สัญญาเลขที่ R2558C014

อภินันทนาการ

โครงการ

Effect of Curcuma Comosa Extract on Pituitary Gland Cells and Ovary of Red Nile Tilapia

ผลของสารสกัดว่านชักมดลูกต่อเซลล์ต่อมใต้สมองและรังไข่ของปลานิลแดงเพศเมีย

คณะผู้วิจัย

1. ดร.อิทธิพล พวงเพชร สังกัด คณะวิทยาศาสตร์การแพทย์
2. นางสาวปวีนันทร์ สินไชย สังกัด คณะวิทยาศาสตร์การแพทย์

สนับสนุนโดย

งบประมาณรายได้ มหาวิทยาลัยนเรศวร

ปีงบประมาณ 2558

สารบัญ

ผลของสารสกัดว่านชักมดลูกต่อเซลล์ต่อมใต้สมองและรังไข่ของปลานิลแดงเพศเมีย

ปวีนันทร์ สินไชย[1], ศุภวัฒน์ สายพานิช[1], อิทธิพล พวงเพชร[1*]

[1]ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร

*Corresponding author, e-mail: ittiponnu47@gmail.com

บทคัดย่อ

ในว่านชักมดลูกมีสารไฟโตเอสโตรเจน ซึ่งมีฤทธิ์คล้ายกับฮอร์โมนเอสโตรเจน ซึ่งเป็นสารมีบทบาทที่เกี่ยวข้องกับการพัฒนาระบบสืบพันธุ์เพศเมียในสัตว์ ในสัตว์น้ำเช่นปลานิล ยังมีการศึกษาการใช้สมุนไพรอยู่น้อยมาก ทางผู้วิจัยสนใจการใช้พืชสมุนไพรพื้นบ้านในประเทศไทย ที่มีสารธรรมชาติ ที่ส่งเสริมและกระตุ้นการพัฒนาการของระบบสืบพันธุ์ปลา ทางผู้วิจัยจึงสนใจศึกษาผลของสารสกัดว่านชักมดลูกที่มีต่อเซลล์ต่อมใต้สมอง การแสดงออกของยีน LH-β และการพัฒนารังไข่ปลานิลแดง โดยแบ่งกลุ่มการทดลองเป็น 6 กลุ่ม คือ กลุ่ม 1 กลุ่มควบคุม กลุ่ม 2 ฉีดน้ำมันงา (50 ul) กลุ่ม 3-4 ฉีด17β-estradiol (65, 130 µg/kg) กลุ่ม 5-6 ฉีดสารสกัดว่านชักมดลูก (65, 130 µg/kg) จากนั้นภายใต้กล้องจุลทรรศน์ นำต่อมใต้สมองมาศึกษาชนิดของเซลล์, ศึกษาโครงสร้างรังไข่และระยะการพัฒนาของไข่ ผลจากการศึกษาทางจุลกายวิภาคศาสตร์พบว่า ต่อมใต้สมองมีเซลล์ 5 ชนิด คือ acidophil, basophil, chromophobe, signet cell และ clear cell รังไข่สามารถแบ่งได้ 5 ระยะคือ ระยะ oogonia, primary oocyte, cortical alveoli, vitellogenic และ mature stage อีกทั้งยังศึกษาการแสดงออกของยีน LH-β ในเซลล์ต่อมใต้สมอง โดยเทคนิค PCR พบว่า มีการแสดงออกของยีนเพิ่มสูงขึ้นในกลุ่มที่ได้รับ 17β-estradiol (เฉพาะ high dose) และกลุ่มสารสกัดว่านชักมดลูก แสดงให้เห็นว่าสารสกัดว่านชักมดลูกมีฤทธิ์ในการเหนี่ยวนำให้มีการเพิ่มเอสโตรเจนที่แปรผันตรงกับฮอร์โมน LHRH และอาจทำให้เกิดการพัฒนาของรังไข่เพิ่มมากขึ้นตามในปลานิลแดงเพศเมีย ซึ่งข้อมูลนี้เราอาจนำไปใช้ในการกระตุ้นรังไข่ให้มีการเจริญสมบูรณ์และอาจนำไปใช้ส่งเสริมการผสมพันธ์นอกฤดูได้

Effect of *Curcuma Comosa* Extract on Pituitary Gland Cells and Ovary of Red Nile Tilapia

Paweenun Sinchai[1], Suphawat Saipanich[1], Ittipon Phoungpet[1*]

[1] Department of Anatomy, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand

*Corresponding author, e-mail: ittiponnu47@gmail.com

Abstract

Curcuma comosa, the Thai herb was contained an estrogen-like chemical (a phytoestrogen) which related to gonadal maturation and also pituitary gland activity. This study investigated effect of *Curcuma comosa* extracted to pituitary gland cells number and LH-β gene expression also oocyte development of red Nile tilapia. The fish were divided into 6 group first group as control group, second as sesame oil injected (50 µl), third and fourth group injected with 17β-estradiol (65, 130 µg/kg), fifth group injected with *C.comosa* extract (65,130 µg/kg). The cell stages and cell types of pituitary gland and oocyte stages were determined under light microscope. LH-β gene expression was analyzed by PCR technique. The results showed that pituitary gland had 5 cell types as acidophil, basophil, chromophobe, signet cell and clear cell. Oocyte can divided into 5 stages as oogonia, primary oocyte, cortical alveoli, vitellogenic and mature stage. By PCR, LH-β gene expression showed significantly increased in 17β-estradiol group (only high dose) and *C.comosa* group. This study suggest that *C.comosa* could promote LH-β gene expression in red Nile tilapia and might stimulate ovary maturation.

Keyword *Oreochromis niloticus*, red Nile tilapia, pituitary gland, *Curcuma comosa*, luteinizing hormone

CHAPTER I
INTRODUCTION

Rationale and significance of the study

Nile tilapia is an economically very important fish of Thailand moreover these fish are in increased demand from both the domestic and international markets such as United States of America, Middle East, Australia, Asia and European Union (FAO Year book, 2006). They were first introduced into Thailand by the Emperor of Japan who gave the fish to H.M. King of Thailand. In this study, we used "Pla nil deng" (Thai red tilapia), a hybrid species between 78% Nile tilapia (*Oreochromis niloticus*) and 22% Mozambique tilapia (*Oreochromis mossambicus*) (Pansri, 1988). As these fish have high quality flesh, they are economically more valuable, making 120 baht/kg more than Nile tilapia at 50 baht/kg (Fish Marketing Organization, June 2015). They are available in all regions of Thailand. They can grow rapidly in all weather conditions to breed at about 4 months, with mature eggs occurring at 6 months (Jarimopas, 1994). Reproduction of teleost fish is controlled by the anterior pituitary regulated by the hypothalamic neuropeptide Gonadotropin Releasing Hormone (GnRH) stimulating release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kawauchi et al., 1989; Swanson et al., 2003). In female fish, LH and FSH act on the ovary follicle to secrete ß-estradiol which stimulates synthesis of vitellogenin in the liver and which in turn results in egg maturation and ovulation. In a previous study, *Curcuma comosa* was found to contain an estrogen-like chemical (a phytoestrogen). The 17β-estradiol (E_2) can directly induce vitellogenin in the tilapia hepatocytes in vitro and in vivo (Akihiro and Byung, 2001) as the phytoestrogen in Fabaceae plants induced vitellogenin synthesis in juvenile brown trout (Holbech et al., 2013). Estradiol benzoate treatment can results in a large increase in gonadotropin-like material in the pituitary gland of brown hagfish (Makoto et al., 2006). Previous studied indicate that phytoestrogen was dose-dependently, cause estrogenic and/or antiestrogenic effects (Yildiz and Fatih, 2005).

The aim of this study is to investigate the effect of a phytoestrogen extract from *Curcuma comosa* on pituitary gland cells, LH hormone production and ovary development in red Nile tilapia.

Main objective

To investigate the effect of *Curcuma comosa* extract on changing pituitary gland cells, LH hormone production and ovary development of female red Nile tilapia.

Specific objectives

1. To examine the effects of *Curcuma comosa* extract to pituitary gland cells number and ovary
2. To examine LH gene expression in control and treatment groups by RT-PCR technique
3. To determine distribution LH peptides in pituitary glands cells and ovary by immunohistochemistry

Keywords

Oreochromis niloticus, red Nile tilapia, pituitary gland, *Curcuma comosa*, luteinizing hormone, phytoestrogens

Scope of this study

This study investigated effect of *Curcuma comosa* extract to pituitary gland cells number and LH production also oocyte development of red Nile tilapia. The Thai red tilapia fish were anesthetized with 0.05% 2-phenoxyethanol for 10 min until the fish were unconscious. The pituitary gland was removed and frozen in dry ice, LHβ mRNA from pituitary was extracted and converted to cDNA, then PCR used for LHβ DNA expression and band optodensity were analyzed. The pituitary glands and ovary were removed and fixed in freshly cool davison's plus 10% glycerol fixative. These specimens were processed to dehydration, embedding and 5-μm sections prepared and placed onto the silane-coated slides. These slides were either stained with Hematoxylin & Eosin to determine cell stage and cell types or were processed for immunohistochemistry to study distribution of LH peptides under light microscopy and then photographed with Nikon digital camera. The results were compared and analyzed between groups.

Hypotheses

The *Curcuma comosa* extract should stimulate pituitary cell proliferation, LH production and also promote oogenesis.

Conceptual framework

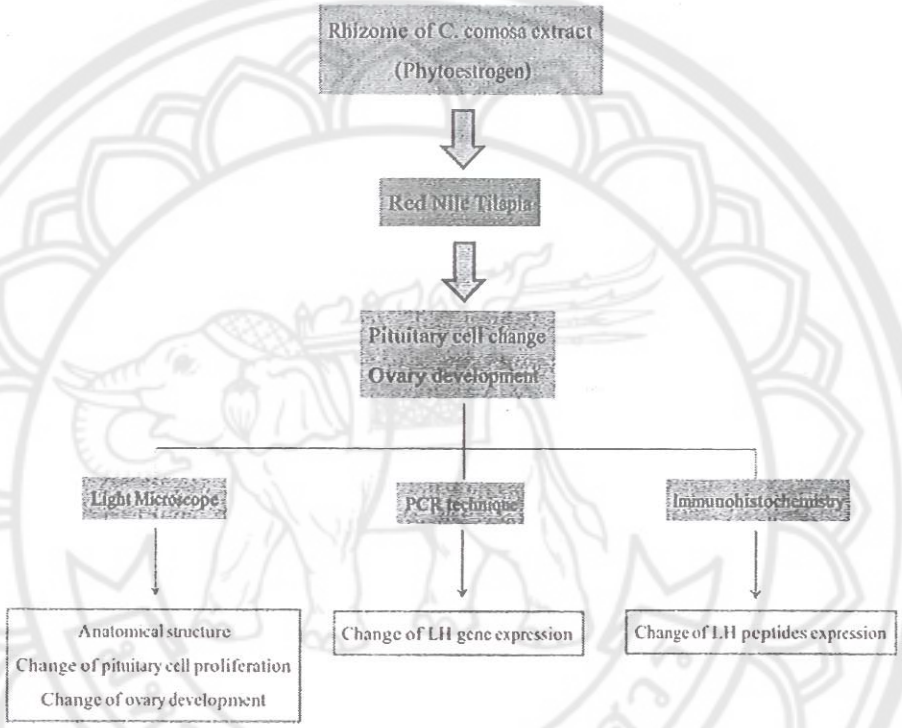


Figure 1 Conceptual framework of this study

CHAPTER II

REVIEW OF RELATED LITERATURES AND RESEARCHS

Historical background

The Nile tilapia (*Oreochromis niloticus*) is not a native species to Thailand but it is actually native from the North Africa and the Middle East (Boyd, 2004) (Figure 2). They were first introduced into Thailand on March 25, 1965 when the Emperor Akihito, as His Royal Highness Crown Prince of Japan at that time gave a fifty fish to H.M. King of Thailand.

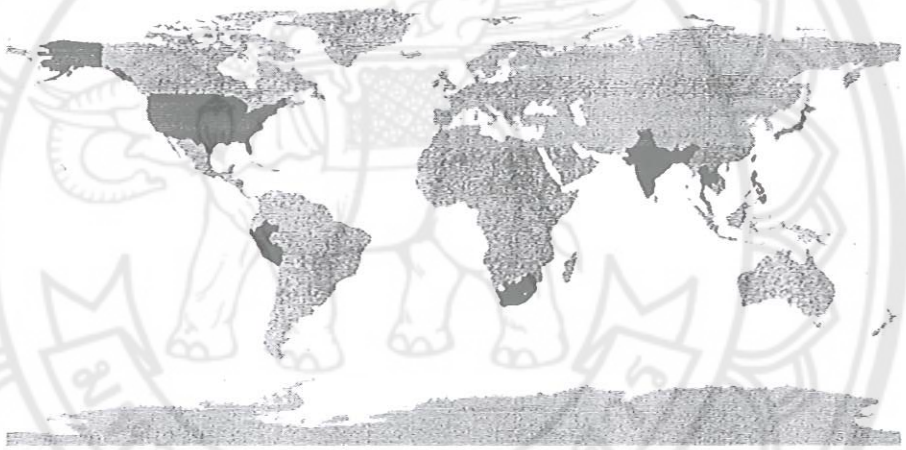


Figure 2 Distribution of *O. niloticus* ranges globally Native (green) and introduced (red) (GISD, 2012).

Fish biology

Scientific classification of *Oreochromis niloticus* was classified by Trewavas, 1983

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Cichlidae

Genus: *Oreochromis*

Species: *niloticus*

Figure 3 External morphology of *Oreochromis niloticus* (FAO, 2012)

Nile tilapia (*Oreochromis niloticus*) (Linnaeus, 1758) is settled both in fresh water and some brackish water species. The tilapia is a genus of fishes in the Cichlidae family of the three genera *Tilapia*, *Sarotherodon*, and *Oreochromis* (FAO, 2011). The most important production is mostly the Nile tilapia (*Oreochromis niloticus*) followed by the Blue tilapia (*Oreochromis aureus*), Mango tilapia (*Sarotherodon galilaeus*) and Sabaki tilapia (*Oreochromis spilurus*).

Red Nile Tilapia

Red Nile tilapia was not only famous in Thai people but also foreigner because they are beautiful color same a red sea bream (*Chrysophrys major*) so that they were raised aquatic red-pink fishes that more look delicious (Sommer et al., 1991). The most of Thai people admired several cooking with them such as a fry, boil and soup. Furthermore, these species is good taste and soft fish meat so they was made a sale at 120 baht/kg more than original Nile tilapia at 50 baht/kg (Fish Marketing Organization,

June 2015) for the fresh fish in Thailand where Nile tilapia and red Nile tilapia were made initial development. Moreover our country is initial exporter of the world.

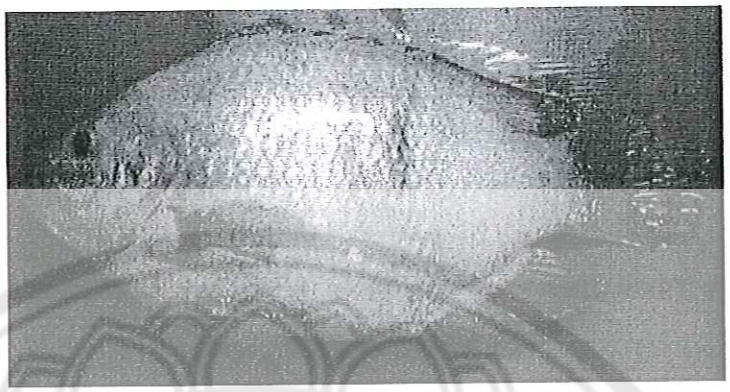


Figure 4 External morphology of Thai red Nile tilapia (*Oreochromis nilotica*)

Sex identification

Specification of sex can identify when the fish lapse into reproductive age of Nile tilapia. Characteristics typical of male and female fish look very similar. But external morphology of the sexual organ is different when looking at the genital area near the anus (Nuanmanee, 2010). The fish are different sex characteristic that is sexual organ (slender) of male fish near anus and 2 apertures (anus and urogenital pore, Fig. 5) but sexual organ of female fish is large aperture, oval shape and 3 apertures (anus, oviduct pore and urinary pore) furthermore sexual organ of male fish is pale color more than female fish (Inland Fisheries Research and Development Bureau).

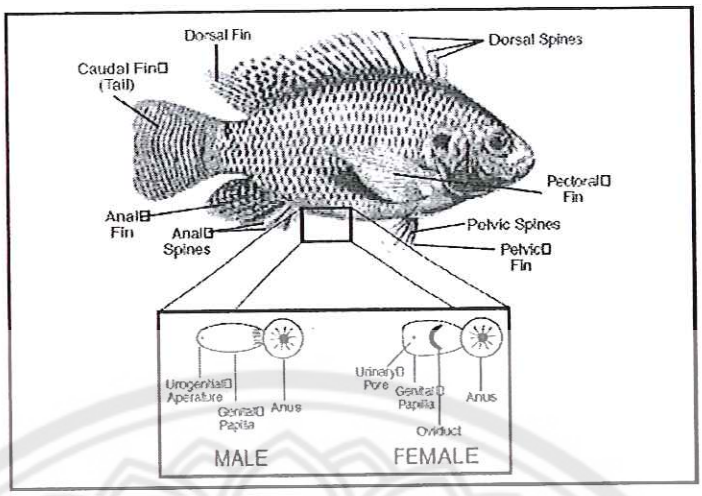


Figure 5 External genital papilla diagram of both the male and female Nile tilapia (*Oreochromis nilotica*) (Thomas and Michael, 1999).

Ovary

The most ovaries of teleost fish were divided 2 types that are general teleost and salmonid fish types (Figure 6) (Hoar W.S. and Randall D.J., 1969). The general teleost type is many their oocytes confederation covering hollow sac-like organ. The salmonid fish type, many their oocytes are covered by clear hollow sac-like organ which opens into the oviduct. The ovaries of red Nile tilapia also are salmonid fish types. The both ovaries are a paired elongate organ within the abdominal cavity, usually of equal length and are covered by a thin sheet of peritoneum which is located the ovary wall a connective tissue structure (Figure 7). The posterior part of each their ovary is elongated by an oviduct directly connected to the genital papilla for transportation to exterior. The ovaries have a fold of the germinal epithelium that is a numerous septa formed and called ovigerous lamellae. At early stage of lamellae as entry into meiotic prophase contain nests of oogonia and oocyte.

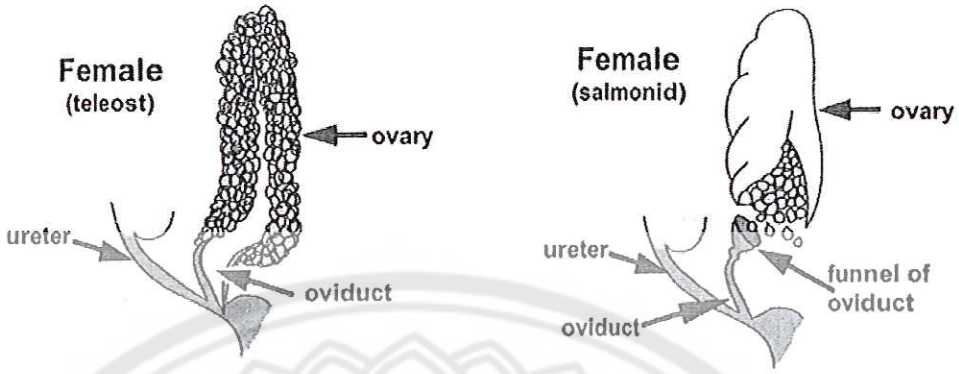


Figure 6 External morphology of ovary in teleost fish. (A) Common pattern in teleosts (B) Salmonids teleost fish (Hoar W.S. and Randall D.J., 1969)

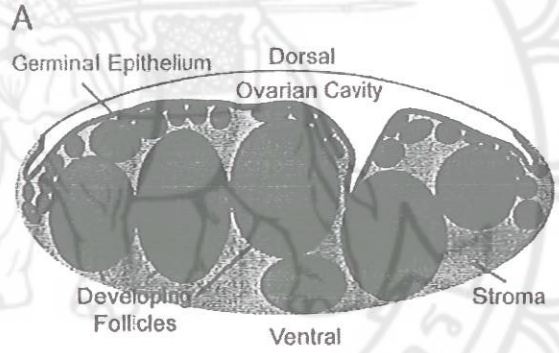


Figure 7 Internal structure of ovarian cord in teleost Medaka (*Oryzias latipes*) (Shuhei et al., 2011)

Oogenesis

Oogenesis in teleost fish is the process of oocyte development by which primordial germ cells become eggs that are ready to be fertilized. Oocytes begin to accumulate neutral lipids and then process, vitellogenin is yolk precursor (induce by estradiol) and stored within yolk granules in oocyte bring to vitellogenic growth (vitellogenesis). The yolk serves as a nutrient store for growing embryo and larvae. Mature oocytes are released from their sac into the ovarian cavity before sent out through the oviduct and the genital papilla or called ovulation (Jalabert B., 2005).

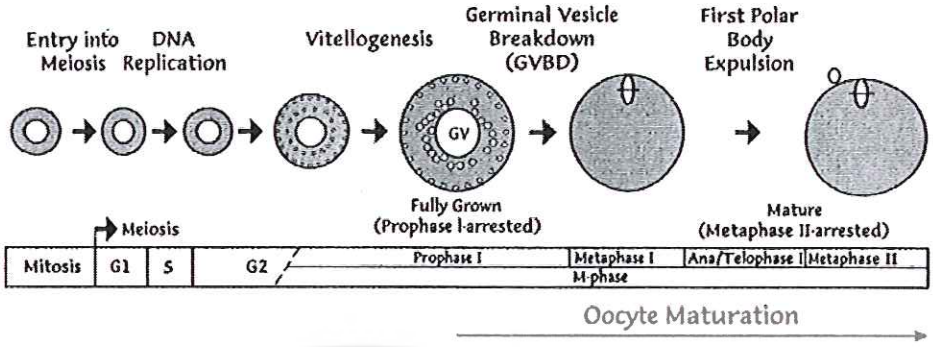


Figure 8 A schematic description of oocyte developmental stages in relation to meiosis in teleost fish, (modified from Suwa and Yamashita, 2007). **From left to right: from primary oocytes, to vitellogenic and mature oocytes. See text for more detailed description.**

Developmental stages of the oocytes in fish

Major stages during egg development in teleost fish include primordial germ-cells (PGCs), oogonia, primary oocytes, secondary oocytes and mature oocytes. Primordial germ-cells generate haploid reproductive cells or gametes occuring transformation by mitotic divisions into oogonia. Oogonia are surrounded by a monolayer of somatic granulosa cells. After that, Proliferation of oogonia begins transition procedure by first meiotic division into a primary oocyte (Selman et al., 1993). First meiotic division involved gene expression encoding the β subunits of the GTHs follicle-stimulating (FSH) and luteinizing (LH) to develop trout ovary. Primary growth contains the period of oocyte development from meiotic chromatin-nucleolus stage to early cortical alveoli stage. In this oocyte stage is surround the follicle layers. Cortical alveoli are displaced to the oocyte periphery during the late stages of oocyte development due to the centripetal accumulation of yolk proteins into secondary growth. The transition from primary to secondary follicular growth increase FSH plasma levels for ready into vitellogenesis. (Swanson, 1991; Breton et al., 1998; Santos et al., 2001; Swanson et al., 2003). Perinucleolar salmon ovarian follicles respond to stimulation with GTHs by increasing E2 production (Swanson et al., 1989). Vitellogenesis procedure in the ooplasm is composed of the lipids accumulation, plasma very low density lipoproteins (VLDL) and vitellogenins (Vtgs), that are phospholipoglycoproteins. The both of vitellogenesis make finally oocyte development

into the mature oocyte accumulated nutritional reserve for the development of the embryo after fertilization (Esther et al., 2010).

Reproduction

Red Nile tilapia, first lay eggs were the average weight 200-500 g, the length 6.5 cm, the fertilization and the spawning 3-4 times in a year which those took the birth about 500-1,000 body in each a generation, was as fertilize as Nile tilapia (Pakorn, 1984 ; Manop et al., 1984 ; Pansri, 1988). The female fish incubates the eggs in her mouth (known as mouth brooding) until they hatch and keeps the young without the yolk sac, that is fully absorbed two weeks later (FAO, 2012). All number of eggs product depended on age and size of the female fish.

The adult egg stages can develop through 5 stages. Time period development stage1 – stage5 of eggs depended on temperature. At 30 ^{0}C first stage, The female fish hatched until was fry a reasonable time 3 days but if decrease temperature is 20 ^{0}C, They hatched to fry within a reasonable time 6 days (MacIntosh and Little, 1995). The development stages for the larval and early juvenile periods of Nile tilapia (*Oreochromis niloticus*) through 5 stages (Figure 9).

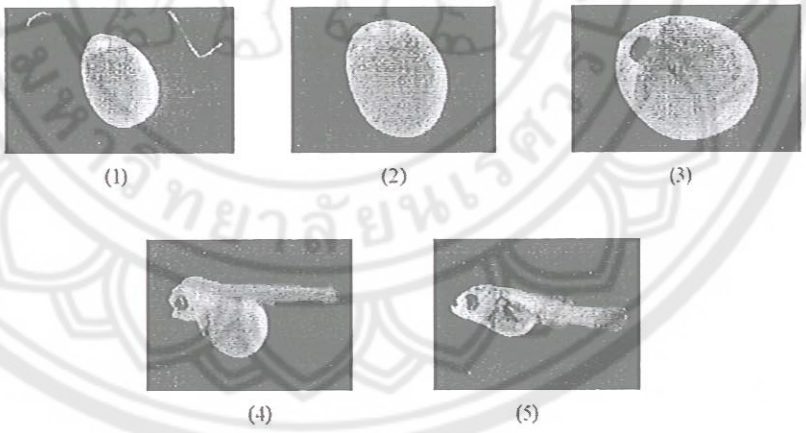


Figure 9 External morphology eggs and larvae in stage 1-5 (Nuanmanee, 2010)

Pituitary gland of fish

The pituitary gland or hypophysis is located at the ventral base of brain (midbrain) called the sella turcica of sphenoid bone, connected to a short infundibulum and suspended from the hypothalamus. It is called the "Master Gland" because it commands directly other organs and endocrine glands, such as the adrenal glands

leading to suppress or induce hormone production. This is cone-shaped structure in zander and sunfish but in carp (*Cyprinus carpio*) similar to an acorn, and as for vimba (*V. vimba*) is round shape (Aygül and Metin, 2013).

The most of early information, the aim of looking at the activity of pituitary cells disconnected from the hypothalamus (Olivereau and Ball, 1966) but this report was showed for the first time that fish hypothalamic extracts only stimulated gonadotropin release in the carp and hagfish (Breton et al., 1971; Breton and Weil, 1973; Masumi, 2013).

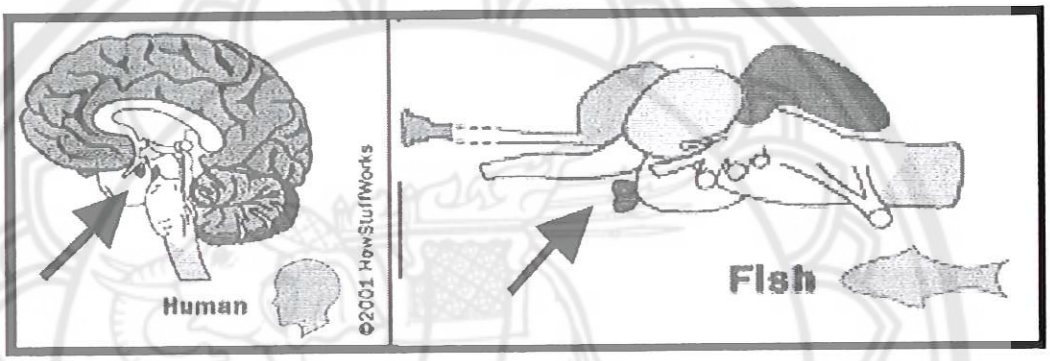


Figure 10 External morphology of the adult vertebrate pituitary show the location of the pituitary gland compare between a humans and fish (black arrows).

Source: Karlstrom Lab Research;
http://www.bio.umass.edu/biology/karlstrom/Karlstrom Lab.html
(Cited: 3/9/56)

The pituitary gland of fish composed of the adenohypophysis, derived from the Rathke's pouch, and the neurohypophysis originating from the diencephalon. The neurohypophysis is believed for controlling the adenohypophysis, some regulation, and some aspects of reproduction. The functions of hormones are promoting survival in fresh water, releasing hormones during stress, stimulating growth, stimulating gonad maturation, stimulating thyroid production, ionic regulation, metabolism and water balance (Mumford et al., 2007; Le Tissier et al., 2012). The adenohypophysis regulates directly gonadal functions in fish that is the site of synthesis, storage and release into circulation of six major hormone producing cell types: lactotrophs producing prolactin

(PRL), somatotrophs producing growth hormone (GH), thyrotrophs producing thyroid stimulating hormone (TSH), gonadotrophs producing gonadotropins (LH and FSH), and corticotrophs and melanotrophs producing proopiomelanocortin (POMC)(Anna Rita Angotzi et al, 2011). The adenohypophysis is divided into the rostral pars distalis, the proximal pars distalis, and the pars intermedia (Ball and Baker, 1969; Schreibman, Leatherl and McKeown, 1973).

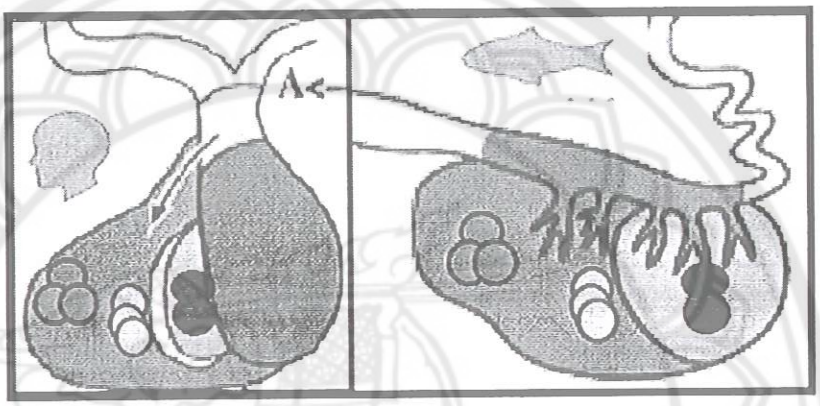


Figure 11 External morphology of the adult both species vertebrate pituitary show structure within the pituitary, the adenohypophysis is divided two lobes (purple and pink) along the anterior-posterior axis. In fish, the neurohypophysis (light blue) is positioned dorsally rather than posterior. Within the adenohypophysis, endocrine cell positions are largely conserved, with PRL and AcTH cells being anterior (green), GH and TSH secreting cells being medial (yellow), and MSH secreting cells being posterior (blue).

Source: http://www.bio.umass.edu/biology/karlstrom/KarlstromLab.html (Cited: September 3rd 2013)

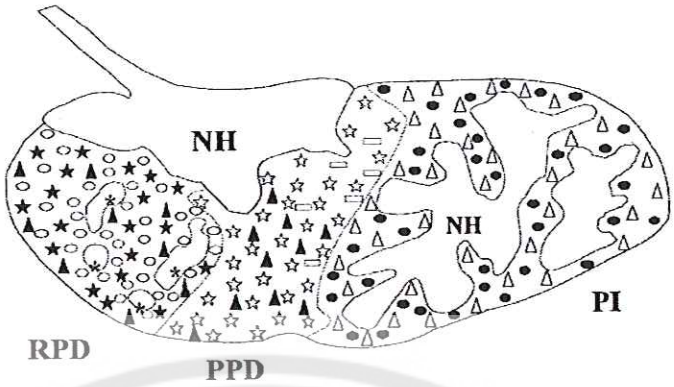


Figure 12 Schematic diagram sagittal representation of the pituitary of *Alosa sapidissima*, showing the distribution of adenohypophyseal cells in juveniles. RPD, rostral pars distalis; PPD, proximal pars distalis; PI, pars intermedia; and NH, neurohypophysis. Cavities (*), PRL (★), ACTH (◦), GH (☆), GTH (▲), TSH (□), SL (•), and MSH (△) cells (Raul Laiz-Carrión et al., 2003).

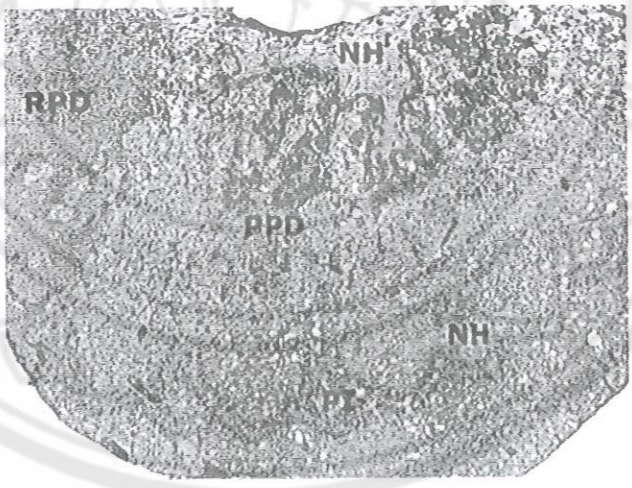


Figure 13 Histological cell types of *Oreochromis niloticus* (Nile tilapia) in each part pituitary gland. NH, Neurohypophysis ; RPD, Rostral pars distalis ; PPD, Proximal pars distalis ; PI, Pars intermediate (El-Sakhawy et al., 2011).

Hypothalamic-pituitary-gonadal axis in fish

Gonadotropin (Follicle stimulating hormone; FSH and Luteinizing hormone; LH)

Gonadotropin-releasing hormone (GnRH) is a central neuropeptide involved in regulation of vertebrate reproduction. Gonadotropin contains a common glycoprotein hormone α subunit (Cgα) and forms a heterodimer with unique β subunits (FSH-β and LH-β) in several tetrapod and teleost species (Yoshiura et al., 1999; Yaron et al., 2001). It stimulated the synthesis of gonadotropins (GTHs) from the adenohypophysis (Breton et al., 1971) that secreted FSH and LH to target organ (Figure 14). In vertebrates, gonadotropin production and release were under the stimulatory control of brain gonadotropin-releasing hormone (GnRH).

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are called gonadotropins stimulating only the gonads in males and females vertebrate. These hormones are considered of central importance in the control of gametogenesis and gonadal steroidogenesis in the fish and other vertebrates (Blázquez et al., 1998). The both hormones were secreted by basophils as poorly basophilic stain. These cells are more concentrated in the central portion of the proximal pars distalis of C. dimerus. They are secreted from these cells in the anterior pituitary essential for reproduction. The most gonadotrophs secrete only LH or FSH, but some appear to secrete both hormones.

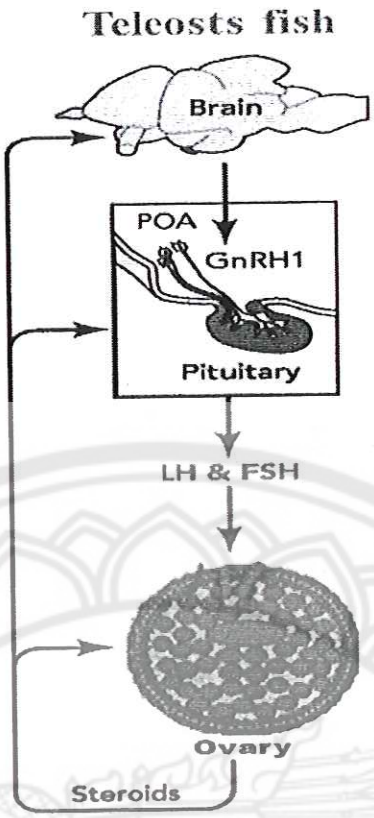


Figure 14 Schematic representation of the hypothalamic-pituitary-gonadal axis in teleost female fish. (Modified from Karen P. et al, 2011)

The roles of pituitary gonadotropins

Control of gonadotropin secretion, GnRH is synthesized and released from neurons in the hypothalamus. GnRH stimulates the synthesis and secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from pituitary gland.

Luteinizing Hormone

In the female, LH stimulates theca cell of ovary to secretion of testosterone, which is converted into estrogen by adjacent granulosa cells. When increasing estrogen, the LH receptors are expressed on the maturing follicle causes produce more estradiol. The full mature follicle inhibits the production of estrogen leading to a decrease in estrogen-mediated negative feedback of GnRH in the hypothalamus as decreasing LH levels in salmon, gold fish and African catfish (Larsen and Swanson, 1997; Kobayashi and Stacey, 1990 and Habibi et al, 1989).

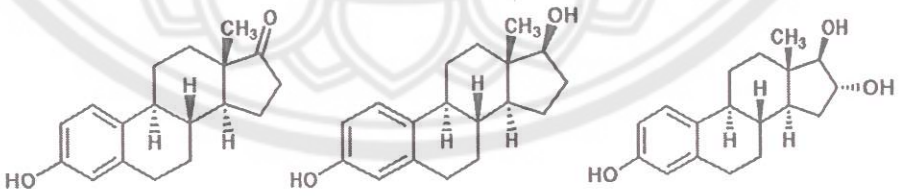
Follicle Stimulating Hormone

In females, FSH initiates for stimulating growth of the ovarian follicle, which includes the developing egg, the cells surrounding the egg (granulosa cells) and the fluid around the egg. When the follicle grows, an increasing amount of the hormone estrogen is produced by the follicle cells and released into the bloodstream. The higher blood levels of estrogen induce the hypothalamus and pituitary gland to slow the production and release of FSH in salmon and gold fish (Dickey and Swanson, 1998 and Kobayashi et al, 2000).

GTH-α, FSH-β and LH-β immunoreactivity were found in oocyte of Gilthead Seabream (Wong and Zohar, 2004) but in hagfish found only one GTHβ. In tilapia, FSH and LH receptors were found to be expressed in gonads as early as 5 days after hatching (dah) (unpublished data). Previous studies indicate LH-β and FSH-β glycoprotein were expressed over the period of development from 1 to 50 dah. cDNAs were cloned from the Nile tilapia and a single band for each subunit was detected (Fan et al., 2003).

Estrogen

Natural estrogens are steroid compounds importance in both menstrual and estrous reproductive cycles effected to development and function of females. The estrogens are synthesized in all vertebrates as well as some insects.



Estrone Estradiol Estriol

Figure 15 Structure of estrogen subtype

Source: http://www.mdpi.com (Cited: September 3rd 2013)

The three major naturally estrogens in the women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the strongest with a potency of approximately 80 times more than estriol. The major only estrogen in female fish, 17β-estradiol (E2), is produced primarily in their ovary. All of the different forms of estrogen are synthesized from androgens, specifically testosterone and androstenedione by the enzyme aromatase. The surge in estrogen level induces the releasing of luteinizing hormone, which triggers ovulation by releasing the egg from the graafian follicle in the ovary.

Estrogen receptor

Estrogen receptor in ray-finned fish (Actinopterygii) and Goldfish (Carassius auratus) species have 3 subtypes including ERα, ERβI and ERβII (Choi and Habibi, 2003; Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2004; Nagler et al., 2007; Tchoudakova et al., 1999; Dereeper et al. 2008). The first form of fish ERβII correlates the most identity with mammalian ERβ, while fish ERβI likely arose from several key amino acid changes (Hawkins and Thomas, 2004; Hawkins et al., 2000). The second form of fish ERα in the rainbow trout likely found in other tetraploid teleost species (Nagler et al., 2007). The ERβ forms are able to bind estradiol with higher affinity than ERα form in Atlantic Croaker, zebrafish and channel catfish (Hawkins and Thomas, 2004; Menuet et al., 2002; Xia et al., 2000, 1999). The affinity of estradiol with ERα is greater at 4^0C than 22^0C in the rainbow trout while the affinity of human ERα showed no change (Petit et al., 1995).

Estradiol (E2) produced by the ovaries and transported via the circulatory system, crosses the nuclear membrane in an inactive conformation in the cytoplasm and the estrogen receptor interacts with a proteins, primarily heat shock proteins Hsp 59, 70, and 90 (Gillesby and Zacharewski, 1998) in the nucleus. Following the transportation of estrogen binds to the estrogen receptor to change its conformation to the active form. The estradiol receptor complex interact specifically DNA segments with the estrogen response elements (EREs) area on the gene to induce transcription of mRNA, followed by production of the target protein (Figure 16) (Nett et al., 2002).

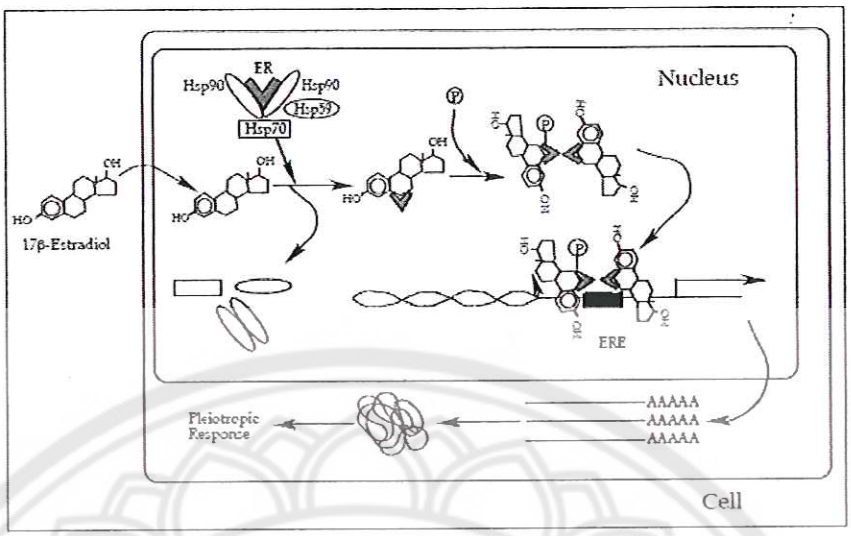


Figure 16 Mechanism of action of the estrogen receptor in fish (Gillesby and Zachrewski, 1998).

Estrogen synthesis

LH stimulates theca interna cells in the ovary synthesis initially of estrogens, by the synthesis of androstenedione from cholesterol. Androstenedione is a substance of androgenic activity which serves a precursor for more potent androgens such as testosterone and estrogen. This compound crosses the basal membrane into the surrounding granulosa cells that were stimulated by FSH. It is converted either immediately into estrone by aromatase, or into testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD).The testosterone is catalyzed by aromatase into 17β-estradiol (E_2) (Pait and Nelson, 2002).

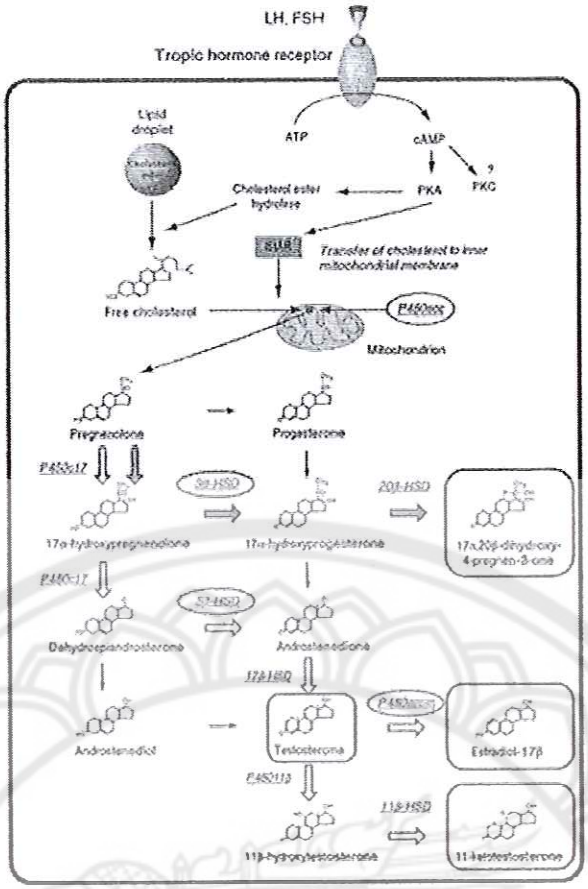


Figure 17 A schematic pathway of steroidgenesis in the gonad of teleost fish. White arrows indicate the proposed androgen synthesis pathway (Modified from Young et al., 2004).

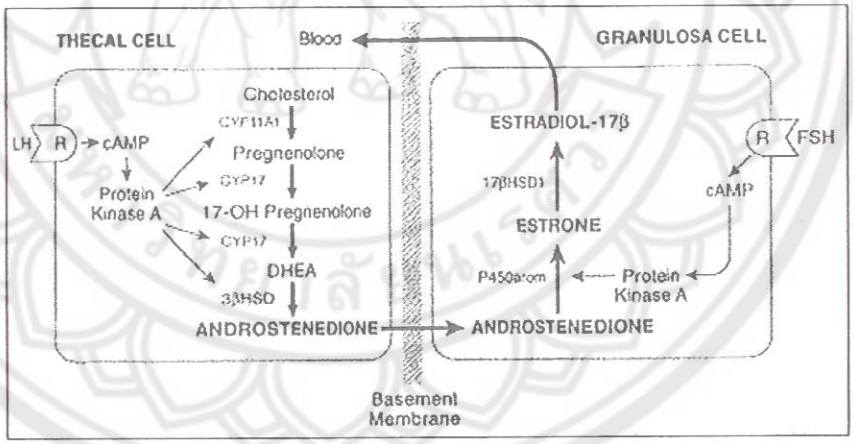


Figure 18 A schematic pathway of estrogen synthesis and secretion of LH and FSH by gonadotropes in the human ovary for easy understand related to fish.

Source:http://www.just.edu.jo/~mafika/733_Reproductive%20Endocrinology/Menstrual %20Cycle_2_733.htm

Endocrine system with oocyte development in fish

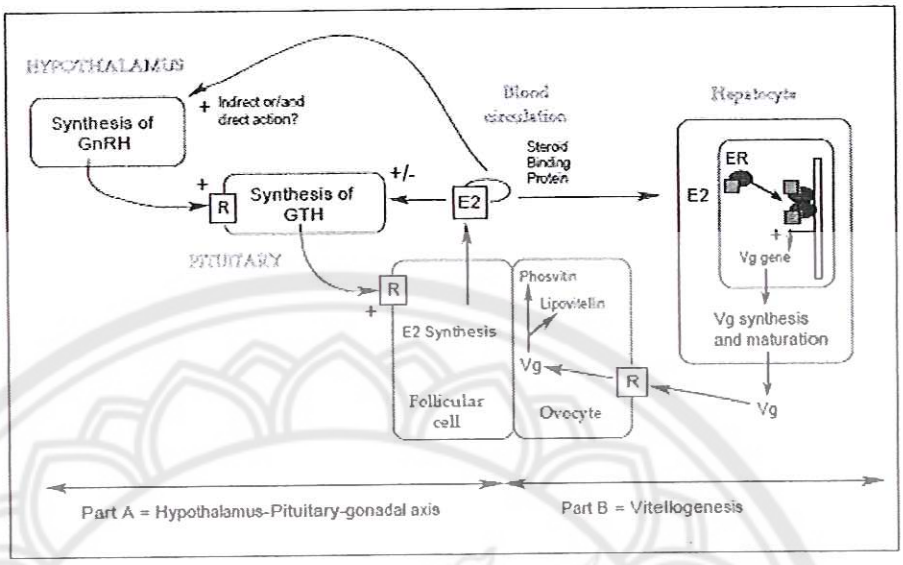


Figure 19 Pituitary-hypothalamic-gonadal axis and the action of estradiol (Drean, 1994).

The gonadotropins released into the systemic circulation increased androgen and estrogen production by the gonads from hypothalamic-pituitary-gonadal axis. 17β-estradiol (E2), is produced primarily in the ovary fish by follicular cells leads to the synthesis of large amounts of vitellogenin by the hepatocytes (liver cells). This high density lipoprotein, the precursor of egg yolk, is transported from the liver via the circulatory system into developing oocytes (Anderson et al., 1996).

Phytoestrogen

Phytoestrogens are estrogen-like chemicals to found in plant foods such as beans, seeds, and grains. The phytoestrogens can be divided into three main classes: flavonoids (flavones, isoflavones, flavanones and chalcones) such as genistein, naringenin, and kaempferol; coumestans (such as coumestrol); and lignans (such as enterodiol and enterolactone) (George G. J. M. Kuiper et al., 1998). The structural of phytoestrogen similarity with estradiol (17β-estradiol), had the ability to cause estrogenic or/and antiestrogenic effects (Yildiz and Fatih, 2005). The ketamine (100 mg/kg, ip) and Rompun (10 mg/kg) were treated in sheep thought to involve a hypothalamic site of action because the positive feedback effect of estrogen to induce LH surges is impaired (Findlay JK et al., 1973). The estrogens regulate follicle-

stimulating hormone (FSH) in a feedback loop, which in turn plays a role in testicular and ovarian development (Jillian E). Previous report, phytoestrogens stimulate in vitro cell proliferation at low concentrations (less than estradiol). They inhibit cell proliferation effect of estradiol at high concentrations (Committee on toxicity, 2012).The effect of estradiol benzoate treatment was found to large increase in amount of gonadotropin-like material in pituitary gland of brown hagfish (Makoto et al., 2006). The 17β-estradiol (E2) induced directly vitellogenin in the tilapia hepatocytes in vitro and in vivo (Akihiro and Byung, 2001) according with the phytoestrogen in Fabaceae plants induced vitellogenin synthesis of juvenile brown trout (Holbech et al., 2013).

The isoflavones, coumestans, lignans, flavonoids, stilbenoids and their metabolites may be considered phytoestrogens also they can interact with estrogen receptors, alter gene expression, and otherwise affect hormones. Some phytoestrogens such as coumestrol, genistein, apigenin, naringenin, and kaempferol compete stronger with E2 for binding to ER beta than to ER alpha (Kuiper et al., 1998).

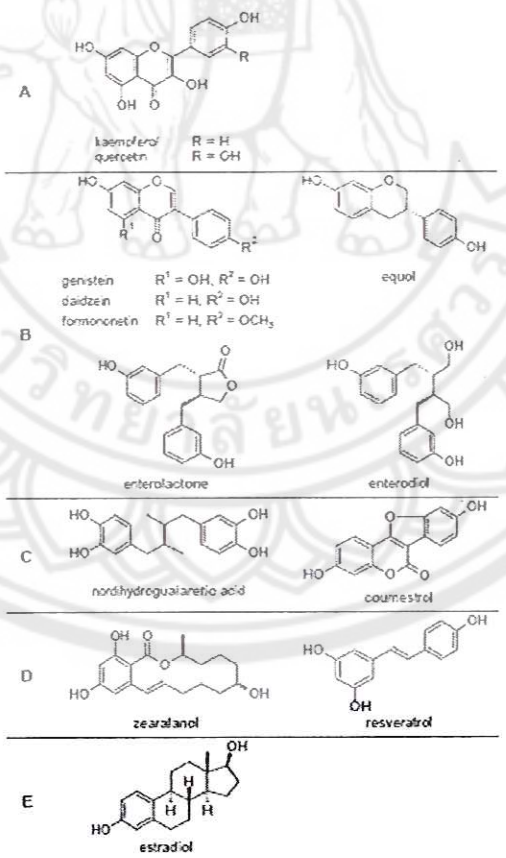


Figure 20 Structures of various phytoestrogen (ZHAO and Qing, 2010)

Curcuma comosa

Curcuma is a plant of about 80 species in Zingiberaceae family. Demonstrative species of Curcuma (Linnaeus, 1753) in Thailand as follows

1. *Curcuma xanthorrhiza* (Indonesia)

2. *Curcuma comosa* (female): Appearance is oval bulb, short branch, central green line on leaf, short peduncle and white-cream matter with pink inner circle.

3. *Curcuma latifolia* (Male): Appearance is oval bulb, long branch, central brown-red line on leaf, long peduncle and white-cream matter with green-gray inner circle (Promjit Saralump, 2012).

Curcuma comosa, commonly known as Wanchakmotluk, is a species of flowering plant in the ginger family. They have been widely used as foods, spice agents and traditional folk medicine in many countries of Asia, including Thailand, Indonesia, Laos and Malaysia (Qu et al., 2009; Boonmee et al., 2011). Active compounds from *curcuma comosa* consist of phytoestrogen such as diarylheptanoid, flavonoid, glycosides and other active constituents such as sesquiterpenes, phloracetophenone glucosides, labdane diterpenes.

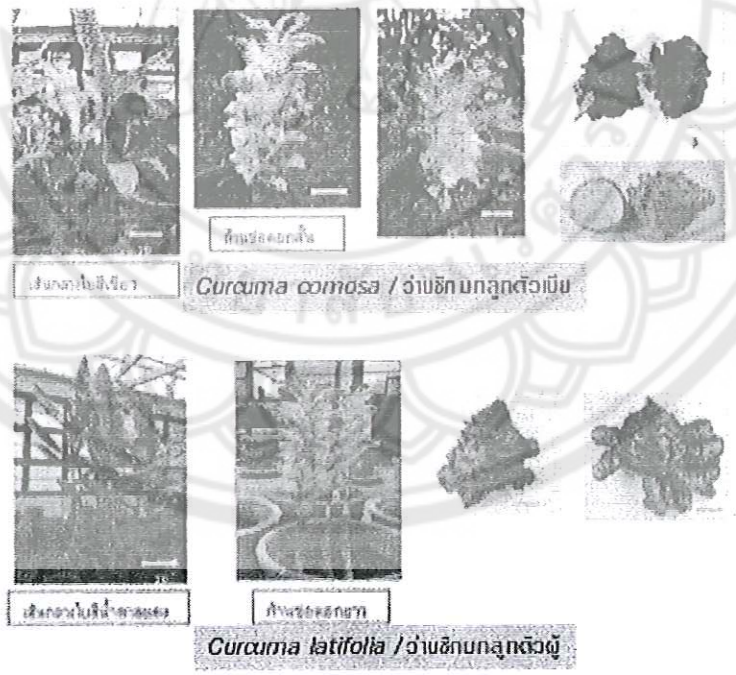


Figure 21 External morphology of *Curcuma comosa* and *Curcuma latifolia*.

Source: http://www.pharmacy.mahidol.ac.th/th/knowledge/article/92/ว่านชักมดลูก (Cited: September 3rd 2013)

Scientific classification of *Curcuma comosa*

Kingdom: Plantae

Order: Zingiberales

Family: Zingiberaceae

Genus: Curcuma

Species: *C. comosa*

Curcuma comosa is an indigenous Thai herb for medicinal plant which is usually used as a food ingredient but it is also used for treatment of ovarian hormone deficiency in Thailand (Weerachayaphorn et al., 2010). It has phytoestrogenic activity, and several diarylheptanoids were found to be the active constituents (Su et al., 2011). In previous study, the C. comosa rhizomes have been found an estrogenic-like action in rats (Piyachaturawat et al., 1995), prevented postmenopausal osteoporosis (Weerachayaphorn et al., 2011), found to have hypocholesterolemic effect (Piyachaturawat et al., 1999), possess an anti-oxidative and anti-atherosclerotic properties (Niumsakul et al., 2007), increased uterine weight and proliferation of the uterine epithelium in ovariectomized mice (Winuthayanon et al., 2009).and in long-term treatment with *Curcuma comosa* has beneficial effects on learning and memory function in rats (Jian et al., 2010) but not have study in female fish especially neuropeptide hormone related with reproductive female fish.

CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research including materials and methods, samples preparation, data analysis. The details of each topic are described below.

Herb samples

C. comosa extract powder was obtained from Assoc. Prof. Dr. Kornkanok Ingkaninan, Faculty of Pharmacy, Pharmaceutical Chemistry and Pharmacognosy, Naresuan University. The fresh rhizomes of *C. comosa* were collected from Naikrajok garden, Ban Khek Noi subdistrict, Khao Kho district, Phetchabun province, Thailand. It was extracted and purified by Assoc. Prof. Dr. Kornkanok Ingkaninan protocol and keep on -20 °C until used.

The fresh rhizome of *C.comosa* (3.8 kg) was sliced and oven-dried at 50 ^{0}C for 3 days. The dry rhizome (800g) was pulverized and extracted successively with the hexane for 3 days. Then solvent was filtrated out by buchner funnel. The *C.comosa* remainder was preserved with the hexane for 2 times. The solvent drain was evaporated hexane out by rotary evaporator at 30 ^{0}C.

Fish samples

The thirty-six female red Nile tilapia age 5-6 months, weight between 300-400 g, size about 12 × 30 cm in length, were cultured at Farming village, Kamphaeng Phet. These fish were fed and recuperate in

floating basket (2×2×2 m) at room temperature under normal light/dark cycle for 1 week with oxygen supply before study.

Experimental design

Fish were divided into six groups; first group as control group, second as negative control, third-forth group as positive control, fifth-sixth group as crude extract injected. The first group was not injected, n=6. The second group was injected with vehicle, the seasame oil, n=6. The third group was injected with low dose (65 µg/kg·BW) commercial 17β-estradiol solve in sesame oil 70 µl as vehicle, n=6. The forth group was injected with high dose (130 µg/kg·BW) commercial 17β-estradiol solved in sesame oil 70 µl as vehicle, n=6. The fifth to sixth group was injected with low and high crude *C. comosa* extracted solved in sesame oil 70 µl as vehicle, each group n=6.

The all of group in this study were collected tissue samples at day 30(D_{30}). At day30 fishes of the each treatment group were anesthetized with 2-phenoxyethanol 1 ml/l (Sigma St. Louis, MO) (G.M. Weber, E.G. Grau, 1999) then measurement length, weight, the ovary, pituitary gland was removed weight and future process.

External morphology of red Nile tilapia and pituitary gland

External and internal morphology of red Nile tilapia and pituitary gland were taken a photograph (digital camera Olympus, FE45, 10.0 million effective pixels). Then fish was weighed by digital kitchen weighing apparatus scale 5 kg/1g (Bradshaw International, CA91730) and pituitary gland was weighed by digital weighing (HR-200, the four digits

after the decimal point). Length of fish was measured by Vernier caliper instrument for measure internal and external distances for accuracy values.

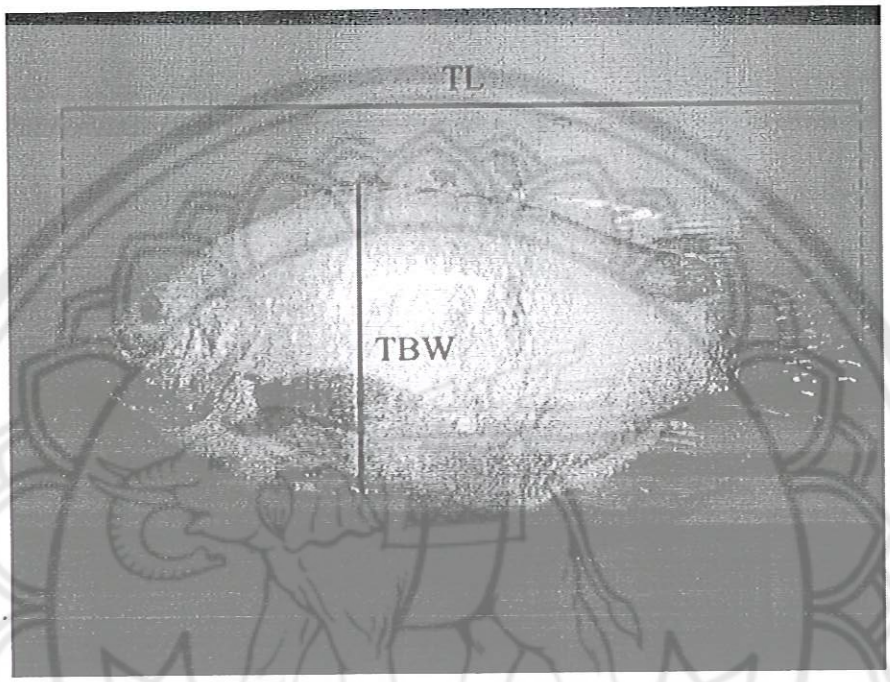


Figure 22 The total length (TL) is maximum length of red Nile tilapia until mouth to caudal fin and total body width (TBW) is maximum and direct width of red Nile tilapia until dorsal fin to abdomen.

Examination external morphology of pituitary gland of red Nile tilapia by stereo microscope

Immediately, after pituitary gland removed, it was fixed in freshly cool Davison's plus 10% glycerol fixative for 30 min before examined. External

morphology of pituitary glands were observed with stereo microscope (Olympus, SZ51) to obtained three-dimensional views and taken a photograph by digital camera (digital Olympus, FE45, 10.0 million effective pixels) and finally data was analyzed.

Examination of pituitary gland and ovary in red Nile tilapia by light microscope Tissue preparation

For light microscopy, the paraffin technique is the most commonly used. This technique used for examined position, appearance, dimension and type of cells study.

The fish were anesthetized by immerged in water adding with 2-phenoxyethanol 1 ml/H_2O 11 (Sigma-Aldrich 0.5g in 5 ml ethanol 95% concentrations) and waited until fish completely unconsciousness. The pituitary glands and ovary were removed rapidly in freshly cool Davison's plus 10% glycerol fixative for overnight at 4 ^{0}C. Then samples were washed with 70 % ethanol 20 min for 3 times to get rid of fixative. Samples were dehydrated with automatic tissue processor (LEICA, TP1020) for 9.45 hr. Briefly, tissues were processed through graded series of alcohol from low to high alcohol concentration (70%, 80%, 90%, 95%, 95%, 100%, 100% - 45 min glass jars) for 5.15 hr and clearing with xylene for 45 min, and finally tissues were infiltrated with melt paraffin to carry off the cassettes into the tissue embedding center (LEICA, EG1160) and tissues were embedded as paraffin blocks. Each paraffin tissues block was cut 5 µm thick by microtome (LEICA, MR2235) as ribbon then folding on warm water and placed to glass slide. After that it was stained by Mayer's hematoxylin and eosin. The glass slide was mounted by a drop of Permount (Fisher Scientific, SP15-500 Toluene Solution UN1294) for adhered, protected tissues on slide and covered with cover slip. Finally slides were examined under light microscopy (Nikon ECLIPSE 80i) and taken a photograph with Nikon digital camera (DXM1200C), using Nikon Act 1C program.

Immunohistochemistry study of LHβ distribution in pituitary gland cells of red Nile tilapia

Immunohistochemistry is an available tool for the detection, localization and quantification of antigens or proteins in tissue by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by an enzyme marker. This research used this technique for examine beta subunit of luteinizing hormone (LHβ) within cell of pituitary gland of red Nile tilapia. Because LH is essential hormone found in humans and other animals which are synthesized by gonadotroph cell in pituitary gland

Briefly, fish were anesthetized with 0.05% 2-phenoxyethanol waiting to until unconsciousness. The pituitary gland and ovary were dissected out and fixed in a freshly cool Davison's plus 10% glycerol fixative for overnight at 4 ^{0}C. After tissue processing and paraffin embedding, the tissues were cut as ribbon and mounted on slides coated with 3-aminopropyl triethoxy-silane solution (Sigma Co., St. Louis, MO, USA). After that, the sections were deparaffinized with xylene for 10 min 3 times and rehydrated through a graded series of ethyl alcohol (100%, 95%, 90%, 80%, and 70%) for 5 min each. Subsequently, endogenous peroxidase and free aldehyde groups were blocked by immersing the sections in 3% H_2O_2 in methanol for 55 min, washed with PBST 3 times (PBS added with 0.5% Triton X-100) and 1% glycine in PBS for 5 min. The sections were washed with PBST 2 times for 5 min and PBS for 5 min. Non-specific binding were blocked by incubating the sections in the blocking solution (containing 1% normal goat serum (NGS) in PBST) for 2 hr at room temperature. The sections were incubated with primary antibodies (rabbit anti-human LHβ diluted at 1:200, US Biological: L7500-27C) in the blocking solution at 4 °C for overnight in a moist chamber. After washing with PBST 2 times for 7 min and with PBS 2 times for 7 min, the sections were incubated with secondary antibody (HRP-conjugated

goat anti-rabbit IgG, Jackson Immuno Research, 111-035-003) diluted at 1:500 in the blocking solution for 1.30 hr. After washing with PBST 2 times for 5 min and with PBS 2 times for 5 min, the color reaction was developed by adding 3, 3'- diaminobenzidin (DAB) for 15 min until brown-red color was observed and reaction was stopped by immersed into distilled water for 5 min. Finally, the sections were counterstained with Mayer's Hematoxylin and rinsed with tap water for 5 min dehydrated, clearing and mounted with Permount (Fisher Scientific, SP15-500 Toluene Solution UN1294). A negative control was incubated with PBS replacing of primary antibodies. Slides were examined under a Nikon ECLIPSE 80i light microscope, and images were photographed using a Nikon digital DXM1200 camera.

Examination of LHβ gene expression in pituitary gland of red Nile tilapia

LHβ gene expression is examined by reverse transcription polymerase chain reaction (RT-PCR). This technique is commonly used in molecular biology for detecting mRNA expression levels. The RNA is reversely transcribed into DNA complementary (cDNA) using enzyme reverse transcriptase and finally cDNA is amplified by PCR machine.

RNA extraction

The pituitary glands at days 30 were rapidly removed and filled into 1 ml cool Trizol® reagent (Invitrogen, CA, USA) in sterile Eppendorf tube. The total RNA from pituitary gland in each group was isolated using Trizol® Reagent. Each pituitary sample was crushed by homogenizer (Micro-Grinder) and keep at -80 ^{0}C until used. When using, the samples were incubated for 5 min at room temperature. Chloroform 0.2 ml was added in tube for RNA isolation and slowly reversed 3 times. The samples were incubated for 3 min at room temperature and centrifuged at 12,000×g for 15 min at 4ºC. The supernatant (colorless) was transferred into a new tube which has RNA pellets. Absolute isopropanol was added to supernatant for RNA precipitation and incubated for 10 min at room

temperature. Then tube was centrifuged at 12,000×g for 10 min at 4°C. The supernatant was taken away from tube, leaving only RNA pellets that were washed with 1 ml of 75% ethanol. RNA pellet tube was rapidly inversed, then centrifuged at 7,500×g for 5 min at 4°C. The supernatant was taken away from tube and washed with 1 ml of absolute ethanol. Tube with RNA pellet was rapidly inversed for RNA washing, then centrifuged at 7,500×g for 5 min at 4°C. The supernatant was completely removed from tube and RNA pellets were leave at room temperature for 10 min until completely dried. Finally the RNA pellets were resolved in sterile DEPC treated water (Amresco) and stored -80°C until used.

Complementary DNA synthesis

A pituitary (about 6.7 mg) of total RNA from each group was used to perform the first strand cDNA synthesis using random primer (Qaigen) and Superscript III kit (Invitrogen, USA) following the manufacturer's protocol. The cDNA was amplified using Go Taq Polymerase (Promega, USA). The primer sequences of the gene encoding LH will be designed from sequence of *Oreochromis niloticus* LH beta subunit mRNA nucleotides sequence in Genbank AY294016.1. The β-actin was used as a control. The PCR cycles for each gene are as follows: 95°C for 30 sec, 55 °C for 45 sec, and 72°C for 1 min for 35 cycles.

Table 1 The sequence of *Oreochromis niloticus* LH beta subunit

and beta actin

Primers	Sequence (5'->3')	Product size (bp)
LH-β	Forward : ACCGGAGAGAATGATGGCAC Reverse : AACTCAAAGCCACGGGGTAG	353
β-actin	Forward : AAGTACCCCATTGAGCACGG Reverse : CAAGGAAGGAAGGCTGGGAG	60

Agarose gel electrophoresis

PCR product was separated by 1.5% agarose gel (Vivantis, USA) and their DNA band size was determined by electrophoresis in 1X TBE buffer at 120 V for 50 min. Each well was loaded with PCR product 12 µl/well and 100 bp DNA Marker (SibEnzyme, Russia) was run on agarose gel along with PCR product for measurement molecular weight of the PCR product. Gel was stained with 0.5 µg/ml ethidium bromide and washed for 40 min. Bands of cDNAs were identified under UV light (Major science) in the dark room box and then photographed using a Canon Powershot G10 camera.

Statistical analysis

All of the data were analyzed with SPSS version 17.0 program using One-way ANOVA followed by LSD post hoc test. Mean and standard deviation of results were presented. The statistically significance was determined as P-values less than 0.05 ($P \leq 0.05$).

CHAPTER IV

RESULTS AND DISCUSSION

External morphology

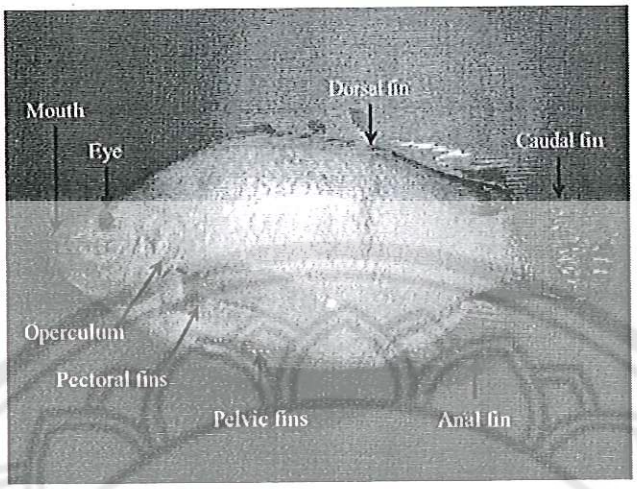


Figure 23 External morphology of red female Nile tilapia (*Oreochromis niloticus*)

External morphology of red Nile tilapia has morphological characteristics that were fusiform body shape, white-orange body, lateral line, orange operculum, orange mouth, oval eyes, swelling abdomen, clear scales and orange fins. They had several fins all of body including appendages the dorsal fin, caudal fin, anal fin and both pair fins which included the pelvic and pectoral fins. Lateral line was a sensory system through long the middle body. The mean body weight was 0.33 ± 0.07 g and width-length ratio $10.33 \pm 1.21 \times 26.83 \pm 1.94$ cm of female red Nile tilapia in control group (n=6).

Gender identification

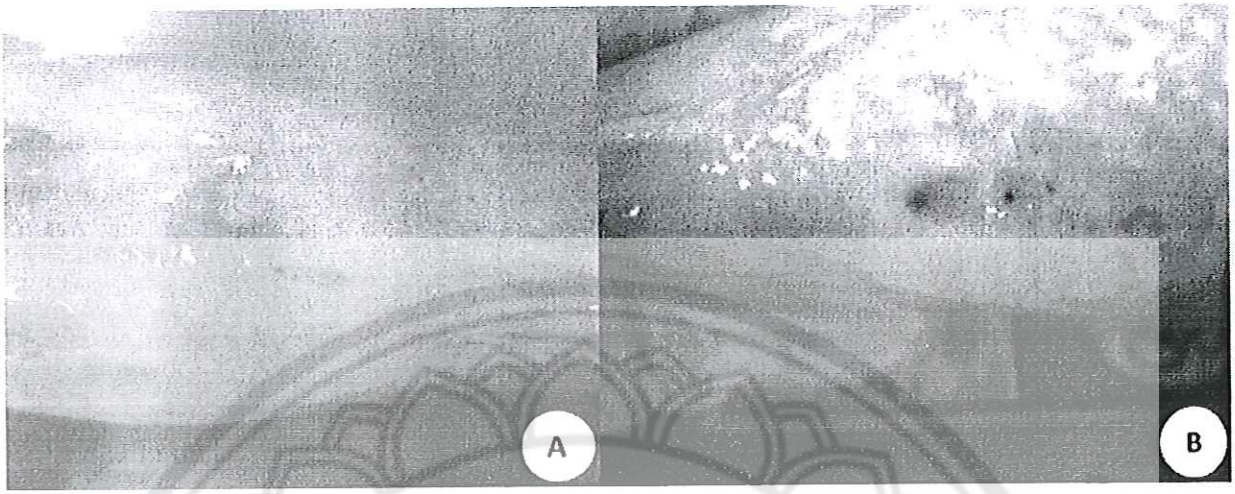


Figure 24 External genital papilla of both the male and female red Nile tilapia (*Oreochromis nilotica*) (A) Male genital papilla (B) Female genital papilla

The both sexual organ were located in the lower back abdominal area. The sexual characteristics of both fish were very similar but genital area near anus was different. The male sexual organ had 2 apertures as anus and urogenital pore that was slender shape (Figure 24A). In contrast, sexual organ of female fish had 3 apertures as anus, urinary pore and oviduct pore to form a short gonoduct leading to the urogenital pore that was large aperture and oval shape (Figure 24B) also male fish was pale color than female fish.

General appearance of Pituitary gland

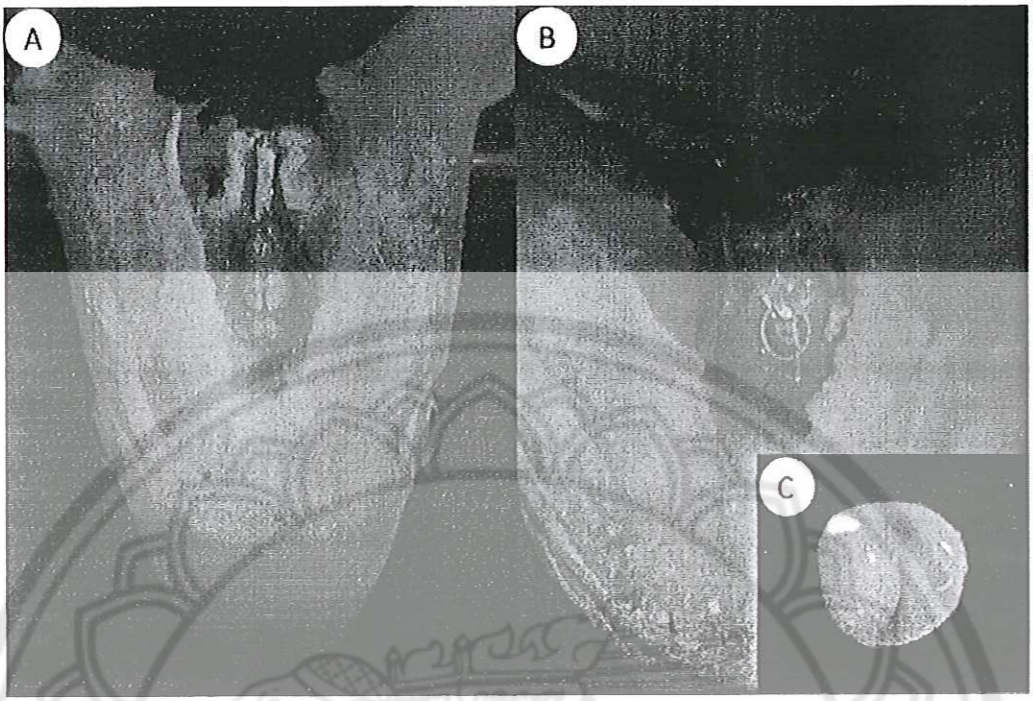


Figure 25 The fish craniectomy, the most common way to removed brain and pituitary gland. (A) Overall image fish head after removed fat (B) Pituitary gland after removed brain (C) Stereo microscopes in posterior pituitary surface

The pituitary gland (hypophysis) was oval shape located at base of the brain. The brain and pituitary gland were covered by thick gel covering (fat) (Figure 25A and 25B). The mean pituitary weight was 6.70 ± 2.33 mg and diameter was 2.53 ± 0.23 mm of female pituitary gland in control group (n=6). The three-dimensional structure of these glands could mainly be divided into 3 parts as anterior, posterior and upper part. Anterior pituitary part (pars distalis)was smooth surface that was attached to sella tunica in the body of the sphenoid bone while posterior pituitary part (pars intermedia) was a middle pit and two gyri (Figure 25C). The last, Upper pituitary part connected to a short pituitary stalk and suspended from the hypothalamus. These part had a pit for pars nervosa.

Histology of the Pituitary Gland

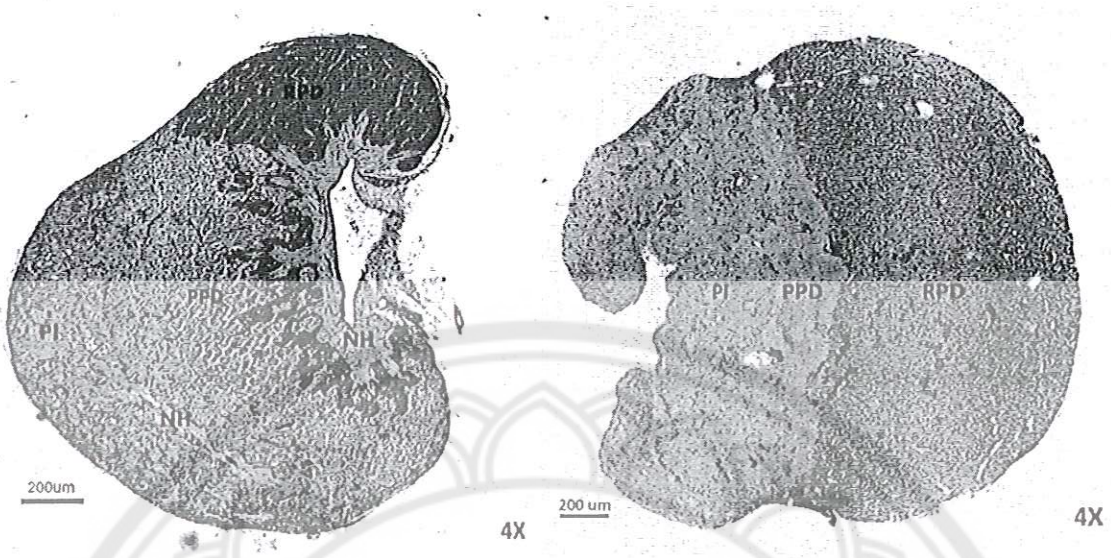


Figure 26 The histological cell types of red Nile tilapia (*Oreochromis niloticus*) in each part of pituitary gland. (A) The mid-sagittal section shown that the pituitary gland completely consist the structure and cell type. (B) The horizontal section shown that the pituitary gland consisted of two main lobes, the anterior lobe (pars distalis) and posterior lobe (pars intermedia). NH, Neurohypophysis; RPD, Rostral pars distalis; PPD, Proximal pars distalis; PI, Pars intermedia. Score bar = 200 um, 4X

The pituitary gland could mainly be divided into 3 parts; Adenohypophysis (Pars distalis), Pars intermedia and Neurohypophysis (Pars nervosa). Histological structure of pituitary gland was divided by the mid-sagittal section. The first started to describe the anterior lobe, the pars distalis area was approximately estimated 50% of all pituitary area. The pars distalis composed of two subclass areas histologically; rostral pars distalis and proximal pars distalis. The second part, the pars intermedia area that located posterior pituitary lobe, was approximately estimated 45% of all pituitary area. Finally part, the

neurohypophysis can mainly found to infiltrate surrounding stalk and a little distribute in pars intermedia (Figure 26).

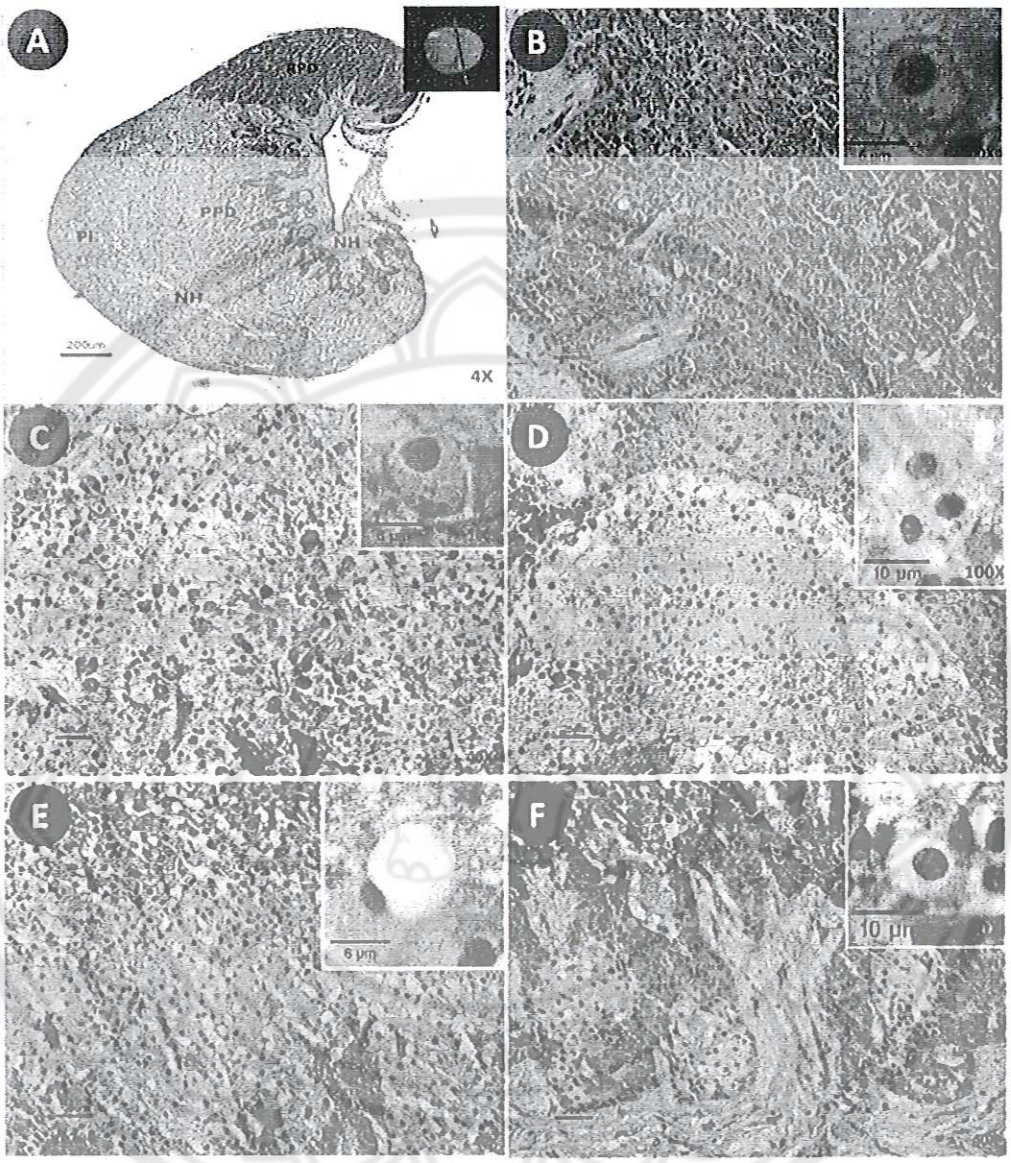


Figure 27 The histological cell types A) The mid-sagittal section of pituitary gland B) acidophil cell in rostal pars distalis C) basophil cell in pars intermidia D) chromophobe cell in proximal pars distalis E) signet cell in proximal pars distalis F) clear cell in surrounding the neurohypophysis stalk. Score bar = 25 um, 40X

Acidophil cells (α cell) were mostly distributed in the rostal pars distalis and infiltrated a little around neurohypophysis, especially near stalk (Figure 27B). The acidophil area was approximately estimated 25% of all pituitary area. Characteristic of acidophil cells were medium oval shape, the cells size were width 8.00 ± 1.63 μm and length 8.54 ± 1.50 μm (n=1,000) that was medium cell when compared with other cell types. The purple-blue concentric nuclei were width 3.94 ± 0.59 μm and length 4.04 ± 0.54 μm (n=1,000). They had pink cytoplasm within consisting of large granules.

Basophil cells (β-cell) were found 45% distribution all pituitary gland, mainly constituted pars intermedia and 10% of pars distalis (Figure 27C). Characteristic of basophil cells were big oval shape, the cells size were width 8.66 ± 1.57 μm and length 9.42 ± 1.56 μm (n=1,000) that was the biggest cell when compared with other cell types. The purple-blue concentric nuclei were width 4.16 ± 0.56 μm and length 4.23 ± 0.50 μm (n=1,000). They have purple-blue cytoplasm within consisting of large granules.

Chromophobe cells were all found in the proximal pars distalis. The chromophobe area was approximately estimated 25% of all pituitary area that depended on infiltration of the signet cell (Figure 27D). Characteristic of chromophobe cells were big oval shape, the cells size were width 8.38 ± 1.60 μm and length 8.92 ± 1.56 μm (n=1,000) that was bigger than the acidophil cells. The purple-blue concentric nuclei were width 4.09 ± 0.57 μm and length 4.13 ± 0.47 μm (n=1,000). They had pale cytoplasm within consisting of small granules.

Signet cells were distributed in the proximal pars distalis, infiltrated chromophobe in this part that depended on the level of secretory activity (Figure 27E). Characteristic of signet cells were small oval shape, the cells size were width 7.90 ± 1.49 μm and length 7.86 ± 1.69 μm (n=1,000) that was smaller than the acidophil cells. The small purple-blue eccentric nuclei were diameter 3.38 ± 0.64 um and clearly large cytoplasm.

Clear cells were infiltrated in the acidophil cell surrounding the neurohypophysis stalk (Figure 27F). Characteristic of clear cells were small oval shape, , the cells size were width 7.51 ± 1.44 μm and length 7.66 ± 1.52 μm (n=1,000) that was the smallest cell when compared with other cell types. The purple-blue concentric nuclei were width 3.98 ± 0.69 μm and length 4.06 ± 0.61 μm (n=1,000). They have clear cytoplasm.

Neurohypophysis

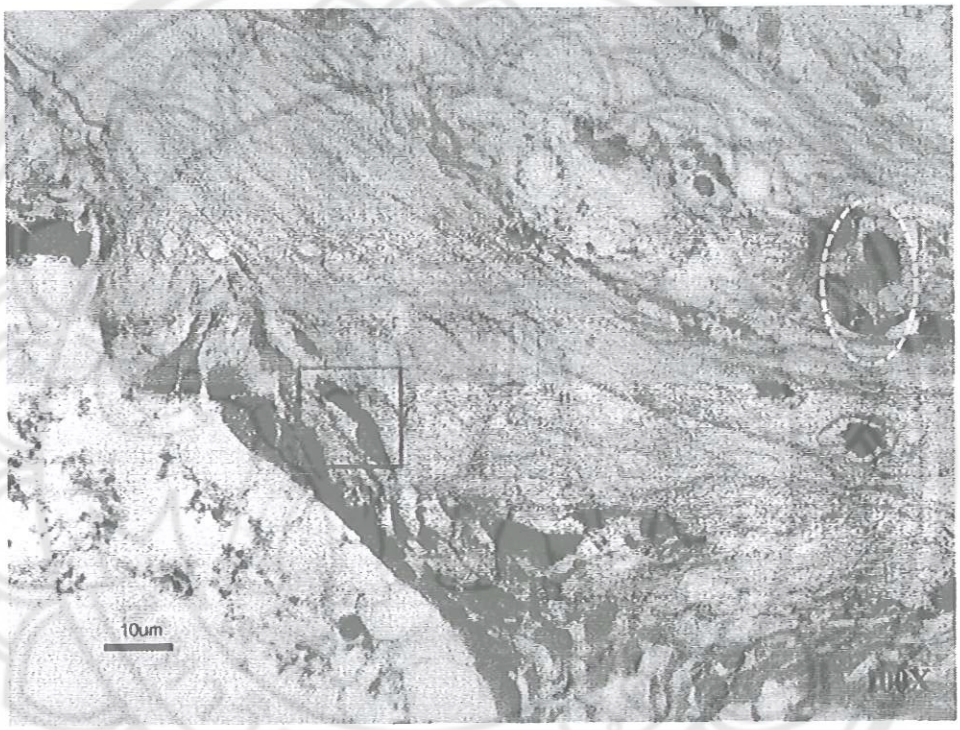


Figure 28 The histological of glia cells and Herring bodies in Neurohypophysis. The glia cells shown in the green square. The Herring bodies was shown in the yellow circle. Score bar = 10 um, 100X

The neurohypophysis found two areas; infiltrate surrounding stalk and distribute in pars intermedia. In this part were consisted numerous glia cells (pituicytes) and Herring bodies (Neurosecretory bodies) that were detected to be located among many the nerve fibers. The glia cells were slender shape to distribute over all this part. The Herring bodies, that three times bigger than glia cells, were a little oval shape within consisting of large granules (Figure 28).

Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RT-PCR assay was used to measure the expression of LH mRNA levels. The level of LHβ mRNA and internal control (beta actin) were measured density band by image J program.

LHβ mRNA expression in control group of female and male red Nile tilapia

Figure 29 Representation pituitary showing agarose electrophoresis band of male and female red Nile tilapia in control group. M (yellow); Marker, P; LHβ primer, β;Beta actin primer, F; Female, M; Male.

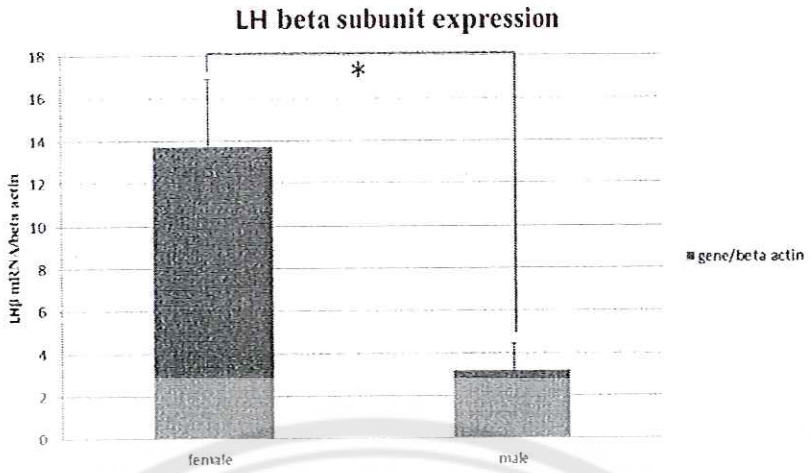


Figure 30 The LHβ gene expression between male and female of red Nile tilapia in pituitary gland. Values are mean±SD. * P-value ≤ 0.05 (N=6)

The three pituitary glands were examined LHβ mRNA expression in each sex (n=6). The mean LHβ mRNA expression level were 13.74 ± 3.14 in female (n=3) and 3.19 ± 1.23 in male (n=3) pituitary (Figure 30).

LHβ mRNA expression in treatment group of female red Nile tilapia

Figure 31 Representation pituitary shown agarose electrophoresis all band in each group with marker. M; Marker, G; LHβ primer, β; Beta actin primer, 1; control, 2; sesame oil, 3; low-dose 17β-estradiol, 4; high-dose 17β-estradiol 5; low-dose *C.comosa* extract, 6; high-dose *C.comosa* extract

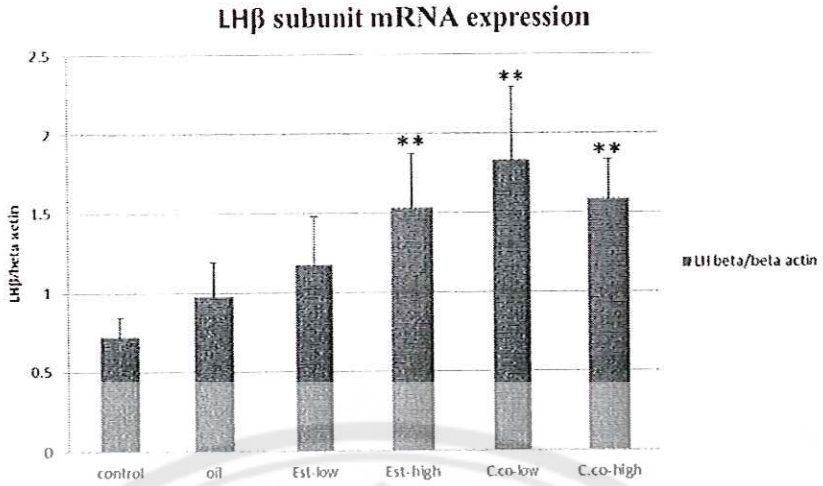


Figure 32 The LHβ relative gene expression between control and treatment group in pituitary red Nile tilapia. Values are **mean±SD. ** P-value ≤ 0.01 (N=18)**

LHβ mRNA expression of three pituitary glands were examined (each group n=6). The mean LHβ mRNA expression level in each group were presented; control=0.72±0.12, sesame oil=0.98±0.22, Est-low=1.17±0.31, Est-high=1.53±0.34, C.co-low=1.83±0.46, C.co-high=1.58±0.25) (Figure 32). It was found that LHβ mRNA expression in the high β-estradiol, both *C.comosa* extract group were increased significantly when compare with control group (P-value ≤ 0.01).

Examination internal morphology in different stage oocytes of red Nile tilapia

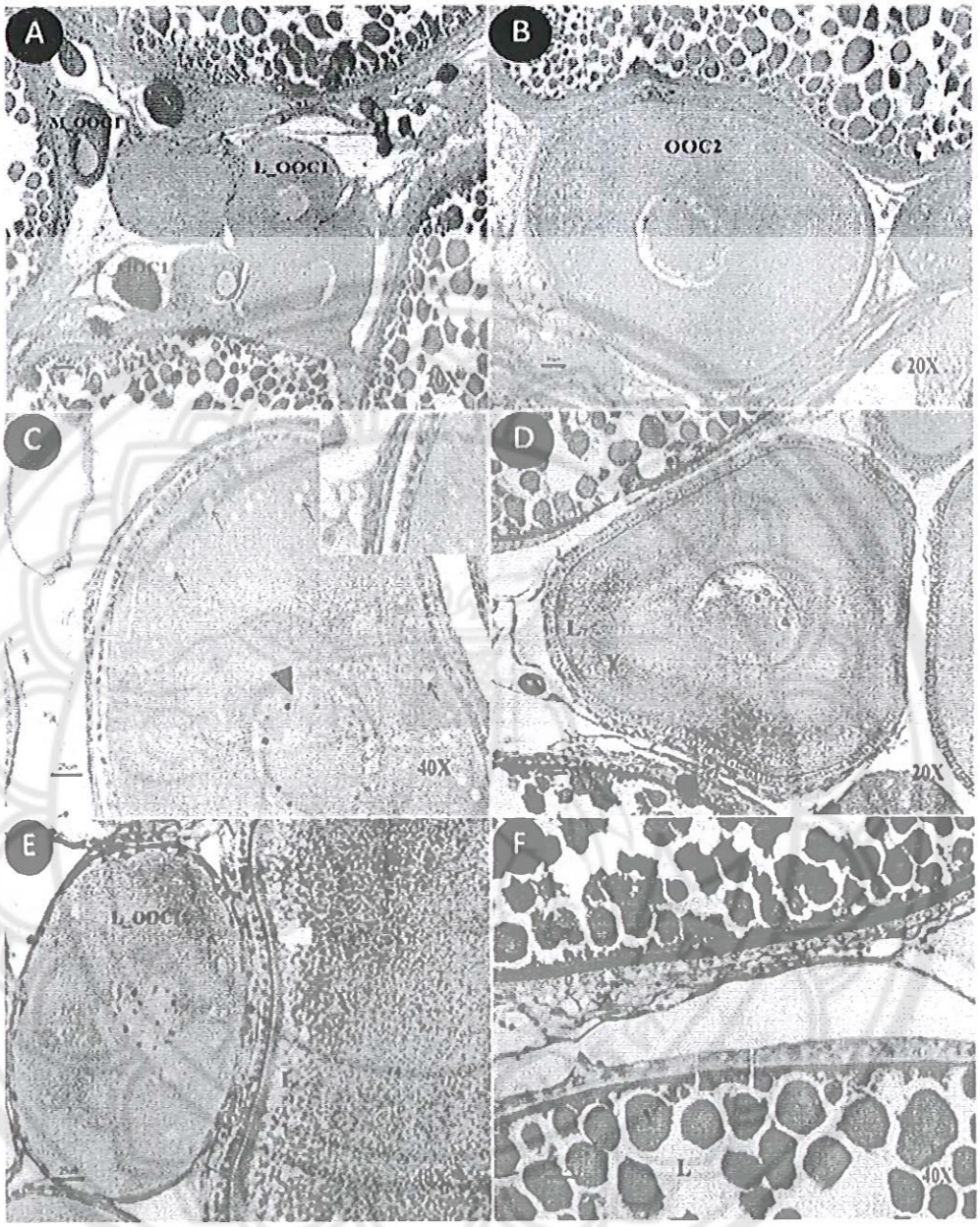


Figure 33 The normal histological of ovaries shown oocytes in different stages of development (H&E stain). Score bar = 50 µm; 20X, Score bar = 25 µm; 40X

Histology structure of the ovary red Nile tilapia and developmental oocyte stages were studied by light microscopy. The ovaries were divided 5 stages as follows.

1. Oogonia in Nile tilapia was first growth phase of the oogenesis in fish. Characteristic of this stage was mostly oval shape, clear ooplasm and strong basophilic nucleus. Moreover surrounding the oocytes were follicular cells that were simple squamous cells.

2. Primary oocyte in oval ovarian follicle was divided into 3 sub-stages.

a) Early primary oocyte (E_OOC1) was overall strong basophilic ooplasm (Figure 33A) and some pale basophilic concentric oocyte.

b) Middle primary oocyte (M_OOC1) was strong basophilic ooplasm and large pale basophilic nucleus (Figure 33A).

c) Late primary oocyte (L_OOC1) was basophilic ooplasm, pale basophilic nucleus and a lot of nucleolus enclosed nuclear membrane in peripheral nucleus. Follicular cells and theca cells were outside surrounding of oocyte respectively. The both cell types also were initially simple squamous cell (Figure 33A).

3. Secondary oocyte (OOC2): Cortical alveoli stage

In this stage that short period of time was third growth phase of the oogenesis in fish. Characteristic of oocytes were large oval shape, pale basophilic ooplasm and nucleus. The diameter ratio nucleus per ooplasm decreased. Nucleus and nucleolus were adjoined nuclear membrane. The dominant feature in this stage, In the periphery zone of the ooplasm had many unstained vacuoles (corticle alveoli) that were small clear oval shape. The outer nuclear membrane layer was follicular layer that were divided into 3 sub-layers; Zona radiata (thin acidophillic vitellogenin membrane begin forming), Follicular cells (simple low cuboidal 4. Vitellogenic stage

In previous growth phase of the oogenesis in fish was developed rapidly get into this stage. Characteristic of oocytes were large oval shape and pale basophilic nucleus. The dominant feature in this stage of ooplasm was divided distinctly into 2 layer; cortical alveoli and yolk granules. Many cortical alveoli layer were small oval shape at the periphery of the ooplasm. Inside zone of ooplasm were many yolk granules layer that small oval shape and strong acidophilic granules (Figure 33D). The follicular trilayer at outer nuclear membrane were divided into 3 sub-layers; zona radiata (thicker acidophillic vitellogenin membrane), follicular cells (simple cuboidal/columnar cells) and theca cells (stratified squamous cells) respectively.

5. Mature stage

In this stage was final growth phase of the oogenesis in fish. Characteristic of oocytes were the largest oval shape, migrated nucleus to peripheral zone and some oocyte no nucleus. The dominant feature in this stage found largest cortical alveoli and yolk granule spread around ooplasm (Figure 33E and 33F). The outer nuclear membrane layer was follicular layer that were divided into 3 sub-layers; zona radiata (the thickest acidophillic vitellogenin membrane), follicular cells (simple cuboidal cells) and theca cells (stratified squamous cells) respectively. (Figure 33B and 33C).

Examination internal morphology of treatment oocytes in red Nile tilapia

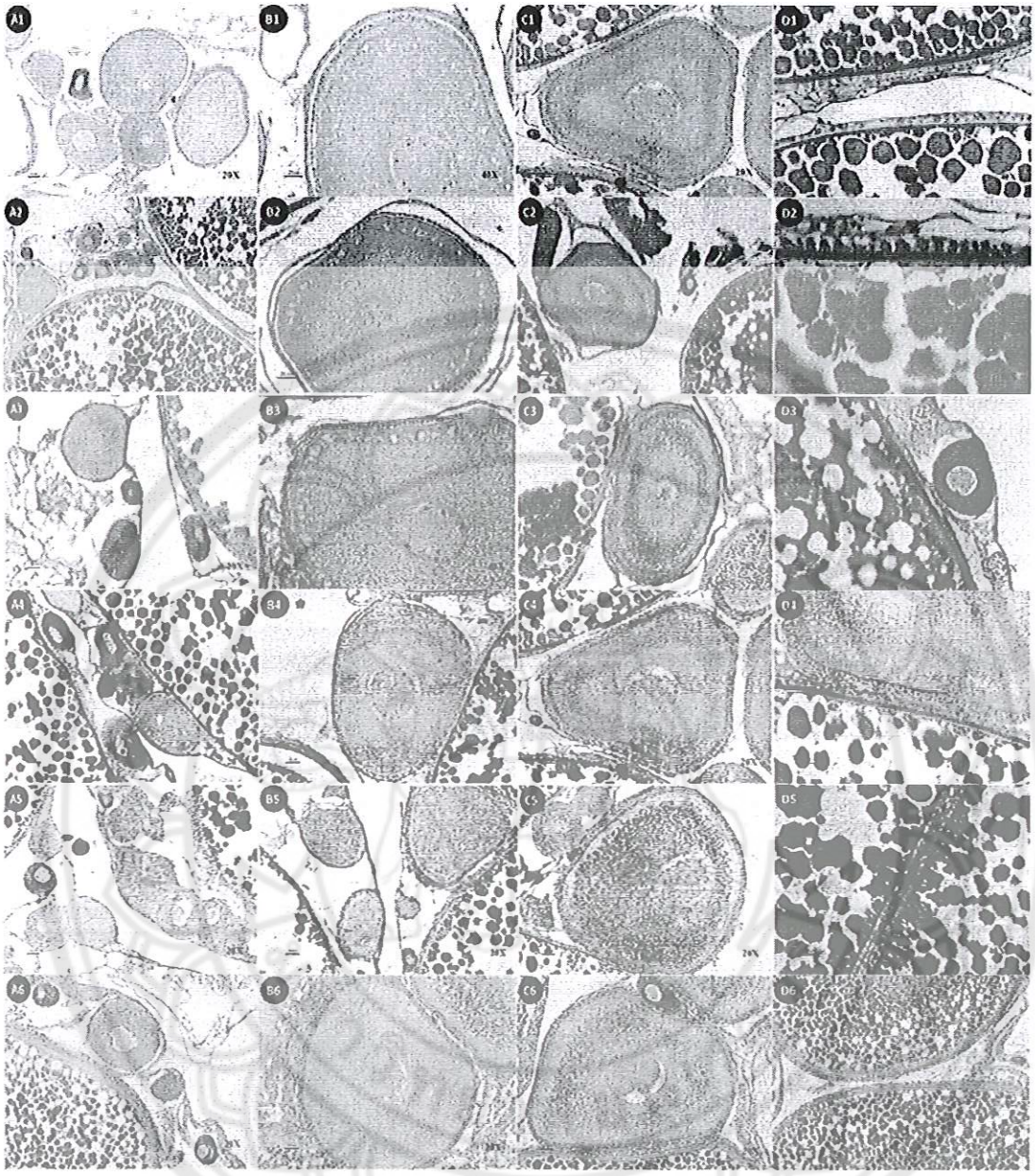


Figure 34 Histology structure of the ovary red Nile tilapia in control and treatment group. A-D shown each oocyte stages (A=Primary oocyte, B=Secondary oocyte, C=Vitellogenin, D=Mature)**; 1-6 shown experimental group** (1=Control, 2=Sesame oil, 3= low-dose 17β-estradiol, 4=high-dose 17β-estradiol, 5=low-dose *C.comosa*, 6=high-dose *C.comosa*)

Overview histology structures of oocytes in comparable experimental groups

1. Comparison in each groups: In each group found equal quantity of oocytes.

2. Comparison in each stages: In each stage mostly found a lot of oocytes, primary oocytes, mature oocytes, vitellogenin and secondary oocytes respectively. The secondary stage was least, only found oocyte about 2-3 oocyte/ovary. Difference size of oocyte, we found oocytes in each stage through the largest size, mature oocyte and the minor size were vitellogenin, secondary oocytes, primary oocytes and oogonia.

3. Comparison in each lot: Oocytes in each lot were not difference in quantity, size, stages and abnormality.

The mature ovary was almost found the mature oocytes and partial follicular atresia. Moreover in follicular space also found yolk materials accumulated and adipose tissue with large fat cells.

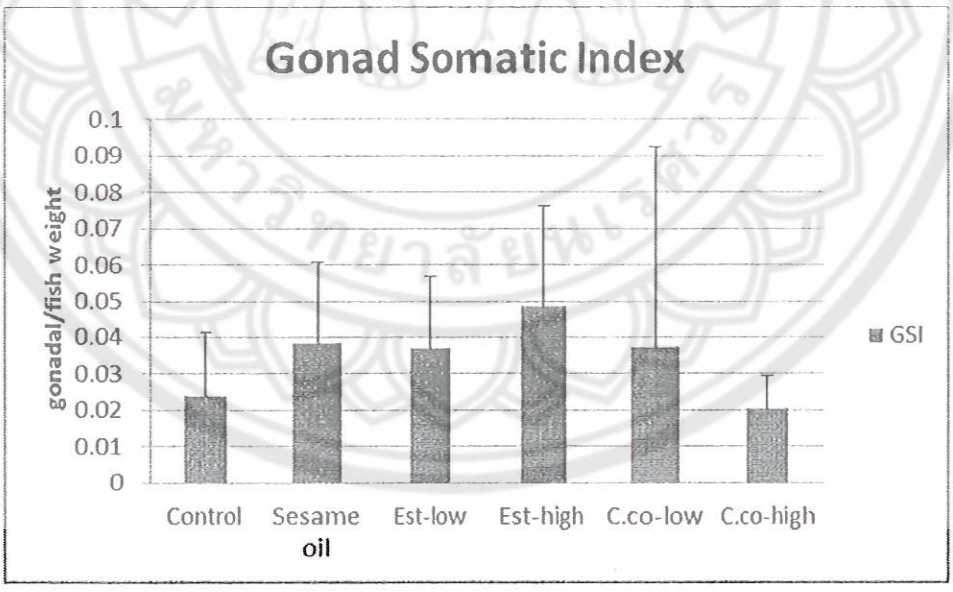


Figure 35 Diagram of gonad somatic index (GSI) in each group (N=6). Values are mean±SD. P-value ≤ 0.05

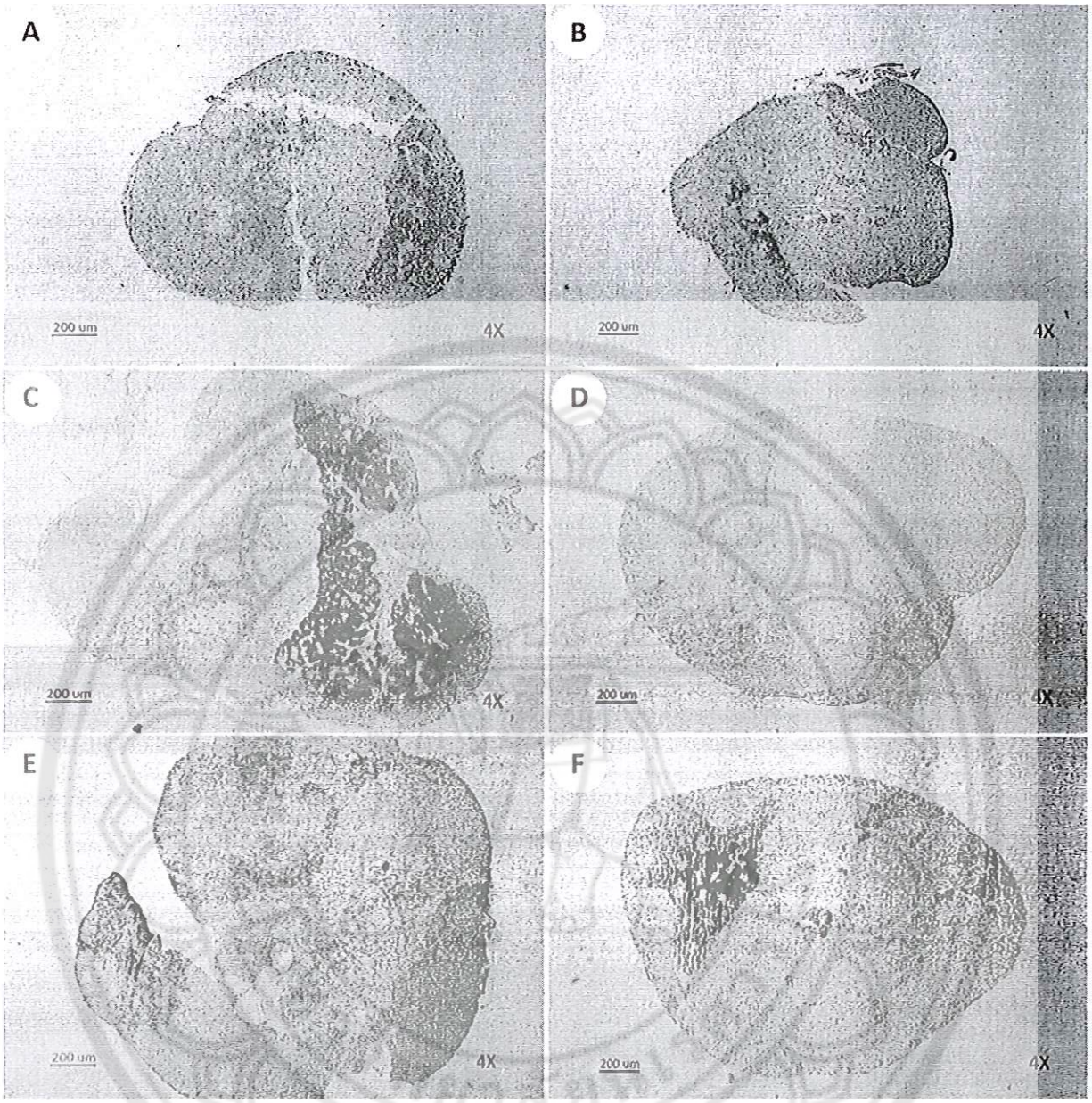


Figure 36 Immunohistochemical reactivity shown distribution of LHβ-like cells the most in Pars intermediate of red Nile tilapia. (A-F) Shown the distribution and accumulation of LHβ-like material of (A) Control group (B) Sesame oil group (C) low-dose 17β-estradiol-treated group (D) high-dose 17β-estradiol-treated group (E) low-dose *C.comosa* extract-treated group (F) high-dose *C.comosa* extract-treated group. Score bar = 200 μm, 4X

The pituitary somatic index (PSI) were examined in each group (n=3). The mean PSI values were (2.66±1.48) $\times 10^{-5}$ in control, (1.46±1.23) $\times 10^{-5}$ in sesame oil, (1.67±0.49) $\times 10^{-5}$ in low-dose 17β-estradiol, (2.20±1.16) $\times 10^{-5}$ in high-dose 17β-estradiol, (1.85±0.52) $\times 10^{-5}$ in low-dose *C.comosa*, (1.08±0.83) $\times 10^{-5}$ in high-dose *C.comosa* treated in pituitaries of female red Nile tilapia. The mean PSI value was not statically significant when compared with both control and between treatment groups.

The pituitaries were examined the relative optical density (ROD) to measure intensity levels in each group (n=3). The mean ROD values were 0.63±0.18 in control, 0.54±0.37 in sesame oil, 0.74±0.29 in low-dose 17β-estradiol, 0.39±0.13 in high-dose 17β-estradiol, 0.48±0.13 in low-dose *C.comosa*, 0.73±0.39 in high-dose *C.comosa* in pituitaries of female red Nile tilapia. The mean ROD value was not statically significant when compared with both control and between treatment groups.

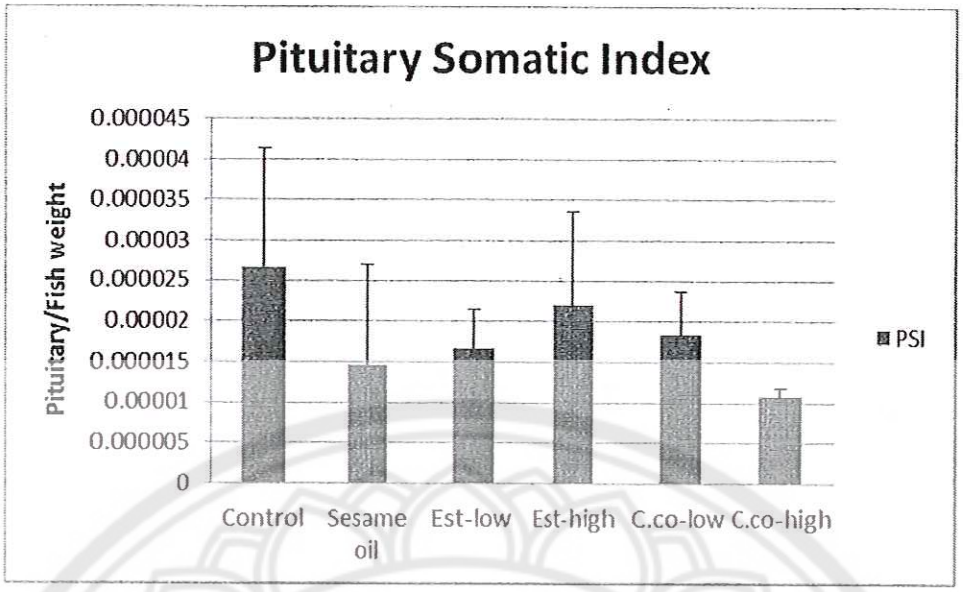


Figure 37 Shown diagram of pituitary somatic index (PSI) in each group (N=3). Values were mean±SD. P-value ≤ 0.05

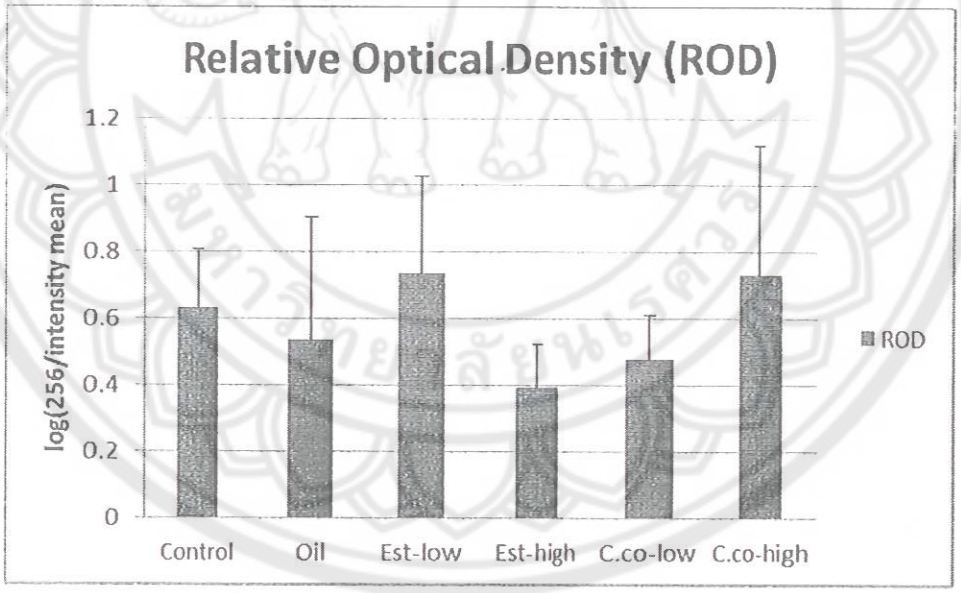


Figure 38 Shown diagram of intensity level of immunohistochemistry staining in each group (n=3).

Discussion

Development stages of the oocytes (Oogenesis) in teleost fish

The oocytes development, reproduction and hormonal relations were investigated in different fish species (Maack, 1964). In the major process of oogenesis of teleost fish can divided to five, six or eight stages (Fishelson et al, 1996; Nagahama, 1983; Unal et al, 1999; West, 1990; Gokce et al, 2003; Isisag, 1996). But the basic stages totally occur as the duplication phase, primary growth phase, follicle development, yolk vesicle formation, vitellogenesis and envelope formation, maturation, ovulation and the spawning-fertilization and egg activation stage. This study, development stages of available oocytes can divided into 5 stages; oogonia, primary oocyte, cortical alveoli, vitellogenin and mature oocyte. The first stage called duplication phase (oogonia), oogonia was the formation of a haploid cell and develop from primodial germ cells (PGCs), that was origin of endodermal, supporting a developing embryo (Foucher and Beamish, 1977). The second stages called primary growth phase (primary oocyte), they were still diploid cells and finish a meiotic division for forming secondary oocyte (oortical alvedi stage). This stage had a polar body that was haploid and vitelline envelop (zona radiate) around the follicle were not thick in this stage. The third stages called cortical alveoli stage (secondary oocyte), the secondary oocyte was a second meiotic division happening a mature an ovum another polar body. Some study report that the first polar body could divided into two polar bodies. The teleost oocytes are surrounded two major cell layers, an outer theca layer and an inner granulosa. Then the oocytes grow, the follicular cells form a follicular layer called the granulosa cell layer (Nagahama et al, 1983; Yueh and Chang, 2000). The vitelline envelop (zona radiate) begin to form thick follicle epithelium. The last this stage, the formation of yolk vesicles contain polysaccharides that were found around the periphery of the oocyte. The forth stages called vitellogenic stage (vitellogenesis), plays an important role in the oocyte development. Vitellogenesis was stimulated by pituitary gonadotropin

follow secretion β-estradiol which stimulates synthesis of vitellogenin in the liver. Vitellogenin (Protein) were carried by blood vessels to follicles in oocyte cytoplasm. Initial vitellogenesis, a germinal vesicle (cortical vesicles), that were located on the periphery of the cytoplasm, migration and breakdown afterward yolk granules forms from inner and push the cortical alveoli to the margin. Then combination of lipid droplets and yolk granules, that found relation, increased both the number and size. They release of the 1st polar body in the process of maturation (Nagahama et al, 1983; Yueh and Chang, 2000). The last stage called mature oocyte, this stage was finally meiotic division and begins taking up ovarian fluid. The lipid droplets and yolk granules were fused and demonstrated a homogenous appearance. An oocyte atresia degenerates before coming to maturity or mature each cycle become atretic. Vitelline envelop breakdown and yolk resorption were an apoptotic process in one of teleost fish (Koc et al., 2008; Ruksana et al., 2011; Neves., 2009; Srijunngam and Wattanasirmkit, 2001; and Fish Reproduction).

LH-β mRNA expression in treatment group of female red Nile tilapia

LH-β mRNA expression was increased in both *C.comosa* extracted group more than high-dose 17β-estradiol group when compared with control group (P-value ≤ 0.01). Previous study, the *Curcuma comosa*, a phytoestrogen as bioactive ingredients, could compete for binding to ERβ than ERα stronger than with 17β-estradiol (George G. J. M. Kuiper et al., 1998). The estrogenic activity of both ER subtypes at high concentrations (1000 nM) of genistein (ERα=198, ERβ= 182) was greater than 17β-estradiol (100 nM) nearly 2 times (George G. J. M. Kuiper et al., 1998). Moreover, teleost fish ERβ forms had higher affinity to bind estradiol than ERα (Kd values: ERα = 0.61, ERβ-I = 0.38, ERβ-II = 0.4 nM). In contrast, human ERα forms had higher affinity to bind estradiol at approximately four times than ERβ (Kd values: ERα = 0.06 and ERβ 0.24 nM) (Hawkins and Thomas, 2004; Menuet et al., 2002; Xia et al., 2000, 1999). Therefore, teleost fish

ERβ forms had high affinity for binding with phytoestrogen more than estradiol. As well as in low-dose 17β-estradiol group was not statically significant when compared with control group that was not probably optimal dose together with phytoestrogen that could bind ERβ greater than estradiol.

LHβ-like distribution in pituitary gland cells

In female red Nile tilapia, Hematoxylin and Eosin stained shown the basophilic cells were found mostly in par intermediate and LH-β immunoreaction was mostly found distributed on basophil cells in the posterior part of female red Nile tilapia pituitary gland (Pars intermedia) and a little distributed in other parts that also had basophilic cells. This result related to basophil cells in human which was a type of cell in the anterior pituitary which manufactures LH hormones. It was called a basophil because it readily takes up bases, and typically stains a relatively deep blue or purple (Vaughan, Deborah, 2002). The mean ROD value was not statically significant compared with both control and between groups.

CHAPTER V

CONCLUSION

Conclusion

In summary, *Curcuma comosa*, the Thai herb was contained an estrogen-like chemical (a phytoestrogen) which related to gonadal maturation and pituitary gland activity. By PCR, LHβ-like gene expression was increased in 17β-estradiol group (only high dose) and *C. comosa* group. The results indicate that a phytoestrogen could induced LH hormone production in red Nile tilapia. The result of pituitary and ovary histology support effect of chemicals non-toxicant on development and reproduction of fish to really use for artificial insemination in other fish with low cost natural hormone.

REFERENCES

Akihiro, T. and Byung, H. Kim. (2001). Effects of estradiol-17β treatment on in vitro and in vivo synthesis of two distinct vitellogenins in tilapia. **Comparative Biochemistry and Physiology Part A**, 129, 641- 651.

Anderson, M.J., Miller, M.R., Hinton, D.E. (1996). In vitro modulation of 17β-estradiol induced vitellogenin synthesis: effects of cytochrome P4501A1 inducing compounds on rainbow trout (*Oncorhynchus mykiss*) liver cells. **Aquatic Toxicology**, 34, 327-350.

Angotzi, AR., Mungpakdee, S., Stefansson, S., Male, R., Chourrout, D. (2011). Involvement of Prop1 homeobox gene in the early development of fish pituitary gland. **General and Comparative Endocrinology**, 171, 332–340.

Ball, J. N. and Olivereau, M. (1966). Experimental identification of ACTH cells in the pituitary of two teleosts, *Poecilia latipinna* and *Anguilla anguilla*: correlated changes in the interrenal and in the pars distalis resulting from administration of metopirone (SU 4885). **General and Comparative Endocrinology**, 6, 5-18.

Ball, J.N. and Baker, B.I. (1969). The pituitary gland: anatomy and histophysiology. **Fish Physiology** (eds. W.S. Hoar and D.J. Randall), 2, 1-110.

Blázquez, M., Zanuy, S., Carillo, M., Piferrer, F. (1998). Effects of rearing temperature on sex differentiation in the European sea bass (*Dicentrarchus labrax* L.). **Journal of Experimental Zoology**, 281, 207–216.

Boonmee, A., Srisomsap, C., Karnchanatat, A., Sangvanich, P. (2011). An antioxidant protein in *Curcuma comosa* Roxb. Rhizomes. **Food Chemistry**, 124, 476–480.

Boyd, E.C. (2004). **Farm-level issues in aquaculture certification: Tilapia.** report commissioned by WWF-US in 2004. Auburn University, Alabama 36831.

Breton, B. and Weil, C. (1973). Effects du LH/FSH-RH synthétiqueetd'extraits hypothalamic quesdecarpesur la secretion d'hormone gonadotrope in vivo chez la carpe *Cyprinus carpio*. C. R. **Academy of Sciences Paris**, 277, 2061-2064.

Breton, B., Govoroun, M., Mikolajczyk, T. (1998). GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. **General and Comparative Endocrinology**, 111, 38-50.

Breton, B., Jalabert, B., Billard, R., Weil, C. (1971). Stimulation in vitro de la libérationd' hormone gonadotrope hypophysaire par unfacteur hypothalamique chez la Carpe *Cyprinus carpio* L. C.R. Hebd. Séances. **Academy of Sciences Paris Part D**, 273, 2591-2594.

Breton, B., Kann, G., Burzawa-Gfrard, E. (1971). Dosage radioimmunology qued'une hormone gonadotrope de carpe (*Cyprinus carpio* L.).C. R. **Academy of Sciences Paris**, 272, 1515-1517.

Choi, C.Y., Habibi, H.R. (2003). Molecular cloning of estrogen receptor alpha and expression pattern of estrogen receptor subtypes in male and female goldfish. **Molecular and Cellular Endocrinology**, 204, 169–177.

Committee on Toxicity Group on Phytoestrogens. **Chemistry and Analysis of Phytoestrogens**. Draft Report. United Kingdom Food Standards Agency. Retrieved November 6, 2012.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. **Nucleic Acids Research**, 36, 465–469.

Dickey, JT., and Swanson, P. (1998). Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). **Journal of Molecular Endocrinology**, 21, 291–306.

Drean, Y.L., Pakdel, F., Valotaire, Y. (1994). Structure and regulation of genes for estrogen receptors. **Fish Physiology**. ed. A.P. Farrell, and D.J. Randall, Volume XII, Chapter 11, 331-366. Academic Press, New York.

Ekici, A. and Timur, M. (2013). An anatomical and histochemical examination of the pituitary gland of carp (*Cyprinus carpio*). **Turkish Journal of Veterinary and Animal Sciences**, 37(4), 399.

El-Sakhawy, M.A., El-Shammaa, M.A., Abd Rabou, M.I., El-Saba, A.A., Hussein, S.H. (2011). Seasonal histology and histochemistry of the adenohypophysis of Nile tilapia (*Oreochromis niloticus*). **Department of Cytology and Histology, Faculty of Veterinary Medicine Cairo University**, 4(2), 39 – 60.

Esther, L., Graham, Y., Julien, B., Joan, C. (2010). Oogenesis in teleosts: How fish eggs are formed. **General and Comparative Endocrinology**, 165, 367–389.

Fan, QIV., Erpool, KM., O'Connor, J., Marsh, JD. (2003). Decoy calcium channel beta subunits modulate contractile function in myocytes. **Molecular and Cellular Biochemistry**, 242, 3–10.

Findlay, J. K., Buckmaster, J. M., Charnley, W. A., Cumming, I. A., Hearnshaw, H., Goding, J. R. (1973). Release of luteinizing hormone by oestradiol-17P and a gonadotrophin-releasing hormone in ewes affected with clover disease. **Neuroendocrinology**, 11, 57-66.

Fish Marketing Organization. (2015). **Aquatic price per day**, Retrieve June 15, 2015, from http://www.fishmarket.co.th/index.php?option=com_jevents&view=month&layout=calendar&Itemid=67.

Fish reproductive. Pdf. 21-22.

Fishelson, L., Goren, M., Van Vuren, J., Manelis, R. (1996). Some aspects of the reproductive biology of Barbus spp., *Capoeta damascina* and their hybrids (Cyprinidae, Teleostei) in Israel. **Hydrobiologia**, 317, 79-88.

Food and Agriculture Organization of the united nations Rome. (2012). **The state of world fisheries and aquaculture**. FAO Department.1-230.

Food and Agriculture Organization. (2006). **FAO yearbook the Fishery and Aquaculture Statistics** [CD-ROM]. Rome, Italy. Food and Agriculture Organization of the United Nations.

George, G. J. M. Kuiper, Josephine, G. Lemmen, Bo Carlsson, J. Christopher Corton, Stephen Safe, Pault, T. Vander Saag, Bart Vander Burg, Jan-Ake Gustafsson. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor b. **Endocrinology**, 139(10), 4252-4263.

Gillesby, B.E., and Zacharewski, T.R. (1998). Exoestrogens: mechanisms of action and strategies for identification and assessment. **Environmental Toxicology and Chemistry**, 17(1), 3-14.

GISD. (2012). **Global Invasive Species Database**. *Oreochromis niloticus*. Available from

http://www.issg.org/database/species/ecology.asp?si=1322&fr=1&sts=sss&lang=EN.

Gokce, M.A., Cengizler, I., Ozak, A.A. (2003). Gonad histology and spawning pattern of the white grouper (*Epinephelus aeneus*) from İskenderun Bay (Turkey). **Turkish Journal of Veterinary and Animal Sciences**, 27, 957–964.

Habibi, HR., De Leeuw, R., Nahorniak, CS., Goos, HJ., Peter, RE. (1989). Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. **Fish Physiology and Biochemistry**, 7, 109–118.

Hawkins, M.B., Thomas, P. (2004). The unusual binding properties of the third distinct teleost estrogen receptor subtype ERbeta-a are accompanied by highly conserved amino acid changes in the ligand binding domain. **Endocrinology**, 145, 2968–2977.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P. (2000). Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. **Proceedings of the National Academy of Sciences**. USA, 97, 10751–10756.

Hoar W.S. and Randall D.J. (1969). **Fish physiology**. London: Academic Press, 325.

Holbech, H., Schröder, KD., Nielsen, ML., Brande-Lavridsen, N., Holbech, BF., Bjerregaard, P. (2013). Estrogenic effect of the phytoestrogen biochaninA in zebrafish, *Danio rerio*, and brown trout, *Salmo trutta*. **Aquatic Toxicology**, 144-145, 19-25.

Inland Fisheries Research and Development Bureau, Phrae. **Nile tilapia**, Retrieved September 22, 2013, from http://www.fisheries.go.th/if-phrae/web2/ index.php? option

=com_content&view=article&id=17:2009-08-19-02-33-00&catid=2:2009-08-19-02-26-29//

Isisag, S. (1996). (Mugilidae, Teleostei) ovaryumlarının gelişimi üzerine histolojik çalışmalar. **Journal of Fisheries and Aquatic Sciences**, 13, 3-4.

Jalabert, B. (2005). Particularities of reproduction and oogenesis in teleost fish compared to mammals. **Reproduction Nutrition Development**, 45, 261-279.

Jarimopas Pansri. (1988). Thai red tilapia species. **Journal of Fisheries Management**, 41-43

Jian, S., Kittisak, S.J., Michael, W., Bungorn, S. (2010). *Curcuma comosa* improves learning and memory function on ovariectomized rats in a long-term Morris water maze test. **Ethnopharmacology**, 130(1), 70–75.

Jillian, E.S. (2009). **Phytoestrogen review**.pdf

Karen, P., Maruska, Russell D., Fernald. (2011). Social Regulation of Gene Expression in the hypothalamic-pituitary-gonadal axis. **Physiology**, 26(6), 412-423.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M. (1989). The duality of teleost gonadotropins. **Fish Physiology and Biochemistry**, 7, 29–38.

Kobayashi, M., and Stacey, NE. (1990). Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. **Zoological Science**, 7, 715–721.

Kobayashi, M., Sohn, YC., Yoshiura, Y., Aida, K. (2000). Effects of sex steroids on the mRNA levels of gonadotropin subunits in juvenile and

ovariectomized goldfish *Carassius auratus*. **Fisheries Science**, 66, 223–231.

Koc, D. N. (Yon), Aytekin, Y., Yucel, R. (2008). Ovary Maturation Stages and Histological Investigation of Ovary of the Zebrafish (*Danio rerio*). **Brazilian Archives of Biology and Technology**, 51(3), 513-522.

Larsen, D., and Swanson, P. (1997). Effects of gonadectomy on plasma gonadotropins I and II in coho salmon, *Oncorhynchus kisutch*. **General and Comparative Endocrinology**, 108(1), 152-160.

Le Tissier, PR., Hodson, DJ., Lafont, C., Fontanaud, P., Schaeffer, M., Mollard, P., (2012). Anterior pituitary cell networks. **Frontiers in Neuroendocrinology**, 33, 252-266.

Linnaeus, C. (1753). **Specius Plantarum**. London

Linnaeus, C. (1758). Tomus I. Systemanaturae per regna trianaturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Editiodecima, reformata. Holmiae. (*Laurentii Salvii*). 1-4, 1-824.

Ma, C.H., Dong, K.W., Yu, K.L. (2000). CDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (*Carassius auratus*). **Biochimica et Biophysica Acta**, 1490, 145–152.

Maack, G. (1964). Estrogen Related Alterations of Gonad Development and of Reproduction in the Zebrafish, *Danio rerio*, **Bilete avion Bucureşti Hamburg**.

MacIntosh, D.J. and Little D.C. (1995). **Nile Tilapia (*Oreochromis niloticus*)**. In: N.R. Bromage and R.J. Roberts (Editors), Broodstock Management and Egg and Larval Quality, Chapter 12. Blackwell Scientific Ltd., Cambridge, Massachusetts, USA, 277-320.

Makoto, M., Toyokazu, S., Katsuhisa, U., Shigeki, H., Masumi, N. (2006). Immunohistochemical detection of gonadotropin-like material in the pituitary of brown hagfish (*Paramyxine atami*) correlated with their gonadal functions and effect of estrogen treatment. **General and Comparative Endocrinology**, 148, 15–21.

Masumi Nozaki. (2013). Hypothalamic-pituitary-gonadal endocrine system in the hagfish. **Frontiers in Endocrinology**, 4, 200.

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F. (2004). Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. **Journal of Molecular Endocrinology**, 32, 975–986.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., et al. (2002). Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. **Biology of Reproduction**, 66, 1881–1892.

Mumford, M.D., Espejo, J., Hunter, S.T., Bedell-Avers, K.E., Eubanks, D.L., Connelly, S. (2007). The sources of leader violence: A comparison of ideological and non-ideological leaders. **The Leadership Quarterly**, 18(3), 217–235.

Nagahama, Y. (1983). The functional morphology of teleost gonads. In: Hoar W.S., Randall D.J., Donaldson E.M. (eds), **Fish Physiology**. Academic Press, New York, 233-275.

Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad, C. (2007). The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERalpha2 and both ERbeta isoforms. **Gene**, 392, 164–173.

Nett, T.M., Turzillo, A.M., Baratta, M., Rispoli, L.A. (2002). Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. **Domestic Animal Endocrinology**, 23, 33–42.

Neves, P.R., Natali, M.R.M., Ribeiro, R.P., Vargas, L., Maehana, K.R., Marengoni, N.G. (2009). Morphological characteristics of ovarian development of two Nile tilapia (*Oreochromis niloticus*) strains in mixed-culture systems. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, 61(5), 1173-1182.

Niumsakul, S., Hirunsaree, A., Wattanapitayakul, S., Junsuwanitch, N., Prapanupun, K. (2007). An antioxidative and cytotoxic substance extracted from *Curcuma comosa* Roxb. **Thai Traditional and Alternative Medicine**, 5(1), 24–29.

Pait, A.S. and Nelson, J.O. (2002). **Endocrine Disruption in Fish**: An Assessment of Recent Research and Results. NOAA Tech. Memo. NOS NCCOS CCMA 149. Silver Spring, MD: NOAA, NOS; Center for Coastal Monitoring and Assessment, 55.

Petit, F., Valotaire, Y., Pakdel, F. (1995). Differential functional activities of rainbow trout and human estrogen receptors expressed in the yeast *Saccharomyces cerevisiae*. **European Journal of Biochemistry**, 233, 584–592.

Piyachaturawat, P., Charoenpiboonsin, J., Toskulkao, C., Suksamrarn, A. (1999). Reduction of plasma cholesterol by *Curcuma comosa* extract in hypercholesterolaemic hamsters. **Ethnopharmacology**, 66(2), 199–204.

Piyachaturawat, P., Ercharuporn, S., Suksamrarn, A. (1995). Estrogenic activity of *Curcuma comosa* in rats. Asia Pacific Journal of Pharmacology, 10, 121–126.

Pongthana Nuanmanee. (2010). ปัจจัยการเพาะเลี้ยงปลานิลและปลานิลแดงให้ประสบผลสำเร็จ. เอกสารเผยแพร่ฉบับที่2/2553. Pathumthani Genetics Research Center. 1-37.

Qu, Y., Xu, F., Nakamura, S., Matsuda, H., Pongpiriyadacha, Y., Wu, L., Yoshikawa, M. et al. (2009). Sesquiterpenes from *Curcuma comosa*. **Natural Medicines**, 63(1), 102-104.

Raul, L.C., Mar del Mar Segura, N., Mardel Pilar Martın del, R., Juan Miguel M. (2003). Ontogeny of adenohypophyseal cells in the pituitary of the American shad (*Alosa sapidissima*). **General and Comparative Endocrinology**, 132, 454–464.

Ruksana, S., Alam, A.M., Kobayashi, Y., Nakamura, M. (2011). Differentiation of steroid-producing cells and folliculogenesis in the developing ovary of the Nile Tilapia *Oreochromis niloticus*. **Zoological Science**, 28(11), 845-852.

Santos, M.C.O., Acuna, L.B., Rosso, S. (2001) Insights on site fidelity and calving intervals of the marine tucuxi dolphin (*Sotalia fluviatilis*) in southeastern Brazil. **Journal of the Marine Biological Association of the United Kingdom,** 81(6), 1049-1052.

Saralump Promjit. (2012). สำนักงานข้อมูลสมุนไพร คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล. Retrieve May 15, 2013, from http://www.medplant.mahidol.ac.th/document/curcuma.asp

Schreibman, M.P., Leatherland, J.F., Mckeown, B.A. (1973). Function morphology of the teleost pituitary gland. **American Zoologist**, 13, 719-742.

Selman, K., R. A. Wallace and QI. X. Sarka. (1993). Stages of oocyte development in the zebrafish, *Brachydanio rerio*. **Journal of Morphology**, 218, 203-224.

Shuhei, N., Kayo, K., Toshiya, N., Minoru, T. (2011). Ovarian germline stem cells in the teleost fish, Medaka (*Oryzias latipes*). **Biological Sciences**, 7(4), 403–409.

Sommer, T.R., Potts, W.T., Morrissy, N.M. (1991). Utilization of microalgal astaxanthin by rainbow trout (*Oncorhynchus mykiss*). **Aquaculture**, 94, 79-88.

Srijunngam, J. and Wattanasirmkit, K. (2001). Histological structures of Nile Tilapia *Oreochromis niloticus* Linn. Ovary. **The Natural History Journal of Chulalongkorn University**, 1(1) ,53-59.

Studies to Biotechnological Applications. **Springer**, Dordrecht, 323–347.

Su, J., Sripanidkulchai, B., Sripanidkulchai, K., Piyachaturawat, P., Wara-Aswapati, N. (2011). Effect of *Curcuma comosa* and estradiol on the spatial memory and hippocampal estrogen receptor in the post-training ovariectomized rats. **Journal of Natural Medicines**, 65, 57–62.

Suwa, K., and Yamashita, M. (2007). Regulatory mechanisms of oocyte maturation and ovulation. **The Fish Oocyte**, 323-347.

Swanson, P. (1991) Salmon gonadotropins: reconciling old and new ideas. In: Scott AP, Sumpter JP, Kime DE, Rolfe MS (eds.), **Reproductive Physiology of Fish**, Sheffield. FishSymp, 2-7.

Swanson, P., Dickey, J.T., Campbell, B. (2003). Biochemistry and physiology of fish gonadotropins. **Fish Physiology and Biochemistry**, 28, 53–59.

Swanson, RA., Sagar, SM., Sharp, FR. (1989). Regional brain glycogen stores and metabolism during complete global ischaemia. **Neurological Research**, 11, 24 –28.

Swanson, WJ., Nielsen, R., Yang, Q. (2003). Pervasive adaptive evolution in mammalian fertilization proteins. **Molecular Biology and Evolution**, 20, 18–20.

Tchoudakova, A., Pathak, S., Callard, G.V. (1999). Molecular cloning of an estrogen receptor beta subtype from the goldfish, *Carassius auratus*. **General and Comparative Endocrinology**, 113, 388–400.

Thomas, P. and Michael M. (1999). **Tilapia life history and biology**. Southern Regional Aquaculture Center, Stone Ville, Ms. Publication No. 283.

Trewavas, E. (1983). **Tilapiine fishes of the genera Sarotherodon, Oreochromis and Danakilia**. Cornell Itahaca, NY: University Press.

Tungtongpiros, M., Uraiwan, S., พรรณศรี เชิดชูพันธ์เสรี. (1984). **Red Nile tilapia**. ฝ่ายวิจัยการเพาะเลี้ยงสัตว์น้ำสถาบันประมงน้ำจืดแห่งชาติกรมประมง,1-20.

Unal, G., Cetinkaya, O., Elp, M. (1999), Histological investigation of gonad development of chalcalburnus tarichi. **Turkish Journal of Zoology**, 23(1), 329–338.

Unprasert Pakorn. (1984). Red Nile tilapia. **Journal of Fisheries Management1**, 37, 229-234.

Vaughan, Deborah (2002). *A Learning System in Histology: CD-ROM and Guide*. **Oxford University Press**. ISBN 978-0195151732

Weerachayaphorn, J., Chuncharunee, A., Jariyawat, S., Lewchalermwong, B., Amonpatumrat, S., Suksamrarn, A., Piyachaturawat, P. (2010). Protection of centrilobular necrosis by *Curcuma comosa* Roxb. in carbon tetrachloride-induced. **Ethnopharmacology**, 129(2), 254-260.

Weerachayaphorna, J., Chuncharuneeb, A., Mahagita, C., Lewchalermwongse, B., Suksamrarn, A., Piyachaturawat, P. (2011). A protective effect of

Curcuma comosa Roxb. on bone loss in estrogen deficient mice. **Ethnopharmacology**, 137, 956– 962.

West, G. (1990). Methods of assessing ovarian development in fishes: a Review. **Australian Journal of Marine and Freshwater Research**, 41, 199-222.

Winuthayanon, W., Suksen, K., Boonchird, C., Chancharunee, A., Ponglikitmongkol, M., Suksamrarn, A., et al. (2009). Estrogenic activity of diarylheptanoids from *Curcuma comosa* Roxb. **Journal of Agricultural and Food Chemistry**, 57, 840–845.

Wong, T.T. and Zohar, Y. (2004). Novel expression of gonadotropin subunit genes in oocytes of the gilthead seabream (*Sparus aurata*). **Endocrinology,** 145, 5210–5220.

Xia, Z., Gale, W.L., Chang, X., Langenau, D., Patino, R., Maule, A.G., et al. (2000). Phylogenetic sequence analysis, recombinant expression, and tissue distribution of a channel catfish estrogen receptor beta. **General and Comparative Endocrinology**, 118, 139–149.

Xia, Z., Patino, R., Gale, W.L., Maule, A.G., Densmore, L.D. (1999). Cloning, in vitro expression, and novel phylogenetic classification of a channel catfish estrogen receptor. **General and Comparative Endocrinology**, 113, 360–368.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Levavi-Sivan, B., Elizur, A. (2001). Regulation of gonadotropin subunit genes in tilapia. **Comparative Biochemistry and Physiology**, 129(B), 489–502.

Yildiz and Fatih (2005). **Phytoestrogens in Functional Foods**. Taylor & Francis Ltd. 3–5, 210–211.

Yoshiura, Y., Suetake, H., Aida, K. (1999). Duality of gonadotropin in a primitive teleost Japanese eel (*Anguilla japonica*). **General and Comparative Endocrinology**, 114, 121–131.

Young, R., Ridding, M., Morrell, T. (2004). Switching skills on by turning off part of the brain. **Neurocase**, 10, 215–222.

Yueh, W. and Chang, C. (2000). Morphological changes and competence of maturing oocytes in the protandrous black porgy, Acanthopagrus schlegeli. **Zoological Studies**, 39(2), 114-122.

Zhao, E. and Qing, MU. (2010). Phytoestrogen Biological Actions on Mammalian Reproductive System and Cancer Growth. **Pharmaceutical Sciences**, 79, 1–20.