by hibernation period

(between November and April) which the frog decrease their activities and food intake. In 1996, Tangpraprutgul *et al.* confirmed that plasma gonadal steroids in both female and male frogs increased during the breeding season (approximately E1,000-2,000 and T1,500-3,000 pmol/L, respectively). Control of the reproduction in this species is the same as in other vertebrate species. There are 2 categories of oocytes in an amphibian ovary. First, the small oocytes (nonvitellogenin) is gonadotropin-independent, increase rapidly in metamorphosed female amphibian. In the resting stage, the oocyte diameter is about 0.3 mm. Second, the larger oocytes or vitellogenic oocytes is gonadotropin-dependent. Growth and maintenance of vitellogenic oocytes were regulated by hypothalamus-pituitary gonadal- liver (HPGL) axis (Lofts, 1974).

2.3 Life cycle of *Hoplobatrachus rugulosus*

Regarding the life cycle of *H. rugulosus*, there are 2 stages of development: embryonic development (1 day) and larval development (28-36 days), followed by brief metamorphic period, then a long, multiyear life on land. The fertilization is external and sexual. Fertilized female frogs will lay large amounts of eggs (jelly) under water. The adults return to the water to court and ultimately lay eggs. Fertilized eggs hatch within 18-28 h. Fertilized egg has round shape, then change to be oval shape and increase length continuously until head, body and tail part appear which look like small fish. About 10 days old, back legs sprout and front legs sprout about 3 days after. About 7-13 days after front legs sprout, their tail will disappear and become frog let (Nootprapan, 1992) (Fig. 2.1).

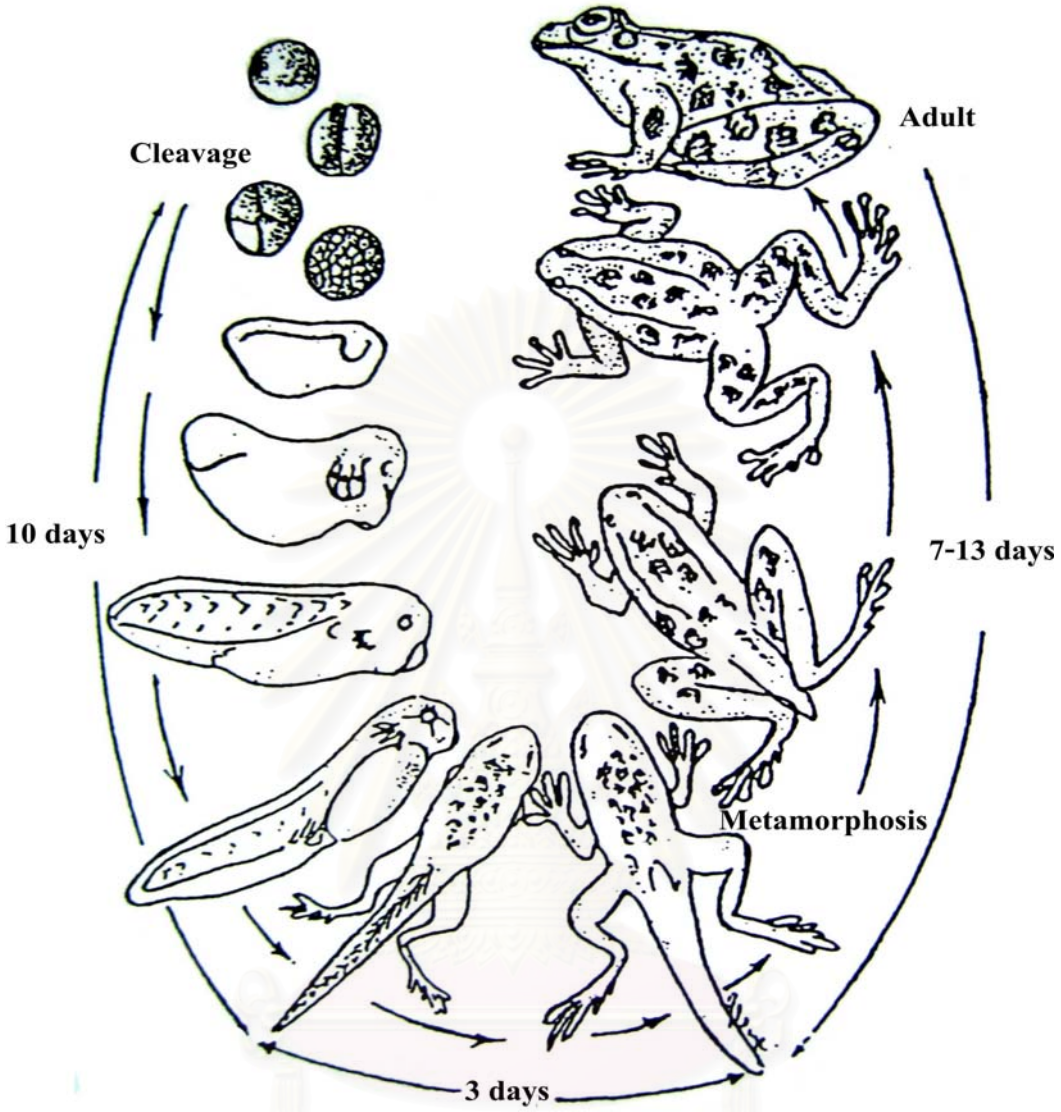


Figure 2.1 Life cycle of *Hoplobatrachus rugulosus* (modified from Nootprapan, 1992).

2.4 Vitellogenesis

Vitellogenin is defined as the precursor of the egg yolk protein. It has molecular weight about 250–600 kDa (according to species) and complex calcium-binding phospholipoglycoprotein. The classification of Vtg as phospholipoglycoprotein indicates the functional groups that are carried on the protein backbone of the molecule are lipids, carbohydrates, and phosphate groups (Mommsen

and Walsh, 1988; Silversand and Haux, 1995). Vtg helps improve fertility of the female frogs by facilitating growth and development of oocytes.

Vitellogenesis occurs in the liver cells of mature oviparous animal and is an estrogen-dependent. The environmental changes, such as water temperature and photoperiod, provide the signal to the central nervous system (Fig. 2.2). The hypothalamus secretes gonadotropin-releasing hormone (GnRH). As the central regulator of hormonal cascades, GnRH stimulates the release of pituitary gonadotropins (GtHs) from the pituitary. There are two GtHs (GtH I & II) structurally similar to human follicle-stimulating hormone (FSH) and luteinising hormone (LH), respectively. GtH I (FSH) is involved in vitellogenesis and zonagenesis, while GtH II (LH) plays a role in final oocyte maturation and ovulation. GtH secretion is regulated through a feedback mechanism by 17β- estradiol (E_2). Several feedback mechanisms regulate the gonadal development through the hypothalamus- pituitary-gonadal-liver axis, because these organs produce substances influencing each other that lead to gonadal development and spawning (Peter and Yu, 1997; Swanson, 1991).

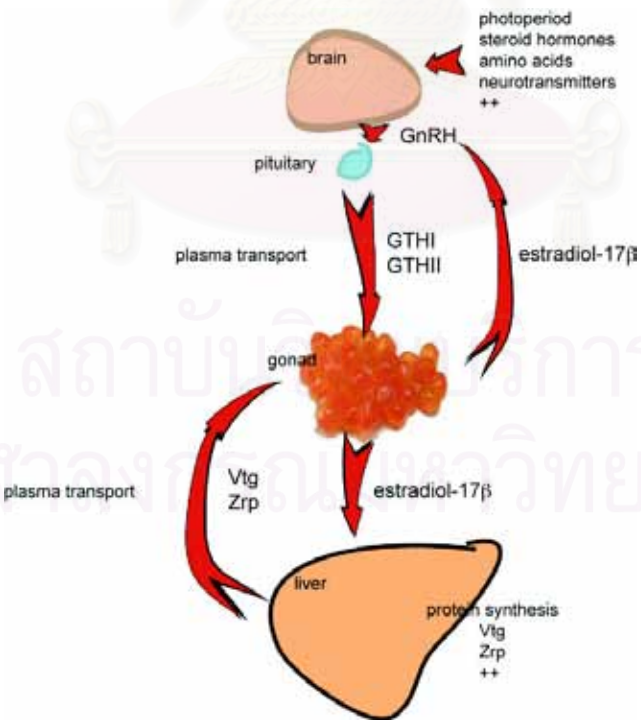


Figure 2.2. The hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in female teleosts. GtH = gonadotropin I & II.

A negative feedback system controls release of GnRH from the hypothalamus. Seasonal changes in gonadotropins and steroid levels in bullfrogs, *R. catesbeiana* do not exhibit a negative feedback relationship (Licht *et al*., 1983). However, dihydroxytestosterone may affect the hypothalamus negatively and the pituitary positively, while estrogen has only negative feedback activity (McCreery and Licht, 1984).

The gonadotropins in amphibians have structural similarities to mammalian gonadotropins. Release of the gonadotropins in amplibians is continuous rather than pulsatile, however, as in mammals (Hubbard and Licht, 1986). The specificity of biological action of FSH and LH in mammalian systems is not necessarily true of amphibians. Both hormones stimulate some physiological responses equally, including testes/ovarian growth, spermiation and estrogen secretion. However, LH and not FSH are required for ovulation, androgen secretion and ovarian progesterone secretion (Licht, 1986). Natural ovulation is induced by a “surge” of LH in the blood. Moreover, a concomitant peak in the blood levels of progesterone, compatible with its supposed role as the hormonal inducer of ovulation (McCreery and Licht, 1983).

Estrogens are important ovarian hormone of reproductive processes in animal. It is produced by the ovary and is believed that the thecal and granulose cells are the source of estrogen production during the follicular phase of the ovarian cycle. Estrogen is released into the blood and transported into the target cells by diffusion and binds with high affinity to the estrogen receptor (ER). There are two subtypes of the ER, the ERα and ERβ. Both receptors have a distinct tissue distribution and play a distinct role in physiology. The detailed mechanisms underpinning their mode of action are under intensive study estrogen receptors are very similar in fish and mammals (Le Roux *et al*., 1993; Pakdel *et al*., 1990), which explains why chemicals that act as estrogens do so throughout the vertebrates. The liver of fish, particularly female fish, contains high concentrations of estrogen receptors (Campbell *et al*., 1994; Pottinger, 1986), which accounts for its ability to synthesize large amounts of Vtg when stimulated by estrogen.

It has been reported that E_2 stimulates Vtg production in female fish (Tata and Smith, 1979; Hyllner *et al*., 1991) and frog (Di Fiore *et al*., 1998; Polzonetti-Magni *et al*., 1998). The molecular mechanisms that lead to Vtg production in the fish hepatocyte have been proposed by Arukwe and Goksøyr (2003) (Fig. 2.3). E_2 produced by the ovarian follicular cells is transported in plasma attached to sex hormone binding globulins (SHBGs) and enters the liver cells by either diffusion or receptor-mediated uptake. In the liver, E_2 binds to E_2-receptor and then the hormone-receptor complex binds tightly in the nucleus at estrogen responsive elements (ERE) located upstream of, or within the estrogen- responsive genes in DNA, resulting in the activation or enhanced transcription of *Vtg* genes and subsequent increase *Vtg* messenger RNA (mRNA). Vtg precursors are synthesized and modified extensively in the rough endoplasmic reticulum (RER). The modified Vtg is secreted into the serum for transport to the ovary.

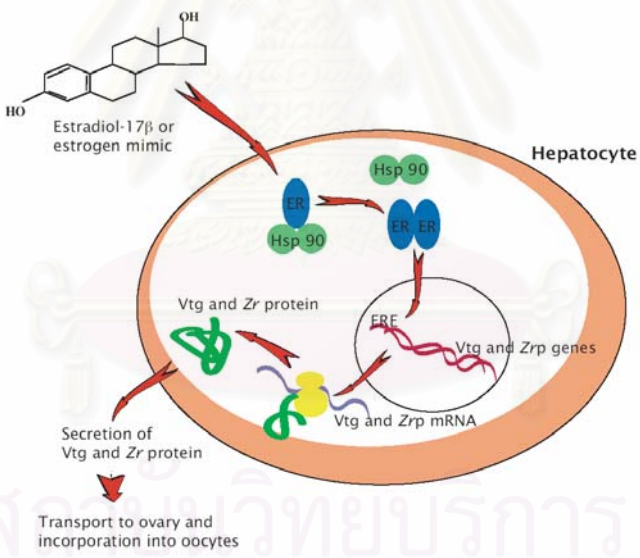


Figure 2.3. The molecular mechanisms that lead to the production of Vtg in the hepatocyte.

Modifications of post-translational Vtg protein occur prior to secretion into the systemic tracks. But the biochemical information of Vtg clearly indicates that the modification must occur in the liver cells. Several metabolic changes occur during Vtg synthesis such as increases in liver weight, RNA contents, lipid deposition, glycogen depletion, increases in plasma protein, calcium and magnesium and

phosphoprotein contents (Wiegand, 1982; Björnsson *et al*., 1986). These parameters can be used as indicators of plasma Vtg levels. The uptake of Vtg into the oocytes occurs by receptor-mediated endocytosis. Vtg receptors (VTGRs) have been identified in the ovary of fish species (Tyler and Lancaster, 1993; Tao, Berlinsky, and Sullivan, 1996), and was recently cloned and sequenced in rainbow trout and winter flounder (Prat *et al*., 1998; Perazzolo *et al*., 1999; Hiramatsu *et al*., 2002). The *X. laevis* VTGRs are 72% similar to the chicken very low-density lipoprotein receptor (VLDLR) (Okabayashi *et al*., 1996).

In amphibian and avian, Vtg is enzymatically cleaved into two major yolk proteins, lipovitellin and phosvitin. In addition to the two yolk proteins, the third yolk protein, β-component, has been identified in salmon and flounder. The immunological study has demonstrated that salmonid fish Vtg is incorporated into oocytes and then dissociated to three yolk components-lipovitellin, phosvitin and β – component (Fig. 2.4). It is also found that a cathepsin-D-like enzyme in the water soluble fraction of salmonid eggs was involved in the cleavage of Vtg into the yolk proteins (Sumpter and Jobling, 1995).

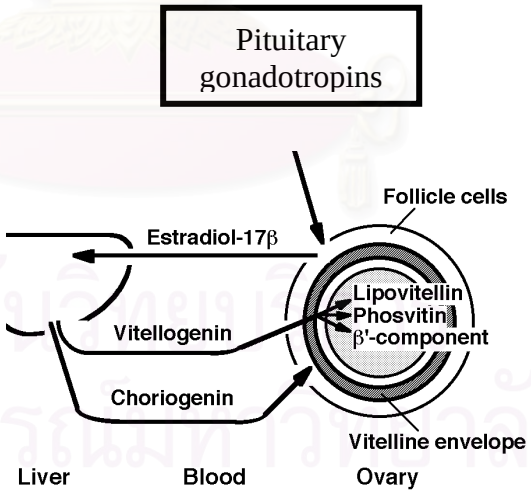


Figure 2.4. Simplified diagram of estradiol-17β (E_2) or E_2-mimic stimulated oogenic protein synthesis. The egg yolk protein precursor, Vtg are synthesized and secreted by the hepatocyte. They are transported in blood to the ovary and incorporated into maturing oocytes in female teleosts.

2.5 Hormonal control of yolk production

In the frog such as *X. laevis*, the leptotene stage of meiosis lasts only 3 to 7 days, zygotene takes from 5 to 9 days, and pachytene persists for roughly 3 weeks. The diplotene stage, however, can last for years. Even so, vitellogenesis occurs in only part of the diplotene, and the signal for the breakdown of the nucleus (the germinal vesicle) occurs after vitellogenesis is completed. The regulation of these events is controlled by the hormonal interactions between the hypothalamus, pituitary gland, and follicle cells of the ovary (Fig. 2.5).

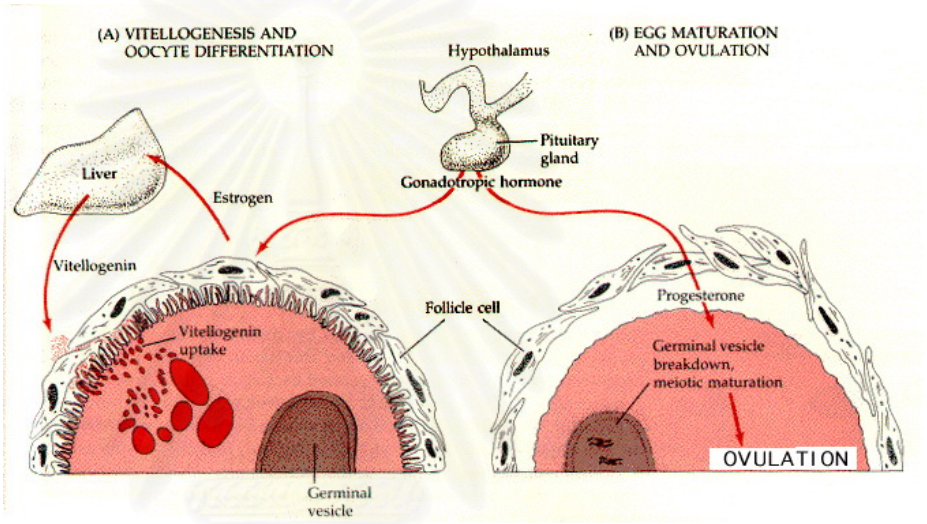


Figure 2.5. Control of amphibian oocyte growth and egg maturation by estrogen and progesterone. (A) Gonadotropic hormone stimulates the follicle cells to produce estrogen, which instructs the liver to secrete vitellogenin. This protein is absorbed by the oocyte. (B) After vitellogenesis, again under the influence of gonadotropic hormone, the follicle cells secrete progesterone. Within 6 hours of progesterone stimulation, the germinal vesicle breaks down, initiating the reactions leading to ovulation.

2.6 Stages of oocytes/follicles

Sretarugsa *et al*. (2001) have classified the stages of oocytes/follicles in the frog into six stages by using size, color and histology as follow:

Stage I oocyte: previtellogenic stage

Oocyte exhibits a translucent cytoplasm with a diameter ranging from 50-350 μm. The nucleus is clearly visible through the cytoplasm and occupies a large portion of the oocyte. At light microscopic level, the cytoplasm of the previtellogenic stage I oocyte appears heavily basophilic. In addition, it also acquires a smooth nuclear membrane and nucleoli of various sizes. In the late stage I oocyte, the cytoplasm stains paler when compared to the early stage.

Stage II oocyte: previtellogenic stage

This stage develops an opaque ring around its concentric nucleus. Its size ranges from 351-550 μm. Towards the end of this stage, the cytoplasm is almost completely opaque so that the nucleus becomes inconspicuous under a stereomicroscope. Histologically, the presence of a few rows of peripheral vacuoles (cortical alveoli) seems to be the most predominant characteristics of stage II oocyte. In addition, numerous nucleoli which vary in size can be observed in each cell.

Stage III oocyte: vitellogenic stage

The opacity is complete in the stage III oocyte as it appears intensely white. The diameter of stage III oocyte is 560-900 μm. Histologically, the number of vacuoles gradually increases, and they become dispersed towards the central area. Yolk platelets are formed and rapidly replace the central vacuoles. The vitelline envelope also becomes conspicuous under the follicle cells. Pigmented granules first appear in this stage and are located at the periphery of the oocyte. The nucleus of the stage III oocyte possesses a convoluted nuclear membrane and numerous nucleoli.

Stage IV oocyte: vitellogenic stage

The distinct morphological feature of the stage IV oocyte is the pigmentation of the surface as lightbrown to brown. The oocyte is 910-1300 μm in diameter. Yolk platelets completely replace the central vacuoles, while the remaining vacuoles are

located around the periphery of oocyte. The nucleus is surrounded by a highly convoluted nuclear membrane and contains a large number of nucleoli.

Stage V and VI oocytes: vitellogenic and fully grown stages

Distinct polarity occurs in the last two stages, ie, stage V (1310-1500 μm) and stage VI (1510-1700μm). This is manifested by the difference in pigmentation underneath the oolemma of the animal pole in contrast to the vegetal pole which contains large-yolk platelets instead; the nucleus also shifts to the animal pole. The vacuoles are decreased in number while the yolk accumulation increases. The animal pole in the stage VI oocyte has only one row of vacuoles on the periphery, whereas two or three rows of vacuoles are present in the vegetal pole.

2.7 The role of E_2 in vitellogenin gene expression

Vitellogenin gene has been reported in a diverse array of species across broad taxa such as frog (Germond *et al*., 1984), fish (Tong *et al*., 2004), insects (Sappington and Raikhel, 1998), and crustacea (Avarre *et al*., 2003; Tsutsui *et al*., 2005; Raviv *et al*., 2006). It is usually found as a multiple gene complex (e.g. Rina and Savakis, 1991; Sappington and Raikhel, 1998; Miracle *et al*., 2006), including frogs with at least four forms encoded for in *X. laevis* (Germond *et al*., 1984). These multiple copy vitellogenin gene complexes can include multiple copies of pseudogenes as well (Trichet *et al*., 2000). There are several reports about *Vtg*-mRNA expression in various species. Tong *et al*. (2004) reported *Vtg* mRNA induction by intramuscular injection of E_2 or immersion of fish in E_2 water in mature male zebrafish (*zvtgl*) and medaka (*mvtg1*). The time course of *zvtgl* mRNA induction is different from that of *mvtg1* mRNA induction. E_2-treated zebrafish increase of *zvtgl* mRNA and *mvtg1* mRNA in 100% was at 24 h and 3 h, respectively. After incubation of *X. laevis*' liver cell with E_2 at doses ranging from 10^{-10} to 10^{-5} M for 36 h, finding indicated that E_2 induced *Vtg* mRNA expression in dose-dependent manner and there were no differences concerning expression of internal control (elongation factor 1α) (Kloas *et al*., 1999). In addition, semi-quantitative RT-PCR revealed a marked increase of *Vtg* mRNA levels in juvenile *X. laevis* with increasing portions of xenoestrogens/estrogen-mimic containing sewage (Bögi *et al*., 2003). Treatment of mature female *Rana esculenta* (*R. esculenta*) frogs with E_2 increased liver *Vtg* mRNA

expression in frogs from both prereproductive and reproductive developmental periods (Carnevali *et al*., 1995).

2.8 The role of E_2 in plasma vitellogenin levels

In adult female green frogs, *R. esculenta* the levels of E_2 progressively increase during recovery period when many follicles in the ovary grow then decrease during spawning and post-reproductive period. The sex pattern matches that of plasma vitellogenin level (Di Fiore *et al*., 1998; Polzonetti-Magni *et al*., 1998). In adult males, the levels of E_2 and androgen also increase markedly during breeding period (Polzonetti-Magni *et al*., 1998). The E_2 treatment of males, *X. laevis* resulted in increased synthesis of Vtg protein in the liver (van Wyk *et al*., 2003). The E_2 treatment of ovariectomized female frogs resulted in increased Vtg level in plasma (Di Fiore *et al*., 1998). Exposure of hepatocytes of *R. temporaria* to both estrone (E_1) and E_2 increased Vtg synthesis in a dose-dependent way (Rankouhi *et al*., 2005). Vitellogenin is generally low in immature and in male carp (Matsumoto *et al*., 2002). The immunohistochemistry staining of Vtg of brown trout was detected in the estradiol-injected juvenile trout but not in uninjected controls (Wahli *et al*., 1998). Werawatgoompa *et al*. (2004) intramusculary injected their fish with E_2 at a dose 500 µg/kg body weight for 5 days for inducing plasma Vtg production.

There are several techniques used for determination of plasma Vtg level such as ELISA (Cellus and Walther, 1998), immunohistochemistry (Arukwe *et al*., 1999), and even immunodiffusion. Immunodiffusion is a basic technique involving diffusion of antigen or antibody through semisolid medium, usually agar or agarose gel, resulting in a precipitin reaction. Precipitin lines or bands form where the concentration of an antigen and antibody are serologically equivalent (Mendoza *et al*., 1986). Vtg assays based on polyclonal antibodies are restricted for using with the homologous species, but some antibodies do cross-react with Vtg in other species (Tyler and Lancaster, 1993; Nilsen *et al*., 1998). Werawatgoompa *et al*. (2004) have reported that antibody of red snapper and grouper could cross react to with Vtg of each other. Recently there was a report that Vtg could be stained with a commercially available fluropore dye (Pro-Q Diamond, Invitrogen), and visualized by ultraviolet transillumination in fish (Van Veld *et. al*., 2005) that described a universal assay that

is based on the high-molecular weight and extensive phosphoserine content of all Vtgs. However, this dye also detects phosphate groups attached to tyrosine or threonine residues. The signal is linear over three orders of magnitude and correlates with the number of phosphates and stained proteins can be accurately identified by mass spectrometry. Thus, it is a revolutionary new fluorescent method for detection of phosphoproteins in gels (http://www.med.uc.edu/proteomics/pro-q.htm).

2.9 The role of E_2 in liver-somatic index, gonadal-somatic index, number and size of oocytes

Vitellogenesis occurs in the liver cells of mature oviparous animal and it was stimulated by estrogen. Several metabolic changes occur during Vtg synthesis in the maturing female oviparous animals such as increases in liver weight, RNA contents, lipid deposition, glycogen depletion, plasma protein, calcium, magnesium as well as phosphoprotein contents (Wiegand, 1982; Björnsson *et al*., 1986). These parameters can be used as indicators of plasma Vtg levels. Some reports show that liver-somatic index [LSI; (liver mass (g)/body mass (g) x 100%)] and gonadal-somatic index [GSI; (gonad mass (g)/body mass (g) x 100%)] related to amount of estrogenic substances in fishes (Banks *et al*., 1999; Noaksson *et al*., 2005). Wiegand (1982) and Björnsson *et al* (1986) have shown that there was an increase in liver weight during Vtg synthesis in the mature female oviparous animals. Thus, LSI indicates vitellogenesis in these animals. GSI indicates sexual ability in frogs of both sexes (McCreery and Litcht, 1983; Tangpraprutgul *et al*., 1996). The egg size and histology of the fish gonad (http://www.fao.org) and several changes in hepatic morphology such as proliferation of rough endoplasmic reticulum and golgi apparatus of *X. laevis* liver cells (http://zygote.swarthmore.edu/germ1.html) have been reported as parameters that cause change to the metabolism. There are two categories of oocytes in an amphibian ovary; nonvitellogenic oocytes (stage I and II) and vitellogenic oocytes (stage III-VI). Vitellogenic oocytes are gonadotropin-dependent. Growth and maintenance of vitellogenic oocytes were regulated by hypothalamus-pituitary gonadal- liver (HPGL) axis (Lofts, 1974). There was a report that ovaries of two-to four-month-old frogs, *R. tigerina* contain only stage I oocytes, while the ovaries of twelve-month-old frogs contain oocytes of all stages, which indicate the maturity of female frogs (Sretarugsa *et al*., 2001).

During ovarian recrudescence, incorporation of oogenic proteins accounts for the major growth of the developing oocytes. An indirect measure of altered hepatic oogenic protein synthesis in fish exposed to xenobiotics (xenoestrogens) is reduced or increased GSI (Kime, 1995). There were several studies have been conducted to evaluate the impact of fish exposure to toxicants (xenobiotics/ xenoestrogens) on ovarian development. Several effects have been observed and these include inhibition of oocyte development and maturation, increased follicular atresia of both yolked and previtellogenic oocytes, abnormal yolk deposition and formation within oocytes, and abnormal egg maturation and production (Arukwe and Goksøyr, 1998; Kime, 1995; Susani 1986; Goksøyr *et al*., 2003).

The native rice field frog, *H. rugulosus* Dubois, 1992 (synonymes; *R. tigerina rugulosa* Fang and Chang, 1931, *R. rugulosa* Wiegmann, 1835), is distributed all over Thailand (Tayler, 1962), and exhibits seasonal breeding, both under natural and farmed conditions (Pariyanonth *et al*., 1985). They reach sexual maturity at the age of 6 and 12 months for males and females, respectively (Sretarugsa *et al*., 1997; 2001). Tangpraprutgul *et al*. (1996) noted that plasma gonadal steroids markedly increased during the breeding season (May to October) in both female and male adult frogs reared under farm conditions.

As mention above, E_2 stimulates vitellogenesis as well as *Vtg* gene expression in adults of a diverse array of species including sexually mature frogs. It is, therefore, interesting to know whether exogenous E_2 can stimulate vitellogenesis in immature frogs, *H. rugulosus*.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

Colonies of *Hoplobatrachus rugulosus* frogs were bred and reared in frog farm at the Huai Sai Royal Development Study Center in Petchaburi province. They were reared in a concrete tank of 2.0 x 2.5 x 1.0 m^3 containing water to a constant depth of 10 cm which was changed every two days (Fig. 3.1). Animals were fed with frog chow twice daily and kept in a natural (outdoor) environment with average ambient temperatures and relative humidity varied from 25.0^0-29.0^0C and 69-81% relative humidity (Chaitiamwong, 1995) throughout the year. The natural daily light and dark cycle were approximately 12 hours each, varying by up to 1 hour over the year. At the age of five-months-old female and male frogs can be distinguished by their size. The male is normally smaller than the female (Fig. 3.2).

Figure 3.1 The concrete tank that used for rearing frog in frog's farm at the Huai Sai Royal Development Study Center in Petchaburi province.

A

B

Figure 3.2 The 5 months old female (A) and male (B) *H. rugulosus*.

3.2 Chemicals and equipments

Chemicals

- Access Quick™ RT-PCR system (catalog # A1702), Promega, USA
- Acrylamide, Promega, USA
- Agarose, Research organics, USA
- Ammonium persulfate, Promega, USA

- Ammonium peroxydisulfate (APS), $(NH_4)_2S_2O_8$, BDH laboratory supplies, UK
- Ammonium sulfate $(NH_4)_2SO_4$, Merck, Germany
- Bromophenol blue, $C_{19}H_{10}Br_4O_5S$, M. W. = 670, BDH laboratory supplies, UK
- Double distilled water, GFL glass water, Germany
- 17-ß estradiol (catalog # 75262), Fluka, Germany
- Ethanol (C_2H_5OH), Thailand
- Formaldehyde, CH_2O, Thailand
- Glycerol, Asia pacific specialty chemicals, Ltd., Australia
- Heparin (5000 U/ml), Leo. USA
- Liquid N_2, Thai industrial gases public Co. Ltd., Thailand
- 2 - Mercaptoethanol, BDH laboratory supplies, UK
- Methanol, CH_3OH, Merck, Germany
- *N, N'* – methylene – bis - acrylamide, Sigma, USA
- PCR purification kit (catalog# 28140), Qiagen, Germany
- Phenylmethylsulfonyl fluoride (PMSF), $C_7H_7FO_2S$, Sigma, Germany
- Phosphoprotein molecular weight standard (catalog # P33350), Invitrogen, USA
- Pro-Q® Diamond phosphoprotein gel stain solution (catalog # P33301), Invitrogen, USA
- RNAlater (catalog # 7020), Ambion, Japan
- SV total RNA isolation system (catalog# Z3100), Promega, USA
- SYPRO® Ruby gel stains (catalog # S12001), Invitrogen, USA
- TEMED (N, N, N', N'- tetramethylenediamine), Amersham biosciences, Sweden
- Tris (hydroxyl methyl) - aminomethane, $NH_2C(CH_2OH)_3$, Promega, USA

Equipment

- Beta liquid scintillation counter, model: 1218-811, Wallac, Finland
- Centrifuge, model: Centrifuge 5410, Eppendorf, Germany
- Centrifuge, model: universal 32R, Hettich zentrifugen GmbH & Co. Kg, Germany

- Centrifuge, model: KR - 20000T; rotor: RA - 6, Kubota, Germany
- Cuvette (Quartz), type: 18/ Q/ 10, Starna, Optiglass Ltd., UK
- Hotplate, Schott, Germany
- Hybridizer, model: HybriLinker HL - 2000, UVP laboratory products, USA
- Microwave, model: Sharp carousel R7456, Sharp, Thailand
- Minishaker, model: MS1, Ika Works Inc., USA
- PCR, model: GeneAmp® PCR system 2400, Applied biosystems, Singapore
- pH meter, Denver instrument, model: 215, Denver instrument Co. Ltd., Taiwan
- Pipette: P10, P20, P200, and P1,000, Gilson, France
- Pipette tip; 10 µl, 200 µl, and 1,000 µl, Sorenson, USA
- Polaroid camera, model: Direct screen instant camera DS 34 H - 34, Peca products, UK
- Polaroid film, Fuji film, Japan
- Poly acrylamide gel electrophoresis model, model: AE - 6530 mPAGE, Atto corporation, Japan
- Power supply, EC 570 - 90 LVD CE, E - C Apparatus Corporation, USA
- Refrigerator centrifuge beckman coulter avanti – J - 30I, Kokusan H - 103N, Germany
- Sonicator, model: BHA - 1000, Branson, USA
- Stereomicroscope, Olympus optical Co. Ltd., Japan
- Vortex mixer, Vortex - genie 2, Scientific industries, USA

3.3 Experimental Design

Experiments were designed in order to study two sets of frogs from different seasons.

Set 1: frogs reared during rainy season (June-October)

Set 2: frogs reared during dry season (November-March)

Four-months-old (completion of metamorphosis designated as age 0) female and male frogs from each season were transferred from the farm rearing facilities to

the animal house at the Department of Biology, Faculty of Science, Chulalongkorn University, and housed under essentially the same conditions as in the farm, for one month prior to the start of the experiment.

Adult female and male *H. rugulosus* and adult female *R. catesbeiana* were used as positive control of *Vtg* gene expression.

Female and male frogs from each season (40 frogs of each sex) were divided into 4 experimental groups (10 each) as follows:

During rainy season (female or male)

1. Control group (olive oil)
2. E_2 50 µg/kg body weight
3. E_2 500 µg/kg body weight
4. E_2 5,000 µg/kg body weight

During dry season (female or male)

1. Control group (olive oil)
2. E_2 50 µg/kg body weight
3. E_2 500 µg/kg body weight
4. E_2 5,000 µg/kg body weight

Frogs were daily intramuscular injected with E_2 (Fluka, catalog # 75262) at a dose of either 0 (control), 50, 500, or 5,000 µg/kg body weight. The injection was performed during 09.00-10.00 a.m. for 5 days. On the next day, animals were then humanely sacrificed by quick decapitation using a guillotine. Blood samples from the trunk were collected in heparin coated tube with 0.4% PMSF (for protein assay). Plasma was separated by centrifugation at 4,000 rpm for 20 min and then aliquoted and stored -20^{0}C for phosphoprotein staining and E_2 assays. The liver and gonad samples were dissected out and weighed. A portion of the gonads was collected and kept in phosphate buffered saline (PBS, pH7.4) for counting and measuring of oocytes. Small pieces of the liver and the gonad were fixed in 10% formalin in PBS for histology procedure. A portion of the liver was immediately transferred to

RNAlater (Ambion, catalog # 7020) and kept at - 20^0C for subsequent RNA extraction for determination of *Vtg*- gene expression levels. The animal remains were eliminated by an incinerator.

3.4 Primer design

Primers to amplify partial sequence of the transcribed *Vtg* gene by RT-PCR were designed from the chromosomal DNA (cDNA) (GenBank accession numbers M18060) sequences of *Vtg* gene of the chicken *Gallus gallus*, and synthesized by the BioService Unit, Thailand. The sequences of the *Vtg* primers are as follows: forward primer (VtgF): 5'-CAAGGTCATTCGAGCAGACA -3' and reverse primer (VtgR): 5'-ACAGCTGGGAACCACGTATC-3'. These primers were designed by using Primer3 software (v. 0.4.0). Primers to amplify a fragment of the transcribed ß-actin gene by RT-PCR were designed from the consensus sequence derived from the alignment of ß-actin cDNA sequences from *Engystomops pustulosus* (AY226144), *Hyla japonica* (AB092520), *Rana lessonae* (AY272629, AY272627) and *Rana catesbeiana* (AB094353); and are as follows: forward primer (ActF): 5'-GATCTGGCATCACACTTTCT- 3' and reverse primer (ActR): 5'-TGGGTGACACCATCACCAGA -3' yielding an expected amplicon size of approximately 230 and 212 bp, respectively.

VtgF

```
1861 gctcctgtcc cccaaactgg acagtatgag ctatcggtac agcaaggtca ttcgagcaga
1921 cacttacttt gataactata gagttggtgc tactggagaa atctttgttg tgaacagccc
1981 aagaactatg ttcccatcag caataatttc caaattgatg gcaaattctg caggttcagt
2041 ggctgatctg gtagaggttg gcatccgagt ggaaggcctc gcagatgtca taatgaaaag
2101 aaacatccca tttgctgaat atcccacata caagcagata aaggagcttg gaaaagctct
2161 gcagggatgg aaagagctgc cgacagaaac ccctttggta tcagcctact tgaaaatact
2221 tggccaagaa gtggccttca tcaacatcaa caaggaactc ctgcaacagg tcatgaagac
2281 tgtagtggaa cctgctgatc gaaacgcagc aataaagaga atcgccaacc agatcctcaa
2341 cagcattgca gggcagtgga cgcagccggt gtggatggga gagctgcgat acgtggttcc
2401 cagctgtctc ggcctgccgc tggagtacgg gtcctacacc accgccctgg cacgagctgc
```

VtgR

Figure 3.3 Location of *Vtg* primers for RT - PCR.

3.5 Isolation of total RNA

Liver tissues were dissected from each individual. Bulk tissue (10 animals) was used for one reaction. The method was due to a protocol of an SV total RNA isolation kit (Promega, catalog# Z3100). Briefly, one hundred mg of the excised liver tissue from each group were ground under liquid nitrogen in mortars. The grinded tissues were mixed by 400 µl of SV RNA lysis buffer and then added 400 µl of RNA dilution buffer. The tube was vortexed 3 – 4 times and then incubated at 70 °C for 3 min. The lysated tissue was then centrifuged at 13,000 rpm for 10 min. The supernatant was pipetted to fresh microcentrifuge tube and added with 300 µl of 95% ethanol. The mixture was transferred to a spin column assembly and centrifuged at 13,000 rpm for 1 min. A flow-through fraction was discarded. The 600 µl of SV RNA wash solution was added to the spin column assembly. Then it was centrifuged at 13,000 rpm for 1 min. The DNase incubation mix was prepared by 40 µl of yellow core buffer, 5 µl of 90 mM $MnCl_2$, and 5 µl of DNase I enzyme per sample. Then, 50 µl of freshly prepared DNase incubation mix was applied to the membrane inside the spin basket. The mixture was incubated for 15 min at RT. Applied 200 µl of SV DNase stop solution to the spin basket and centrifuged at 13,000 rpm for 1 min. Then, 600 µl of SV RNA wash solution were added and centrifuged at 13,000 rpm for 1 min. Empty the collection tube and then added 250 µl of SV RNA wash solution again and centrifuged at 13,000 rpm for 2 min. Then, spin basket was transferred to the elution tube. Later, 100 µl of nuclease - free H_2O was added to the membrane for eluting RNA. The centrifuged RNA was then stored at -20^oC until use.

3.6 Quantity and quality of RNA

RNA quantified by O.D. at 260 nm incident light, (concentration (µg/ml) of total RNA was calculated using A_{260} x 40 x dilution) and integrity checked by electrophoresis through a 1% (w/v) formaldehyde MOPS-agarose denaturing gel with visualization by UV-transillumination with Ethidium bromide (EtBr) staining. The purity of the product was assayed by the ratio of O.D. at 260/280 which was in range 1.8-2.0.

3.7 Formaldehyde gel electrophoresis

For Formaldehyde gel preparation, 1% (w/v) agarose was melt in 1× MOPS buffer (40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA). After it was being cool, the gel was mixed by 1.2% formaldehyde. For RNA preparation, RNA sample was mixed by 1× formaldehyde loading dye (95% formamide, 18 mM EDTA, 0.025% xylene cyanol, 0.25% (v/v) glycerol, and 0.025% bromophenol blue). Then it was incubated at 65^{o}C for 10 min. The sample was quick spun and cool on ice for 3 min. Then sample was loaded on 1% formaldehyde gel. The gel was covered by 1× MOPS buffer. The electrophoresis ran at 50 V for 50 min. Then, a gel was stained by EtBr for 30 h and destained for 20 min.

3.8 Native agarose gel electrophoresis

Each extracted RNA solution was checked by electrophoresis in 1.2% (wlv) agarose gel in 1× TBE buffer (0.05M Tris-HCl, O.05M Boric acid and 0.65M EDTA) as a running buffer. Loading sample composed of 5 µl of the extracted RNA and 1 µl of a loading dye [5X loading dye: 25 mM Tris – HCl (pH 7), 0.05% bromophenol blue, 150 mM EDTA, and 25% glycerol]. The electrophoresis was performed by 1× TBE at 100 V for 50 min. After that the gel was stained with ethidium bromide solution for 3 min and destained in d-H_2O water. The RNA will then be visibled under UV light with a UV transilluminator.

3.9 RT - PCR amplification

Semi-quantitative RT-PCR was performed using an Access Quick^{TM} RT-PCR system (Promega, catalog # A1702) with 200 ng of total RNA used in the reaction. The first strand cDNA was synthesized at 48^{o}C for 45 min. Then, the PCR amplification was as followed: 1 cycle of denaturation at 94^{o}C for 2 min; 30 cycles of denaturation at 94^{o}C for 30 sec, of annealing at 54^{o}C for 30 sec, and of extension at 72^{o}C for 2 min. At last, the final extension was at 68^{o}C for 5 min. The PCR product was separated on TBE-1.2% (w/v) agarose gel, and visualized by UV-transillumination with Ethidium bromide staining (2 µg/ml).

RNA extracted from mature female and male *H. rugulosus*, and mature female *R. catesbeiana* control groups, were used as for testing the designed *Vtg* and β-

actin primer pairs and optimization of the semi-quantitative RT-PCR assay conditions for the determination of *Vtg* gene expression levels.

3.10 Purification of RT - PCR product for cDNA sequencing

Before sequencing the PCR products, any potential contaminants in the PCR mixture must be removed by purification. The purification was performed by using a QIAquick PCR purification kit (Qiagen, catalog # 28104). Five volumes of buffer PB were mixed with one volume of the PCR product. The mixture was then applied to a QIAquick spin column placed in a 2 ml collection tube, and centrifuged at 13000 rpm for 1 min. The flow-through was discarded. A 0.75 ml of buffer PE was added to the column, which then was centrifuged at 13000 rpm for 1 min. The flow-through was discarded again and the column was centrifuged at 13000 rpm for 1 min again. The column was transferred to a new Eppendorf tube and 50 µl of buffer EB or sterile distilled water was added to the center of the column, which was left standing for 1 minute before centrifuging for 1 min at 13000 rpm. The purified cDNA was stored at -20^{o}C.

3.11 Sequencing of cDNA

The purified product was sequenced by an automated DNA sequencer (BioService Unit, Thailand).

3.12 Alignment of cDNA sequences and phylogenetic analyses

The nucleotide sequences were blasted against nucleotide database (NCBI; BLASTn, default parameters, against all sequence entries) to elucidate *Vtg*-like gene products and then aligned by Clustal X program. Aligned cDNA of adult female *H. rugulosus* were compared to the sequences of adult male *H. rugulosus*, adult female *R. catesbeiana, G. gallus* (M18060) and X. *laevis* (M18061) and then checked % G + C for finding out appropriate annealing temperature and sequence similarity with eye. The data matrix from Clustal X was converted to a NEXUS file format before reconstructing a phylogenetic tree. Phylogenetic trees of *Vtg* among these animals were made by using program phylogenetic analysis using parsimony (PAUP, version 4.0b). A neighbour joining (NJ) tree was preliminarily constructed.

3.13 Total protein measurement by using absorbance at 280 nm (A_{280})

Protein was diluted by dd-H_2O. Concentration of total protein was calculated using the following formula:

Concentration of protein (mg/ ml) = A_{280} × dilution

Total protein (mg) = concentration of protein × total volume

3.14 SDS - polyacrylamide gel electrophoresis

An SDS - polyacrylamide gel (8 × 9 cm size; 1 mm thick; and 10 wells) was prepared as a discontinuous gel. A 12% separating gel was prepared by 30% bis - acrylamide solution, 1 M Tris - HCl buffer (pH 8.8), 10% (w/v) SDS, dd - H_2O, 10% fresh ammonium persulfate (APS), and 0.05% TEMED. A 4% stacking gel was prepared by the composition of 30% bis - acrylamide, 0.5 M Tris - HCl (pH 6.8), 10% (w/v) SDS, 10% fresh APS, and 0.1% of TEMED. For sample preparation, 20 µg of crude protein were mixed with 1× loading dye [For 5× loading dye: 1 M Tris - HCl (pH 6.8), 50% (v/v) glycerol, 10% (w/v) SDS, and 1% bromophenol blue]. The phosphoprotein molecular weight standard (Invitrogen, catalog # (P33350) was also loaded. The electrophoresis was performed by 1× electrode buffer [25 mM Tris (hydroxylmethyl) - aminometane, 192 mM glycine, and 0.1% (w/v) SDS]. The power supply was used at 100 V. The gel was run until the dry front reaches the bottom of the gel, approximately 1.30 h. After that the gel from the glass plate was removed into a staining dish.

3.15 Phosphoprotein staining

After electrophoresis, the gel was fixed in fixative solution [50% methanol and 10% acetic acid] overnight. The gel was washed with d-H_2O for 10 min and repeated this step twice. Then, the gel was incubated in 60 ml of Pro-Q® Diamond phosphoprotein gel stain solution (Invitrogen, catalog # P33301) and gently shaken for 90 min. Then, the gel was washed several times in 100 ml destain solution [5% of 1 M sodium acetate, 20% of acetonitrile, and 75% of d-H_2O] until the background was clear and then imaged. Washed the gel with d-H_2O 3 times for 5 min each. The same gel was stained for total protein again by using SYPRO® Ruby gel stain solution

(Invitrogen, catalog # S12001). A 60 ml of SYPRO® Ruby gel stain was added to the gel and agitated on an orbital shaker overnight. Wash gel with wash solution [10% methanol and 7% acetic acid] for 30 min. Finally, the intensity of each expected band in the gel was measured by Quantity one software (version 4.6.1) (www.bio-rad.com).

3.16 Peptide sequence determination from mass spectra

The light bands (expected band) were excised from SDS-PAGE. The excised gels were searched for mass spectra by Bioservice unit, Thailand. Mass spectra of protein was determined by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF). The peptide mass mapping obtained from protein was searched against protein database via the MASCOT program (http://www.matrixscience.com). The searching parameters were trypsin enzyme, two missed trypsin cleavages, +1 Da mass accuracy, and doublet charged peptides.

3.17 Measurement of oocyte/ovary

A proper portion of ovaries from individuals (5 animals) was sampled 3 areas and then weighed 30 mg each. The number of oocytes was counted from each piece and then analyzed into mean. Total number of oocytes per each animal was calculated by oocytes number × total gonad weight / 30 mg. The size of oocytes was measured from 20 oocytes of each animal by using ocular scale under the light microscope.

3.18 Histology of the liver and ovary

The small pieces of liver and ovarian tissues were fixed in 10% formalin in PBS (1:10; w/v), and subsequently processed for paraffin wax embedding. Briefly, fixed tissues were washed with tap water. After that, tissues were dehydrated with 70% ethanol for 2 h, 90% ethanol for 6 h, 95% ethanol for 12 h and n-butyl for 1 h (absolute ethanol) and then clearing the tissues with xylene for 1 h. Tissues were infiltrated with embedding media (paraffin) and then embedded in paraffin in the block. About three longitudinal sections per liver or ovary were cut at 10 µm thick and stained with hematoxylin and eosin. Staining procedure was as follows: the slides were deparaffinized and hydrated to water. Sections were slightly overstained with hematoxylin for 3-5 min. Excess stain was removed in running tap water for 2 min. It was then counterstained with eosin for 2 min. Slides were taken through 3 changes of

95% ethanol, 5 min. each or until excess eosin is removed (check under microscope), then transferred to the first absolute ethanol of the clearing, clear in xylene, two changes of 2 min each and then mounted.

3.19 Liver-somatic index (LSI) and gonadal-somatic index (GSI)

LSI and GSI were calculated using the following formulas: LSI = liver mass (g)/body mass (g) x 100% and GSI = gonad mass (g)/body mass (g) x 100%, respectively.

3.20. Determination of plasma E_2 levels

Plasma E_2 level was measured by using radioimmunoassay technique as described by Tangpraprutgul *et al*. (1996). The assay was performed as follows: duplication of 200 µl of plasma from each sample was extracted in 5 ml diethyl ether. After drying, each sample was dissolved in 500 µl phosphate buffer saline (0.05 M, pH 7.4). The 100 µl of hormone tracer ($^3H.E_2$) and 100 µl of antiserum were added. The mixture was incubated at 4^0C for 18-20 h. Bound and free forms were separated by dextran-coated charcoal. Then, the bound form was transferred into scintillation counting fluid and counted in the beta counter. The percent coefficient variation of intra-assay and inter-assay were 7.3 and 13.8, respectively.

3.21 Data analysis

Results were reported as mean ± standard error of mean (SEM). The comparison of the difference between the means of treatments was analyzed by the analysis of variance (ANOVA). The statistical significance ($p < 0.05$) was determined using a post-hoc LSD's test. Different comparison between seasons by using paired-samples T test

CHAPTER 4

RESULTS

4.1 Quantity and quality of RNA extraction

Total RNA was extracted from the bulk liver tissue of each group. The 10 µl extracted RNA was dissolved in 990 µl of d-H_2O and then measured by the absorbance at 260/280 nm. The amount of RNA extracted was determined by O.D. 260 nm and then calculated (Table 4.1). The quality of RNA extracts was found to be satisfactory (1.8-2.0) as determined by a 260/280 ratio and that the 28S and 18S rRNA bands were intact (clear and sharp) (Table 4.1 and Fig. 4.1).

Table 4.1. The Quantity and purity of RNA measured by O.D. 260/280 nm.

Samples (control group)	Weight of liver (mg)	RNA (µg/ml)	O.D. 260/280
Immature female *H. rugulosus*	100	1858.7	1.90
Immature male *H. rugulosus*	100	566.2	1.79

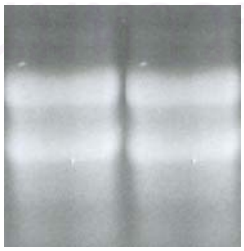


Figure 4.1 Total RNA extracted from liver was electrophoresed on 1% formaldehyde gel.

4.2 Partial sequence of *Vtg* gene

Total RNA extracted from each frog's liver was used as template for RT-PCR amplification. RT-PCR products from immature and mature female and male H. rugulosus, and from mature female R. catesbeiana, of approximately 230 bp were obtained with the VtgF/VtgR primer pair (Fig. 4.2).

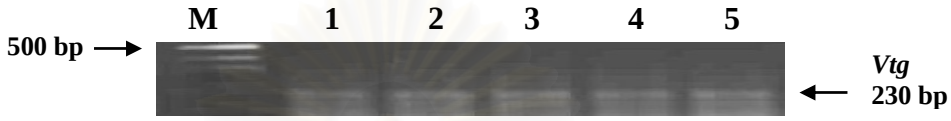


Figure 4.2. RT-PCR amplification of partial *Vtg* cDNA sequence using the VtgF/VtgR primer pair. Lane M indicates 100 bp DNA ladder as a marker. Lanes 1-2 contained PCR products amplified from immature female and male *H. rugulosus*, respectively. Lanes 3-4 contained PCR products amplified from mature female and male *H. rugulosus*, respectively. Lane 5 contained PCR products amplified from mature female *R. catesbeiana*.

RT-PCR products from immature and mature female and male *H. rugulosus*, and from mature female *R. catesbeiana*, of approximately 230 bp were purified and sequenced. Partial sequence of mature female *H rugulosus* as show in figure 4.3. It matched the NCBI database sequence of chicken vitellogenin gene (X13607, NM_001031276, AB185211, and M18060 with score 42.1 (E value 0.76). The percentage G + C composition of the obtained amplicon sequences from female and male *H. rugulosus* and female *R. catesbeiana* were 53.0%, 58.1%, and 55.9%, respectively. The obtained nucleotide was aligned and calculated for percentage of similarity. The nucleotide sequence of *Vtg* from female *H. rugulosus* showed 89.9% and 85.7% sequence similarity with that from male *H. rugulosus* and female *R. catesbeiana*, respectively (Fig. 4.4; and Table 4.2), suggesting likely similarity.

```
            ....|....|....|....|....|....|....|....|....|....|
                      10        20        30        40        50
H.rugu_f_   AAAATAGCAGCCTCCTGTGAACATGACCACTCTGTGTCGATTGAGGAACT

            ....|....|....|....|....|....|....|....|....|....|
                      60        70        80        90        100
H.rugu_f_   AGTTACATTCGCGTCTGGTATGTCTTTTTATCCGCAGCTCCCACCGAGGT

            ....|....|....|....|....|....|....|....|....|....|
                     110       120       130       140       150
H.rugu_f_   CTGGCGGGGCTCCTCTGAGGACAGCCGGGCGCTGGGGAGGCTGCAGGATA

            ....|....|....|....|....|....|....|....|....|....|
                     160       170       180       190       200
H.rugu_f_   CGTGGTTCCCAGCTGTATTGATGATGACTGTTGATACGTGCTTCCCAGCT

             ....|....| ....|....| ....|....| ....|
                      210       220       230
H.rugu_f_    GTAGGTTGATACGTGGTTCCCAACTGTAAA
```

Figure 4.3. The partial sequence of vitellogenin gene (about 230 bp in length) after mRNA amplification of *H. rugulosus*. *H. rugu_f = H. rugulosus female*.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
H. rugu_m       CCTACTGT-- AACT-----G ACCACTT-CT GCT--T---- -TGATGAAC-
R. cate_f       CCAACTGTGA AACT-----G GCCACTTGCT TCT--T---- -TGGTCAAC-
H. rugu_f       CCTCCTGTGA ACAT-----G ACCACTCTGT GTCGAT---- -TGAGGAAC-
G. gall         TCAACATCAA CAAG-----G AACTCCTGCA ACAGGTC--- ATGAAGACTG
X. lae          CCAACTTTGA AAATCTTCAG GCACTTTGGA AACAGTTTGC ACAAAGAACT
Clustal A        *  *               *   * *           *      **   *
Clustal Co       *  *               *                 *           *
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
H. rugu_m       TCG-AACATT CGC--GCTGT A-TGTCTTTT TATCCGCAGC TCCCGGC--A
R. cate_f       TAGGAACATT CTCTGGCTGT A-TGTCTTTT TATCCGCAGC TCCCGGC--A
H. rugu_f       TAGTTACATT CGCGTCTGGT A-TGTCTTTT TATCCGCAGC TCCCACCG-A
G. gall         TAGTGGAACC TGCTGATCGA AACGCAGCAA TAAAGAGAAT CGCCAACC-A
X. lae          CAG-TATAGA CGCTGTTTGC T-TGATGCTC TCCCTATGGC TGGTACAGTG
Clustal A       * *    *     *     *  *  *       **     *     **  *  *
Clustal Co        *    *     *     *     *       *
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
H. rugu_m       GGTCAGGCGG GGCTCCTCTG AGGA------ -CAGCCGGGC --GCTGGGGA
R. cate_f       GGTCAGGCGG GGCTCCTCTG AGGA------ -CAGCCGGGC --GCTGGGGA
H. rugu_f       GGTCTGGCGG GGCTCCTCTG AGGA------ -CAGCCGGGC --GCTGGGGA
G. gall         GATCCTCAAC AGCATTGCAG GGCAGTGGAC GCAGCCGGTG TGGATGGGAG
X. lae          GATTGTCTGA AGTTCATCAA ACAA-----C TTATTCATAA T-GAGGAGCT
Clustal A       * **         **    * *  * *       *******     * ****
Clustal Co      * *          *     *     *        *  *        *  * *
                ....|....| ....|....| ....|....|
                       160        170        180
H. rugu_m       GGCTGC--AG GATACGTGGT TCCCAGCTGT
R. cate_f       GGCTGC--AG GATACGTGGT TCCCAGCTGT
H. rugu_f       GGCTGC--AG GATACGTGGT TCCCAGCTGT
G. gall         AGCTGC---- GATACGTGGT TCCCAGCTGT
X. lae          GACTACTCAG GAGGCAGCAG TTCTAATTAC
Clustal A        *****
Clustal Co      ** *     ** *     * * * *
```

Figure 4.4. A multiple alignment of nucleotide sequences deduced from a cDNA fragment of the *Vtg* gene after RT-PCR amplification with VtgF/VtgR primer pairs. *H. rugu_ m = H. rugulosus male*; *H. rugu_f = H. rugulosus female; R. cate_f = R. catesbeiana female; G. gall = G. gallus* (M18060), *X. lae = X. laevis* (M18061)**.** Conserved sequences between *H. rugulosus*, *R. caresbeicana*, *G. gallus* excluding (cluster A) or including (cluster co) *X. laevis* are indicated by *. Note that the sequences of the PCR primers used (underlined) are excluded from homology analysis accordingly**.**

Table 4.2. Percentage of similarity of bases from various species

	*H. rugulosus*_m	*R. catesbeiana*_f	*H. rugulosus*_f	*G. gallus*	*X. laevis*
*H. rugulosus*_m	-	95.3	89.9	53.4	45.3
*R. catesbeiana*_f	-	-	85.7	50.6	48.1
*H. rugulosus*_f	-	-	-	53.5	47.8
G. gallus	-	-	-	-	40.5

Aligned sequences were imported into a phylogenetic analysis program, PAUP (version 4.0b). A phylogenetic tree was preliminarily constructed by maximum parsimony using heuristic search (Fig. 4.5). Results from rooted tree indicated that male, *H. rugulosus* and *R. catesbeiana* are clustered together with high bootstrap supporting-values (98%) Moreover, these two species were also grouped with female, *H. rugulosus* with high bootstrap supporting-values (100%).

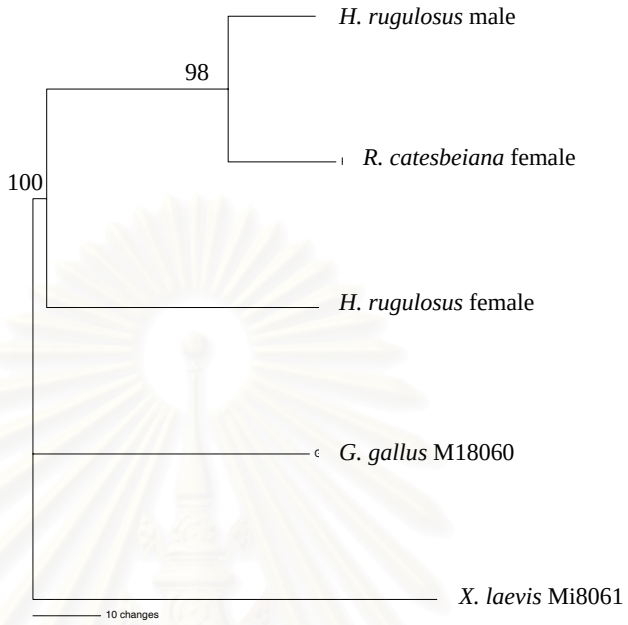


Figure 4.5. A phylogenetic tree of vitellogenin gene from five groups of animals. *H. rugulosus* male, *R. catesbeiana* female, *H. rugulosus* female, *G. gallus*, and *X. laevis.*

4.3 The effect of E_2 on experimental parameters in the immature frogs during rainy season

4.3.1 The effect of E_2 on liver vitellogenin gene expression in the immature female frogs

The *Vtg*-gene expression in immature female frogs was determined by RT-PCR (Fig. 4.6A)**.** The β-actin, the internal control (Fig. 4.6B) is the same in treated as in control groups. The results showed that E_2 at a dose of either 50, 500 or 5,000 µg/kg body weight significantly decreased *Vtg* gene expression when compared with the control (0.65 ± 0.02, 0.60 ± 0.02 and 0.58 ± 0.02, respectively vs. control 0.85 ± 0.03) (Fig. 4.6C).

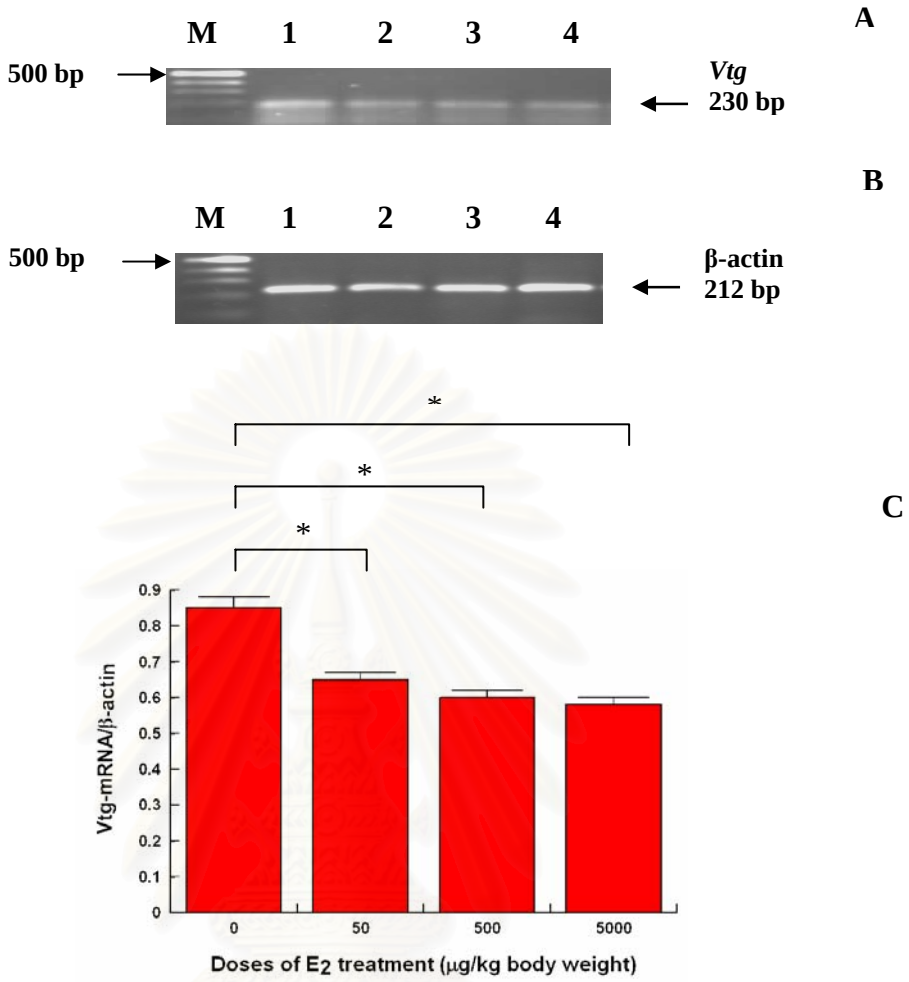


Figure 4.6. Vitellogenin gene (A) expression levels determined by RT-PCR after treatment of immature female *H. rugulosus* for 5 days. β-actin, the internal control (B) was the same in treated as in control. Lane M indicated 100 bp DNA ladder as a marker. Lanes 1-4 were RT-PCR (VtgF/VtgR) amplicons from the liver of *H. rugulosus* treated with E_2 at a dose of either 0, 50, 500 or 5,000 μg/kg body weight, respectively. Expression of *Vtg* -gene levels detected by semi-quantitative RT-PCR using β-actin as the internal reference standard (C). Data were presented as the mean ± SEM (n = 6). Significant difference from the control group: *$p < 0.05$.

4.3.2 The effect of E_2 on liver vitellogenin gene expression in the immature male frogs

The *Vtg* gene expression in immature male frogs was determined by RT-PCR (Fig. 4.7A)**.** The β-actin, the internal control (Fig. 4.7B) was the same in treated as in control. In contrast to the immature female, E_2 stimulated *Vtg* gene expression in immature male frog at a dose of 500 µg/kg body weight when compared with the control (0.80 ± 0.06 vs 0.61 ± 0.04). The E_2 at a doses of 50 and 5,000 µg/kg body weight tended to increase *Vtg* gene expression (0.70 ± 0.05, and 0.74 ± 0.05, respectively) (Fig. 4.7C)**.**

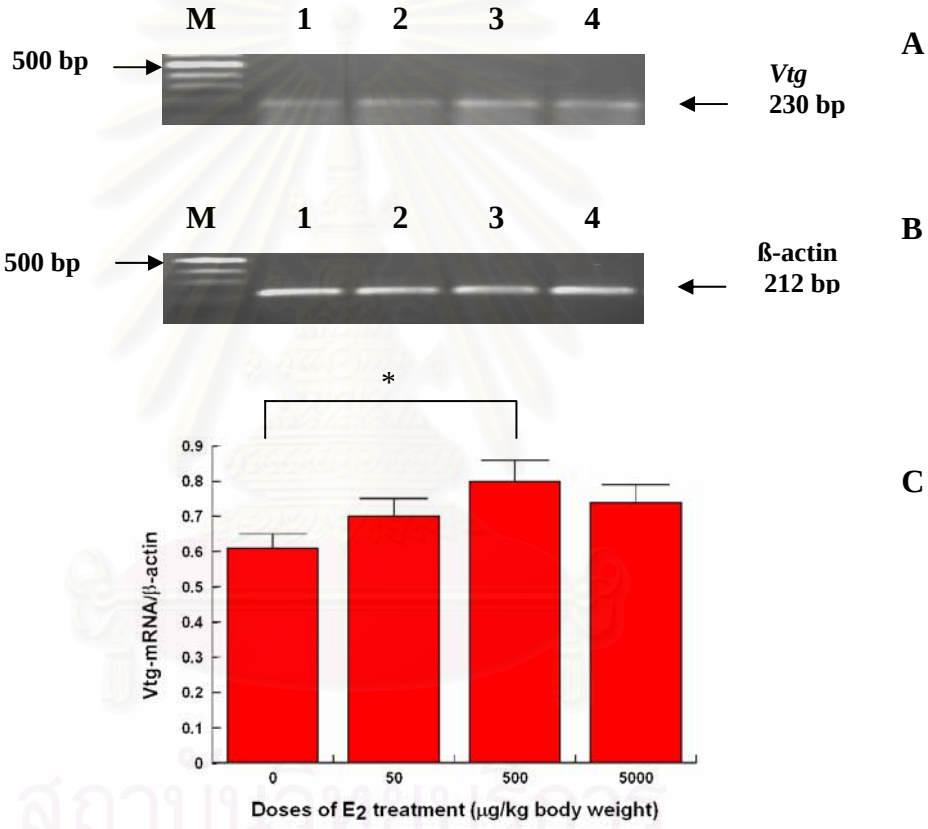


Figure 4.7. Vitellogenin-gene expression levels determined by RT-PCR after treatment of immature male *H. rugulosus* for 5 days (A). β-actin, the internal control was the same in treated as in control (B). Lane M indicates 100 bp DNA ladder as a marker. Lanes 1-4 were RT-PCR amplicons from the liver of *H. rugulosus* treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively. Expression of *Vtg* gene levels detected by semi-quantitative RT-PCR using β-actin as the internal reference standard (C). Data were presented as the mean ± SEM (n = 6). Significant difference from the control group: *$p < 0.05$.

4.3.3 The effect of E_2 on plasma phosphoprotein in immature female and male frogs

After SDS-PAGE, the gel was incubated in Pro-Q® Diamond phosphoprotein gel stain solution. Only phosphoprotein would be stained by this procedure. Thus it was found several bands of the phosphoprotein on the gel after staining (Fig. 4.8A). The protein bands of interest were manually excised and in-gel digested with trypsin. Therefore, in-gel digestion was used for the peptide mass mapping technique (MALDI-TOP). The molecular mass of the expected Vtg is higher than 116,250 Da (Fig. 4.8B). It matched the NCBI database sequence of a member of vitellogenin precursor - *Oreochromis aureus* (T31095) with score 38 (mass values matched: 14).

The gel was measured the intensity each bands. The data showed that there was a significant increase in intensity of expected Vtg bands in immature female frogs treated with E_2 at a dose 5,000 µg/kg body weight (2308.13 ± 135.11) for 5 days when compared with control groups (1657.83 ± 98.46). The intensity of expected Vtg bands was also higher in frogs treated with E_2 at a dose of either 50 or 500 µg/kg body weight (1841.81 ± 95.48 and 1912.84 ± 148.17), but these differences were not statistically significant (Fig. 4.9A).

In immature male groups, data showed that frogs treated with E_2 at a dose 500, or 5,000 µg/kg body weight significantly increased the intensity of expected Vtg bands when compared with control groups (2019.65 ± 85.81, and 2170.40 ± 122.96 vs. 1775.44 ± 142.68). The intensity of expected Vtg bands tended to be increased in frogs that treated with E_2 at dose of 50 µg/kg body weight (2019.65 ± 85.81) (Fig. 4.9B).

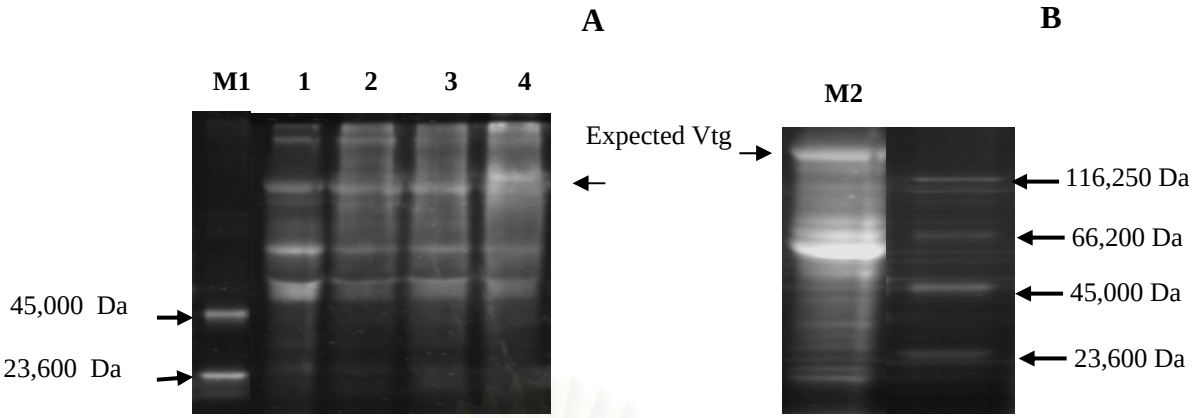


Figure 4.8. Phosphorylated (lane M1) and nonphosphorylated (lane M2) proteins in the Peppermint Stick phosphoprotein molecular mass standards. Fig. 4.8A indicated phosphorylated protein. Fig. 4.8B indicated both phosphorylated and nonphosphorylated proteins. Lanes 1-4 (the product from frog plasma) was treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively.

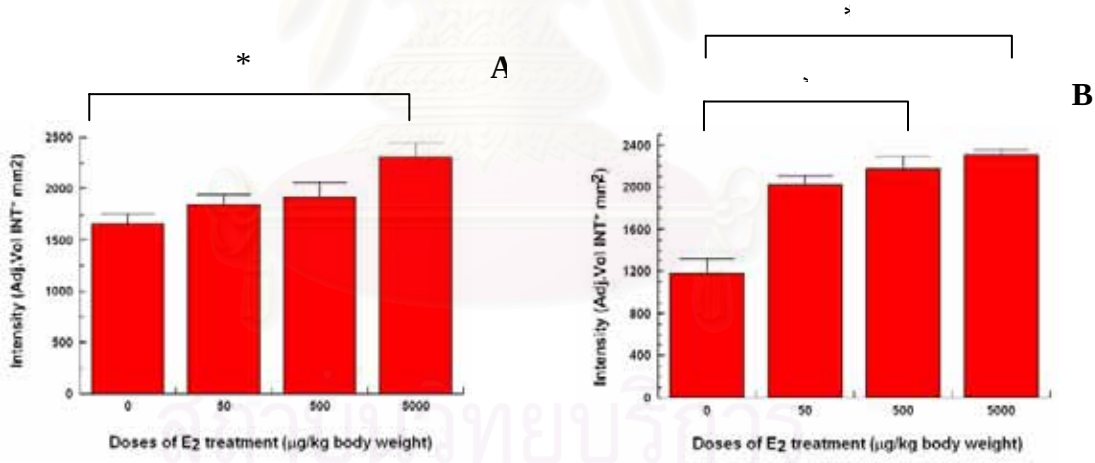
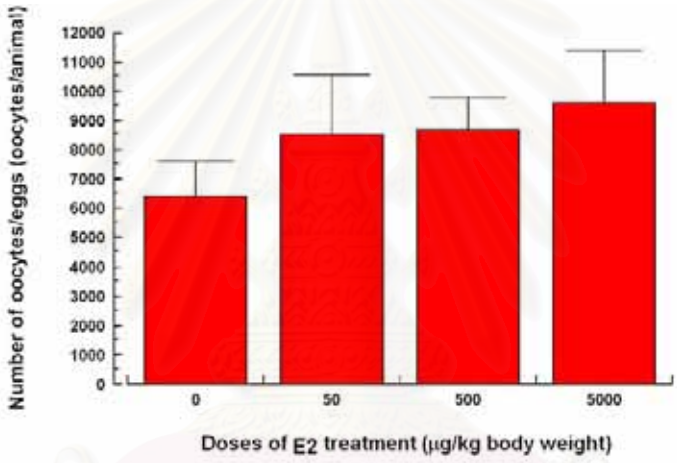


Figure 4.9. The intensity of expected–Vtg bands from immature female (A) and male frog (B) that were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively. The data represent the mean ± SEM (n = 6). Significant difference from the control group: *$p < 0.05$.

4.3.4 The effect of E_2 on the number and the size of oocytes

The oocytes were counted and then measured by using ocular scale under light microscope. The data were statistically analyzed by one way ANOVA as presented in figure 4.10. The data showed that there was no significant differences in number of oocytes of each experimental group (8516.44 ± 2056.73, 8688.60 ± 1101.18 and 9591 ± 1798.69 oocytes/animals, respectively) compared with the control group (6397.33 ± 1210.97 oocytes/animals) (Fig. 4.10A). In addition, the size of oocytes from each experiment group (1.38 ± 0.01, 1.47 ± 0.01 and 1.48 ± 0.009 mm, respectively) was not significantly different from the size of control groups (1.45 ± 0.04 mm) (Fig. 4.10B).

A



B

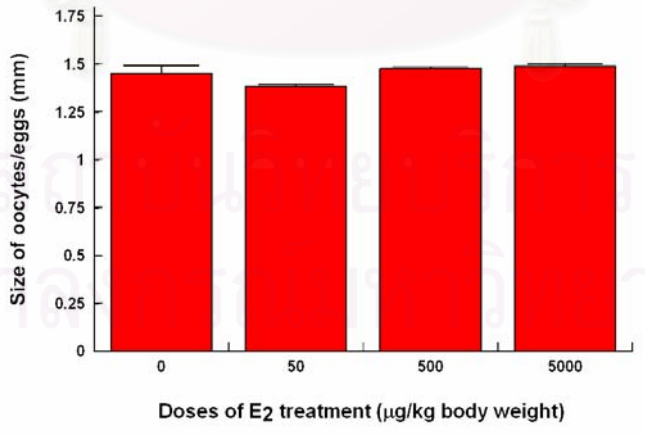


Figure 4.10. This figure represented the number (A) and the size of oocytes (B) of female frogs which were treated with E_2 at a dose of either 0, 50, 500, or 5,000 μg/kg body weight, respectively. The data represented the mean ± SEM (n = 5).

4.3.5 The effect of E_2 on the weight of body, liver, gonad and LSI and GSI in immature female and male frog

The body, liver and gonad weight of the immature female frogs were analyzed (Fig. 4.11). The data showed that body weight of the immature female frogs that treated with E_2 at different doses studied were not significantly different among treatment groups (182.00 ± 4.89, 176.00 ± 5.61, and 177.00 ± 7.15 g, respectively) and control group (183.00 ± 8.17 g) (Fig. 4.11A). The liver weight was not significantly different among treatment groups (8.12 ± 0.46, 8.42 ± 0.66 and 9.57 ± 0.62 g, respectively) and control group (8.50 ± 0.64 g) (Fig. 4.11B). The gonad weight was not significantly different among treatment groups (16.49 ± 1.86, 15.33 ± 1.54 and 15.97 ± 1.59 g, respectively) and control group (12.81 ± 2.14 g) (Fig. 4.11C).

The body, liver and gonad of the immature male frogs were shown in figure 4.10. The data showed that body weight of the male frogs that treated with E_2 at a dose of either 50 or 5,000 µg/kg body weight were significantly decreased (103.33 ± 4.08, and 101.25 ± 7.42 g, respectively) when compared with control group (121.42 ± 6.33 g) (Fig 4.11A). The liver weight was not significantly different among treatment groups (4.57 ± 0.35, 5.10 ± 0.49, and 5.44 ± 0.57 g, respectively) and control group (5.33 ± 0.58 g) (Fig. 4.11B). The E_2 at a dose of either 500 or 5,000 µg/kg body weight could significantly decrease the gonad weight (0.13 ± 0.01, and 0.13 ± 0.01 g, respectively) compared with control group (0.17 ± 0.01 g) (Fig. 4.11D).

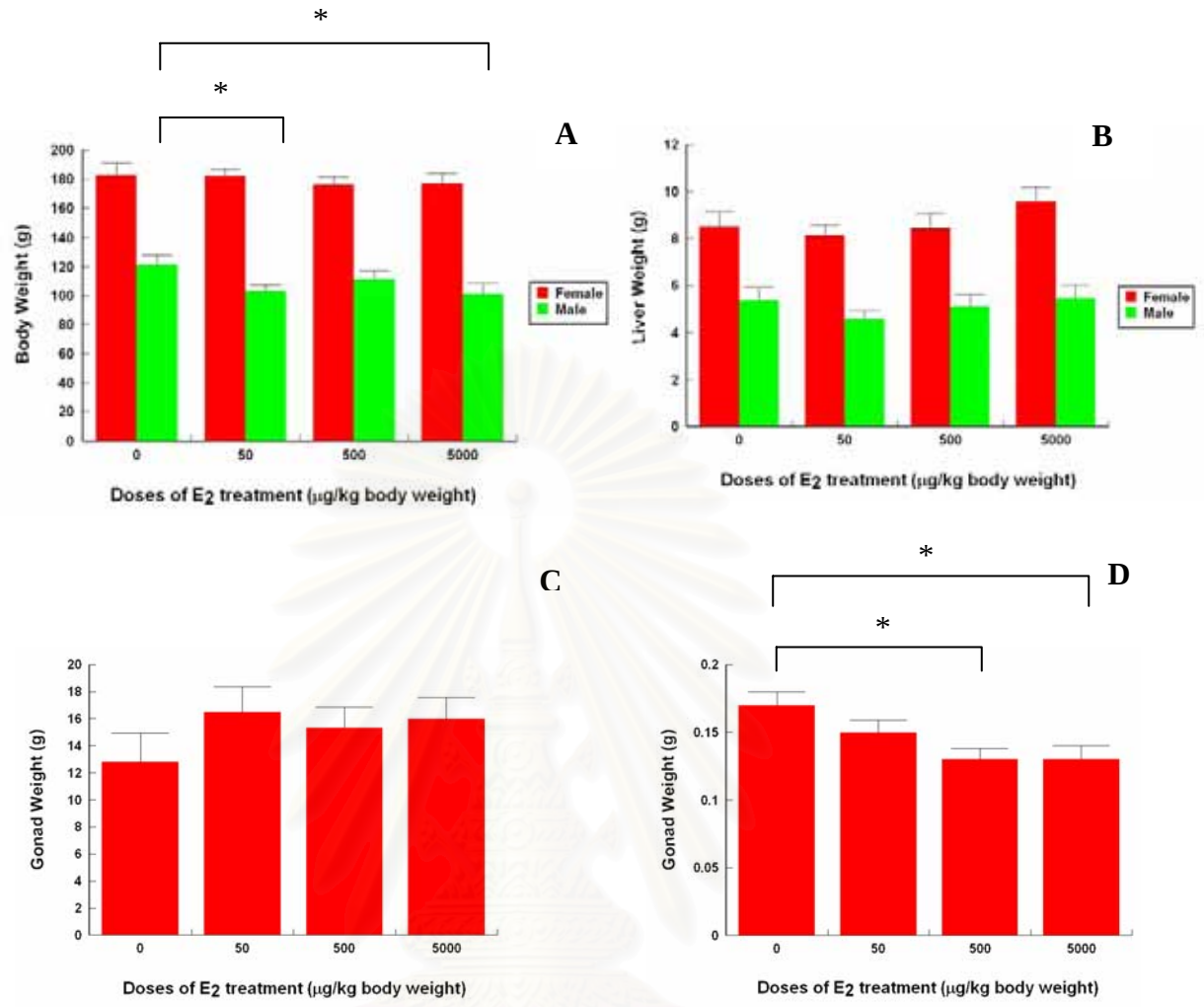


Figure 4.11. This figure represented the body weight of female and male frogs (A), the liver weight of female and male frogs (B) and female (C) and male gonad (D) weight of the frogs which were treated with E_2 at a dose of either 0, 50, 500, or 5,000 μg/kg body weight, respectively. (Female; n = 10, 10, 10, and 10 animals respectively Male; n = 7, 9, 7 and 8 animals, respectively). The data represented the mean ± SEM. Significant difference from the control group: *p < 0.05.

Liver-somatic index (LSI) and gonadal-somatic index (GSI) (weight of liver or ovary/total body weight x 100) of the immature female frogs were analyzed (Fig. 4.12A-B). The data showed that LSI of the immature female frogs treated with E_2 at different doses studied were not significantly different among treatment groups (4.45 ± 0.21, 4.77 ± 1.33, and 5.42 ± 0.30, respectively) and control group (4.65 ± 0.29)

(Fig. 4.12A). The GSI was not significantly different among treatment groups (9.03 ± 0.99, 8.96 ± 0.92 and 9.11 ± 0.91, respectively) and control group (7.07 ± 1.19) (Fig. 4.12B).

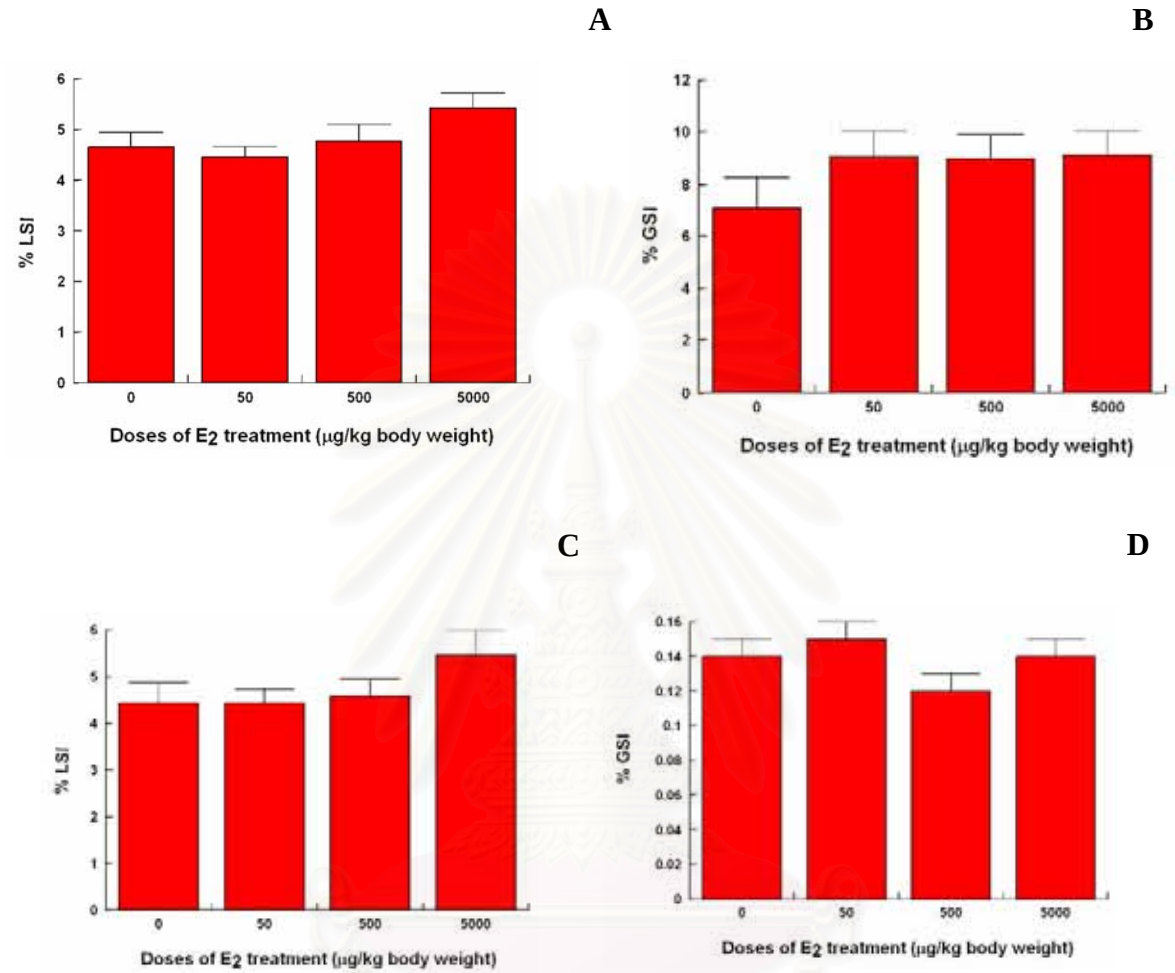


Figure 4.12. Liver- and gonadal-somatic index in immature female (A-B) and male (C-D) frogs which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight for 5 days.(Female; n = 10, 10, 10, and 10 animals, respectively Male; n = 7, 9, 7 and 8 animals, respectively). The data represented the mean ± SEM.

Liver-somatic index and gonadal-somatic index (weight of liver or testis/total body weight x 100) of the immature male frogs were analyzed (Fig. 4.12). The data showed that LSI of the frogs treated with E_2 in different doses studied were not significantly different among treatment groups (4.43 ± 0.30, 4.58 ± 0.36, and 5.46 ± 0.53, respectively) and control group (4.43 ± 0.44) (Fig 4.12C). The GSI was not

significantly different among treatment groups (0.15 ± 0.01, 0.12 ± 0.01. and 0.14 ± 0.01, respectively) and control group (0.14 ± 0.01) (Fig. 4.12D).

4.3.6 The effect of E_2 on histology of the liver and the ovary

According to histology of liver and gonad the results showed that E_2 at all doses of treatment had no effect on structure of neither liver cells nor gonads, so only the histology results of control and highest dose treatment group were represented. Figure 4.13A-B showed a high power view of a central vein (CV) surrounding parenchyma. The arrangement of the liver cells or hepatocytes (H) into cords appeared. Sinusoids (S) could be seen entering the central vein in several places. The central vein was supported by a small amount of connective tissue, here visible as a pinkish bar along its lower edge, and lined by endothelial cells. Flattened endothelial cell nuclei could be seen in the central vein and in the sinusoids. Red blood cells were present in the central vein. They appeared very pale toward the centre of the vein, but were more darkly stained around its edges. Liver cells usually appear vacuolated because the glycogen and lipids were removed during processing**.**

Histological analysis of ovarian indicated that most female frogs were in late vitellogenesis with the remaining in early vitellogenesis (Fig. 4.13C-D). Vitellogenic female gonads had with characteristic yolk vesicles (YV) containing bright pink. Pre-vitellogenic cells were stained purple and were characterized by a germinal vesicle (GV) and cortical alveoli (CA). A thin layer of flat follicle cells surrounded the oocyte. Egg yolk granules filled almost all the space outside the nucleus, with only a little cytoplasm spread around the nucleus and near the egg membrane. The nucleus edge was wavy, with a few nucleoli inserted in the troughs; most of the nucleoli have moved toward the centre of the nucleus.

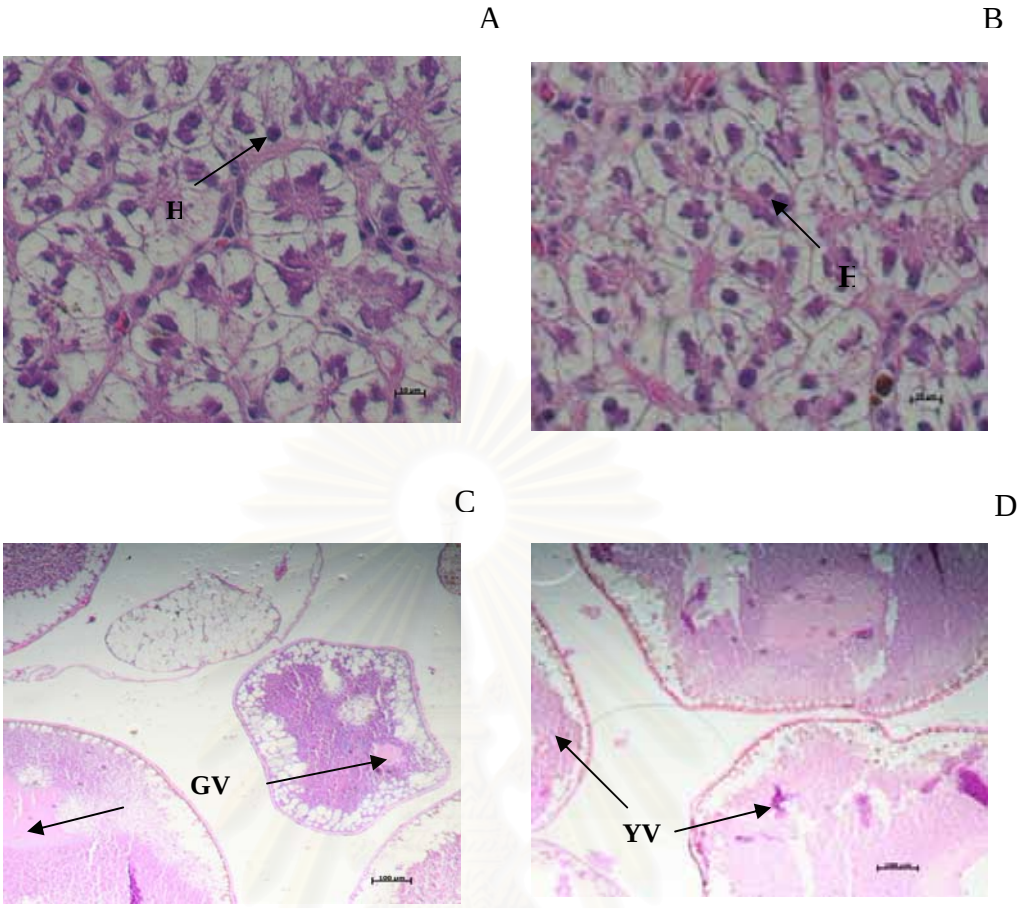


Figure 4.13. Histological section of frog liver (A and B) and ovary (C and D)(10% formalin-fixed, H&E-colored) as basis of determination of effect of E_2: (A,C) control group, (B,D) groups were treated with E_2 at a dose of 5,000 µg/ body weight. H = hepatocytes, YV = yolk vesicles, GV = germinal vesicle, Scale bar = 10 (A,B) and 100 µm (C,D).

4.3.7 Plasma E_2 levels in immature female and male frogs

As shown in figure 4.14, E_2 of immature female frogs that reared during rainy season had significantly higher than male group (327.24 ± 188.61 and 17.58 ± 4.46 pg/ml, respectively).

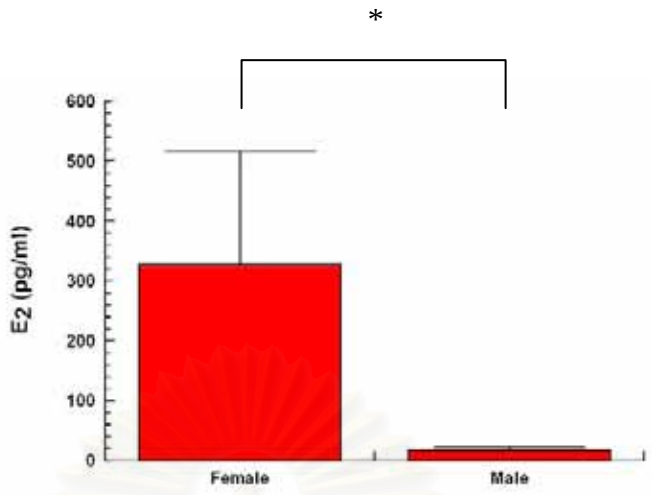


Figure 4.14. Plasma E_2 level in untreated-immature female and male frogs (n = 5 animals). Data are presented as the mean ± SEM. Significant difference: *p < 0.05.

4.4 The effect of E_2 on experimental parameters in the immature frogs during dry season

4.4.1 The effect of E_2 on liver vitellogenin gene expression in immature female frogs

The *Vtg* gene expressions in immature female frogs were determined by RT-PCR (Fig. 4.15A)**.** The ß-actin, the internal control was the same in treated as in control groups (Fig. 4.15B). The results from experiments showed that E_2 at a doses of either 50, 500 or 5,000 µg/kg body weight (0.68 ± 0.04, 0.78 ± 0.05 or 0.72 ± 0.04, respectively) could not stimulate *Vtg* gene expression when compared with the control (0.65 ± 0.04) (Fig. 4.15C).

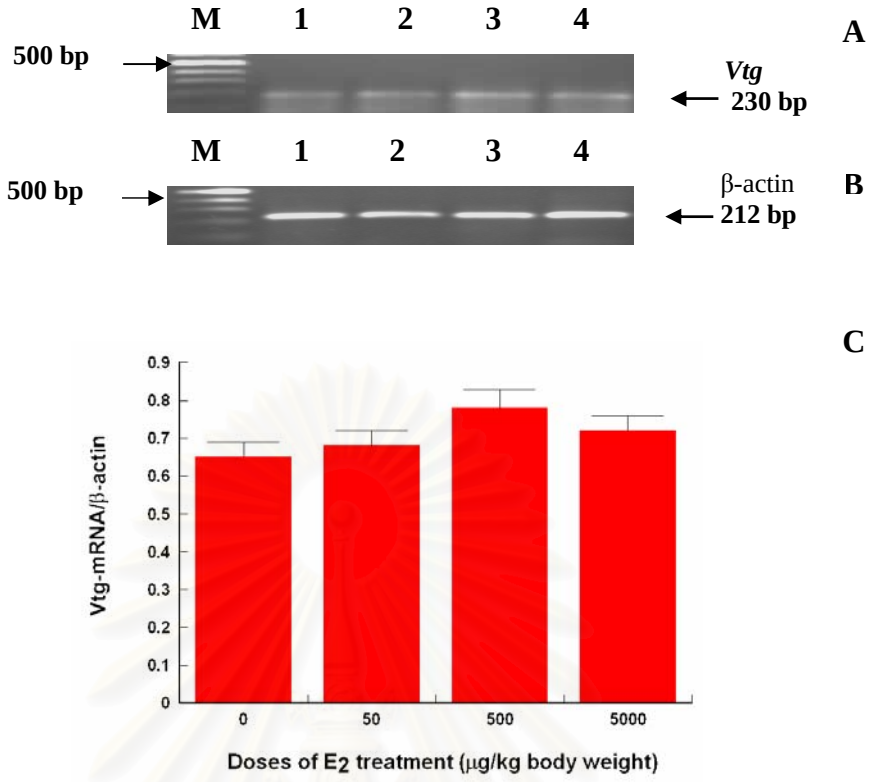


Figure 4.15. Vitellogenin gene expression levels determined by RT-PCR after treatment of immature female *H. rugulosus* with E_2 for 5 days A). β-actin, the internal control as the same in treated as in control (B). Lane M indicated 100 bp DNA ladder as a marker. Lanes 1-4 were RT-PCR (VtgF/VtgR) amplicons from the liver of *H. rugulosus* treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively. Expression of *Vtg* gene levels detected by semi-quantitative RT-PCR using β-actin as the internal reference standard (C). Data are presented as the mean ± SEM (n = 6).

4.4.2 The effect of E_2 on liver vitellogenin gene expression in immature male frogs

The *Vtg*-gene expression in immature male frogs was determined by RT-PCR (Fig. 4.16A). The ß-actin, the internal control (Fig. 4.16B) was the same in treated as in control groups. The results from experiments showed that E_2 at a dose of 50, 500 and 5,000 µg/kg body weight (0.70 ± 0.06, 0.72 ± 0.05 and 0.73 ± 0.03, respectively) could not stimulate *Vtg* gene expression when compared with the control (0.68 ± 0.04) (Fig. 4.16C).

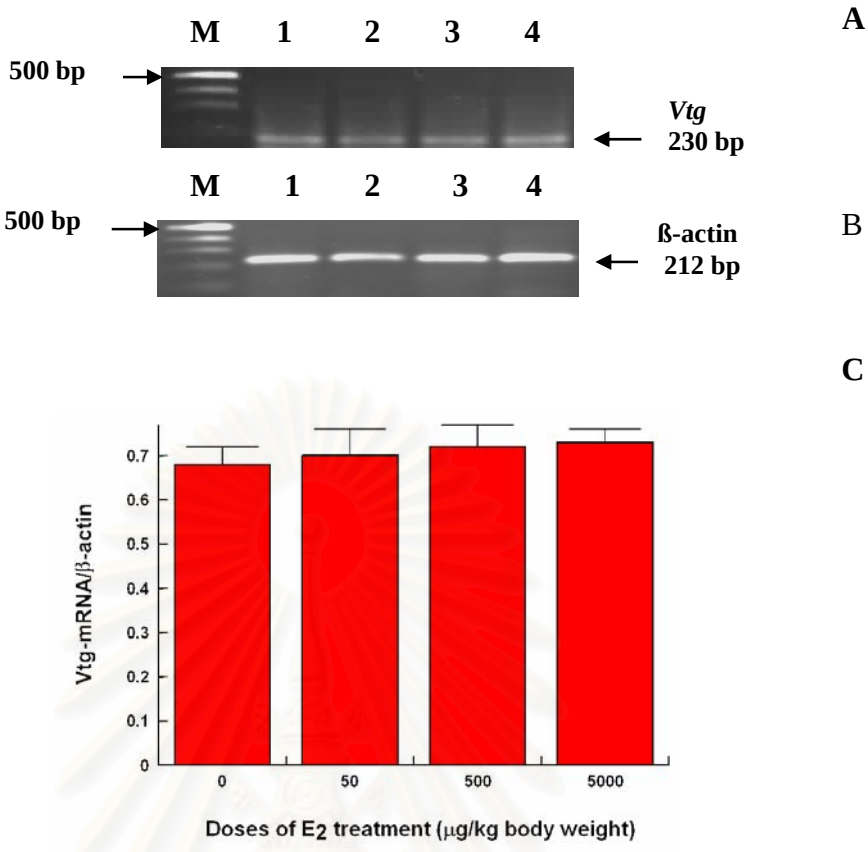


Figure 4.16. Vitellogenin gene expression levels determined by RT-PCR after treatment of immature male *H. rugulosus* with E_2 for 5 days (A). β-actin, the internal control was the same in treated as in control group (B). Lane M indicated 100 bp DNA ladder as a marker. Lanes 1-4 were RT-PCR (VtgF/VtgR) amplicons from the liver of *H. rugulosus* treated with E_2 at a dose of either 0, 50, 500 or 5,000 μg/kg body weight, respectively. Expression of *Vtg* gene levels detected by semi-quantitative RT-PCR using β-actin as the internal reference standard (C). Data were presented as the mean ± SEM (n = 6).

4.3.3 The effect of E_2 on plasma phosphoprotein levels in immature female and male frogs

The molecular mass of the expected Vtg is higher than 116,250 Da (Fig. 4.17). The data from immature female group showed that there was a significant increase in intensity of expected Vtg bands in immature female frogs exposed to E_2 all doses studied when compared with control groups (3055.16 ± 167.20, 3314.31 ± 173.73, 3414.23 ± 228.82 vs. 2071.25 ± 157.52) ($P<0.05$) (Fig. 4.18A). In immature male

frogs that were treated with E_2 at a dose of either 500 or 5,000 µg/kg body weight significantly increased the intensity of expected Vtg bands when compared with control group. (2555.97 ± 278.96, and 2647.97 ± 168.84 vs. 1499.44 ± 129.54) (Fig. 4.18B).

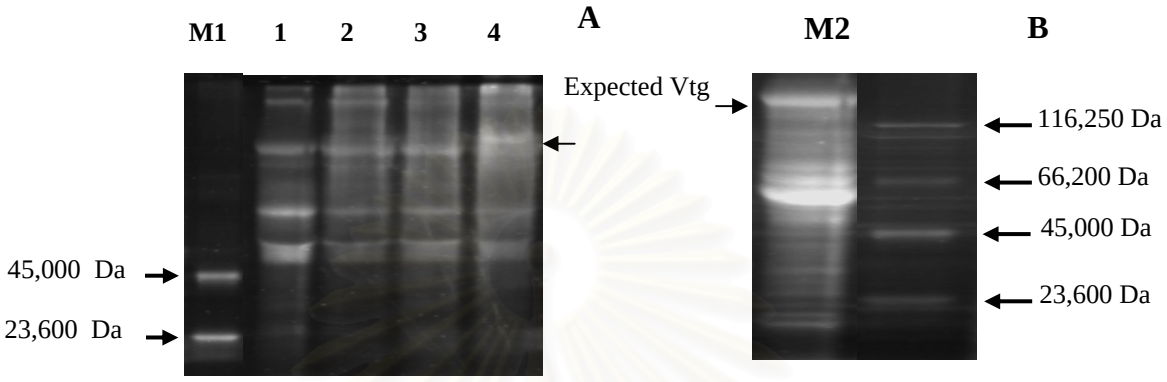


Figure 4.17. Phosphorylated (lane M1) and nonphosphorylated (lane M2) proteins in the Peppermint Stick phosphoprotein molecular mass standards. Fig. 4.17A indicated phosphorylated protein. Fig. 4.17B indicated both phosphorylated and nonphosphorylated proteins. Lanes 1-4 (the product from frog plasma) treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively.

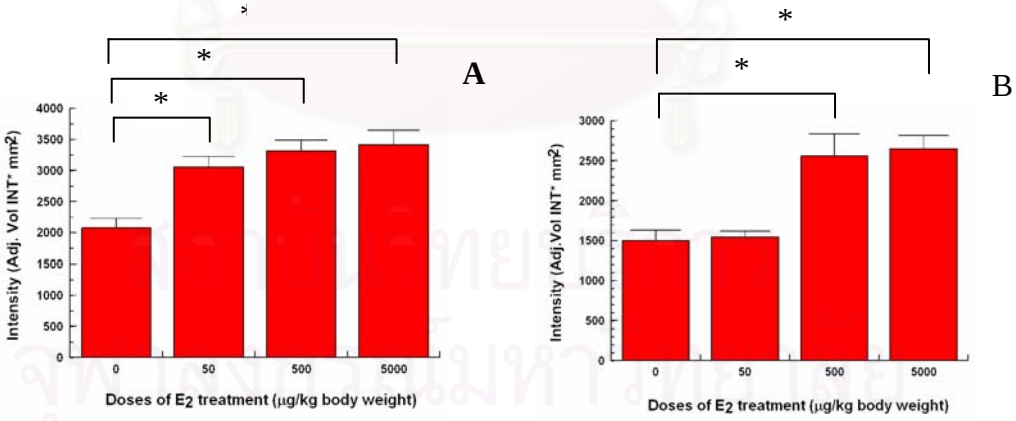


Figure 4.18. The intensity of expected Vtg bands from immature female (A) and male (B) frog that were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively. The data represented the mean ± SEM (n = 6). Significant difference from the control group: *$p < 0.05$.

4.4.4 The effect of E_2 on the weight of body, liver, gonad and LSI and GSI in immature female and male frog

The body, liver and gonad weight of the immature female frogs that were reared during dry season were analyzed (Fig. 4.19). The data showed that body weights of the immature female frogs treated with E_2 in different doses studied were not significantly different among treatment groups (147.77 ± 7.02, 108.88 ± 9.92, and 115.00 ± 14.43 g, respectively) and control group (140.00 ± 20.70 g) (Fig. 4.19A). The liver weights were not significantly different among treatment groups (4.51 ± 0.45, 3.39 ± 0.48 and 3.09 ± 0.53 g, respectively) and control group (3.50 ± 1.23 g) (Fig. 4.19B). The gonad weights were not significantly different among treatment groups (6.40 ± 2.14, 2.17 ± 0.79, and 2.91 ± 1.64 g, respectively) and control group (4.58 ± 2.14 g) (Fig. 4.19C).

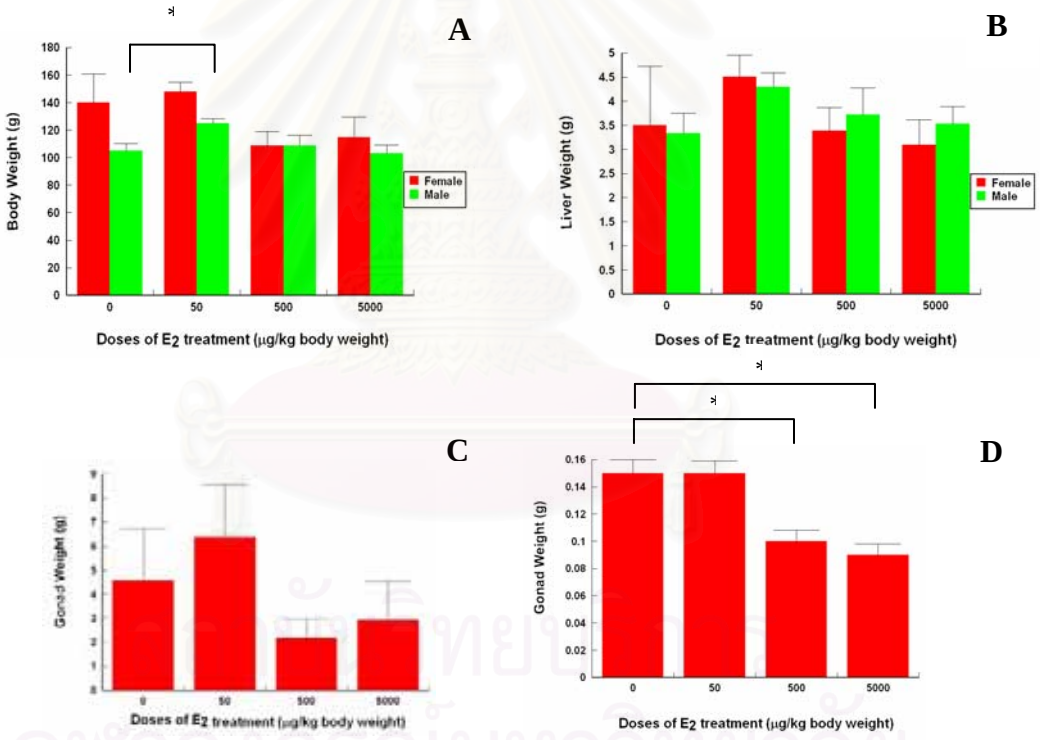


Figure 4.19. This figure represented the body weight of female and male frogs (A), the liver weight of female and male frogs (B) and female (C) and male gonad (D) weight of the frogs which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively. (Female; n = 7, 9, 9 and 4 animals, respectively Male; n = 10, 10, 8 and 10 animals, respectively). The data represented the mean ± SEM. Significant difference from the control group: *p < 0.05.

The body, liver and gonad of the immature male frogs that were reared during dry season were shown in figure 4.18. The data showed that body weight of the male frogs that treated with E_2 at a dose of 50 µg/kg body weight significantly higher than control group (125.00 ± 3.41 vs 105.00 ± 5.21, respectively). There were no significant change in body weight of the groups that were treated with E_2 at doses of 500 and 5,000 µg/kg body weight (Fig 4.19A). The liver weights were not significantly different among treatment groups (4.30 ± 0.29, 3.72 ± 0.56, and 3.53 ± 0.36, respectively) and control group (3.33 ± 0.42) (Fig. 4.19B). The E_2 at a dose of 500 or 5,000 µg/kg body weight significantly decreased the gonad weight (0.10 ± 0.01 and 0.09 ± 0.01, respectively) when compared with control group (0.15 ± 0.01 (Fig. 4.19D)

Liver-somatic index (LSI) and gonadal-somatic index (GSI) (weight of liver or ovary/total body weightx100) of the immature female frogs were analyzed (Fig. 4.20). The data showed that LSI of the immature female frogs treated with E_2 in different doses studied were not significantly different among treatment groups (3.03 ± 0.27, 3.02 ± 0.24, and 2.72 ± 0.39, respectively) and control group (2.22 ± 0.38) (Fig. 4.20A). In the same way, the GSI was not significantly different among treatment groups (3.97 ± 0.95, 1.96 ± 0.72, and 2.21 ± 1.01, respectively) and control group (2.86 ± 0.99) (Fig. 4.20B).

After treatment immature male with E_2, the data showed that the LSI of the frogs that treated with E_2 in different doses was not significantly different among treatment groups (3.43 ± 0.20, 3.31 ± 0.28, and 3.40 ± 0.24, respectively) and control group (3.13 ± 0.30) in frogs that were reared in dry season (Fig. 4.20C). The GSI was not significantly different among treatment groups (0.12 ± 0.006, 0.09 ± 0.007. and 0.09 ± 0.006, respectively) and control group (0.14 ± 0.009) (Fig. 4.20D).

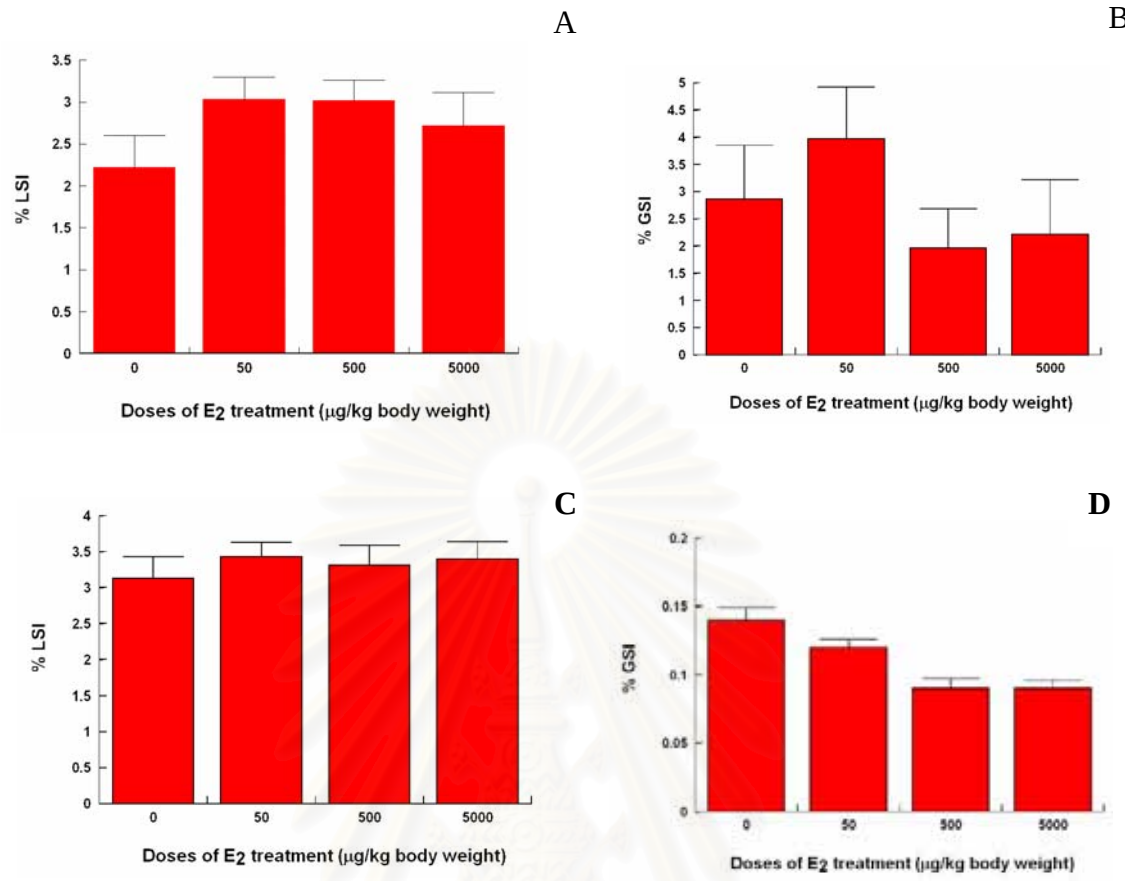


Figure 4.20. Liver- and gonadal-somatic index in immature female (A-B) and male (C-D) frogs which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 μg/kg body weight for 5 days. (Female; n = 7, 9, 9 and 4 animals, respectively Male; n = 10, 10, 8 and 10 animals, respectively). The data represented the mean ± SEM.

4.4.5 Plasma E_2 levels in immature female and male frogs

As shown in figure 4.21, plasma E_2 levels in immature female and male frogs were determined. The data showed that there was no significant difference between female (87.33 ± 24.18 pg/ml) and male (68.03 ± 23.81 pg/ml) group.

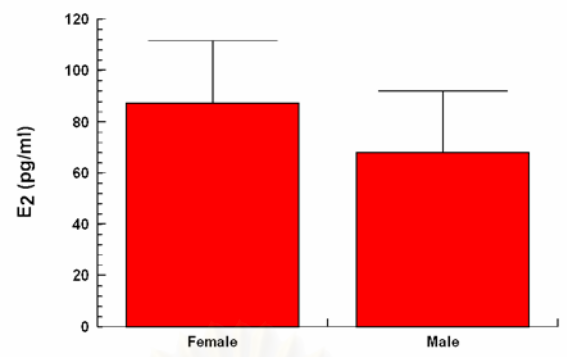


Figure 4.21. Plasma E_2 level in untreated immature female and male frogs (n = 5 animals). Data are presented as the mean ± SEM.

4.5 The comparison of some experimental parameters in the frog of the two seasons

4.5.1 The comparison of the *Vtg* gene expression in immature female and male frogs in the two seasons

Comparison of the *Vtg*-gene expression in immature female frog between rainy season and dry season with paired-samples T test. Data showed that *Vtg*-gene expression in frog treated with E_2 at a dose either of 0, 50, 500 or 5,000 µg/kg body weight in both season was not significantly different (0.85 ± 0.03 vs 0.65 ± 0.04, 0.65 ± 0.02 vs 0.68 ± 0.04 , 0.60 ± 0.02 vs 0.78 ± 0.05 and 0.58 ± 0.02 vs 0.72 ± 0.04, respectively) (Fig. 4.22A).

Comparison of the *Vtg*-gene expression in immature male frog between rainy season and dry season. Data showed that *Vtg*-gene expression in frog treated with E_2 at a dose either of 0, 50, 500 and 5,000 µg/kg body weight in both season was not significantly different (0.61 ± 0.04 vs 0.68 ± 0.04, 0.70 ± 0.05 vs 0.70 ± 0.06 , 0.80 ± 0.06 vs 0.72 ± 0.05 and 0.74 ± 0.05 vs 0.73 ± 0.03, respectively) (Fig. 4.22B).

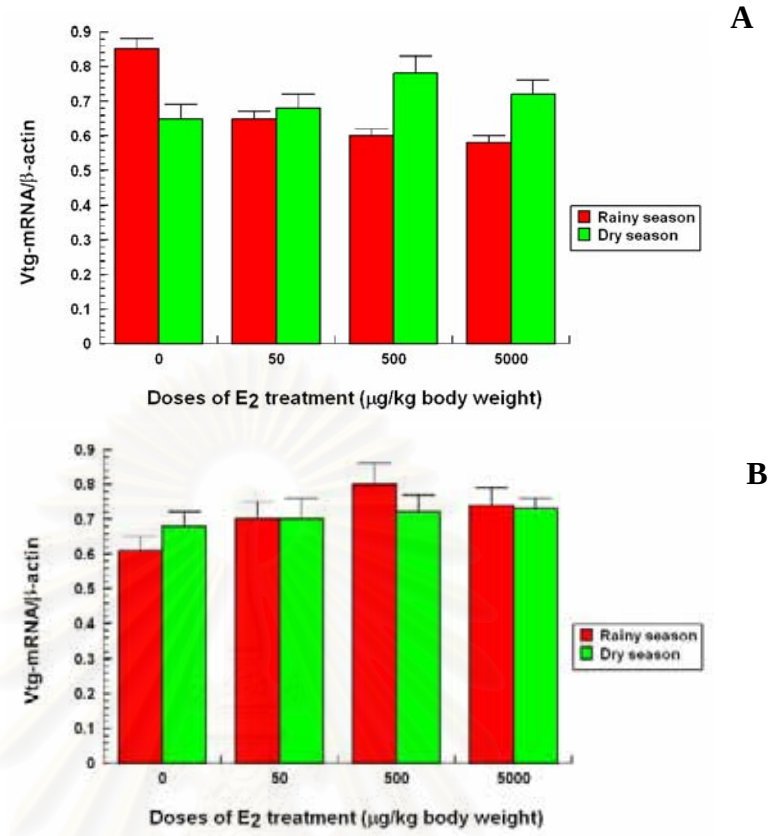


Figure 4.22. Vitellogenin gene expression levels determined by RT-PCR after treatment of immature female (A) and male (B) *H. rugulosus* with E_2 at a dose either of 0, 50, 500 or 5,000 µg/kg body weight for 5 days. Expression of *Vtg* gene levels detected by semi-quantitative RT-PCR using β-actin as the internal reference standard. Data are presented as the mean ± SEM (n = 6).

4.5.2 The Comparison of intensity of expected Vtg bands in immature female and male frogs in the two seasons

Comparison of the intensity of expected Vtg bands in immature female frog between rainy season and dry season with paired-samples T test, data showed that intensity of all expected Vtg bands from frog which reared during dry season was higher than frog which reared during rainy season. Frogs were treated with E_2 at doses 50, 500,and 5,000 was significantly different from the rainy season groups (1657.83 ± 98.46 vs 2071.25 ± 157.52, 1841.81 ± 95.48 vs 3055.16 ± 167.20 , 1912.84 ± 148.17 vs 3314.31 ± 173.73 and 2308.13 ± 135.11 vs 3414.23 ± 228.82, respectively) (Fig. 4.23A).

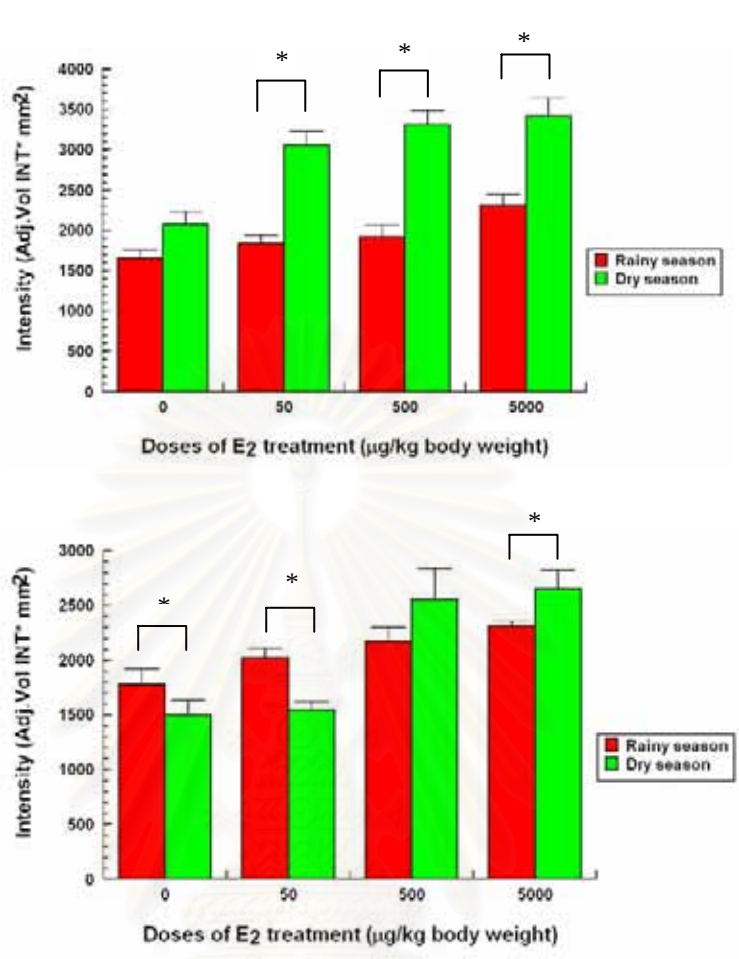


Figure 4.23. The intensity of expected Vtg bands from immature female (A) and male (B) frog that were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight, respectively. The data represented the mean ± SEM (n = 6). Rainy season vs dry season: *p< 0.05.

Comparison of the intensity of expected Vtg bands in immature male frog between rainy season and dry season, data showed that intensity of all expected Vtg bands was not significantly different (1775.44 ± 142.68 vs 1499.44 ± 129.54, 2019.65 ± 85.81 vs 1543.85 ± 75.84, 2019.65 ± 85.81 vs 2555.97 ± 278.96 and 2170.40 ± 122.96 vs 2647.97 ± 168.84, respectively) (Fig. 4.23B).

4.5.3 The comparison of the body weight in immature female and male frog in the two seasons

Comparison of the body weight of the immature female and male frogs was shown in figure 4.23. The data showed that body weight of the female frogs which reared during rainy season and treated with E_2 at doses were significantly higher than frogs which reared during dry season (183.00 ± 8.17 vs 140.00 ± 20.70, 182.00 ± 4.89 vs 147.77 ± 7.02, 176.00 ± 5.61 vs 108.88 ± 9.92 and 177.00 ± 7.15 vs 115.00 ± 14.13 g, respectively) (Fig. 4.24A)

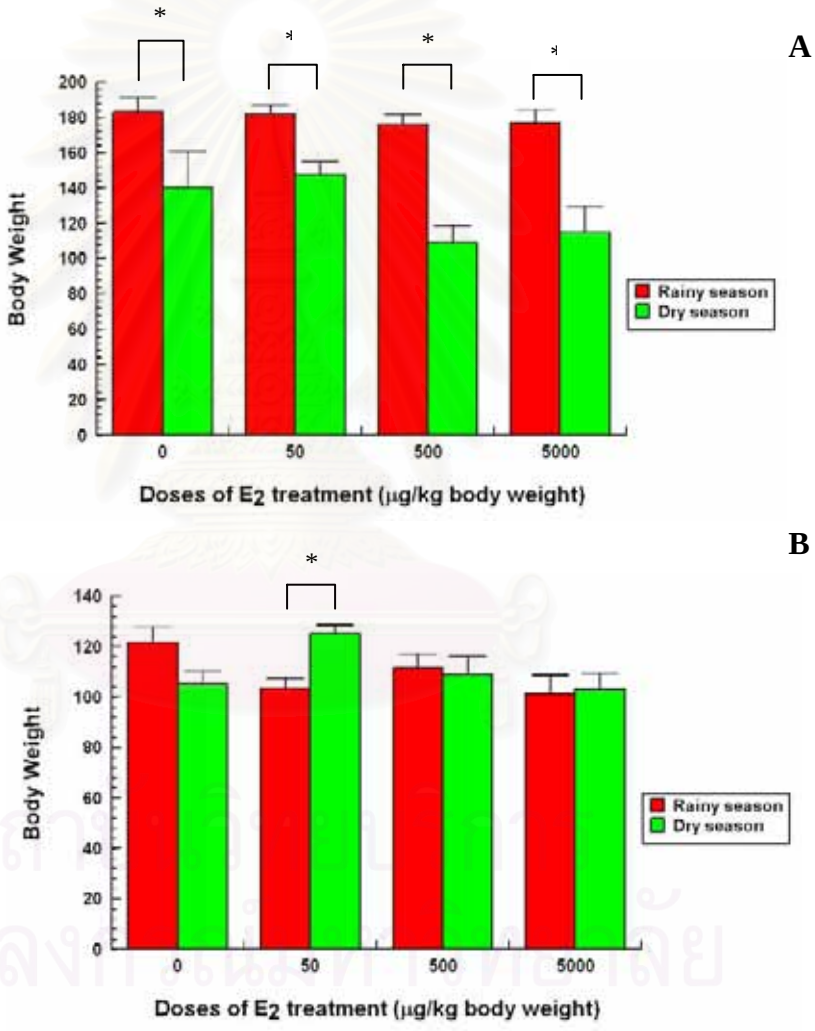


Figure 4.24. This figure represents the body weight of immature female (A) and male (B) frogs which were treated with E_2 at a dose of either 0, 50, 500, or 5,000 µg/kg body weight, respectively. (Female; n = 10, 10, 10, and 10 animals, respectively Male; n = 7, 9, 9 and 4 animals, respectively). The data represented the mean ± SEM. Rainy season vs dry season: *p< 0.05.

Comparison of the body weight of the immature male frog was shown in figure 4.22B. The data showed that there were no differences in body weight of all treated male frogs which reared in both seasons (103.33 ± 4.08 vs 125.00 ± 3.41 , 111.42 ± 5.53 vs 108.75 ± 7.42 and 101.25 ± 7.42 vs 103.00 ± 6.15 g, respectively), except male frog which reared during dry season and treated with E_2 at a dose of 50 µg/kg body weight was significantly higher than same treated-frog which reared during rainy season (121.42 ± 6.33 g vs 105.00 ± 5.21 g) (Fig. 4.24B).

4.5.4 The comparison of the liver weight in immature female and male frogs in the two seasons

Comparison of the liver weight of the immature female and male frogs was shown in figure 4.24. The data showed that liver weight of the female frogs which reared during rainy season and treated with E_2 at all doses were significantly higher than frog which reared during dry season (8.50 ± 0.64 vs 3.50 ± 1.23, 8.12 ± 0.46 vs 4.51 ± 0.45, 8.42 ± 0.66 vs 3.39 ± 0.48 and 9.57 ± 0.62 vs 3.09 ± 0.53 g, respectively) (Fig. 4.25A).

Comparison of the liver weight of the immature male frog was shown in figure 4.23B. The data showed that liver weight of the male frogs which reared during rainy season tended to be higher than frog which reared during dry season. E_2 at dose only 5,000 µg/kg body weight and untreated group was significantly higher than frog which reared during dry season. (5.33 ± 0.58 vs 3.33 ± 0.42, 4.57 ± 0.35 vs 4.30 ± 0.29, 5.10 ± 0.49 vs 3.72 ± 0.56 and 5.44 ± 0.57 vs 3.53 ± 0.36 g, respectively) (Fig. 4.25B).

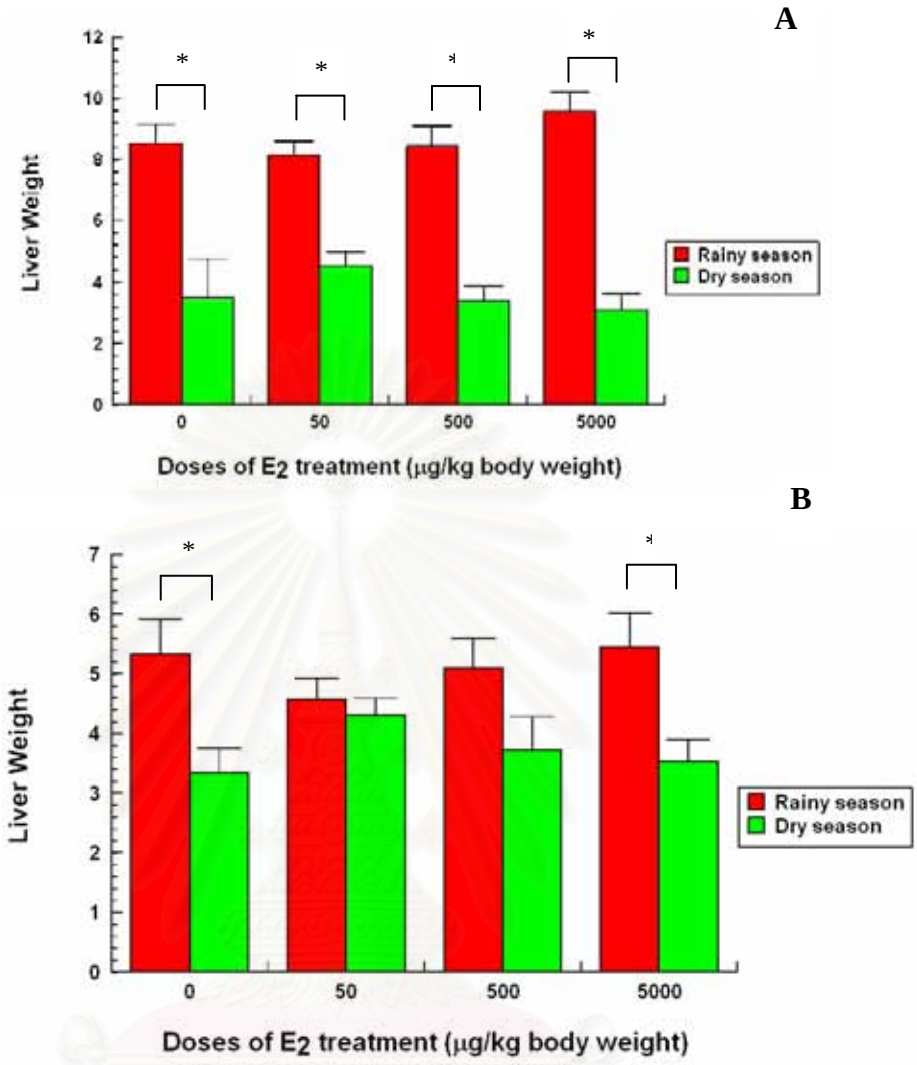


Figure 4.25. This figure represented the liver weight of immature female (A) and male (B) frog which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 μg/kg body weight, respectively, (Female; n = 10,7, 10,9, 10,9 and 10,4 and Male; n = 7,10, 9,10, 7,8 and 8,10 animals, respectively). The data represented the mean ± SEM. Rainy season vs dry season: *p < 0.05.

4.5.5 The comparison of the gonad weight in immature female and male frogs in the two seasons

Comparison of the gonad weight of the immature female and male frogs was shown in figure 4.26. The data showed that gonad weight of the female frogs which reared during rainy season and exposed to E_2 all doses was significantly higher than frog which reared during dry season (12.81 ± 2.14 vs 4.58 ± 2.14, 16.49 ± 1.86 vs

6.40 ± 2.14, 15.33 ± 1.54 vs 2.17 ± 0.79 and 15.97 ± 1.59 vs 2.91 ± 1.64 g, respectively) (Fig. 4.26A).

Comparison of the gonad weight of the immature male frog was shown in figure 4.24B. The data showed that gonad weight of the male frogs which reared in both season was not significantly different (0.17 ± 0.01 vs 0.15 ± 0.01, 0.15 ± 0.01 vs 0.15 ± 0.01, 0.13 ± 0.01 vs 0.10 ± 0.01 and 0.13 ± 0.01 vs 0.09 ± 0.01 g, respectively) (Fig. 4.26B).

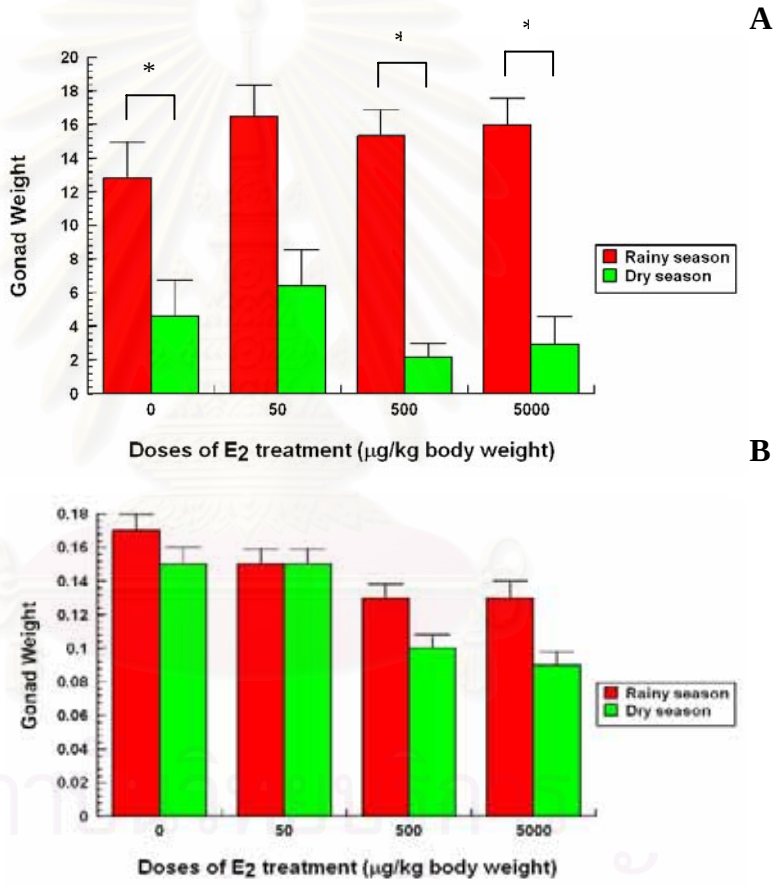


Figure 4.26. This figure represented the gonad weight of the immature female (A) and male (B) frog which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 μg/kg body weight, respectively, (Female; n = 10,7, 10,9, 10,9 and 10,4 and Male n = 7,10, 9,10, 7,8 and 8,10 animals, respectively). The data represented the mean ± SEM. Rainy season vs dry season: *p < 0.05.

4.5.6 The comparison of the liver-somatic index in immature female and male frogs in the two seasons

Liver-somatic index (LSI) of the immature female and male frogs was analyzed (Fig. 4.27). The data showed that LSI of the immature female frogs which reared during rainy season both control and exposed to E_2 in different doses was significantly higher than groups which reared during dry season (4.65 ± 0.29 vs 2.22 ± 0.38, 4.45 ± 0.21 vs 3.03 ± 0.27, 4.77 ± 1.33 vs 3.02 ± 0.24 and 5.42 ± 0.30 vs 2.72 ± 0.39, respectively) (Fig. 4.27A).

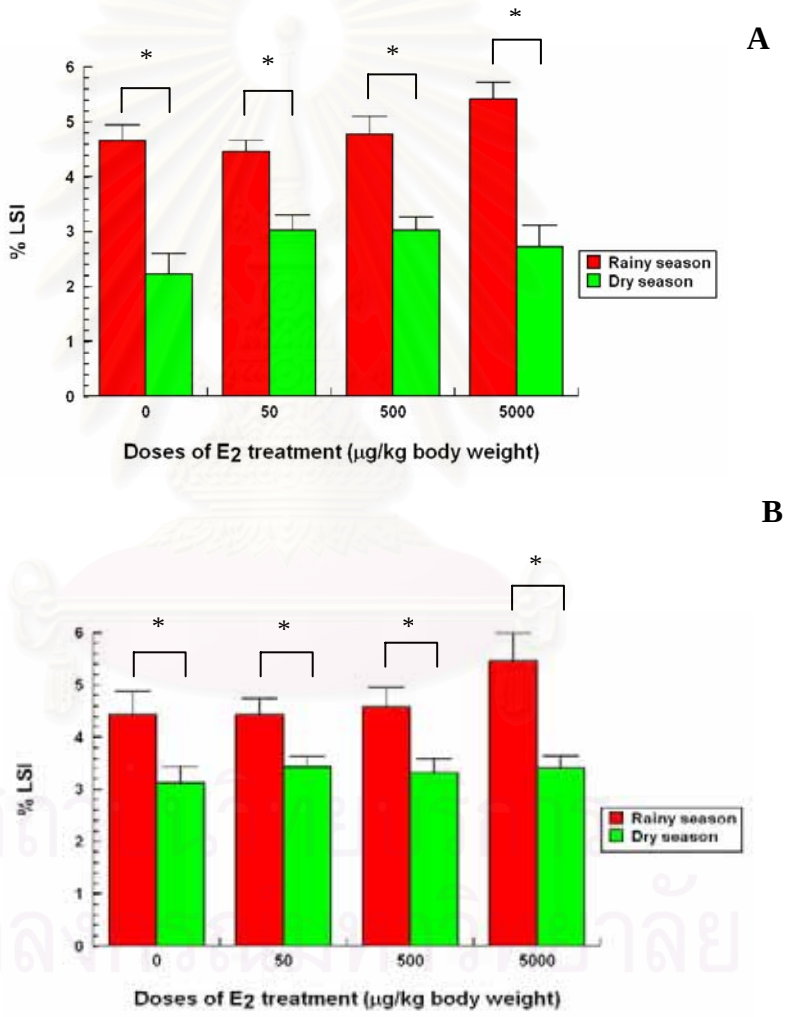


Figure 4.27. Liver-somatic index of immature female (A) and male (B) frog which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight for 5 days, (Female; n = 10,7, 10,9, 10,9 and 10,4 and Male; n = 7,10, 9,10, 7,8 and 8,10 animals, respectively). The data represented the mean ± SEM. Rainy season vs dry season: *p< 0.05.

The data showed that LSI of the immature male frogs which reared during rainy season both control and exposed to E_2 in different doses was significantly higher than groups which reared during dry season (4.43 ± 0.44 vs 3.13 ± 0.03, 4.43 ± 0.03 vs 3.43 ± 0.02, 4.58 ± 0.36 vs 3.31 ± 0.28 and 5.46 ± 0.53 vs 3.40 ± 0.24, respectively) (Fig. 4.27B).

4.5.7 The comparison of the gonadal-somatic index in immature female and male frogs in the two seasons

Gonadal-somatic index of the immature female and male frogs were analyzed (Fig. 4.28). The data showed that GSI of the immature female frogs all groups tended to be higher than frog which reared during dry season. The GSI of frog which reared during rainy season and treated with E_2 at a dose of either 500 or 5,000 µg/kg body weight was significantly higher than same group from dry season (7.07 ± 1.19 vs 2.86 ± 0.99, 9.03 ± 0.99 vs 3.97 ± 0.95, 8.96 ± 0.92 vs 1.96 ± 0.72 and 9.11 ± 0.91 vs 2.21 ± 1.01, respectively) (Fig. 4.28A).

Gonadal-somatic index of the immature male frogs were analyzed (Fig. 4.26B). The data showed that GSI of the immature male frogs treated with E_2 at different doses were not significantly different among both groups (0.14 ± 0.01 vs 0.14 ± 0.01, 0.15 ± 0.01 vs 0.12 ± 0.006, 0.12 ± 0.01 vs 0.09 ± 0.007 and 0.14 ± 0.01 vs 0.09 ± 0.006, respectively) (Fig. 4.28B).

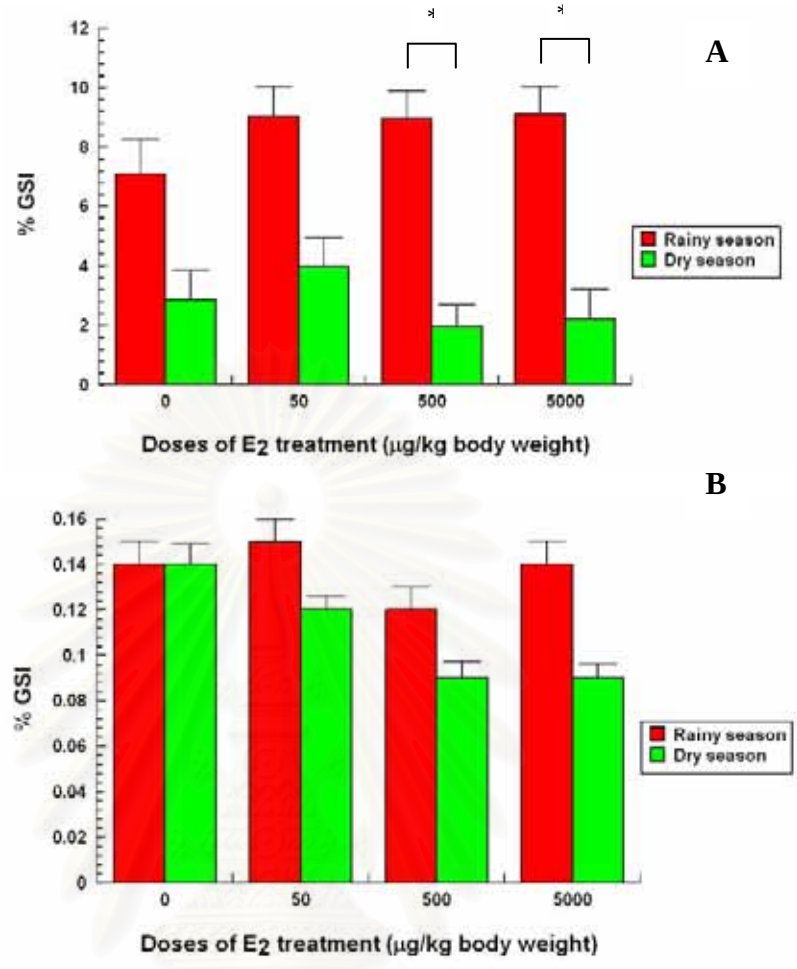


Figure 4.28. Gonadal-somatic index of immature female (A) and male (B) frog which were treated with E_2 at a dose of either 0, 50, 500 or 5,000 µg/kg body weight for 5 days, (Female; n = 10,7, 10,9, 10,9 and 10,4 and Male; n = 7,10, 9,10, 7,8 and 8,10 animals, respectively). The data represented the mean ± SEM. Rainy season vs dry season: *p< 0.05.

4.5.8 The comparison of plasma E_2 levels in immature female and male frog in the two seasons

Comparison of plasma E_2 levels of the immature female and male frogs were shown in figure 4.29. The data showed that plasma E_2 levels of the immature female frogs (327.24 ± 188.61 pg/ml) which reared during rainy season was significantly higher than immature female (87.33 ± 24.18 pg/ml) which reared during dry season. In contrast, plasma E_2 levels in untreated-immature male frog (17.59 ± 4.47 pg/ml)

which reared during rainy season was significantly lower than frog (68.03 ± 23.81 pg/ml) which reared during dry season.

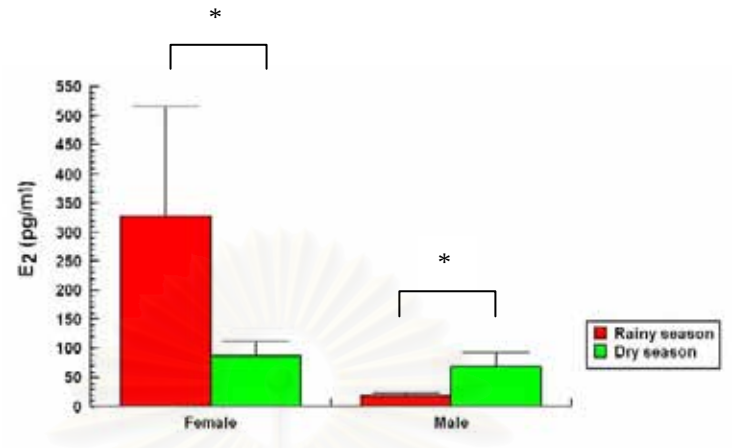


Figure 4.29. Plasma E_2 level in untreated-immature female and male (n = 5 animals). Data are presented as the mean ± SEM. Significant difference: *p < 0.05.

CHAPTER 5

DISCUSSION

5.1 The cDNA sequence of *Vtg* gene in *Hoplobatrachus rugulosus*

An attempt was made to amplify *Vtg* genes by following the methods of other successful reports including Yseng *et al*. (2001), Kloas *et al*. (1999), and by designing primers from the consensus sequences derived from the alignment of *Vtg* cDNA sequences from *Xenopus laevis* (M18061, Y00354), *Oncorhynchus mykiss* (S82450), and *Gallus gallus* (M18060, X13607) in the GenBank nucleotide database. Unfortunately all these experimental approaches failed to yield RT-PCR-products from *H. rugulosus*. Finally, the last primers were designed from *G. gallus* (M18060).

The results of this study showed that a pair of primers designed from part of cDNA sequence of vitellogenin gene of *G. gallus* (GenBank accession number M18060) was able to amplify a fragment of at least one of the *Vtg* genes in the frogs, *H. rugulosus* and *R. catesbeiana*. Indeed, the sequences obtained from part of the *Vtg* gene of *H. rugulosus* and *R. catesbeiana* were well conserved with the avian *G. gallus* sequence (chicken; GenBank accession number M18060) (Fig. 4.4), supporting the claimed conservation among vertebrate species (Mouchal *et al*., 1997). However, this was not the case for the *X. laevis* sequence (GenBank accession number M18161, Fig. 4.4) which was poorly conserved at the 3' end of the aligned sequence. Indeed the VtgF/VtgR primer pair in this study would be unlikely to amplify this particular *X. laevis Vtg* gene/exon. This may represent allelic sequence divergence within a gene/exon, or differential exon splicing, and thus that the VgtF-VgtR primer pair may not amplify across all frogs let alone amphibians. However, it is probably more likely that this is the result of comparison between different *Vtg* gene paralogs or exon shuffled orthologs and thus this primer set may preferentially or specifically amplify a subset of *Vtg* gene(s)/exons in *H. rugulosus* and *R. catesbeiana*. The nucleotide sequence obtained from the female frogs (230 bp) was longer than those obtained from the male frogs which might be due the different exon splicing in the female, or different isoform of the genes. These results indicated that *Vtg* mRNA in male *H. rugulosus* may be non-functional, or otherwise, may function in a different manner.

Certainly, this notion is consistent with, but not conclusive for, a role of estrogens in the control of sex-specific gene expression (Gahr and Metzdorf, 1997; Kalsotra *et al*., 2002). Therefore, further experiment isolation of the whole coding sequence of *Vtg* gene should be required. A phylogenetic tree resulted from *Vtg*-mRNA sequence data revealed that the frogs in Ranidae family (*H. rugulosus*: male and female, *R. catesbeiana*) had most closed genetic relationship compared to other animals from other families.

5.2 The effect of E_2 on *Vtg* gene expression

It was found that frogs injected with E_2 significantly decreased the level of hepatic *Vtg* gene expression in immature female *H. rugulosus* but, increased the level of hepatic *Vtg* gene expression in immature male frogs that were reared during rainy season. Tangpraprutgul *et al*. (1996) reported increasing E_2 levels in the plasma of *H. rugulosus* and *R. catesbeiana* in the breeding seasons (May to October), indicating the sexual ability in the mature female frogs. However, in this case exogenously injected E_2 had different effects between the two sexes. Highly endogenous E_2 levels in females might have induced a negative feedback to the higher centers such as pituitary glands and the hypothalamus. Thus when endogenous E_2 declined, it resulted in *Vtg* gene expression in female frog liver. On the other hand, present study revealed a decreased *Vtg* gene expression level whereas Carnevali *et al*. (1995) showed that mature female *R. esculenta* livers' cultured with E_2 (1 nM) increased *Vtg* gene expression levels in frogs from both prereproductive and reproductive periods. However, exogenous xenoestrogens and estrogen-mimic containing sewage increased *Vtg* mRNA expression levels in female juvenile *X. laevis* (Bögi *et al*., 2003). Although it was contrary to the present results and those of Carnevali *et al*. (1995), this might likely be due to either different experimental procedures used in the *in vitro* assays, or to the different developmental stages (age) of the animals studied (Bögi *et al* used animals after completion of metamorphosis). However, a real difference among each frog species could not be excluded formally at this stage.

The E_2 levels in mature males are normally lower than those in mature females both in fishes (Kawai *et al*., 2003) and *X. laevis* (Hecker *et al*., 2004). In this study, when E_2 was injected, usually binds to receptor and acts directly upon liver cells, it

resulted in up-regulation of *Vtg* gene expression in the male liver. This result agreed with reports showing that *Vtg* gene expression can be induced in mature male *R. esculenta* frogs by injection of E_2 (Carnevali *et al*., 1995), as well as in mature male zebrafish (*zvtg)* and medaka (*mvtg)* by either intramuscular injection of E_2 (1 µg/animal; body weight ~ 200 mg) or immersion of fish in water containing exogenous E_2 at 1 nM (Tong *et al*., 2004). Moreover, *in vitro* incubation of liver cells freshly explanted from mature male *X. laevis* with E_2 (1 nM) induced *Vtg* gene expression (Kloas *et al*., 1999). Taken together, these results strongly support that E_2 stimulates *Vtg* gene expression in mature and immature male frogs and fish both *in vivo* and *in vitro,* including immature male frogs.

Endogenous hormone levels seem to be one of the factors that can cause differences in sensitivity to exogenous endocrine active substances including xenoestrogens and estrogen-mimics. When exogenous hormones are administered to animals with different concentrations of the endogenous hormone(s) in the blood, the effects of the administered hormone would be expected to be higher in the animal with low levels of hormone than in the one with high levels due to the competition between the exogenous and endogenous hormone for binding to the receptor (Kawai *et al*., 2003). Thus, results obtained in this study may thus be due to the fact that exogenous E_2 might interfere with the endogenous level of E_2, resulting in decreased *Vtg* gene expression in immature females but increased *Vtg* gene expression in immature males frog that were reared during rainy season. Present study found that injected E_2 tended stimulate the level of hepatic *Vtg* gene expression in both immature female and male *H. rugulosus* that were reared during dry season. Tangpraprutgul *et al*. (1996) reported decreasing E_2 levels in the plasma of *H. rugulosus* and *R. catesbeiana* in the hibernation period (November to April). However, when E_2 was injected exogenously, similar results could be observed between the two sexes. Low endogenous E_2 levels in frogs might be including exogenous E_2, and then have directly induced *Vtg* gene expression on the liver. It resulted in increased expression of *Vtg* gene expression in the both immature female and male liver which is in agreement with reports that *Vtg* gene expression can be induced in female juvenile *X. laevis* (Bögi *et al*., 2003), mature male *R. esculenta* frogs (Carnevali *et al*., 1995) and fishes (Tong *et al*. (2004). Taken together, these results supported that E_2 stimulates

Vtg gene expression in immature and mature frog and fish including within immature female and male frogs which were reared in dry season.

To confirm the levels of E_2 in plasma, plasma E_2 was collected from frogs in both season. The E_2 levels in mature males are normally lower than those in mature females both in fishes (Kawai *et al*., 2003) and *X. laevis* (Hecker *et al*., 2004). The results from the experiment indicated that E_2 level of untreated immature male was lower than untreated-immature female as well. Moreover, E_2 in untreated-immature female that reared during rainy season was also higher than untreated-immature female that reared during dry season. Tsai and Jones (2005) reported that after mature male frog, *Rana pipiens* were implanted subcutaneously with silastic capsules containing E_2 (crystalline E_2) for 20 days decreased circulation levels of LH that reflects the level of GnRH output. Along the hypothalamic-pituitary-gonadal axis, the pituitary has been identified as a direct target of E_2 action in the frogs (Pavgi and Licht, 1989; 1993). However, LH levels that were not detectable after treatment the frogs with E_2 from Tsai and Jones experiment could due to E_2 action on GnRH system as well. Moreover, Tsai and Jones also reported that E_2 induced a significant enlargement of GnRH neurons in hypothalamus, thus supporting the role of E_2 in feedback regulation of the GnRH system. These results indicate clearly that E_2 act both the gonadotropes and the GnRH system. Larger GnRH neurons might be fewer secretaries, so LH also decreased. Thus a possible reason about the experiment conducted in immature female frog group which were reared during rainy season was, high exogenous (endogenous) E_2 levels that applied to in immature female frogs might act as negative feedback that resulted to decrease total plasma E_2 levels in the experiment, reflect to decrease *Vtg* gene expression in this group.

The results reported here support the notion that in the frog *H. rugulosus*, exogenous E_2 acts to decrease liver *Vtg* gene expression in immature females which reared during rainy season but stimulates liver *Vtg* gene expression in immature female and male which reared during dry season and rainy season. Thus it is likely that xenoestrogens and estrogen-mimics in the aquatic environment will potentially interfere with the frog's sexual development at the early stage.

5.3 The effect of E_2 on plasma phosphoprotein levels

Vitellogenin is usually measured in blood by immunoassays that require species-specific antibodies. Analytical ELISA kits for the determination of Vtg are available for several fish species, including salmon, medaka, zebrafish, fathead minnow, carp, rainbow trout, and cod (ICES WKIMON II Report 2006). However, it was reported that there was some antibodies do cross-react with Vtg in other species (Tyler and Lancaster, 1993; Nilsen *et al*., 1998). Werawatgoompa and colleagues (2004) have reported that antibody of red snapper, *L. campechanus* and grouper, *C. pachycentron* could cross react to with Vtg of each other. However, heterologous ELISAs are not as sensitive as homologous ELISAs that use species-specific antibodies and antigen (ICES WKIMON II Report 2006).In this study antibody of red snapper and grouper was used to test cross react to Vtg of the frog by using immunodiffusion technique. The results showed that both anti-Vtg of red snapper and grouper did not react to frog's Vtg, as observed to have no precipitin bands (data not shown). This might confirm the knowledge that Vtg measurements need specific antibodies for the species.

This study has tried to approach plasma Vtg and recently, there was a report that Vtg could be stained with a commercially available fluropore dye (Pro-Q Diamond, invitrogen) in fish (Van *et. al*., 2005). It was described as a universal assay that is based on the high-molecular weight and extensive phosphoserine content of all Vtgs. The above reports have showed that Vtg seems to have a common structure in several species in vertebrates since its structure include phospholipoglycoprotein. In this study was interested to follow Van *et. al*. (2005)' method by using crude protein from frog plasma which were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then stained with a commercially available fluropore dye (Pro-Q Diamond), and visualized by ultraviolet transillumination. The method seemed to be a sensitive detection of Vtg frog tested, since mass spectra from expected band (protein) matched to vitellogenin precursor of fish, *Oreochromis aureus* (T31095) with score 38. Results obtained from experiment showed that intensity of bands from immature frogs reared in both seasons was higher than immature control group. These results might indicate that there are higher phosphoprotein (Vtg precursor) levels in immature frogs treated with E_2 than immature control. However, the pattern seems

uncertain since the bands did not have only Vtg (phosphoserine) but it also included other kinds of phosphoprotein (tyrosine or threonine residues), showing that there was no pure frog Vtg in those bands. The results indicated that the phosphoprotein assay is at least as sensitive as antibody-based methods but it is universal (Van Veld *et al.*, 2005).

5.4 The effect of E_2 on number and size of oocytes

After vitellogenin has been synthesized, Vtg will normally transport along blood system and up take in vitellogenic oocytes during oogenesis. Plasma Vtg concentrations increase during sexual maturation in female fishes (Scott and Sumpter, 1983) and at this time seems Vtg is the major blood protein. Plasma Vtg concentrations increase around one million fold during the seasonal reproductive cycle of female fishes (Arukwe and Goksøyr, 2003). After treatment with E_2, however, the results showed that E_2 had no effect on number and size of oocytes. Sretarugsa *et al*. (2001) have reported that diameters of follicles/oocytes in frogs, *R. tigerina* were 1310-1500 µm and 1510-1700 µm in stage V and VI respectively which their size were close to these experiments (1380-1480 µm). Thus the results may be caused either by the duration of treatment which was rather short, or most of the oocytes were at the vitellogenic and fully grown stages already (stage V and VI). There was evidence supporting the uptake of vitellogenin *in vitro* by injection of vital dye trypan blue into the dorsal lymph sac of female *X. laevis* (Wallace and Dumont, 1968; Dumont, 1972). These studies showed that the uptake of trypan blue began in stage III oocytes and reached the maximal level in stage IV oocytes while the activity decreased in the stage V and VI oocytes.

This study did not find oocytes (by eyes) in frog which reared during dry season. The gonads that were found had white color. However, nonvitellogenic stage of oocytes should be found (Sretarugsa *et al*., 2001) but be resting stage, since this stage of oocytes is gonadotropin-independent. These results indicated that even Vtg was synthesized after exogenously E_2 but it did not effect to the size of oocytes. Another season might due to short period of treatment.

5.5 The effect of E_2 on the weight of the body, liver, gonad and LSI and GSI

The body weight in mature male is normally lower than those in mature female frog (Tangpraprutgul *et al*., 1996) because male frog has smaller size than the female frog. The results from our experiment also showed that body weight of immature male was lower than female. Wiegand (1982) and Björnsson *et al* (1986) have shown that there was an increase in liver weight during Vtg synthesis in the mature female oviparous animals. However, data from our experiment showed that there were no changes in liver weight in both female and male frogs which were reared in both seasons and data quite vary in each group, even though Vtg was synthesized continuous. These results might be able to be explained by the short number of treatment days also. There was a significant decrease of gonad weight in immature male which were treated with E_2 at doses of 500 and 5,000 µg/kg body weight in both seasons. These results might be due to E_2 action as negative feedback at GnRH and pituitary system also, reflects to size of gonad but these results were unclear in immature female. However, the body, liver and gonad weight of frogs which were reared during rainy season were higher than those of dry season. The results might be due to characteristics of amphibians, of which reproductive cycle change depends on the variation in environmental or seasonal condition (Jørgensen, 1992).

There is an increase liver weight during Vtg synthesis in the mature female oviparous animals (Wiegand, 1982; Björnsson *et al*., 1986). Thus, functional E_2 receptors are present in the liver because Vtg was produced. Vtg is produced in the liver in response to E_2, and is transported via the bloodstream to the ovary where it is incorporated into the developing oocyte (Wallace, 1985). As a result mature females normally have a higher LSI (an indication of hepatic Vtg production) and GSI (an indication of sexual maturity) than would normally be present in immature females (Thompson *et al*., 2002). Our data about GSI in male frogs at age of 5 months, which seemed slightly lower than those in mature male group where as GSI in female frogs at age of 5 months had higher than those in mature female (Chaitiamwong, 1995). The results obtained may be due to characteristics of amphibians is change of reproductive cycle which depend on the variation in environmental or seasonal condition (Jørgensen, 1992).

The pre-spawning development of the female teleost oocyte occurs in two phases, a period of bulk growth and a period of final maturation (Wallace, 1985). Oocyte growth is primarily the result of the uptake of Vtg (Wallace, 1985). The data from our experiment showed that there were no changes in LSI and GSI obtained in E_2 treated frogs (both immature female and male), though in the immature females *Vtg* gene expression decreased, and in the immature males, *Vtg* gene expression increased. In an experiment performed by Thompson *et al*. (2002), immature female squirrelfish, *Holocentrus adscensionis* were given interperitoneal injections of 5 mg E_2 kg body weight^{-1} (1 ml kg^{-1} in peanut oil) for 4 days. Their results showed that the E_2 treatment increased LSI (different from our results) but no effect on GSI (which coincides with our results). The increased LSI was due to increase Vtg production in fish liver during the treatment period. According to Thompson *et al*. (2002), no change in the GSI was due to the short duration of treatment. This is confirmed by the results of Tsai and Jones (2005) that implanted male leopard frogs, *Rana pipiens* with silastic capsules containing E_2 for 20 days but GSI had no correlation to the size of GnRH neurons. They suggested that changed GSI was only manifested after being exposed to elevated steroid hormone levels for prolonged periods. Thus in this case if we expose animals to E_2 longer than 5 days, the LSI and GSI should be changed.

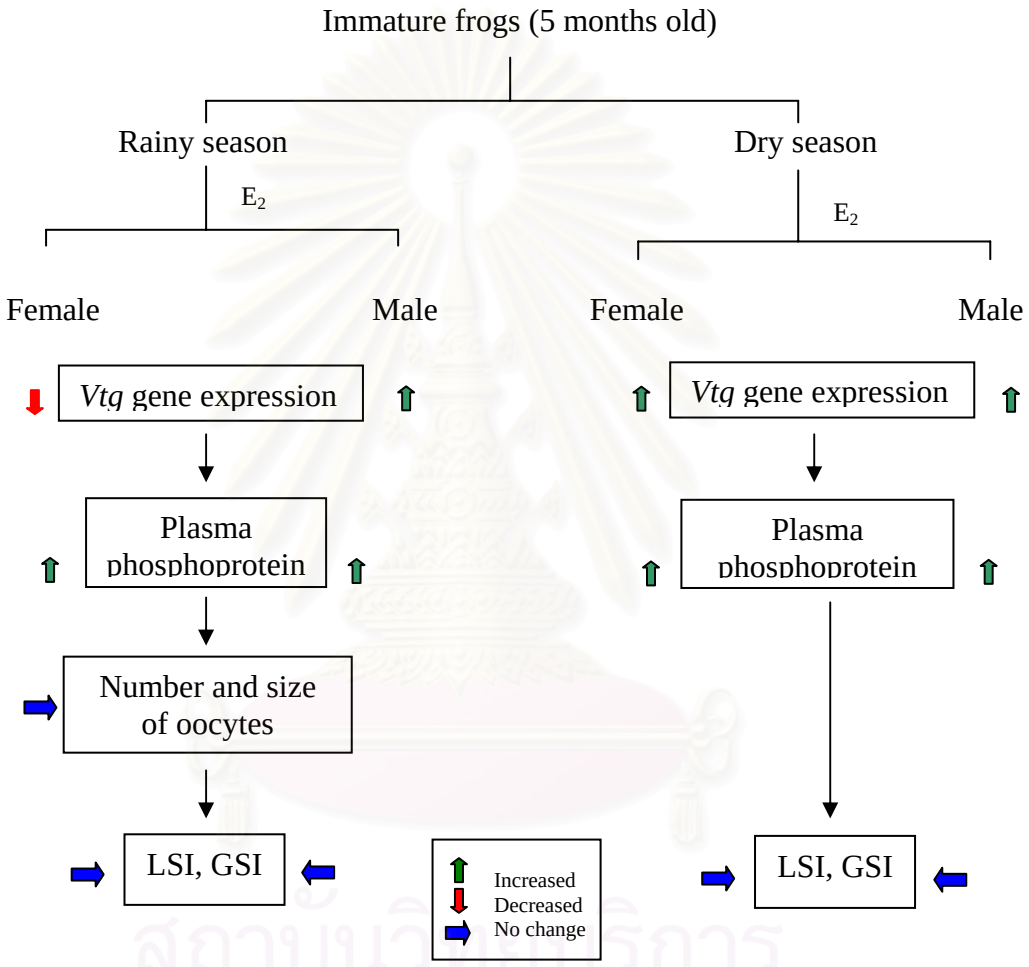
5.6 The effect of E_2 on histology of live and gonad

There were reports that egg size and histology of the fish gonad (http://www.fao.org; Sretarugsa *et al*., 2001) and several changes in hepatic morphology such as proliferation of rough endoplasmic reticulum and golgi apparatus of *X. laevis* liver cells (http://zygote.swarthmore.edu/germ1.html) during vitellogenesis. The histology showed that there was no change in structure of both liver and oocytes. These results might be able to be explained by the short duration of treatment. However, some intrastructure changes such as rough endoplasmic reticulum and golgi apparatus might be able to be observed by using electron microscope (http://zygote.swarthmore.edu/germ1.html). The study found that almost of oocytes were filled with yolk and germinal vesicle was pressed to periphery. The size of oocytes in both untreated group and treated group looked similar.

CHAPTER 6

CONCLUSIONS

From the study, it could be concluded as follows:



The primers designed from part of cDNA sequence of vitellogenin gene of *G. gallus* were able to amplify a fragment of at least one of the *Vtg* genes in the frogs, *H. rugulosus* and *R. catesbeiana*. It is therefore confirmed that this part of the gene is well conserved among vertebrates. E_2 inhibited *Vtg* gene expression in the immature female frog and stimulated *Vtg* gene expression in the immature male frog, hence it is possible that the reproduction of frog exposed to high exogenous E_2 such as xenoestrogen in the environment would be impaired.

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APPENDICES

APPENDIX A

Preparation of polyacrylamind gel electrophoresis

A) Stock reagents

1) 30% Acrylamide and 0.8% bis – acrylamide 100 ml

Acrylamide 29.2 g

N, N' – methylene – bis – acrylamide 0.8 g

Adjusted volume to be 100 ml by d - H_2O.

2) 1.5 M Tris – HCl, pH 8.8 100 ml

Tris (hydroxymethyl) – aminometane 18.17 g

Adjusted pH to be 8.8 by 1 M HCl and adjusted volume to be 100 ml by d - H_2O.

3) 0.5 M Tris – HCl, pH 6.8 100 ml

Tris (hydroxymethyl) – aminometane 6.06 g

Adjusted pH to be 6.8 by 1 M HCl and adjusted volume to be 100 ml by d - H_2O.

4) 1 M Tris – HCl, pH 6.8 100 ml

Tris (hydroxymethyl) – aminometane 12.1 g

Adjusted pH to be 6.8 by 1 M HCl and adjusted volume to be 100 ml by d - H_2O.

5) 10% ammonium persulfate (APS)

APS (10% w/v) 0.05 g

d - H_2O 500 µl

Fresh ammonium persulfate prepared just prior to use.

B) SDS-PAGE followed from Srimawong (2003)

1) 12% Separting gel

30% Acrylamide solution (12%)	6 ml
1 M Tris-HCl, pH 8.8 (0.375 M)	5.6 ml
10% (w/v) SDS	150 µl
d.H_2O	3.16 ml
10% APS	75 µl
TEMED (0.05%)	7.5 µl

2) 4% Stacking gel

30% Acrylamide solution (4%)	0.80 ml
0.5 M Tris-HCl, pH 6.8 (0.125 M)	0.75 ml
10% (w/v) SDS	60 µl
d.H_2O	4.3 ml
10% APS	30 µl
TEMED (0.1%)	6 µl

3) Sample buffer (5X loading dye)

1 M Tris-HCl, pH 6.8 (0.312M)	0.6 ml
Glycerol (50 %v/v)	5.0 ml
10% (w/v) SDS	2.0 ml
2-Mercaptoethanol	0.5 ml
1% Bromophenol blue	0.1 g
d.H_2O	0.9 ml

One part of sample buffer was added to four parts of sample.

The mixture was heated for 5 min in boiling water before loading to the gel.

4) Electrophoresis buffer (25 mM Tris and 192 mM glycine)

Tris (hydroxymethyl) – aminometane	3.0 g
Glycine	14.4 g
SDS	1.2 g

Adjust volume to be 1 liter by d.H_2O and adjusted pH

to be approximately 8.3

C) Preparation for tissue fixation and satining

1) 10% Buffer formalin

40% formalin	100 ml
$NaH_2PO_4.H_2O$	4 g
Na_2HPO_4	6.5 g
H_2O	900 ml

2) Heamatoxylin

Heamatoxylin	8.0 g
95% ethanol	400 ml
Ammonia alum	8.0 g
d-H_2O	400 ml
Glycerin	400 ml
Glacial acetic acid	40 ml

Leave the stain for at least 6 weeks before use

3) Eosin

Eosin	0.5 g
95% ethanol	100 ml

D) Preparation of solution for formaldehyde and agarose gels

1) MOPS (10X) for formaldehyde gel

MOPS	16.74 g
Sodium acetate	7.72 g
EDTA	0.74 g

Adjust volume to be 200 ml by d.H_2O

2) 1 % formaldehyde gel

MOPS (1X)	40 ml
Agarose	0.4 g
Formaldehyde	1.2 ml

3) Tris-borate buffer (10X TBE)

Tris	10.8 g
Boric acid	5.5 g
EDTA	0.93 g

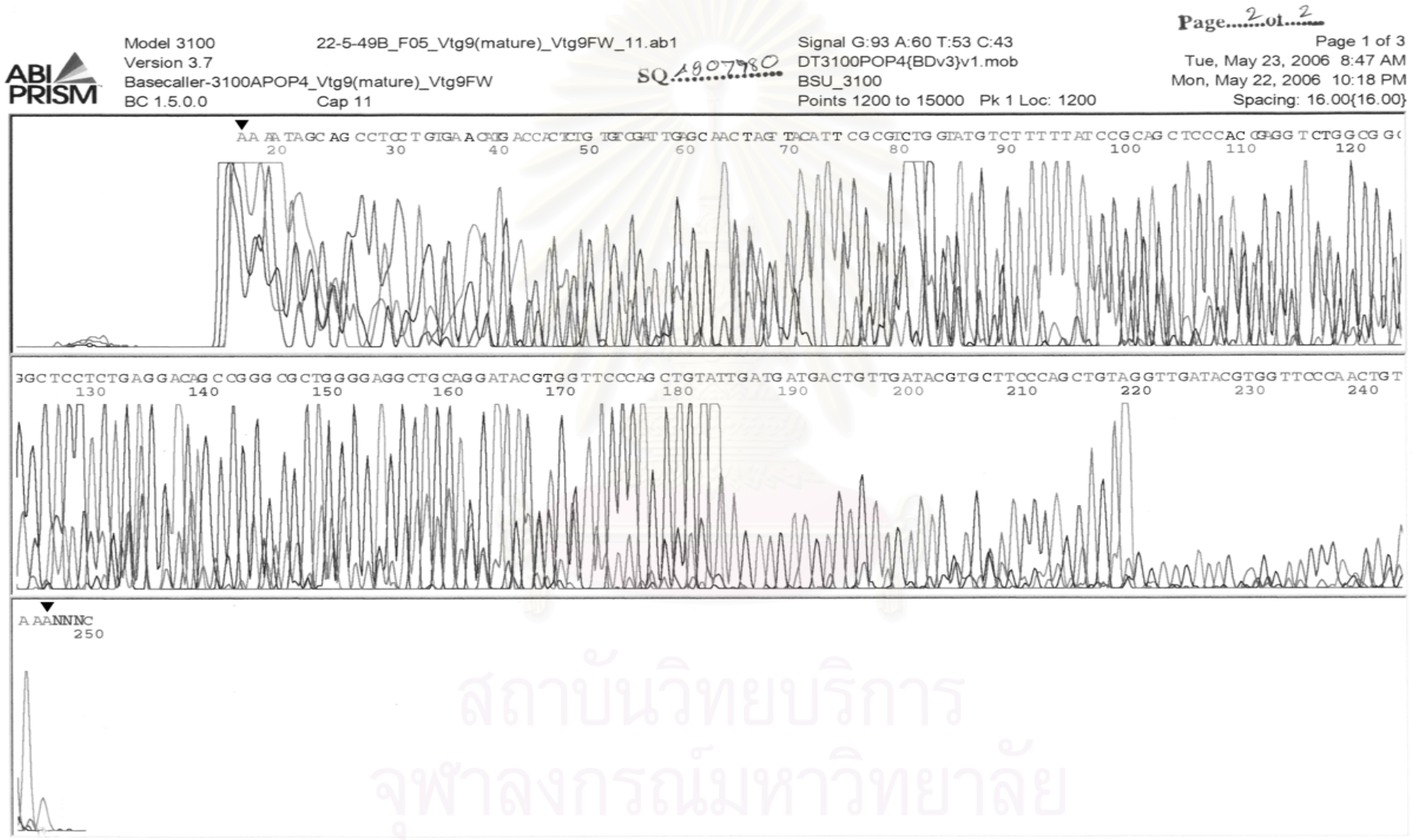
Adjust volume to be 100 ml by d.H_2O .

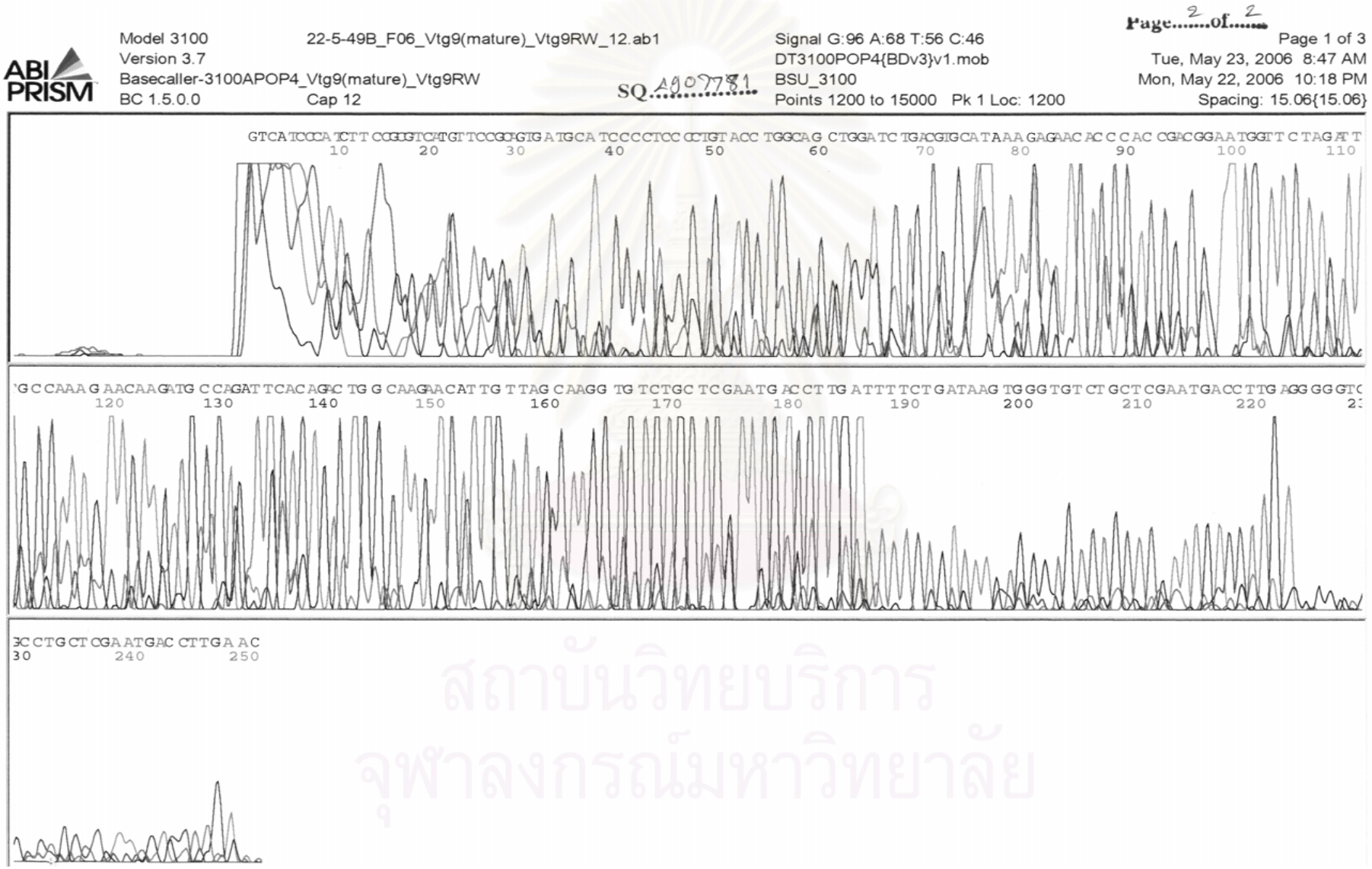
APPENDIX B

The DNA sequencing profiles

A) *Vtg*: mature female and male *H. rugulosus* and mature female *R. caresbeicana*

B) ß-actin: mature female *H. rugulosus* and mature female *R. caresbeiana*

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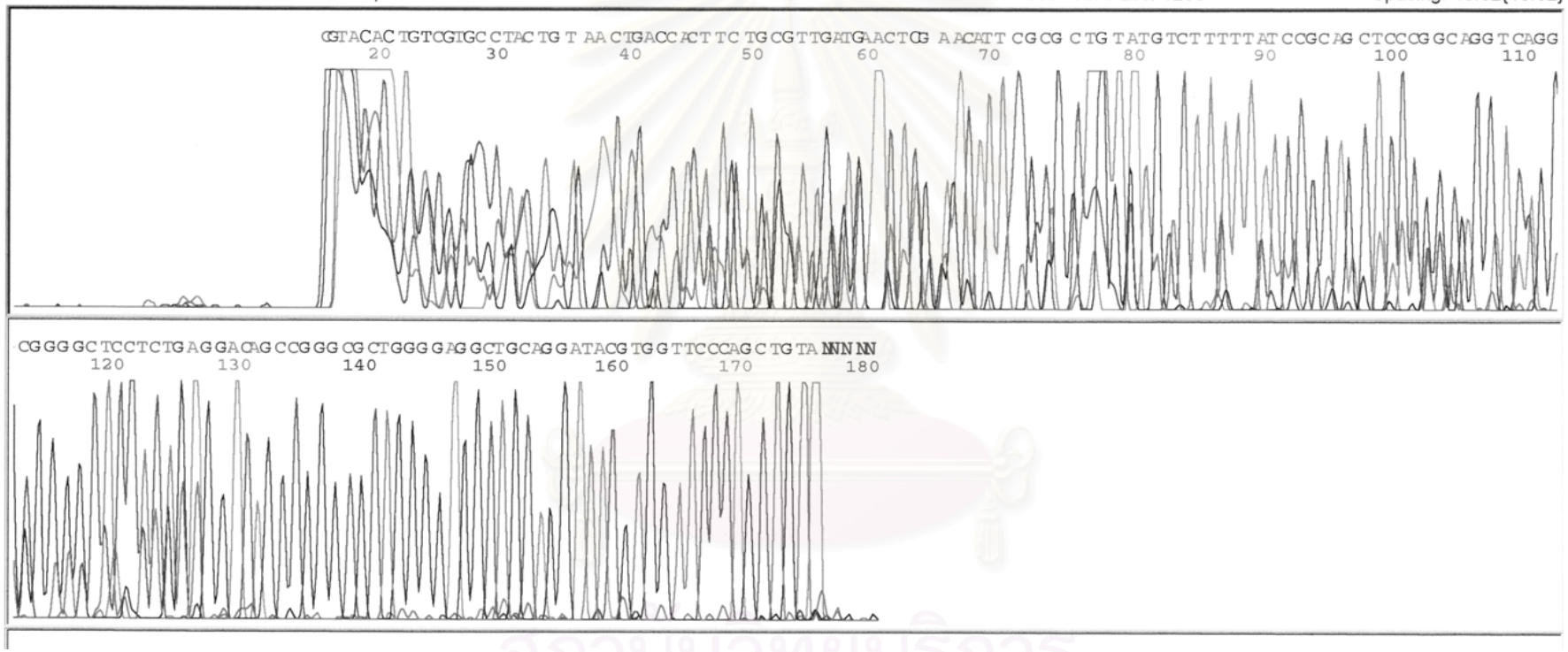

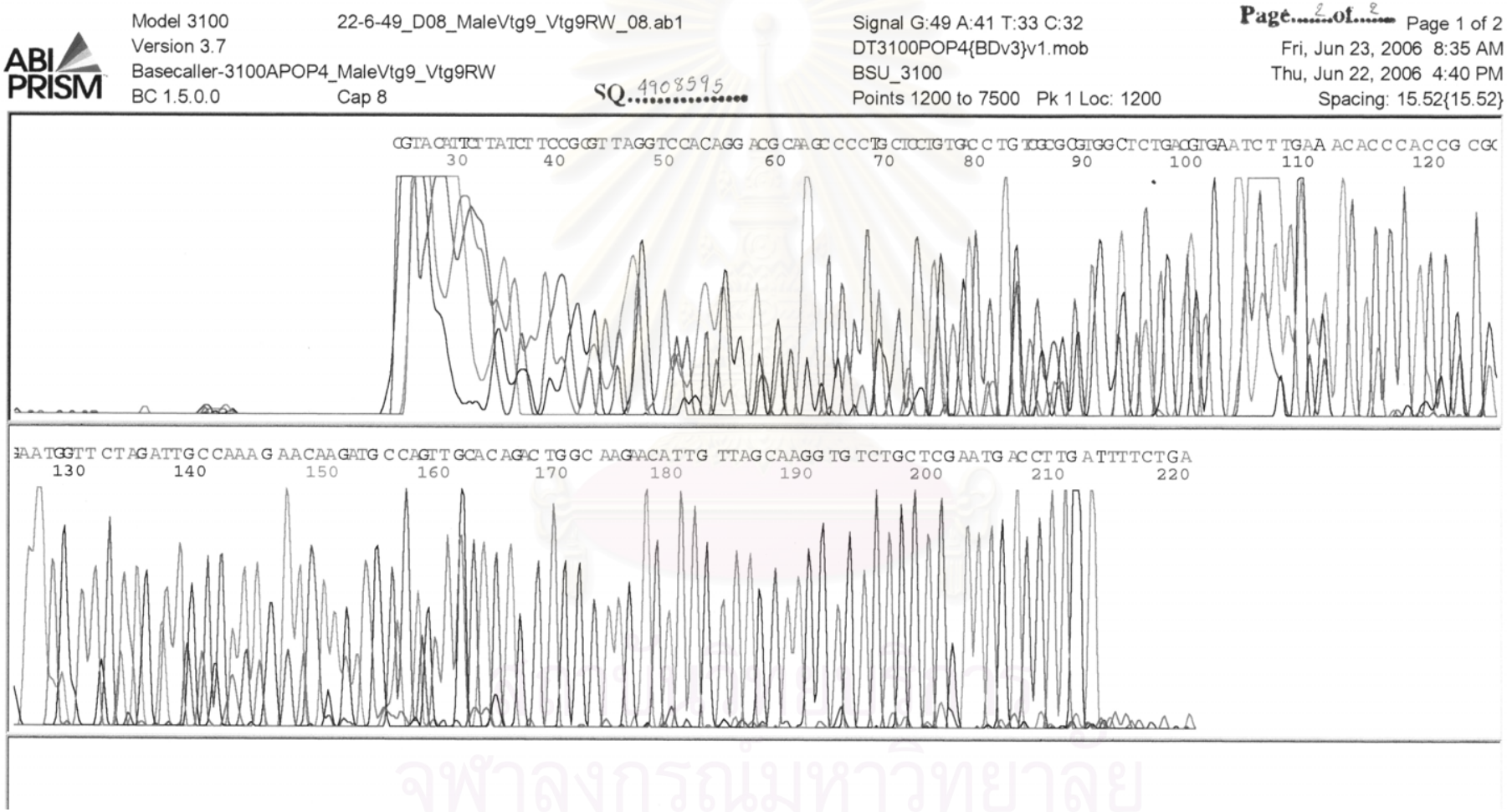
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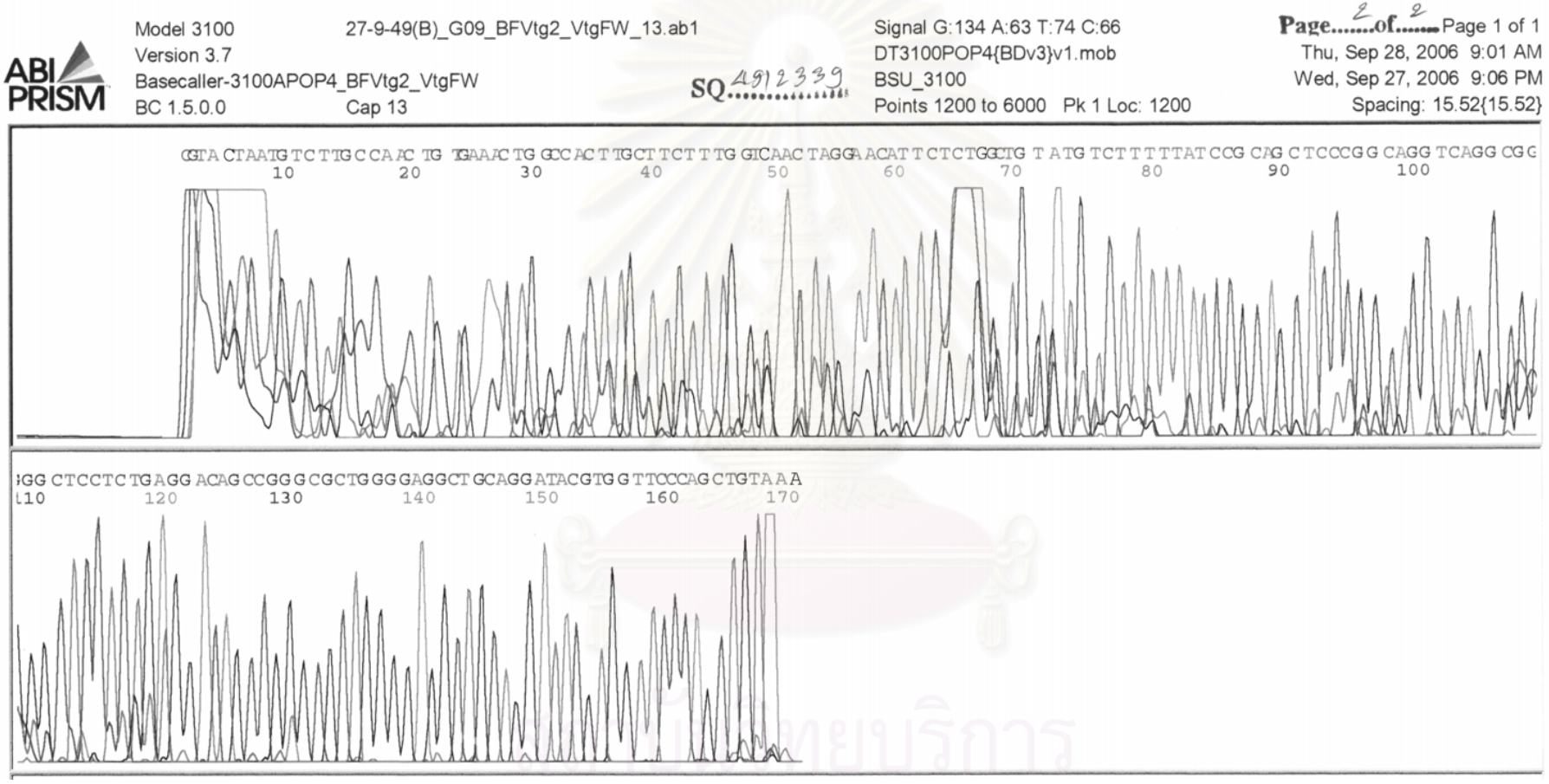
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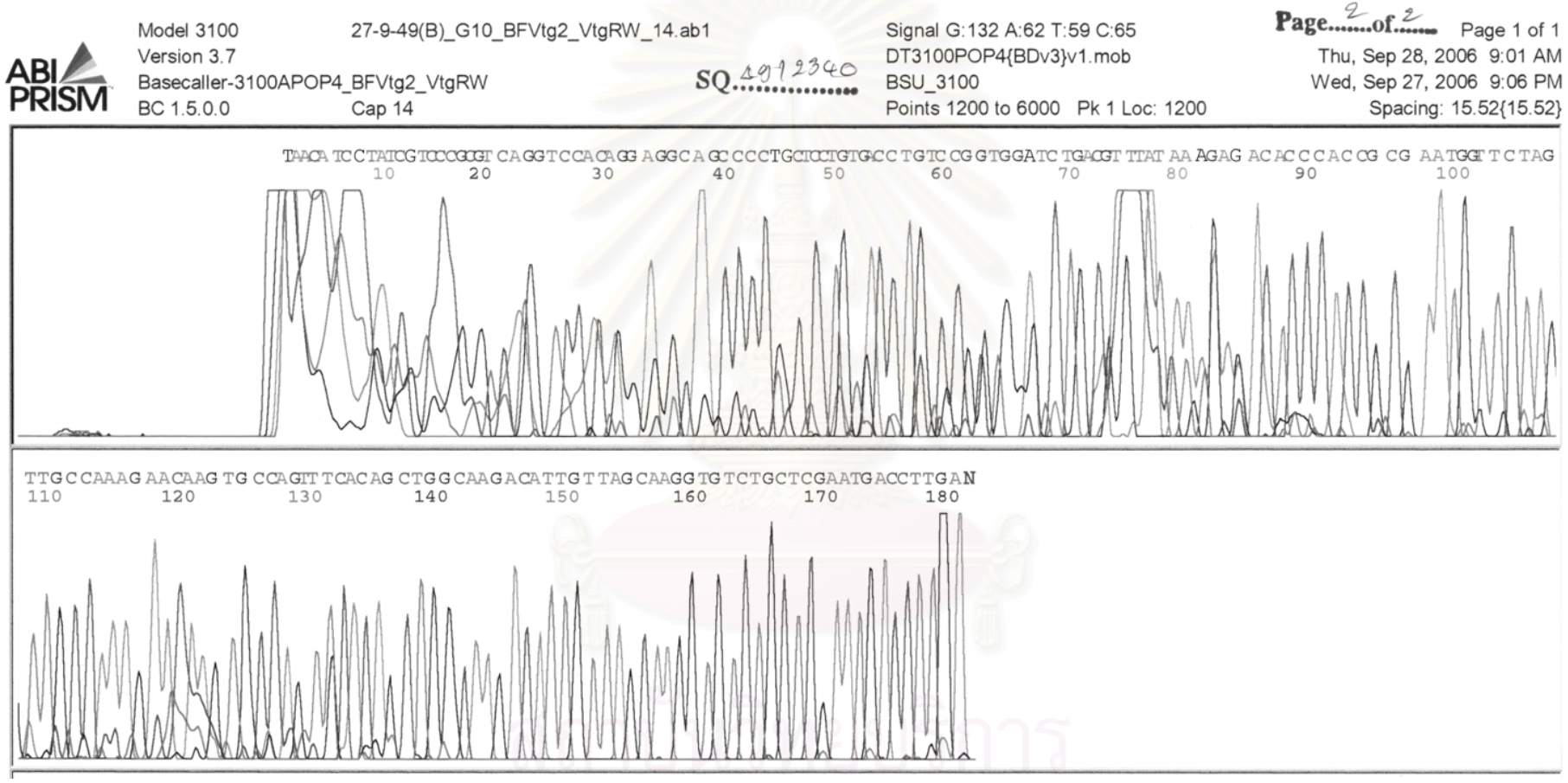
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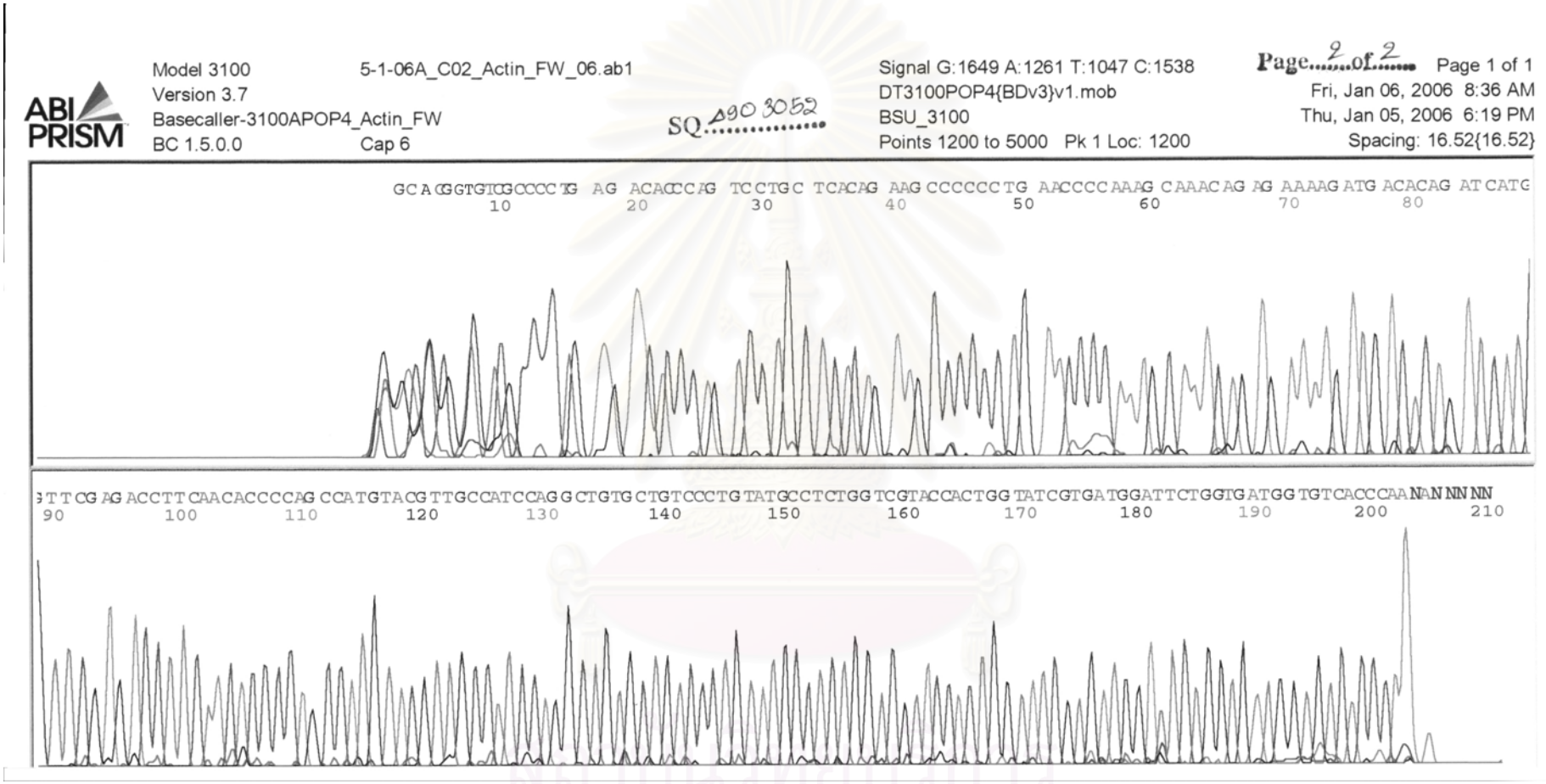
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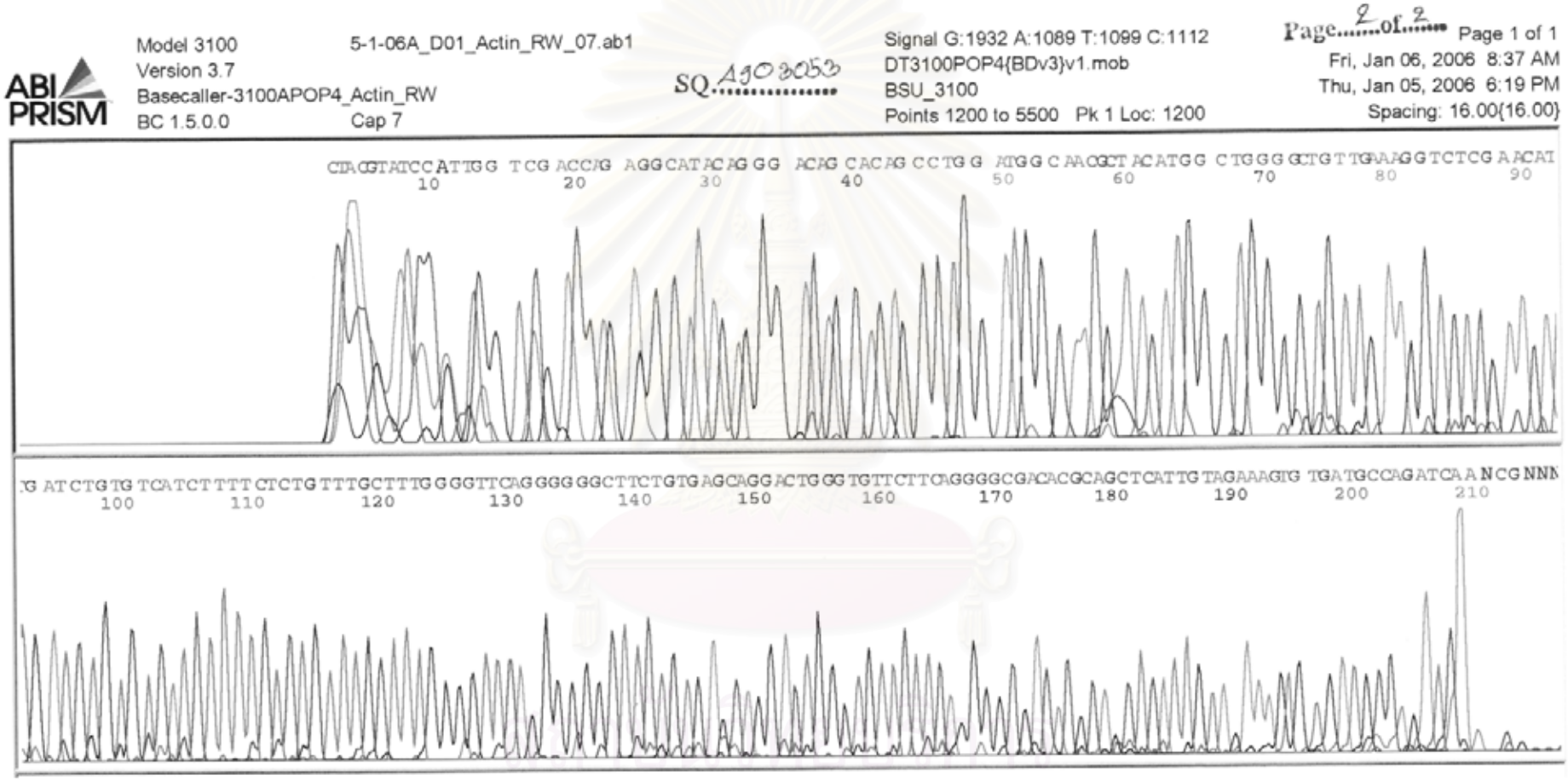

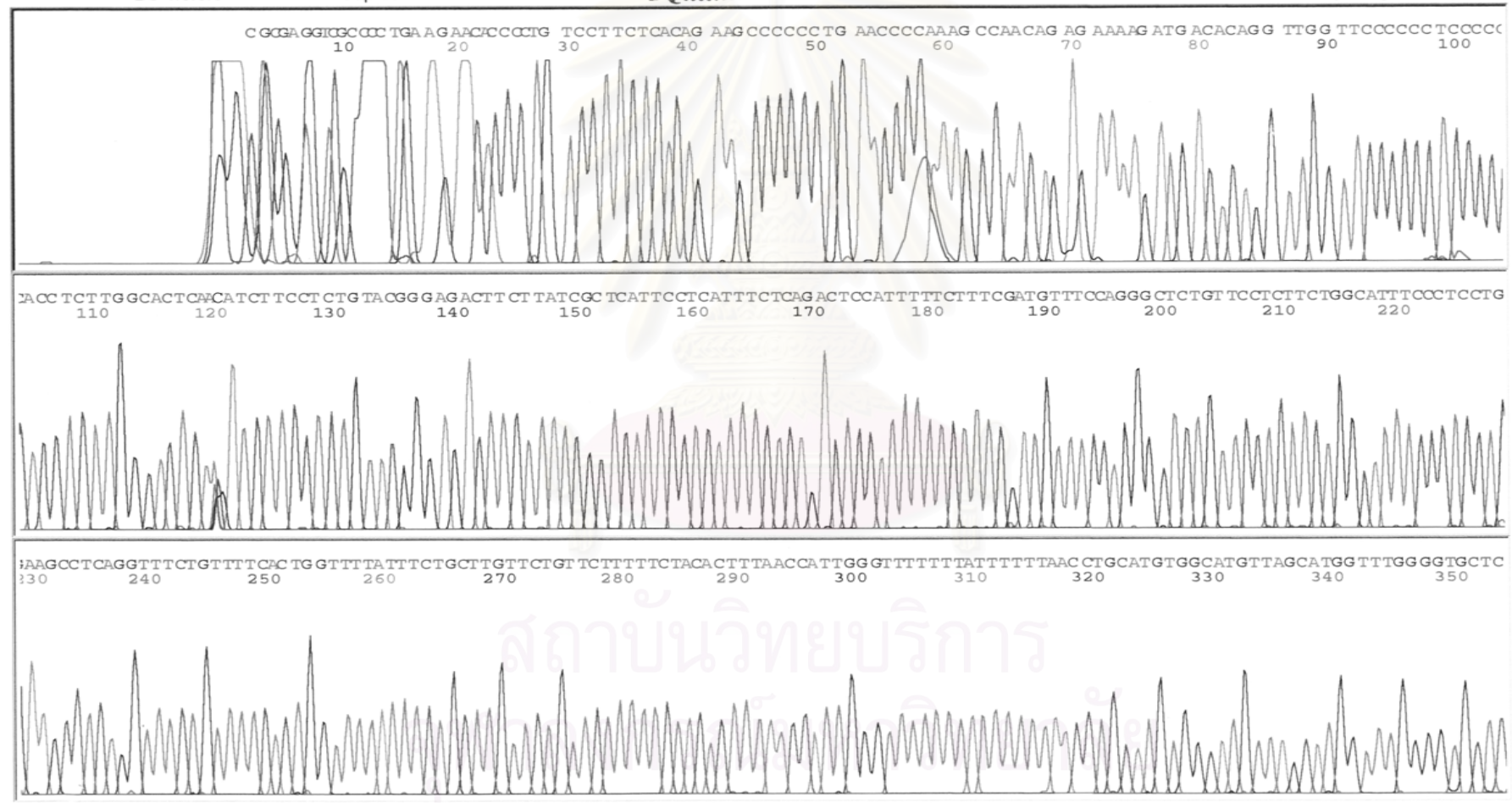
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Version 3.7
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BC 1.5.0.0
Cap 15
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SQ 491234
Signal G:301 A:184 T:233 C:236
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BSU_3100
Points 1200 to 9500 Pk 1 Loc: 1200
Page 2 of 3
Page 1 of 2
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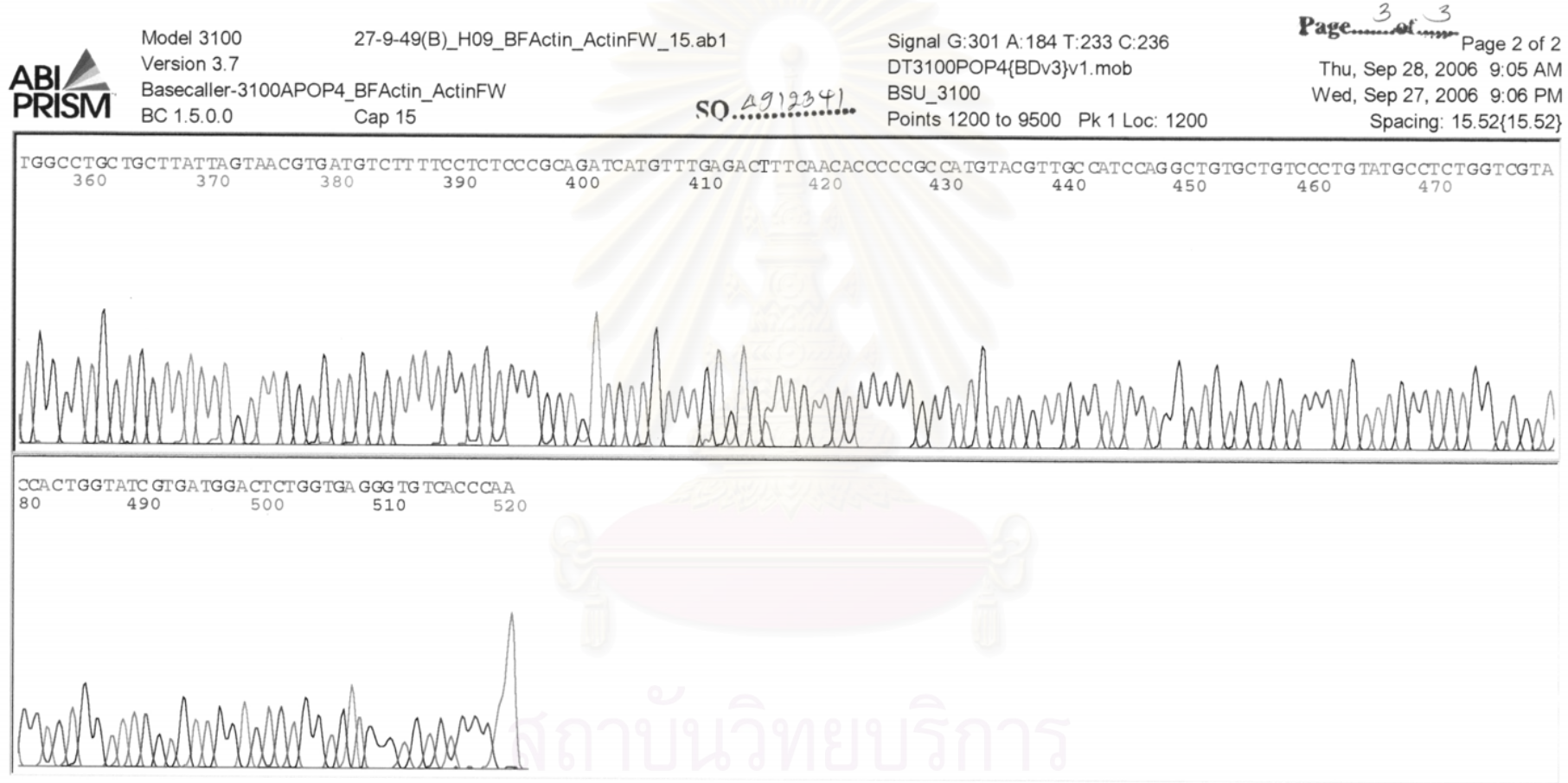
Wed, Sep 27, 2006 9:06 PM
Spacing: 15.52{15.52}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Version 3.7
Basecaller-3100APOP4_BFActin_ActinFW
BC 1.5.0.0
27-9-49(B)_H09_BFActin_ActinFW_15.ab1
Cap 15
SQ.491234
Signal G:301 A:184 T:233 C:236
DT3100POP4{BDv3}v1.mob
BSU_3100
Points 1200 to 9500 Pk 1 Loc: 1200
Page 3 of 3
Page 2 of 2
Thu, Sep 28, 2006 9:05 AM
Wed, Sep 27, 2006 9:06 PM
Spacing: 15.52{15.52}
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360 370 380 390 400 410 420 430 440 450 460 470
CCACTGGTATCGTGATGGACTCTGGTGAGGGTGTCACCCAA
80 490 500 510 520

]

Page 2 of 2

ABI PRISM

Model 3100
Version 3.7
Basecaller-3100APOP4_BFActin_ActinRW
BC 1.5.0.0

28-9-49(A)_F09_BFActin_ActinRW_11.ab1
Cap 11

SQ 491342

Signal G:103 A:53 T:26 C:27
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Page 1 of 2
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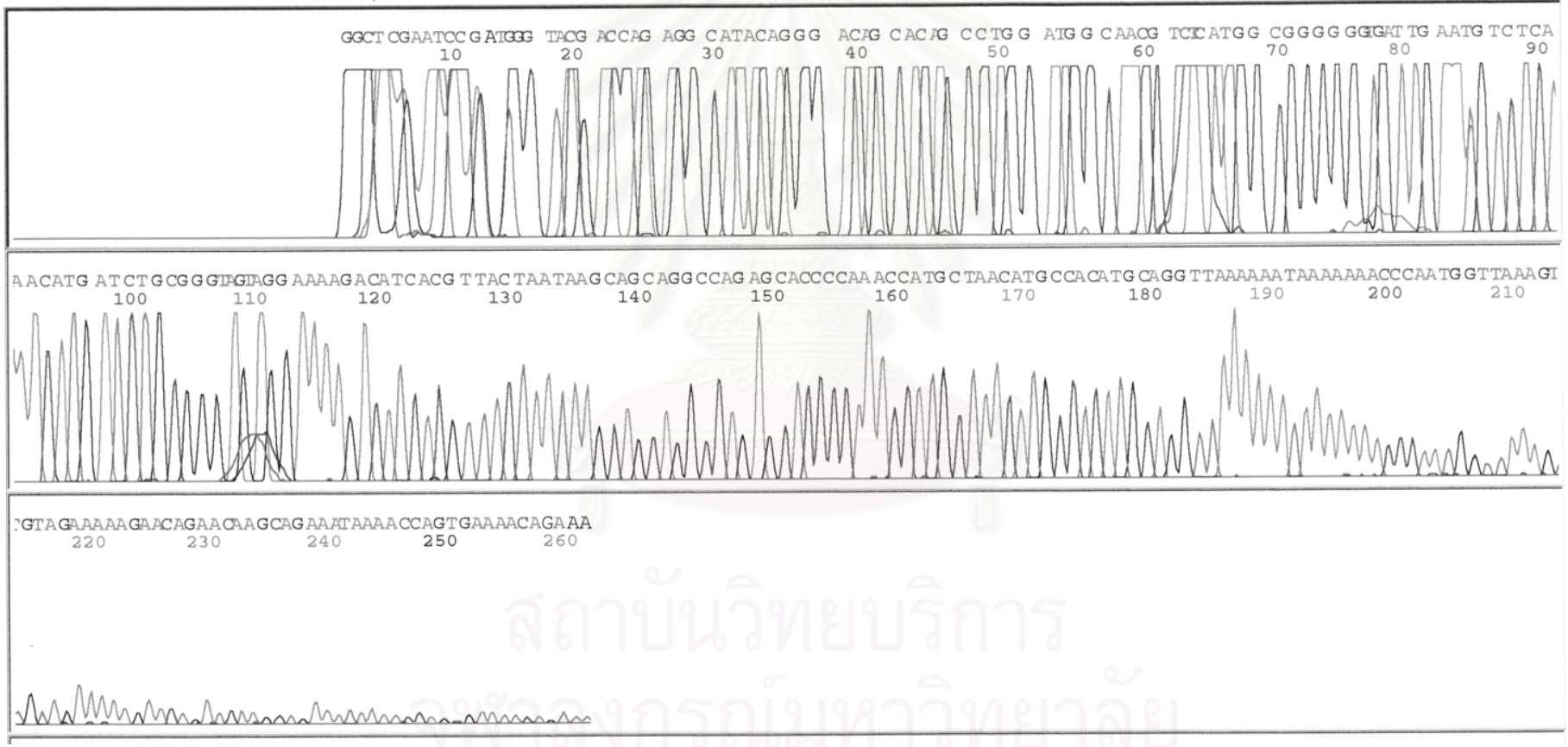
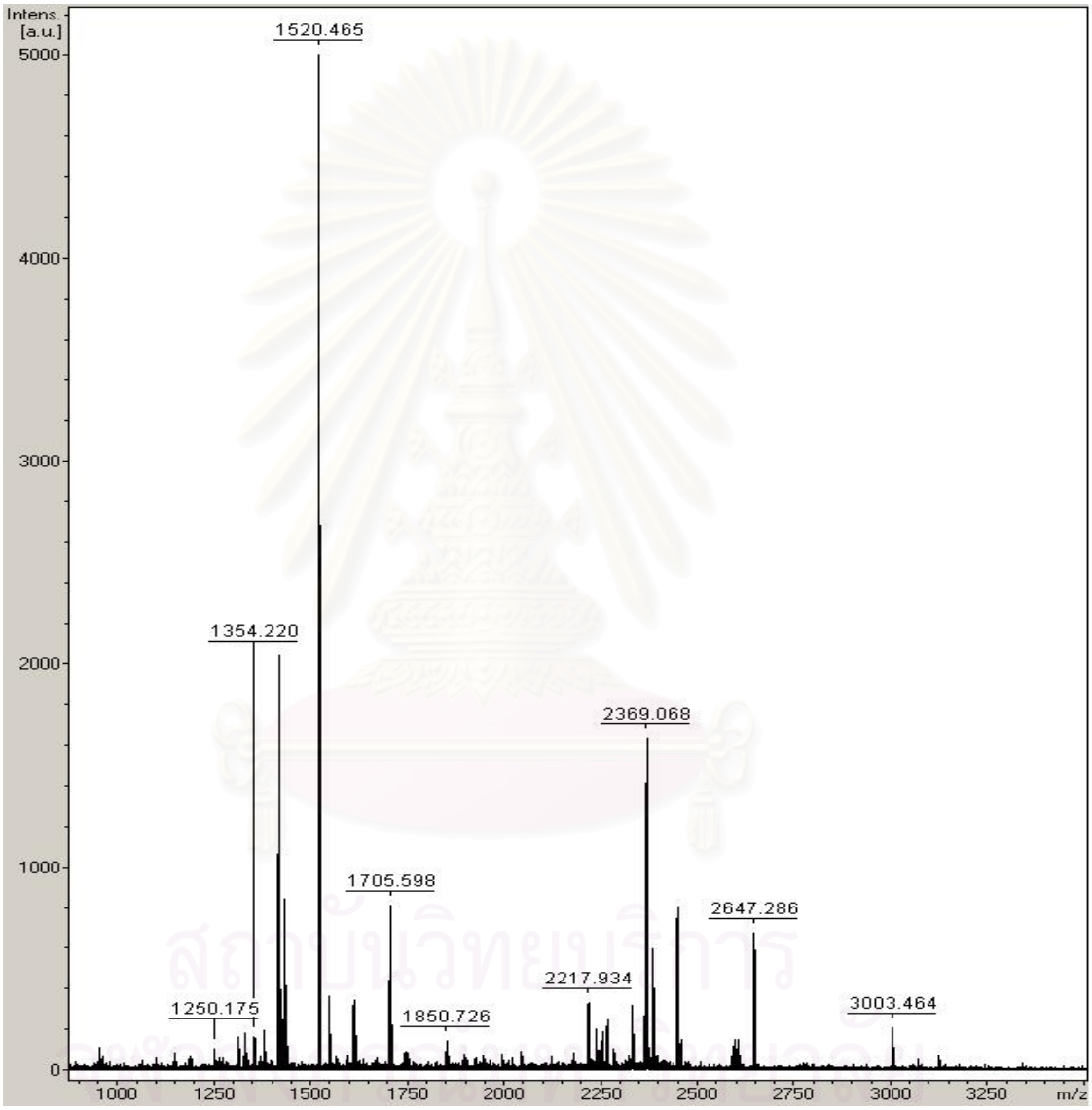


APPENDIX C

MALDI-TOP mass spectra of tryptic fragments

A) Mass spectra of tryptic fragments from in gel digestion



B) The amino acid sequence of vitellogenin precursor-*Oreochromis aureus*.

```
   1 MRVLVLALAV ALAVGDQSNL APGFASVKTY MYKYEAVLMG GLPEEGLARA
  51 GVKIRGKVLI SATSANDYIL KLVDPQLLEY SGIWPKDPFH PATKLTTALA
 101 TQLSTPIKFE YTNGVVGRLA APPGVSTTVL NIYRGIINLL QLNVKKTQNV
 151 YEMQESGAHG VCKTNYVIRE DARAERIHLT KTKDLNHCQE KIMKAIGLEH
 201 VEKCHDCEAR GKSLKGTASY NYIMKPAPSG SLIMEAVARE VIEFSPFNIL
 251 NGAAQMESKQ ILTFLDIENT PVDHARYTYV HRGSLQYEHG SEILQTPIHL
 301 LRVTHAEAQI VSTLNHLVAS NVAKVHEDAP LKFVELIQVM RVARFETIES
 351 LWAQFKSRPD HRYWLLNAVP HIRTHAALKF LIEKLLANEL SETEAAMALL
 401 ECLHSVTADQ KTIELVRSLA ENHRVKRNAV LNEIVMLGWG TVISRFCKAQ
 451 PSCSSDLVTP VHRQVAEAVE TGDIDQLTVT LKCLDNAGHP ASIKTIMKFL
 501 PGFGSAAARV PLKVQVDAVL ALRRIAKREP KMVQEIAAQL LMEKHLHAEL
 551 RMVAAMVLFE TKLPVGLAAS ISTALIKEKN LQVVSFVYSY MKAMAKTTSP
 601 DHVSVAAACN VALRFLNPKL GRLNFRYSRA FHVDTYNNAW MMGAAASAVL
 651 INDAATVLPR MIMAKARTYM AGAYVDAFEV GVRTEGIQEA LLKRRHENSE
 701 NADRITKIKQ AMRALSEWRA NPSSQALASM YVKVFGQEIA FANIDKSKVD
 751 QLIQFASGPL RNVFRDAVNS VLSGYATHFA KPMLLGELRL ILPTTVGLPM
 801 EISLITSAVT AASVDVQATV SPPLPVNYRV SQLLESDIQL RATVAPSLAM
 851 QTYAFMGVNT ALIQAAVMTK AKVYTAVPAQ IKARIDIVKG NLKVEFLSLQ
 901 GINTIASAHA ETVAIARNVE DLPAARSTPL ISSETASQLS KASLNSKISR
 951 MASSVTGGMS ASSEIIPADL PSKIGRKMKL PKTYRKKIRA SSRMLGFKAY
1001 AEIESHNAAY IRDCPLYALI GKHAASVRIA PASGPVIEKI EVEIQVGDKA
1051 AENMIKAIDM SEEEEALEDK NVLLKIKKIL APGLKNTTSS SSSSSSSSSS
1101 SSSSNKSSSS SSRSSSSQSS SSRSHRSRSR KSQSSSSQSS RSPSSSSSSS
1151 SSSSSRSSSR SSSRSSSRSS SRSSSRSRTK MADIVAPIIT TSTRVSSSSS
1201 RSASNSSSSS ASYLLSSSKR RSRSRSSSSS SSSSSSSSSS SSSSSSSSKN
1251 SKRSKSSNSK SSSSRSSRRS AQSKQQLLAL KFRKNHVHRH AISTQRGSSH
1301 SSARSFDSIY NKAKYLANTL TPAMSIAIRA VRVDHKVQGY QLAAYLDKQT
1351 NRLQLIFARV AEKDNWRICA DIVQLSSHKL MAKTAWGAEC KQYSTMIVAE
1401 TGLLGHEPAA RLKLTWDKLP GSIKHYAKRA LKSIVPIAQE YGVNYAKAKN
1451 PRNQIKLTVA VATETSMNIV LNTPKAIVYK RGVCLPVALP IGNTAAELQA
1501 TRDNWADKMS YLVTKANAVE CSLINNTLTT FNNRKARDEL PHSCYQVLAQ
1551 DCTPELKFMV LLKKDQIQDQ NQINVKISDI DVDMYRKNNA IAVMVNGVEI
1601 PNSNLPYLHP SGNIHIRQSN EGITLNAPSH GLQEVFLGFN ELRVKVADWM
1651 KGKTCGACGT ASGNVGDEYR TPSEQVTKDA ISYAHSWVLS SNTCRDPSEC
1701 SIKQESVKLE KRVIFEGVES KCYSVEPVLQ CLPGCIPVRT TTVNVGFHCL
1751 PSDTTVDRSG LSSFFEKSID LRDTAEAHLA CRCTPQCA
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*** Matched peptides shown in underline**

1. Alanine Ala (A)
2. Asparagine Asn (N)
3. Cysteine Cys (C)
4. Glutamine Gln (Q)
5. Glycine Gly (G)
6. Isoleucine* Ile (I)
7. Leucine* Leu (L)
8. Methiomonine* Met (M)
9. Phenylalanine* Phe (F)
10. Proline Pro (P)
11. Serine Ser (S)
12. Threonine* Thr (T)
13. Trypophan* Try (W)
14. Tyrosine Tyr (Y)
15. Valine* Val (V)
16. Aspartic acid Asp (D)
17. Glutamic acid Glu (E)
18. Arginine* Arg (R)
19. Histidine* His (H) 155
20. Lysine* Lys (K)

APPENDIX D

The data from the experiments

Table 1. Vitellogenin gene expresion and intensity of phosphoprotein of immature female and male frog.

Season	Sexes	Group (µg/kg)	n	Vtg/ß-actin	Phosphoprot. Int. (Adj. Vol. INT* mm2)
Rainy	Female	0	6	0.85 ± 0.03	1657.84 ± 98.46
		50	6	0.65 ± 0.02	1841.81 ± 95.48
		500	6	0.60 ± 0.02	1912.84 ± 148.17
		5,000	6	0.58 ± 0.02	2308.13 ± 135.11
	Male	0	6	0.61 ± 0.04	1175.44 ± 142.68
		50	6	0.70 ± 0.05	2019.65 ± 85.81
		500	6	0.80 ± 0.06	2170.40 ± 122.96
		5,000	6	0.74 ± 0.05	2306.88 ± 48.02
Dry	Female	0	6	0.65 ± 0.04	2071.25 ± 157.52
		50	6	0.68 ± 0.04	3055.16 ± 167.2
		500	6	0.78 ± 0.05	3314.31 ± 173.76
		5,000	6	0.72 ± 0.04	3414.23 ± 288.82
	Male	0	6	0.68 ± 0.04	1499.44 ± 129.54
		50	6	0.70 ± 0.06	1543.85 ± 75.84
		500	6	0.72 ± 0.05	2555.97 ± 278.96
		5,000	6	0.73 ± 0.03	2647.97 ± 168.84

Table 2. Show the body, liver, ovarian, and testicular weight, %LSI and %GSI of immature female and male frog which reared during rainy season.

Season	Sexes	Group (µg/kg)	n	BW (g)	Liver wt. (g)	Ovarian wt. (g)	Testicular wt. (g)	% LSI	% GSI
Rainy	Female	0	10	183 ± 8.17	8.5 ± 0.64	12.81 ± 2.14		4.65 ± 0.29	7.07 ± 1.19
		50	10	182 ± 4.89	8.12 ± 0.46	16.49 ± 1.86		4.45 ± 0.21	9.03 ± 0.99
		500	10	176 ± 5.61	8.42 ± 0.66	15.33 ± 1.54		4.77 ± 0.33	8.96 ± 0.92
		5,000	10	177 ± 7.15	9.57 ± 0.62	15.97 ± 1.59		5.42 ± 0.30	9.11 ± 0.91
	Male	0	7	121.42 ± 6.33	5.33 ± 0.58		0.17 ± 0.010	4.43 ± 0.44	0.14 ± 0.01
		50	9	103.33 ± 4.08	4.57 ± 0.35		0.15 ± 0.009	4.43 ± 0.30	0.15 ± 0.01
		500	7	111.42 ± 5.53	5.1 ± 0.49		0.13 ± 0.008	4.58 ± 0.36	0.12 ± 0.01
		5,000	8	101.25 ± 7.42	5.44 ± 0.57		0.13 ± 0.010	5.46 ± 0.53	0.14 ± 0.01

Table 3. Show the body, liver, ovarian, and testicular weight, %LSI and %GSI of immature female and male frog which reared during dry season.

Season	Sexes	Group (µg/kg)	n	BW (g)	Liver wt. (g)	Ovarian wt. (g)	Testicular wt. (g)	% LSI	% GSI
Dry	Female	0	7	140 ± 20.7	3.5 ± 1.23	4.58 ± 2.14		2.22 ± 0.38	2.86 ± 0.990
		50	9	147.77 ± 7.02	4.51 ± 0.45	6.4 ± 2.14		3.03 ± 0.27	3.97 ± 0.950
		500	9	108.88 ± 9.92	3.39 ± 0.48	2.17 ± 0.79		3.02 ± 0.24	1.96 ± 0.720
		5,000	4	115.00 ± 14.43	3.09 ± 0.53	2.91 ± 1.64		2.72 ± 0.39	2.21 ± 1.010
	Male	0	10	105.00 ± 5.21	3.33 ± 0.42		0.15 ± 0.01	3.13 ± 0.30	0.14 ± 0.009
		50	10	125.00 ± 3.41	4.3 ± 0.29		0.15 ± 0.009	3.43 ± 0.20	0.12 ± 0.006
		500	8	108.75 ± 7.42	3.72 ± 0.56		0.10 ± 0.008	3.31 ± 0.28	0.09 ± 0.007
		5,000	10	103.00 ± 6.15	3.53 ± 0.36		0.09 ± 0.008	3.4 ± 0.24	0.09 ± 0.006

Table 4. Plasma E2 levels of immature female and male in both seasons.

Season	Sexes	Group (µg/kg)	n	Plasma by E2 (pg/ml)
Rainy	Female	control	5	327.24 ± 188.61
	Male	control	5	17.58 ± 4.46
Dry	Female	control	5	87.33 ± 24.18
	Male	control	5	68.03 ± 23.81

BIOGRAPHY

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