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Determination of differential expression of sex-related genes in the brain-pituitary-gonad axis during the ovarian development of Japanese eel (*Anguilla Japonica*)

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DETERMINATION OF DIFFERENTIAL EXPRESSION OF SEX-RELATED GENES IN THE BRAIN-PITUITARY-GONAD AXIS DURING THE OVARIAN DEVELOPMENT OF JAPANESE EEL (*Anguilla Japonica*)

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요약문

극동산 뱀장어(*Aguilla japonica*)는 일본, 대만, 중국, 필리핀, 배트남 그리고 한국을 포함한 동아시아에서 경제적적으로 중요한 부분을 차지하는 뱀장어과중 하나이다. 극동산 뱀장어는 강하회유성 생활사를 갖고 있으며, 대부분은 담수에서 생활하다가 산란을 위하여 하천에서 바다로 내려간다. 뱀장어는 양식분야에서 경제적으로 가치가 있는 주요어종이며, 자연산 실뱀장어를 활용한다. 뱀장어 양식을 위한 종묘는 모두 실뱀장어의 회유성을 활용하여 포획하여 양식한다. 지난 25년간, 자연산 실뱀장어의 남획으로 인하여 아시아와 유럽에서의 포획율이 급감하였다. 그러한 이유로 2014년, 세계자연보전연맹(the International Union for Conservation of Nature, IUCN)에서는 극동산 뱀장어를 멸종될 위기에 이른 종으로 분류를 하였다. 그러므로 자연산 실뱀장어의 증식을 위하여 인공적으로 번식을 유도하는 것이 최근 중요하게 여겨지고 있다.

이러한 이유로, 극동산 뱀장어의 생식과 이에 따른 기초적이고 근본적인 메커니즘을 이해하기 위하여 본 연구에서는 성선자극호르몬 (GnRH1, GnRH2), 성샘자극호르몬 (GTH)과 수용기 (FSHb, LHb, FSHr, LHr), 성호르몬과 수용기 (E2, ERa, ERb, ARa, ARb) 그리고 방향화효소 (CYP19A1, aromatase)를 뇌와 뇌하수체에서 발현을 분석하였다. 그리고 연어 뇌하수체 추출물 (SPE)를 극동산 뱀장어에 주입하여 생식선 조직으로부터 다양한 생식조직의 발단단계를 관찰하였다. 앞선 인자들을 분석하기 위하여 본 연구에서는 해부학적 관찰을 통해 perinucleolar stage (PN), nucleolar stage (N), early-vitellogenic stage (EV), mid-vitellogenic stage (MV), late-vitellogenic stage (LV), migratory nucleus stage (MN) 그리고 after ovulation (AO) stage로 총 7 단계로 성주기를 구별하였다. 뇌, 뇌하수체, 생식선 그리고 간 조직에서 RNA를 분리하여 대상 유전자의 발현을 측정하였다. 또한, 추가적으로 비텔로제닌 전사조절인자

와 ERs를 간 조직에서 정량 PCR(qPCR)방법으로 측정하였다. 뇌조직으로부터 cDNA를 합성하였고, Kiss1 유전자의 부분 서열을 확보한 뒤에 Kiss1 과 Kiss1r mRNA 발현을 뇌와 생식선에서 qPCR 방법을 통하여 측정하였다.

본 연구에서 우리는 뱀장어과에서 최초로 Kiss1 유전자의 부분서열을 확인하였다. Kiss1 mRNA 발현량은 MN 단계에 유의적인 변화가 관찰되었으며, 뇌와 생식선에서는 Kiss1r의 발현이 두드러지게 관찰되었다.

뇌에서의 GnRH1과 GnRH2 mRNA는 다른 성주기에 비하여 MN 단계에서 높게 발현이 되었다. GnRH1의 발현과 유사하게 CYP19A1과 ERa mRNA 발현 또한 최종 성숙단계에서 유의적인 변화가 관찰되었다.

뇌하수체에서 FSHb 조절인자는 난황형성 시기와 MV 단계에 유의적으로 높게 나타났고 그 후 LV단계에서 AO단계까지 유의적으로 서서히 감소하는 것을 관찰되었다. 이와 대조적으로 LHb mRNA 발현은 휴지기에 비하여 LV, MN 그리고 AO 단계에서 유의적으로 상승되었고 MN 단계에서 가장 높게 관찰되었다.

생식선의 FSHr와 LHr 전사인자 수치는 난모세포가 발달됨에 따라 유사하게 나타나며 난모세포 성숙시기에는 두 전사인자 모두 유의적으로 높게 나타났다. 또한 CYP19A1 전사인자 수치는 다른 성숙 단계에 비하여 MN 단계에 급격히 유의적으로 상승하였다.

간에서는 vitellogenic을 주입하지 않은 암컷 뱀장어에서 상대적으로 매우 적은 수치의 Vtg 발현량을 확인하였다. 그러나 SPE를 주입 후 Vtg mRNA 발현은 상승하기 시작하여 LV 단계에서 가장 높게 나타났고 그 후 MN 단계에서 유의적으로 낮게 발현되었다. 이와 유사하게, ERs 전사인자 수치 또한 LV 단계에서 유의적으로 상승하였으나 MN 단계에서는 유의적인 감소가 관찰되지 않았다.

본 논문에서는 극동산 뱀장어에서 처음으로 생식선의 발달과 BPG 축과 관련하여 Kisspeptin (Kiss1) 체계에 대해서 관찰하였다. 추가적으로 우리는 극동산 뱀장어의 LH 주기를 결과적으로 완전하게 확인하였다. 또한, 다른 문헌에 근거하여 LV단계 이후에 aromatase가 감소할 것이라고 예상했지만 유의적인 변화를 관찰하지 못하였다.

Summary

Japanese eels (*Anguilla Japonica*) are one of the most commercially important eel groups, distributed in East Asian countries including Japan, Taiwan, China, Korea, Vietnam and Northern Philippines. This fish has a catadromous life cycle; which lives a part of the life cycle in the fresh water and migrates to sea for spawn. Eels are commercially produced by aquaculture of which majority is represented by of natural glass eels (Tsukamoto et al. 2009). Seedlings for eel aquaculture are totally dependent on capturing of glass eels during their upstream migration. During last 25 years, the rate of glass eel capturing has been reduced in Asia and Europe, because of the sharp decreasement of glass eel in natural habitats. Due to those reasons in 2014, Japanese eels were included in to the IUCN rest list as an endangered species. Therefore, the artificially induced breeding is essential to full fill the great demand of glass eel, and thereby develop the better method for eel production in order to protect the natural glass eels.

Hence, to understand the fundamental mechanisms involved in Japanese eel reproduction and provide a broad basis for comparative study of eel, we have analyzed the expression levels of gonadotropins (*GnRH1, GnRH2*), GTHs & their receptor (*FSHb, LHb, FSHr, LHr*), sex steroids & their receptor (E2, *ERa, ERb, ARa, ARb*) and aromatase (*CYP19A1*) enzyme in brain, pituitary and gonadal tissues during different gonadal development stages of immature and Salmon pituitary extract (SPE) injected Japanese eels. To analyze the transcriptional levels of aforementioned molecules, we have divided the complete estrus cycle into seven stages, including perinucleolar stage (PN), nucleolar stage (N), early-vitellogenic stage (EV), mid-vitellogenic stage (MV), late-vitellogenic stage (LV), migratory nucleus stage (MN) and after ovulation (AO) stage according to the histological observations. Brain, pituitary, gonad and liver tissues were isolated and RNA was extracted to investigate the above target gene expression levels. Furthermore, extra qPCRs were performed to examine the transcriptional

modulations of vitellogenin (Vtg) and ERs in the liver tissue of above eels. Thereafter, cDNA was synthesised from brain tissues and amplified segment of Kiss1 gene partial sequence with degenerate primers using PCR, and examined the mRNA expression of *Kiss1* in the brain and *Kiss1r* in brain & gonad during the oocyte development in Japanese eel by qPCR assays using gene specific primers.

To the best of our knowledge, we are the first group that cloned the partial sequence (120bp) of *Kiss1* from the brain of anguillids. The *Kiss1* mRNA expression was significantly elevated in the MN stage of the Japanese eel, where brain and gonad showed prominent *Kiss1r* expression.

The brain *GnRH1* and *GnRH2* mRNA were highly expressed during the MN stage compared to the other stages. Similar to the GnRH1 expression, *CYP19A1* and *ERa* mRNA expression was also significantly pronounced in the brain during final oocyte maturation in Japanese eels.

In the pituitary, *FSHb* transcripts were abundant in vitellogenesis and significantly elevated in MV stage. After that, the expression was significantly and gradually decreased from LV to AO stages. In contrast to the FSH, pituitary *LHb* mRNA expression was significantly increased during LV, MN and AO stages than rest of the stages and reached the peak in MN stage. Pituitary *CYP19A1* mRNA expression was also significantly elevated in LV and MN stages during gonadal development of Japanese eel.

The gonadal *FSHr* and *LHr* transcript level showed a synchronized fluctuation along the oocyte development in Japanese eel. Transcript levels of both genes were significantly increased during final oocyte maturation. Furthermore, *CYP19A1* transcript levels were robustly and significantly increased in MN, compared to the other development stages in ovary of Japanese eels.

In the liver, comparatively very low *Vtg* expression was observed in un-injected (pre-vitellogenic) females. But after the SPE injection *Vtg* mRNA expression was started to increase and it was peaked in LV stage then significantly decreased in MN stage. As similar to the *Vtg*, ERs transcript levels also significantly increased in LV stage but not significantly decreased in MN stage.

Our present study provides the first insights into the kisspeptin (Kiss1) system of Japanese eel and its involvement in the BPG axis during gonadal development. In addition to that, our findings collectively complete the LH cycle of the Japanese eel. Furthermore, we speculate that failure to decrease the aromatase activity after the LV stage may be attributed to the suspension of the ovulation of Japanese eels.

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ABBREVIATIONS

°C	Degrees of calculus
µL	Microliter
µmol	Micromolar
AjEF1a	*Anguilla japonica* Elongation factor 1 alpha
Kiss1	Kisspeptine
Kiss1r	Kisspeptine1 receptor
GnRH1	Gonadotropin releasing hormone 1
GnRH2	Gonadotropin releasing hormone2
CYP19A1	Cytochrome P450 aromatase
ERa	Estrogen receptor alpha subunit
ERb	Estrogen receptor beta subunit
ARa	Androgen receptor alpha subunit
ARb	Androgen receptor beta subunit
FSH	Follicle stimulating hormone
FSHr	Follicle stimulating hormone receptor
LH	Luteinising hormone
LHr	Luteinising hormone
Vtg	Vitellogenin
T	Testosteron
E2	Estrodol-17b
11KT	11- keto testosterone
GTH	Gonadotrophins
ANOVA	Analysis of variance
cDNA	complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-triphosphate
FAO	Food and Agriculture Organization of the United Nations
g	Gram
hrs	Hours
mg	Milligram
mM	Milimolor
mRNA	Messenger ribonucleic acid
pmol	picomol
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minutes
SE	Standard error
SPE	Salmon pituitary extract
PN	Perinucleolar

N	Nucleolar
EV	Early vittelogenic
MV	Mid vitellogenic
LV	Late vitellogenic
MN	Migratory nucleos
AO	After ovulation
BPG axis	Brain pituitary gonad axis
GV	Germinal vesicle
IUCN	International Union for Conservation of Nature
ER	Estrogen receptor
AR	Androgen receptor
GPR54	G-protein coupled receptor
ERI	Environmental Research Institute

1. Introduction

The Japanese eel

There are nineteen species and six sub species of fresh water eels, distributed in all over the world. They are primitive group of fish belong to the genus *Anguilla* and family Anguillidae. Freshwater eels are elongated, cylindrical, snake-shaped animals with large, pointed head. Moreover, their dorsal fins are usually continuous with their caudal and anal fins to form a fringe lining the posterior end of their bodies.

Japanese eels (*Anguilla Japonica*) are one of the most commercially important eel group, distributed in East Asian countries including Japan, Taiwan, China, Korea,Vietnam (Sang et al., 1994; Beentjes, 1999) and Northern Philippines.

Fig 1: The Japanese eel (*Anguilla Japonica*)

Kingdom:	**Animalia**
Phylum:	Chordata
Class:	Actinopterygii
Order:	Anguilliformes
Family:	Anguillidae
Genus:	*Anguilla*
Species:	***A. japonica***

Life cycle

The life cycle of the eels is catadromous, which means that they live a part of the life cycle in the fresh water and, spawn in the sea. According to the Beentjes (1999) and (2009), catadromous Japanese eels spawn at the offshore in the west of the Mariana Islands of the Pacific Ocean (14–16 ºN; 134–143ºE). After hatching eggs, the larvae called leptocephali

drift to the growing grounds at East Asia using the North Equatorial current and the Kuroshio Current. In the Kuroshio Current, leptocephali metamorphose into glass eels on the continental shelf (Beentjes, 1999). Afterward they migrate to the brackish and fresh waters for their settlement, and become pigmented elvers (Fukuda et al. 2013). The migration period from spawning ground to the estuaries is around 5-6 months (Beentjes, 1999). These pigmented elvers are turned into yellow eels by changing the pigment color into yellow, and they migrate to the fresh water up streams and some of them stay at the brackish water. After 5-12 years, the yellow eels grow up to the breeding size and migrate to downstream in autumn for start to the journey of spawning (Tsukamoto, 2009). During the yellow eel stage, sexual maturation can be observed, and after the second metamorphosis they become as silver eels who migrate back to the ocean to spawn in offshore (Beentjes, 1999; Tsukamoto, 2009). Silver eels have to migrate across the strong Kuroshio Current, and it takes them nearly 9 month to reach their spawning grounds at a distance of 2000-3000 km (Beentjes, 1999).

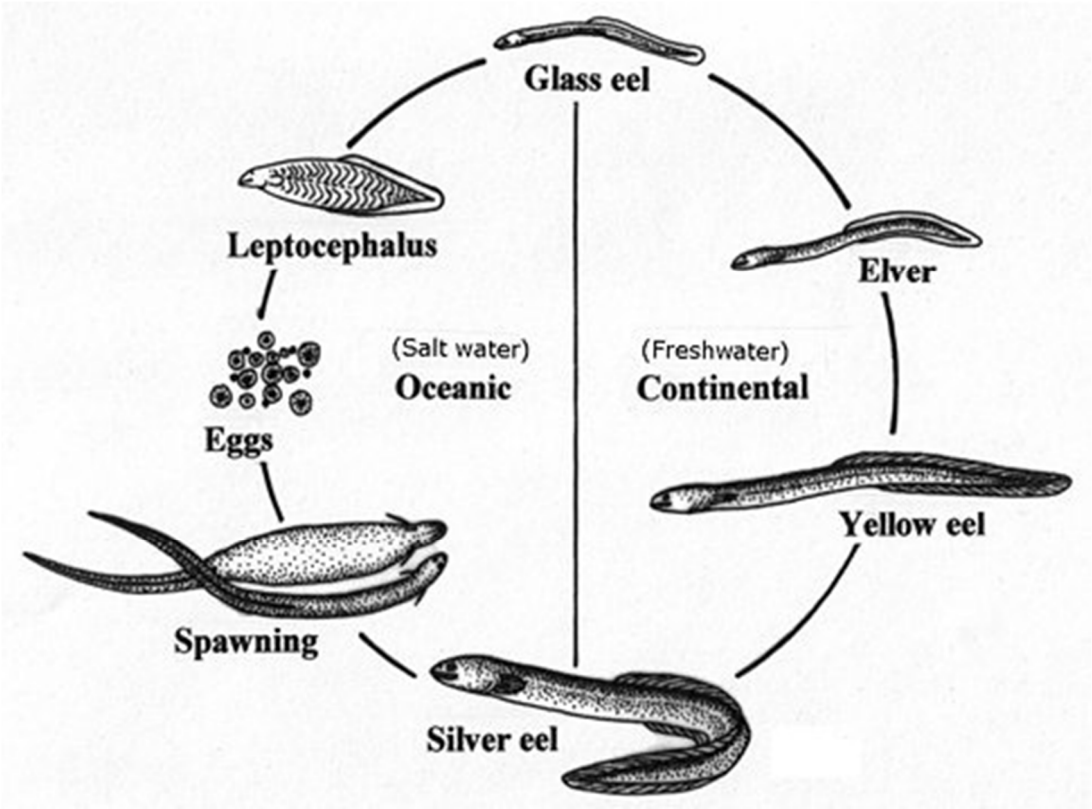


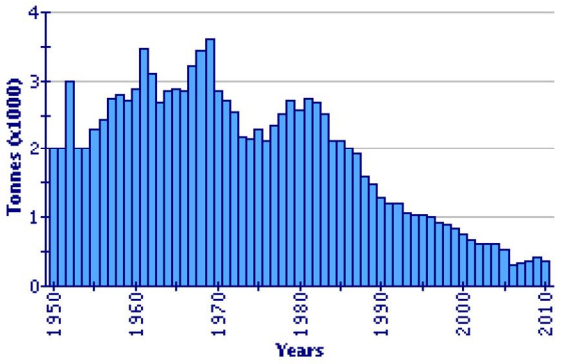
Fig 2: Life cycle of the Japanese eel
Source: (http://bioweb.uwlax.edu/bio203/s2009/peaslee_alex/Anguilla_Japonica/Life_Cycle.html)

Demand on Eels

The Japanese eel (*Anguilla Japonica*) is a commercially important culturing species in Asia and Europe. However, the most of the eel productions supplied as the product of aquaculture are generally depend on the natural glass eels (Tsukamoto, 2009). Seedlings for eel aquaculture are totally dependent on glass eels which are captured during their upstream migration between November to March (Beentjes, 1999; Tsukamoto, 2009). During last 25 years, the rate of glass eel capture has been reduced in Asia and Europe, because of the sharp decrement of glass eel in natural habitats (Kagawa et al., 2005). In addition, the overfishing, loss of habitat, and degradation of the growing habitats by human activities also caused to decrease the natural yellow eels (Tatsukawa., 2003). In the beginning, wild eels harvested to accomplish the growing demand were mostly females due to their greater size and long fresh water life (Beentjes, 1999). These reasons are also collectively contribute to the decrement of natural eels. Due to those reasons, Japanese eels were included in to the IUCN Red list as an endangered species in 2014.

It has been reported that the total production of Japanese eel in Japan was 30,000 tons per year, and additionally they import 80,000 tons from China, Taiwan and other countries annually (Kagawa et al., 2005). According to the data of Food and Agriculture Organization (FAO) in 2014, during the last 60 years the global capture production (Fig. 3a) of Japanese eel has been decreased annually, and the global aquaculture production (Fig. 3b) was annually increased. It indicates that total larvae capture was increased and yellow eel & silver eel capture were decreased during that period of time.

(a) Global Capture production for *Anguilla japonica*



(b) Global Aquaculture production for *Anguilla japonica*

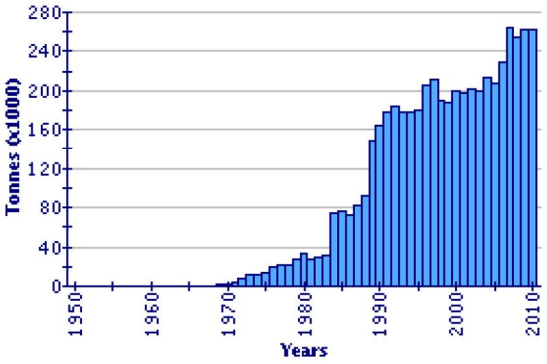


Figure 3: - Global capture and culture production of Japanese eel during past 6o years (Food and Agriculture Organization (FAO) 2014).

In recent years, the population of Japanese eel has dramatically dropped due to the lack of sustainably developed eel fishing practices. As a result of that numbers of fish those can reproduce successfully have been reduced. The main issue of the eel culture is the availability of fry. Therefore, breeding of eels in the captivity is essential for overcome this problem. However, the successful results have not been achieved so far since eels cannot get matured under normal culturing condition (Okuzawa, 2002). On the other hand, captured female eels were in early development stage of oocytes, while males were in sexually immature stage (Yamamoto et al., 1992, 1994a). Consequently, the artificially induced breeding is essential to full fill the great demand of glass eel, and thereby develop the better method for eel production in order to protect the natural glass eels.

Hence, investigation of the importance of the bottlenecks in puberty, and sex related gene expression during oocyte maturation in female eels were highly essential. Therefore, the techniques for artificial breeding of the Japanese eel have been intensively studied since 1960s. Yamamoto and Yamauchi (1974) have been succeeded by obtaining fertilized eggs and larvae through repeated injection of salmon pituitary extraction (SPE). However, during past 41years scientists have failed to obtain mass production of glass eels. In this study our

main objective was to get the clear understand about the correlation between Brain-Pituitary-Gonad (BPG) axis and sex related genes during gonadal maturation

General sex related hormones and their function during oocyte maturation and ovulation

During natural condition the brain secrets the kisspeptin to release the gonadotropin releasing hormone (GnRH), and it stimulates the pituitary to secrete follicle stimulating hormone (FSH) and Luteinizing Hormone (LH). FSH is the main controller of puberty as well as gametogenesis while LH controls the gonadal maturation and spawning (Schulz and Miura, 2002). Above 2 hormones (FSH & LH) stimulate the ovaries to secrete estrogen and testosterone; however, estrogen is the key player of synthesis of the Vitellogenin (Vtg) from the liver. Then the Vtg produce the oil droplet and are deposited in the maturing eggs as yolk. After the egg was released, the ovary begins to produce progesterone and inhibits the production of hormones of hypothalamus and pituitary to complete the ovarian cycle. At the stage of ovulation, progesterone becomes decrease and hypothalamus re-start to produce GnRH. This hormone level controls the uterine (menstrual) cycle causing the proliferation phase after ovulation, the secretary phase after ovulation and menstruation when conception does not occur. However, The triggers and mechanisms involved in its activation at the start of puberty are not well known for any fish species (Weltzien et al., 2004).

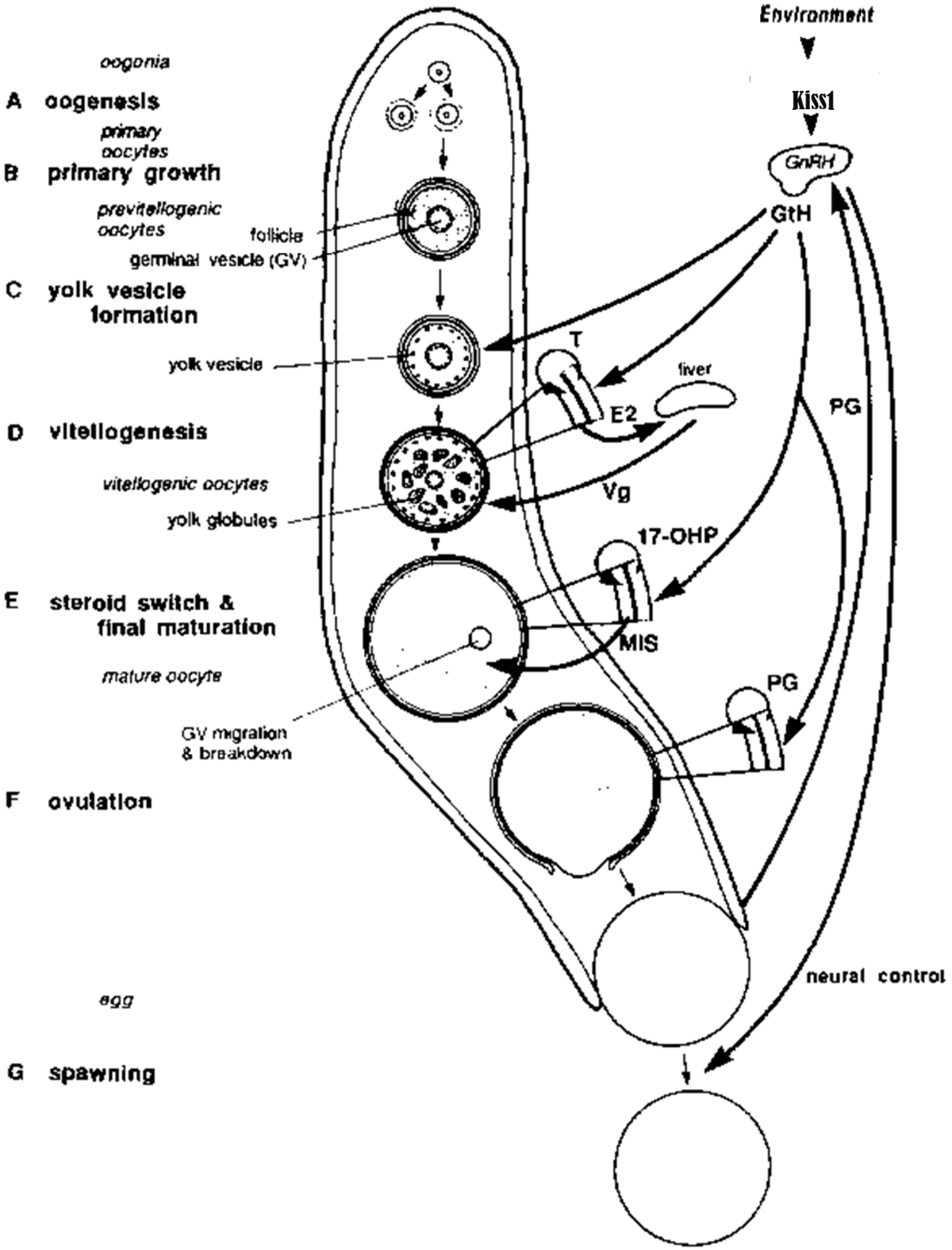


Fig4: BPG axis involvement in gonadal development and maturation of teleost- gonadotropins (GtH), testosterone (T), estradol (E2), 17 hydroxy- progesterone (17OHP), maturation-inducing steroids (MIS), vitellogenin (Vg)

(Source: http://www.nzdl.org/gsdlmod?e=d-00000-00---off-0hdl--00-0----0-10-0---0---0direct-10---4-------0-1l--11-en-50---20-home---00-0-1-00-0-0-11-1-0utfZz-8-00&a=d&cl=CL1.9&d=HASH016eeb3fd93e4e349784808a.4.7)

However, cultivated and captured silver eels do not mature and ovulate under normal culture conditions. Therefore, until now some aspects of the life cycle, such as sexual maturation and spawning are still unknown under natural conditions. Therefore indeed understanding in this process and BPG axis hormone fluctuation during oocyte maturation in eel is very important for the aquaculture industry for modify the timing of puberty (Okuzawa, 2002) protect the natural eel population to obtain reliable supplies of glass eels for aquaculture and development of artificial breeding procedure.

Eel maturation

Puberty

Puberty is the embryonic development process in the achievement of full reproductive capacity of sexually immature individuals, and encompasses various morphological, physiological and behavioral changes. Herein different metamorphic changes are induced by the sexual steroids. (Weltzien et al., 2004). Also whole process is induced by the BPG axis (reviewed by Josephine et al, 2008). This system involves complex regulation of neuroendocrine, endocrine, paracrine and autocrine signals and also this is the common processes which can be seen within the all vertebrates in the planet.

Commencement of spermatocytes in males and vetellogenesis of females initiate the puberty and it is ended by the ovulation of females and sparmiation of males among the teleosts (revived by Dufour and Rousseau 2007). Puberty is not fully described yet in any invertebrates. Therefore, currently many scientists focused to understand the puberty and reproduction of vertebrates. In the natural conditions the yellow stage eels do not become mature and when they only reach the silvering stages they becmaome mature and ready to

migrate for spawning. According to the definition, puberty occurs only once in the life cycle after a certain period of juvenile growth (Okumura et al., 2001).

Role of the brain during oocyte maturation

Similar to that of other vertebrates, the brain of teleost is a main center of regulation of sex and adrenal steroids effects of which have been examined mainly in the context of the neuroendocrine control of reproduction (Zohar et al., 2010).

In the beginning Kiss1 have been investigated as a human malignant melanoma metastasis-suppressor gene by Lee et al. (1996) from the tumour cells. After that, in 2003, de Roux et al. (2003) investigated the regulation of reproduction and puberty by the Kiss1/ GPR54 system. They investigated on inactivating mutations in the G-coupled protein receptor 54 (GPR54 or Kiss1r) which results in idiopathic hypogonadotrophic hypogonadism, by which the affected individuals did not undergo puberty. The kisspeptins are the products of the Kiss1 gene, which encodes for a 145 amino acid precursor protein and were later found to be the ligand for the Kisspeptin, which is called G- protein coupled receptor 54; now named as Kiss1r. Kiss1 involves in control of LH and FSH secretion, acting via the GnRH system. The differential distribution of estrogen receptor (ER) a and b is associated with the specific modulations of LH response to kisspeptins and the generation of preovulatory LH surge (Roa et al., 2008)

However, whether those effects were caused by direct activation of gonadotropin release or indirectly via the GnRH system is unknown. It is however worth to mention that a potential anatomical association of Kiss1r with the GnRH system was identified through the work of (Parhar et al., 2004). Using laser capture technology and RT-PCR in single cells, the co-expression of GnRH and Kiss1r was suggested in tilapia (Parhar et al., 2004). And also the majority of expression was found to be in the telencephalonand the olfactory bulbs, where

GnRH expression was also detected, consistent with their linked functionality and further supporting the conserved role of the Kiss system in fish.

There are 3 types of GnRH released by the brain; GnRH1 (mammal type GnRH) released by Hypothalamus, GnRH2 (chicken type GnRH) released by the mid brain and GnRH3 (salmn type GnRH) released by the caudal olfactory bulb (Okumura et al., 2001; Filby et al., 2008). Among these,GnRH1 maintain the gonadotrophic role (Sherwood and Wu, 2005). On the other hand,GnRH2 and GnRH3 promote the reproductive (Uchida et al., 2005). GnRH1 is synthesized and released from GnRH neurons within the hypothalamus. This peptide belongs to gonadotropin-releasing hormone family. Indeed, the actions of GnRH are mediated through binding to membrane receptors (GnRHr) belonging to the rhodopsin family of G-protein coupled receptors. It constitutes the initial step in the BPG axis. GnRH plays a central role in the neuroendocrine control of the reproductive process in vertebrates, notably by stimulating synthesis and release of pituitary gonadotropins (Weltzien et al., 2004).

Aromatase (CYP19A1 / Cytochrome P450 aromatase) is the key enzyme for oestrogen biosynthesis and is mainly distributed in the brain, pituitary, and gonad in vertebrates (Simpson et al., 1994). In tetrapod, neural aromatase may be involved in the production of neurosteroids and in neural development, brain sex differentiation, sexual behaviour, the initiation of puberty, and the regulation of gonadotropin secretion (Choate and Resko, 1994; Lephart, 1997). In mammals, only one copy of aromatase can be found (except in pig) (Graddy et al., 2000). But in teleost there are two types of aromatases can be found due to the duplication of aromatase gene named as CYP19A1a in brain and CYP19A1b in gonads. Interestingly, only one type of CYP19A1 is conserved in eels throughout the evolution and it is more related to the gonad type (Jeng et al., 2012). Therefore, brain aromatase expression is comparatively lower than that in the gonads.

In the brain, E2 is best known for its organizing and regulatory effects on the neuroendocrine circuits which controls the reproductive functions (Beyer., 1999). There are three types of ER possessed in teleost called as ERγ, ERα and ERβ (Hawkins et al., 2000) which belong to the superfamily of nuclear receptors these ERs can act as ligand inducible transcription factors. In the brain of vertebrates, many effects of oestrogens are prominent due to the local conversion of C19 androgens into estrogens (Diotel et al., 2010). Aromatization ultimately lead to form estrogens and most of estrogen effects are mediated through specific nuclear estrogen receptors (ER). In rainbow trout, brain ERa are responsible for the inhibition of LH release during vitellogenesis (Linard et al., 1996). Moreover, zebrafish and European sea bass have demonstrated that Kiss neurons in the mediobasal hypothalamus are targets for estrogens (Servili et al., 2011). According to Diaten et al., (2010) estrogens in neurogenesis is the unique feature of the adult fish brain suggests that, regulating the classical functions on brain sexual differentiation and sexual behavior.

There is only limited data on the distribution of androgen receptor mRNA or protein in the brain of fish. However, at least two androgen subtypes (ARa andARb) in teleosts, likely resulting from the whole genome duplication in the teleost lineage. However, the central or peripheral origins of aromatizable androgens are used as substrates for brain aromatization (Diotel et al., 2011a).

Role of the pituitary during oocyte maturation

Gonadotropins, FSH and LH are secreted by pituitary and act through binding to their specific receptors (FSHr and LHr) in the gonads of vertebrates, to induce steroidogenesis and gametogenesis (Nagahama et al., 1995; Dufau, 1998).In teleosts, the presence of two gonadotropins were first identified from salmonids (Yan et al., 1992). These gonadotropins are categorized under the pituitary glycoprotein hormone family and they are heterodimers

with two subunits, a common a-subunit and a hormone specific b-subunit. These subunits are synthesized as separate glycoproteins from different genes but in the cytoplasm they are bound with non-covalent bonds and form a biologically active dimer molecules (Gharib et al., 1990). These glycosylation is essential for their specific biological activity (Combarnous, 1992).

Gonadotropin receptors (FSHr and LHr) belong to the superfamily of G-protein couple receptors and consist with seven transmembrane domains. Depending on the gonadal development stage, positive and negative feedback effects of sex steroids on FSHr and LHr have been reported in teleost by Zohar et al. (2010) using *invivo* conditions. In generally, duty of the FSH is to control the puberty and gametogenesis whereas LH controls the final gonadal maturation and ovulation in vertebrates (Prat et al., 1996) Schulz and Miura, 2002). Consequently, FSHβ highly expressed in the pre-gametogenesis and early gametogenesis, compare to the LHβ. Whereas LHβ mRNA was significantly increased at the end of the reproductive cycle in rainbow trout (Gomez et al., 1999). Miwa et al. (1994) has been indicated that FSHr transcript levels has been higher than LHr in immature ovary of salmons. However in zebra fish highest LHr expression could be observed at the end of the full grown oocyte stage (Kwok et al., 2005). In addition, androgens (ARα & ARβ), oestrogens (ERα & ERβ) and aromatase (CYP19A1) also can be found in the pituitary of vertebrates. In fish the sex steroid feedback is further increased by the high capacity of the brain and the pituitary in fish to convert aromatizable androgens into oestrogens.

Role of the gonads during oocyte maturation

Fish gonadotropins (GTHs) act via binding to two gonadal gonadotropin receptors called as LHr and FSHr. In contrast to the situation in mammals, the interactions of gonadotropins and

their receptors are highly specific (Okumura et al., 2001). But in teleosts FSHr and LHr can be cross-activated by their ligands. Hence, the receptor specificity is yet unclear (Levavi-Sivan et al., 2010). Indeed, the catfish FSHr is highly responsive to both catfish LH and FSH (Bogerd et al., 2001; Vischer et al., 2003). Whereas, the LHr is rather specific to LH (Vischer et al., 2003). However, another studies in Japanese eel (Kazeto et al., 2008), amago salmon (Oba et al., 1999) and also zebrafish (Kwok et al., 2005) showed that FSHr is specific to FSH while LHr is activated by both LH and FSH.

According to the Francois et al., (2010), Senegalese sole's FSHr expression was closely associated with vitellogenesis, because its expression level increased significantly when the follicles were recruited from the primary growth stage continuing to rise throughout vitellogenesis. In contrast, LHr expression becomes prominent during late vitellogenic stage in Senagalese sole. Same observations have been reported in several other teleosts such as zebrafish (Kwok et al., 2005), Atlantic cod (Mittelholzer et al., 2009), Sea bass (Rocha et al., 2007), Atlantic halibut (Kobayashi et al., 2008). Nevertheless, in Japanese eel FSHr transcripts were strongly elevated during the immature eels with compare to the LHr (Jeng et al., 2007). In contrast, after the SPE treatment, both FSHr and LHr levels were significantly increased in Japanese eels (Jeng et al., 2007).

Sex steroid hormones are also play an important role in the reproduction of vertebrates under the control of pituitary gonadotropins. There are three different sex steroid hormones [estradol -17β (E2), 11-ketotestosteron (11-KT) and 17α20β-dihydroxy-4-pregnen-3one (DHP)] can be identified in teleost gonadal tissues are essential for critical steps of gametogenesis (Nagahama and Yamashita, 2008).

E2 stimulates the liver vitellogen expression to promote the oocyte growth in teleost as same in higher vertebrates (Wallace et al., 1985). Furthermore, the artificial maturation (SPE injection) increased the liver Vtg transcriptional level correlated with the serum levels of E2 and Vtg during oogenesis in Japanese eel (Okumura et al., 2002). *In* (Wang and Lou, 2006) and in vitro (Kwon and Mugiya, 1994) experiments also have been demonstrated the E2 induced vitellogenesis during oocyte maturation in Japanese eel. In addition, early stage ovarian follicles induced the secretion of E2 due to the stimulation of FSH and in turn increased the transcripts levels of androgen subtypes (*ARa* and *ARb*) during the late oil droplet and early-vitellogenic stage in Japanese eel (Tosaka et al., 2010).

Gonadal cytochrome P450 aromatase (CYP19A) is also important to the ovarian development in chordates. Therefore, aromatase is a key steroidogenic enzyme can be used for elucidate the control mechanism of oocyte development in Japanese eel (Ijiri et al., 2003). In human and rat FSH and forskolin, an activator of adenylate cyclase, increase the expression of CYP19 mRNA in ovarian granulosa in culture (Hickey et al., 1990). According to the Ijiri et al. (2003), CYP19 was elevated in gonad samples of the migratory nucleus stage eels due to the abnormal development process in final maturation.

In addition to the BPG axis, liver plays a vital role by providing egg yolk precursor vitellogenin for gonadal development through stimulation of E2 in oviparous vertebrates. Vitellogenin gene expression is regulated by the binding of estrogen receptor-E2 complex to the estrogen responsive elements at the promoter site of the Vtg (Wahli, 1988). Normally Vtg is undetectable in male and immature female fish.

In order to understand the fundamental mechanisms involved in Japanese eel reproduction and provide a broad basis for comparative study of eel, we have investigated the potential role of gonadotropins, GTHs and sex steroids and their receptor's differential regulation

along the BPG axis according to the histological observation during gonadal maturation. Furthermore, we analysed the involvement of liver vitellogenin and ERs in different oocyte development stages during the artificially maturing Japanese eel. In order to that we have investigated the partial cDNA sequence encoding Kisspeptin precursor molecule from *A. japonica* brain using degenerate primers and analysed the *Kiss1* gene expression alone the gonadal development in artificially matured Japanese eels.

2. Material and Method

2.1. Animals

Feminized female Japanese eels (by feeding commercial diet containing estradiol-17β for five months: Tachiki and Nakagawa, 1993) were purchased from the Eel Fisheries Cooperatives farm in Younggwang, South Korea. Prior to the experiment they were transferred in to Environmental Research Institute (ERI) of Jeju National University, Republic of Korea. Fish were kept in freshwater tanks for two days and gradually acclimatized in to circulating sea water (salinity 34 ~). Water temperature maintained as 20 °C. Tank was covered to maintain constant darkness, thereby reducing the stress.

2.2. Induction of oocyte maturation and ovulation

According to the investigation of Tachiki and Nakagawa (1993) and Kagawa et al., (1995), eels were weekly delivered (for 13 weeks) 20mg/Kg salmon pituitary extraction (SPE) suspended in saline (0.9% NaCl) by intraperitoneal injections. 18hrs after the last SPE injection, DHP (17 alpha,20 beta-Dihydroxy-4-pregnen-3-one) (2μg/g per body weight) was injected in to the remaining eels for stimulate the ovulation. (Fig 5.) Eels were not fed throughout the experimental period of 13 weeks.

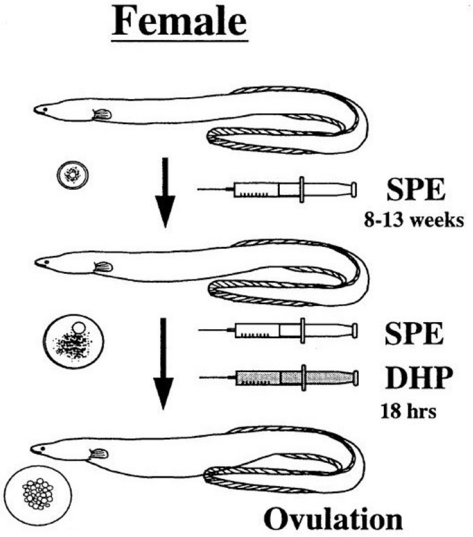


Fig.5. SPE injection procedure
Source: Kagawa et al., 2005

2.3. Tissue collection

Un-injected and SPE treated eels were anesthetized with MS222 before scarification. Fifteen female eels were sacrificed to serve as pre-nucleolus (PN), nucleolus (N) at the two days after arrived to the ERI. After the 2^{nd}, 4^{th}, 6^{th}, 13^{th} injections and after ovulation (AO) five eels from each step were sacrificed for tissue collection. Total body weight, liver and gonad weights were measured to calculate gonadosomatic index (GSI = 100 gonad weight × total body weight^{-1}) and hepatosomatic index (HSI = 100 liver weight ×total body weight^{-1}). For RNA extraction and mRNA expression, brain, pituitary, gonad and liver were isolated from each sacrificed Japanese eels and tissues were immediately immersed in TaKaRa RNAisoplus solution (300µl) and homogenised well. Subsequently mixtures were stored at 4 $^{\circ}$C until RNA extraction. The blood was centrifuged at 3000 rpm for 10 min at 4°C and the separated serum was stored at - 80 $^{\circ}$C until it was subjected to the ELISA assay. A piece of gonad sample was preserved in Buin's fluid for histological observation.

2.4. Histological observation

Dehydration of tissues for histological analysis (already in Buin's fluid) was carried out by using the Tissue Tec® VIP™ 5Jr (Japan) and after that samples were embedded in paraffin. Sections were cut 7µm thickness with a manual microtome (LEICA RM 2235- Germany). Slides were stained with water based haematoxylin and eosin. Stained slides were observed with an Olympus CX 31RTSF (Tokyo, Japan) microscope and pictures were taken with a cannon digital camera attached to the microscope. There are 7 oogenesis stages were determined according to Kayaba et al, (2001),Mazzeo et al. (2012), Hirohiko Kagawa (2013), and Thulasitha W.S. and Sivashanthi K (2013) as follows, perinucleolar (PN), nucleolar (N), early-vitellogenic stage (EV), mid-vitellogenic stage (MV), late-vitellogenic stage (LV), migratory nucleus stage (MN) and after ovulation (AO). Their deacriptional identification characters were categorized in table 1 and histological images were shown in fig 6.

Table 1: Histological criteria for classification of gonadal development stages in Japanese eel

Stages	Description	Category
Perinucleolar	Mostly single conspicuous spherical nucleus present in the oocyte	Pre-vitellogenic
Nucleolar	Several nucleoli present in the peripheral region of the germinal vesicle and increase the size of the nucleus and cell	
Early-vitellogenic	Cortical alveoli were observed in the oocyte cytoplasm and yolk deposition initiate periphery of the oocyte	Vitellogenesis
Mid-vitellogenic	Large yolk globules can be observed throughout the oocyte	
Late-vitellogenic stage	• Nucleus membrane and nucleolus become disappear • Cell membrane become thick • Yolk globules are tightly packed in the cytoplasm (comparatively higher than MV)	
Migratory nucleus stage	oocyte hydration and the migration of the nucleus towards the animal pole	Oocyte maturation and ovulation
After ovulation	Can be identified using the ruptured follicles	

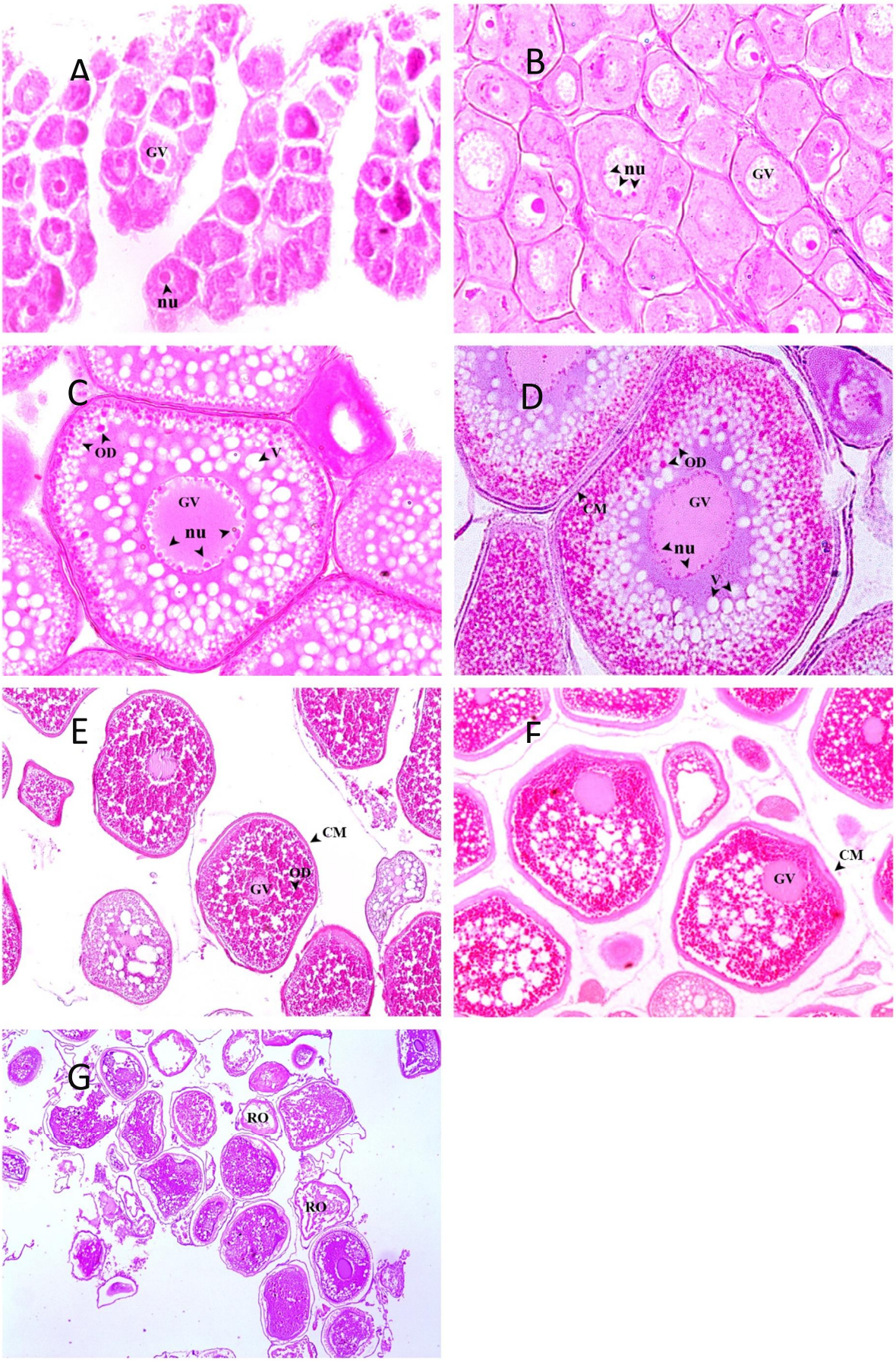


Fig.6. Light microscope photograph of different developing stages of an ovary of Japanese eel (*Anguilla japonica*). A: perinucleolar, B: nucleolar, C: Early-vitellogenic, D: mid-vitellogenic, E: late-vitellogenic, F: migratory nucleos, G: after ovulation. GV, germinal vesicle; nu, nucleolus; od, oil droplet; RO, ruptured oocytes; CM, cell membrane: Mag, magnification

2.5. RNA extraction

Total RNA was extracted from homogenized brain, pituitary, gonad and liver tissues using TAKARA RNAiso plus Total RNA extraction reagent according to the manufacturer's protocol. Extracted RNA was purified after DNase treatment (Portage, USA) according to the manufacturer's instructions. The RNA concentration was assessed before and after DNase treatment, spectrophotometrically by measuring the absorption at 260 and 280 nm on Nanodrop 2000C spectrophotometer (Thermo Scientific, USA). To check the quality of RNA samples (Before and after) gel-electrophoresis was carried out using 1% agarose gel stained with ethidium bromide. Simultaneously working RNA stock was prepared using DNase treated RNA samples by adding nuclease free water. Finally, all RNA samples were stored at -80°C until use.

2.6. Kiss1 partial sequence cloning

Degenerate primer sets (Table 2) were used for amplification of *A. Japonica* Kisspeptin containing cDNA fragments, based on the conserved Kisspeptin regions from *Epinephelus bruneus* (accession no GU984382), *Scomber japonicas* (GU731672) and S*eriola lalandi* (HC449729). Degenerate primers were used exclusively or in combination to obtain the initial PCR products. A 50µl total volume PCR mixture using a Prime tag polymerase kit (Tech and Innovation ^{TM}, Korea) was subjected to a conventional PCR amplification.

Thermal cycling conditions were as follows, initial denaturation at 95°C for 3 min, 35 cycles followed by 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec, final elongation 72°C for 5 min with brain tissue as template. PCR products were electrophoresed in 1% agarose gels with a 100 kb DNA ladder (Enzynomic^{TM}, Korea) and visualized by ethidium bromide staining. PCR products of expected size were electrophoresed in 1% low melt agarose gels, excised and purified using BiIOFACT, HiGene^{TM} Gel & PCR purification system (Korea). Purified PCR products were ligated to a T-Blunt vector using T- Blunt^{TM} PCR cloning kit (BIOFACT, Korea) and vector has been transformed into competent cells. The plasmid DNA was then extracted from the bacterial cells using Gene ALL® Hybrid-Q^{TM} plasmid extraction kit (Korea). Plasmid samples were quantified with the Nanodrop 2000C spectrophotometer (Thermo Scientific, USA) and sent for sequencing. Sequencing was analysed using the sequence scanner version 1. Then the gene specific primers designed for the obtained partial sequence (Table 2).

Table2.

Description of degenerate primers used in this study

	Forward primers (5'-3')	Revers primers (5'-3')
Degenerate primers	**F1**-TTGTTGCTYTGAYGRTGGCT	**R1**- CGGARACCAAAGGAGTTGA RGTTGTA
	F2-TGCTRCTTTGTCAACAGAGGT	**R2**- CACCATCYTGHCCTGGGAA ACTTT
	F3-ATCCACCHACYWCAGTGAAG ATCAG	**R3**- TGHCCTGGGAAACTTTCYA CAGCT

2.7. Quantification of Brain, Pituitary, Gonad and Liver target gene transcripts by real time PCR analysis.

The qPCR was carried out to determine the target gene expression in brain, pituitary, gonad and liver (Table 3.) during different oocyte maturation stages in Japanese eel. Total 15 µL of reaction mixture contained 5.75 µL of template RNA (20ng) in nuclease free water, 0.75 µL of TOPreal™ One-step RT qPCR Enzyme MIX, 7.5 µL of 2× TOPreal™ One-step RT qPCR Reaction MIX and 0.5µL of each primers (10 pmol/µL) (Table 2.).The qPCR experiment was performed using the TaKaRa TP850 Thermal Cycler Dice ^{TM} Real Time System (TaKaRa, Japan) and thermal cycling conditions were as follows: hold at 50°C for 30 min (cDNA syntheisis), initial denaturation at 95°C for 10 min, 45 cycles followed by 95°C for 20 sec, 60°C for 20 sec, 72°C for 30 sec, and final cycle of 95°C 15 sec, 60°C 30 sec and 95°C 15 sec. Same qPCR profiles was carried out for Japanese eel Elongation Factor 1-Alpha (*AjEF1-a*) to measure the relative expression. The relative mRNA expression of *AjCYP19A1* was determine by the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001).

Table 3. Description of target specific primers used in current study

Target gene	Forward primers (5'-3')	Revers primers (5'-3')	Accession no.
KiSS1	CTACACCTCCAAAGATAAGG CAATACTCAAAGCC	ACATCAGTTGAATGGAT CATATATGCTGGCAAA	
KiSS1r	TGCCGCTGCTCTTCG CTATTT	TGGAACACAGCACA CCAGGAAA	JX913802
GnRH1	TGTGGCACTGGTGTG TCAGG	AGGTCATTGCAGCT GGGCAAA	AB026989
GnRH2	CGGCAGGCTGGTGCT GATTT	TTCCAATACCTCGG CGGTGCT	AB026990
GnRHr	TGTTTGCGGCGTTCA GCAATCT	ACCATGTCCAGCGG CATCAC	AB041327
ERa	GTTCCGCGCTCTCAA	CCAGCATGCACTGC	HM545084

	GCTGAA	ACCGTAAA	
ERb	CGCCAGCACCATGTC GAAGATT	TGTTGGCTGGAGGT GCTGATG	AB003356
ARa	ACCTGTGCGCCAGCA TCAAT	GGCCCTCATTTGCCC GATCTT	AB025361
ARb	ATGCAGCGCGTTCTG GTTTCT	ATGCTCGACTGCCTC GTTGG	AB023960
CYP19 A1	CAACGAAAAGGAGCT TCTGG	TCAGTGGCAAAGTCA GCATC	YA540622
FSHb	AAGAATGCGGTGGCT GCATCA	CCAGGCAAGTGCACA GTCTCA	AB016169
FSHr	CTGCATGGGCGTCTA CCTGTT	TGCATGGCGAAGGTG ATGGT	AB360713
LH b	TGCACCTGGCTCCTC TTTGTAT	AGTGACCACTGCAGA TGGATGT	AB175835
LHr	CGCTACGCATTCAAC GGGACAA	GAGGGAGTGGAGAGC CGTAGAA	EU635883
DHPr	AGACATTGCCGGTGC ACAAGAG	GTGGAGTCCAGCGGGA GATAGA	AB032075
Vtg	ATGCAGCAGTTGTCA AGGCAGAG	ATGAGAGCAGCACCAG GGATGTA	AY775788
EF1a	GAGACTGGAATCCTGAAGCC TGG	GATCTGTCCAGGGTGAT TCAGGATG	AB593812

2.8. ELISA analysis

Estradol (E2) ELISA

The E2 ELISA kit (Cat. No. SB-E13017Fh) was purchased from CUSABIO Company, Japan. The competitive inhibition enzyme immunoassay technique was employed using this kit. The microtiter plate provided in this kit has been pre-coated with goat-anti-rabbit antibody. The serum E2 levels were measured according to the product manual and the optical density (OD) was determined using a microplate reader (TriStar Multiple reader LB 942, Korea) through the 450nm. The sensitivity of the assay was 38.46pg/ml.

2.9. Statistical analysis

All the data were collected from five individual samples and presented as mean standard error (SE) of five replicates (n=5). To determine the statistical significance between the experimental and control groups, all the mRNA expression analysis data were subjected to either Student's t-test or one-way analysis of variance (ANOVA) in SPSS 16.0 for Windows. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. PART 1: The involvement of BPG-axis sex related genes into oocyte development in Japanese eel.

3.1.1. Involvement of the brain

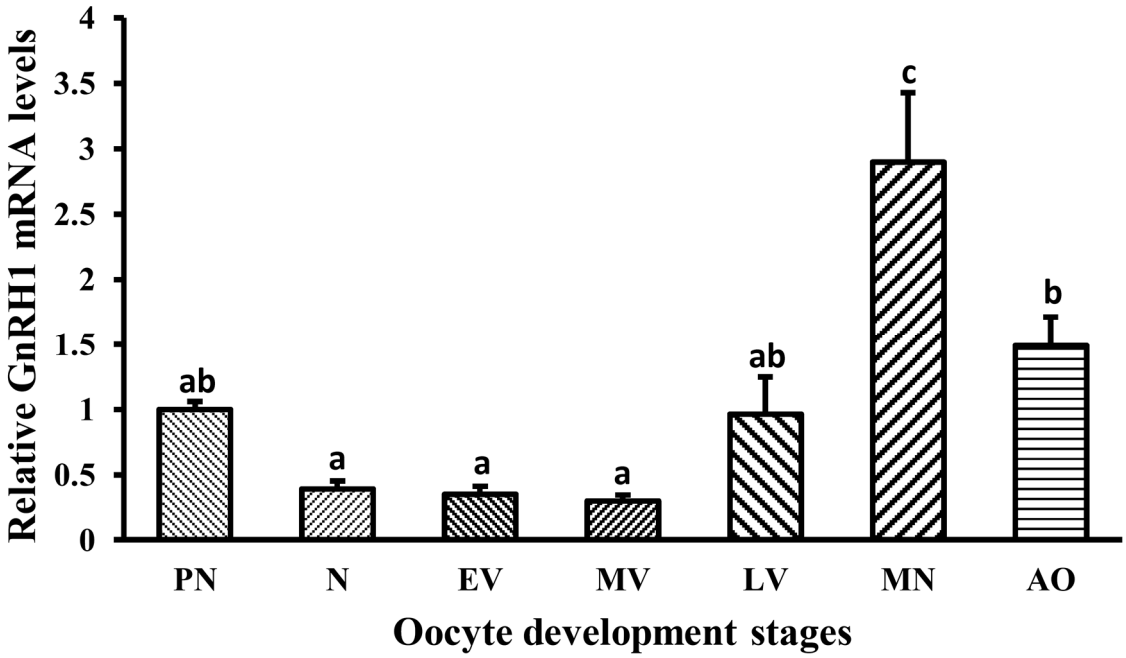


Fig 7. Brain *GnRH1* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *GnRH1* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The brain *GnRH1* mRNA transcriptional levels were not significantly fluctuate during pre-vitellogenic and vitellogenic stages. However, it was significantly increased in MN stage and significantly decreased after ovulation.

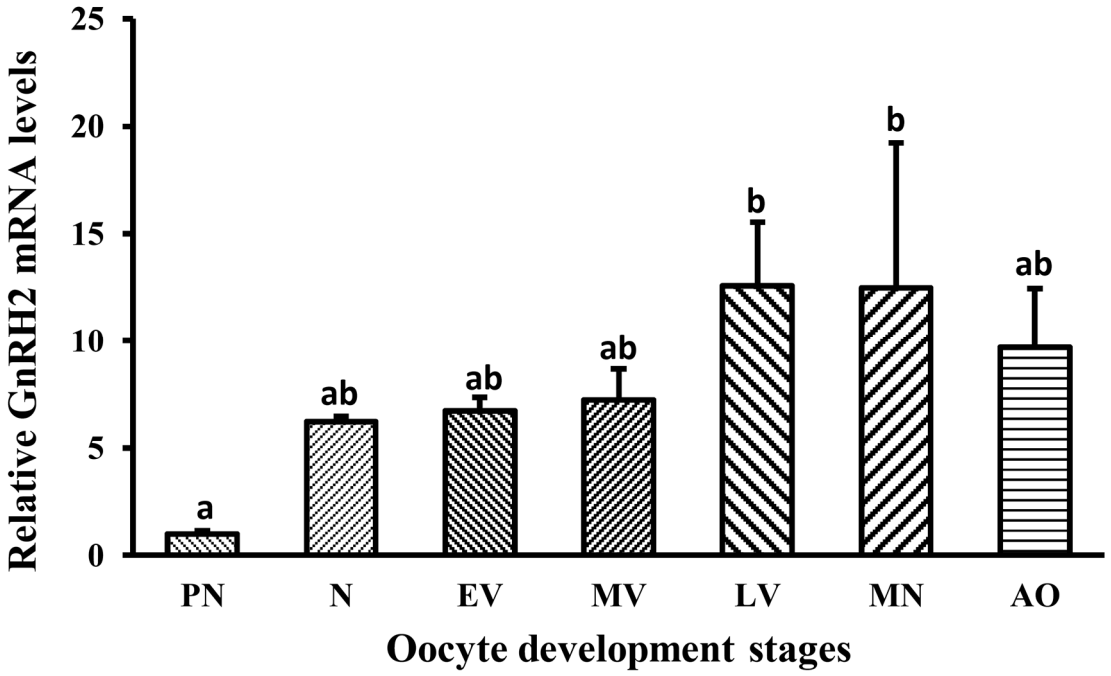


Fig 8. Brain *GnRH2* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *GnRH2* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a, b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The lowest brain *GnRH2* transcript levels were shown in PN stage and highest transcript value was shown in MN stage. However, there was no significant variation was observed alone the oocyte development in female Japanese eels (except PN).

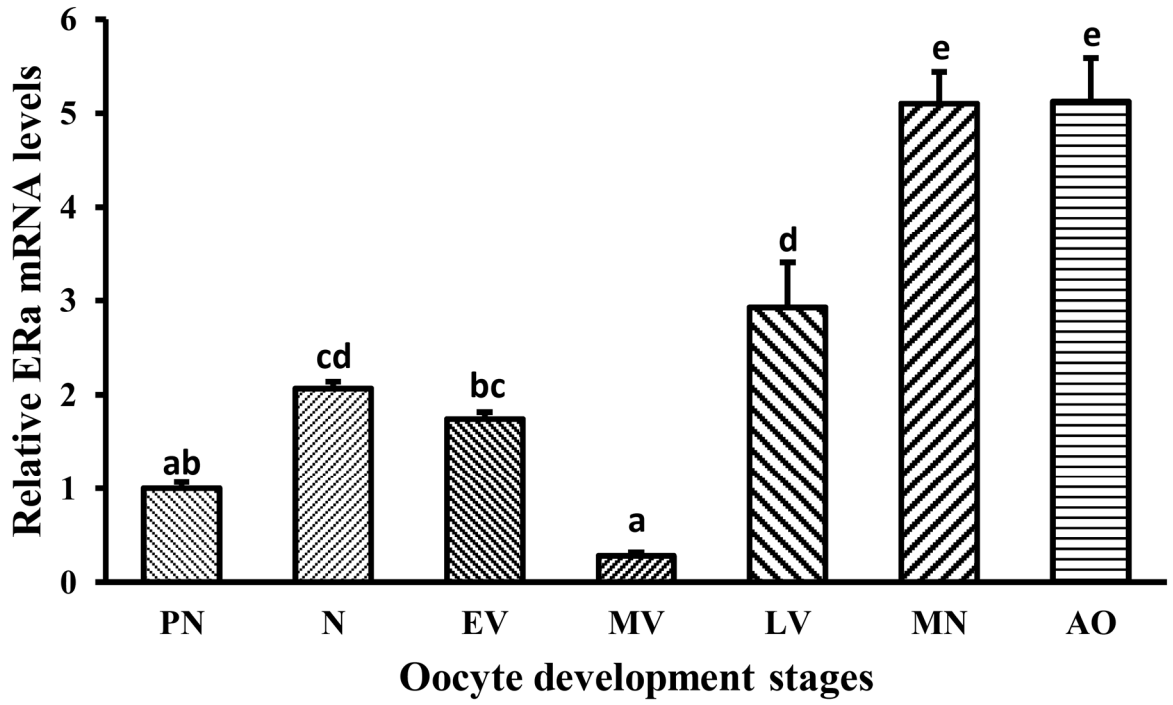


Fig 9. Brain *ERa* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *ERa* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c,d,e) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The ANOVA test indicate the prominent significant effect of *ERa* on end of the gonadal development and final maturation with compare to the other developmental stages. The lowest expression was obtained in the MV stage.

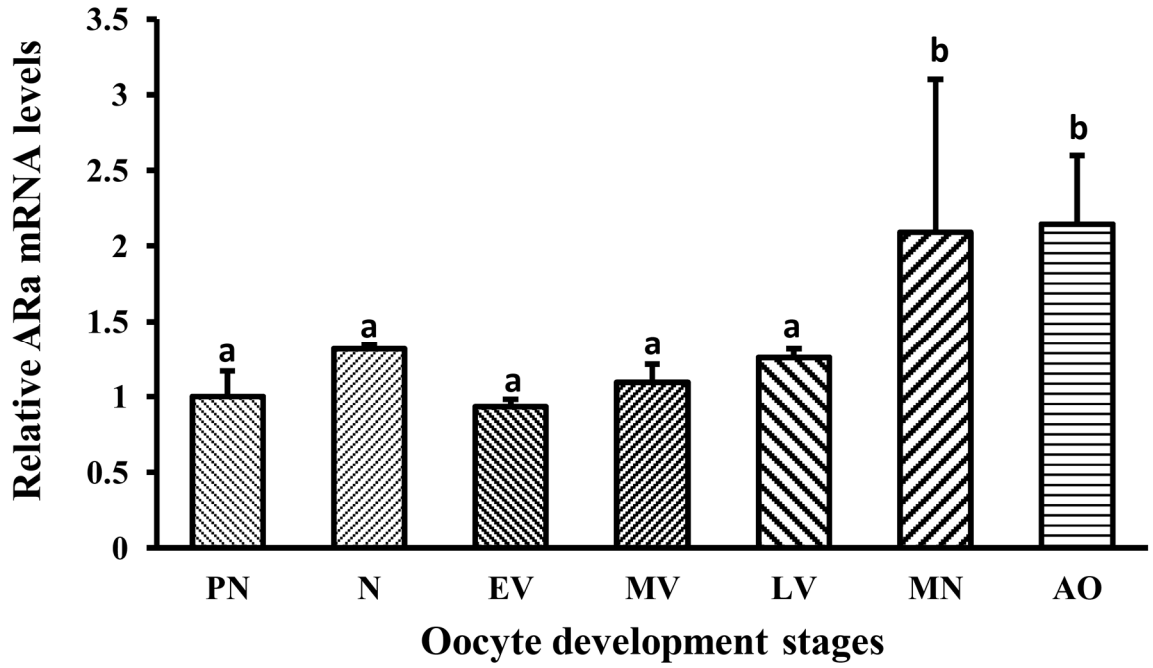


Fig 10. **Brain *ARa* transcription levels in female Japanese eels during different gonadal development stages.**

The expression analysis of *ARa* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

ARa expression was not significantly elevated in previtellogenic stage and vitellogenic stages in the brain but a significant boost can be found during final oocyte maturation and post ovulation.

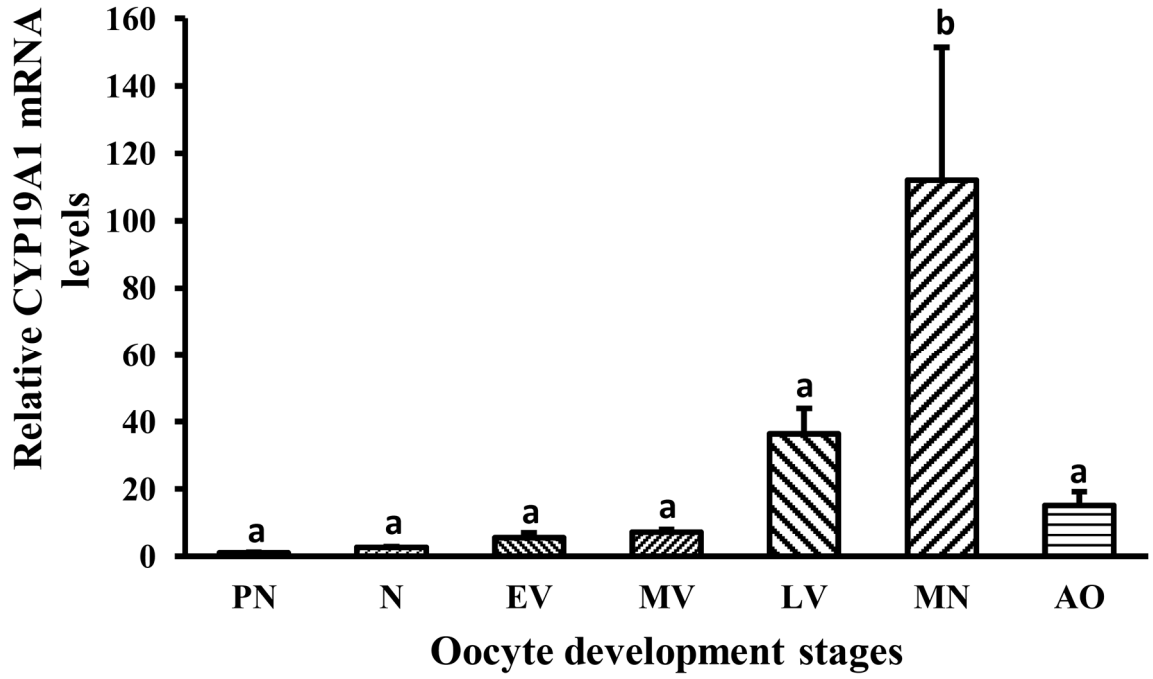


Fig 11. Brain *CYP19A1* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *CYP19A1* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The brain *CYP19A1* indicate a significant expressional incensement in brain during the MN stage with compare to other all development stages.

3.1.2. Involvement of the Pituitary

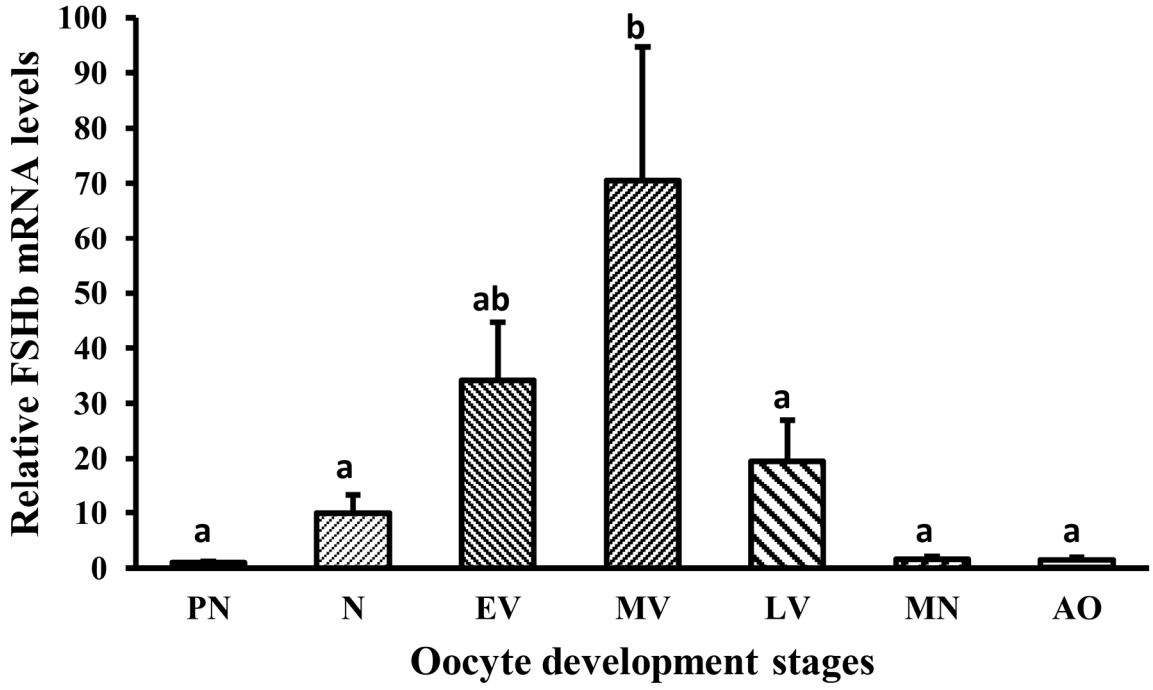


Fig 12. Pituitary *FSHb* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *FSHb* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The pituitary *FSHb* transcriptional level is significantly increased in MV stage; overall, it is gradually increased until MV stage and then gradually decreased.

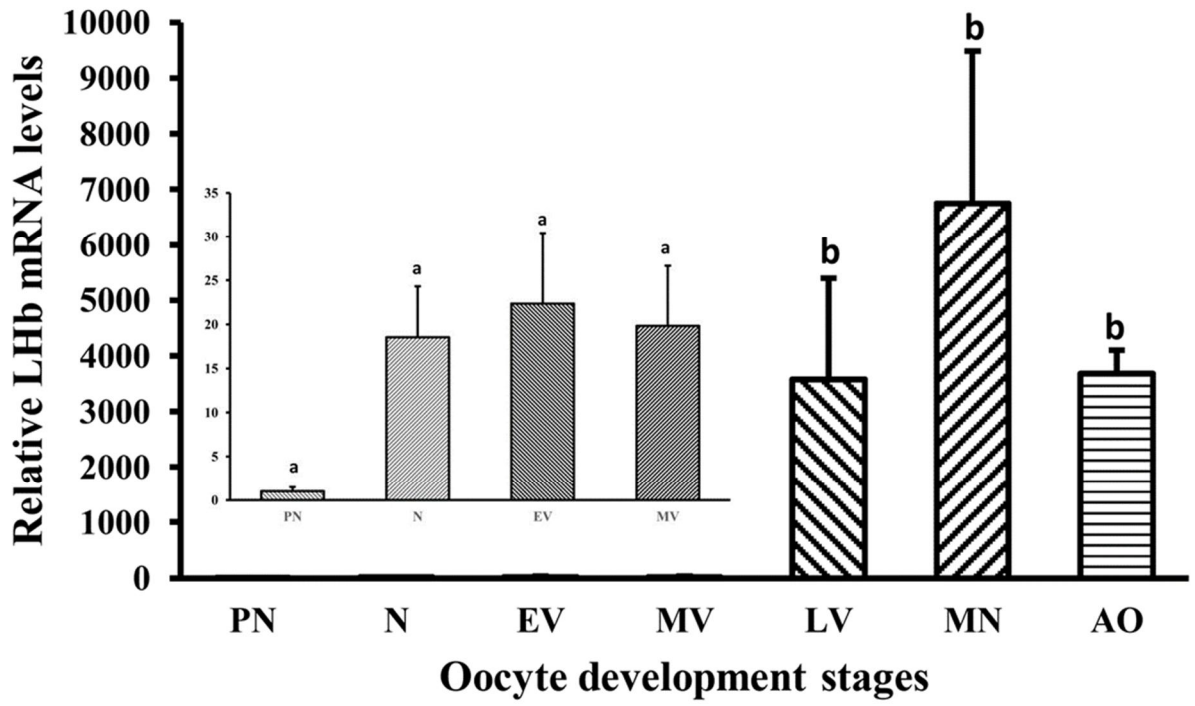


Fig 13. **Pituitary *LHb* transcription levels in female Japanese eels during different gonadal development stages.**

The expression analysis of *LHb* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

LHb showed extremely lower expression until MV stage and then transcript levels were significantly increased up to AO stage of the oocyte development. However, the highest *LHb* expression was observed in the MN stage.

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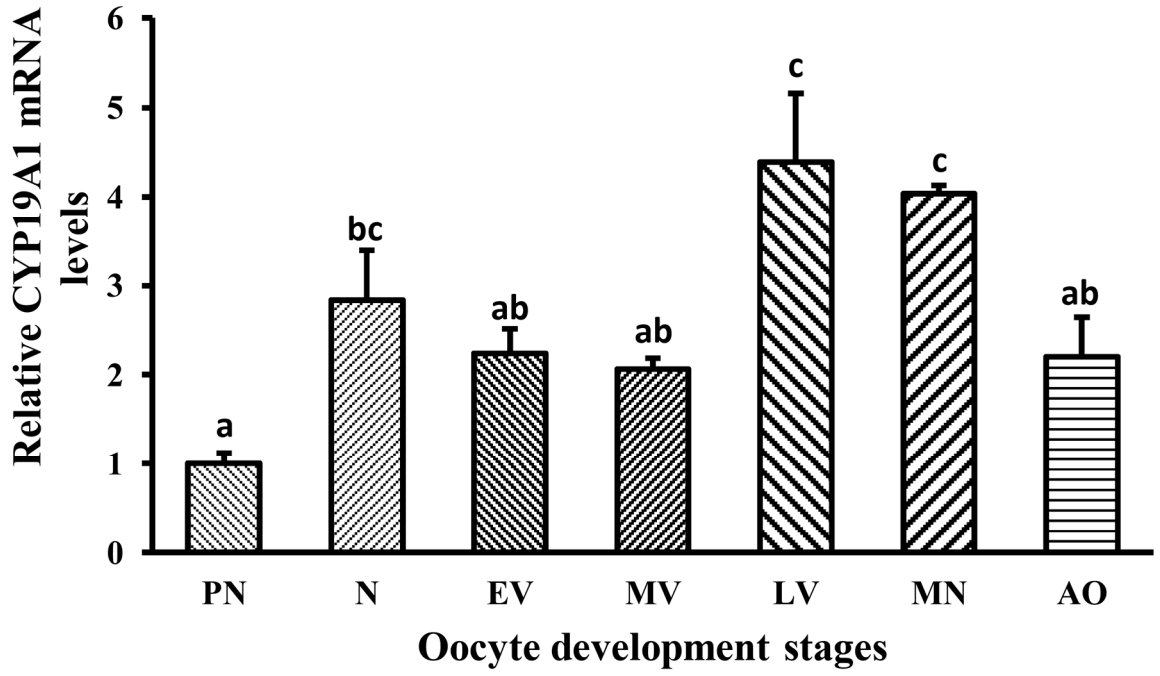

Fig 14. Pituitary *CYP19A1* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *CYP19A1* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

CYP19A1 transcript levels were significantly increased in LV & MN stage of the oocyte development, compare to the other development stages (except N).

3.1.3. Involvement of the Gonad

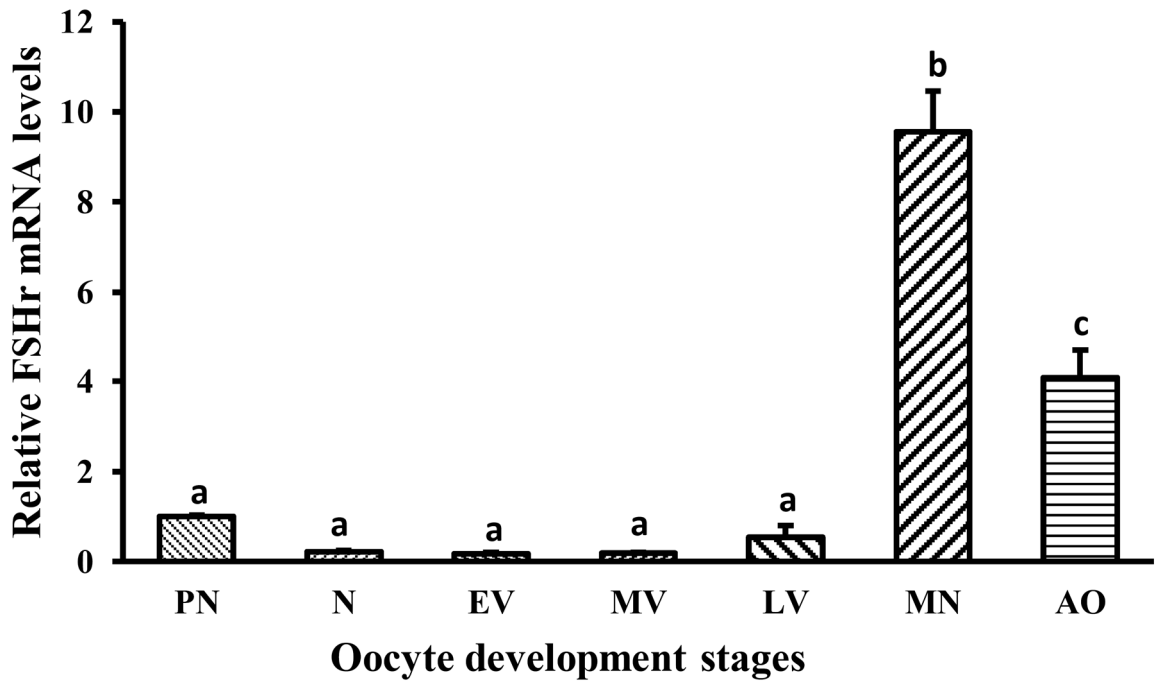

Fig 15. Gonad *FSHr* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *FSHr* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

There was no significant fluctuation on *FSHr* expression in pituitary during pre-vitellogenic and vitellogenic stages in Japanese eel. However, it was significantly increased in MN stage and then significantly decreased during AO stage.

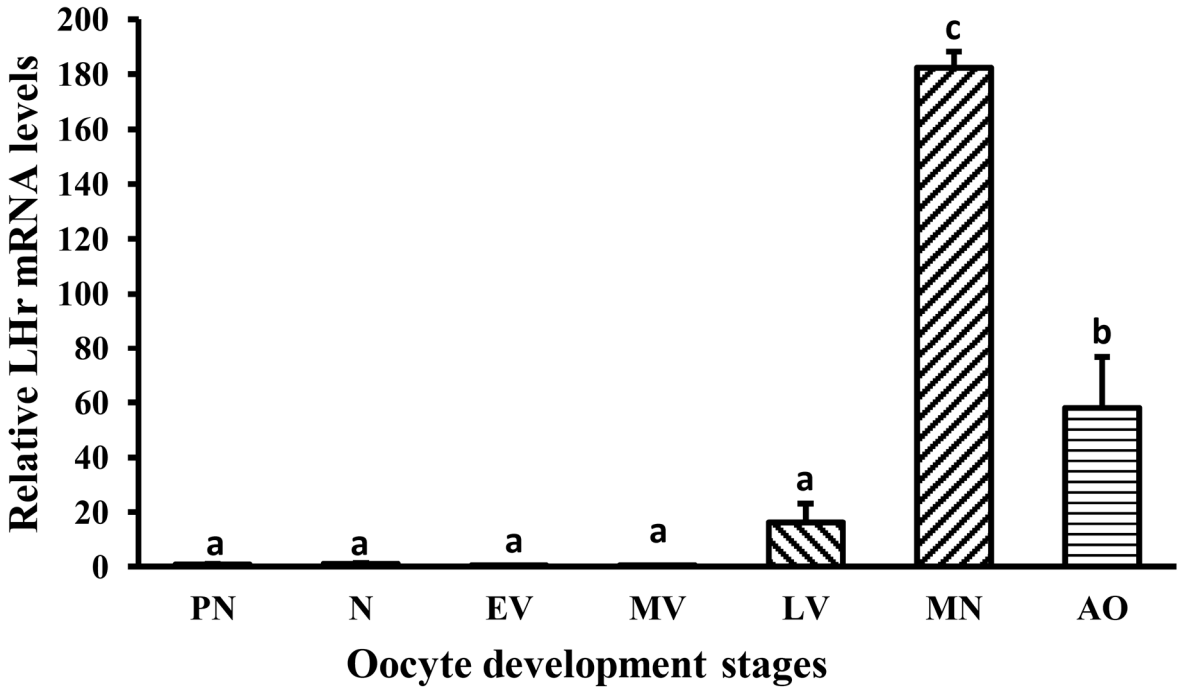

Fig 16. Gonad *LHr* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *LHr* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

There was no significant fluctuation on *LHr* expression in pituitary during pre-vitellogenic and vitellogenic stages in Japanese eel. However, it was significantly increased in MN stage and then significantly decreased in AO stage.

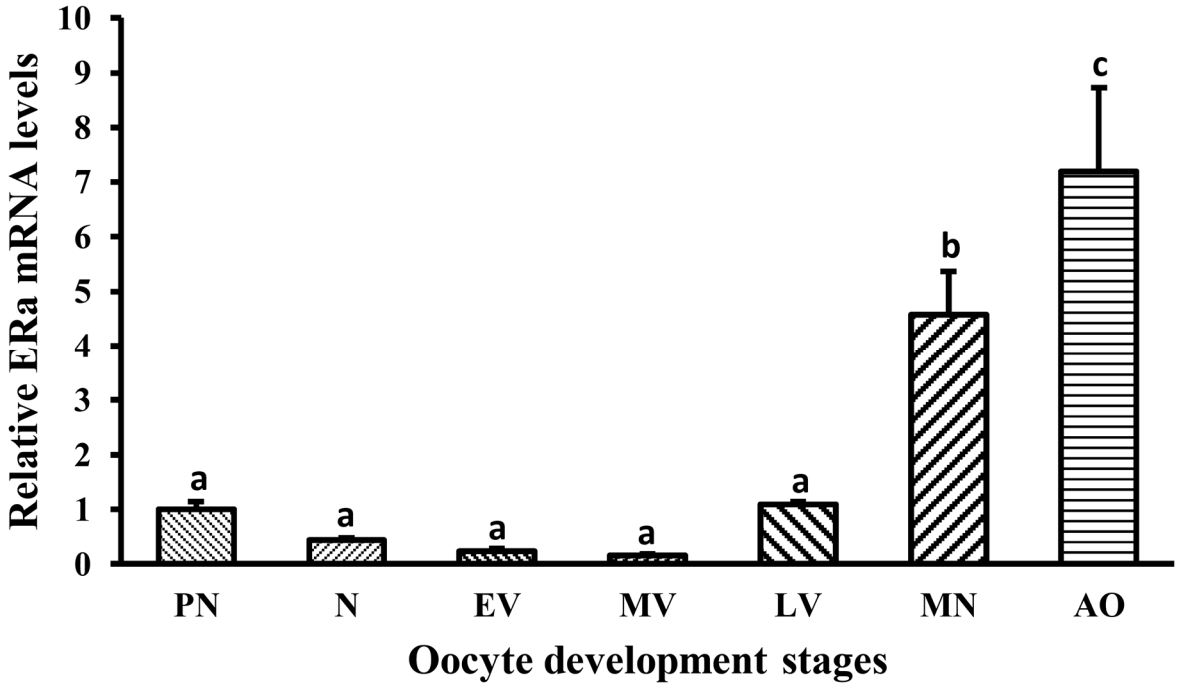

Fig 17. Gonad *ERa* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *ERa* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

There was no significant fluctuation on *ERa* expression in pituitary during pre-vitellogenic and vitellogenic stages in Japanese eel. However, it was gradually and significantly increased during final maturation and AO stage.

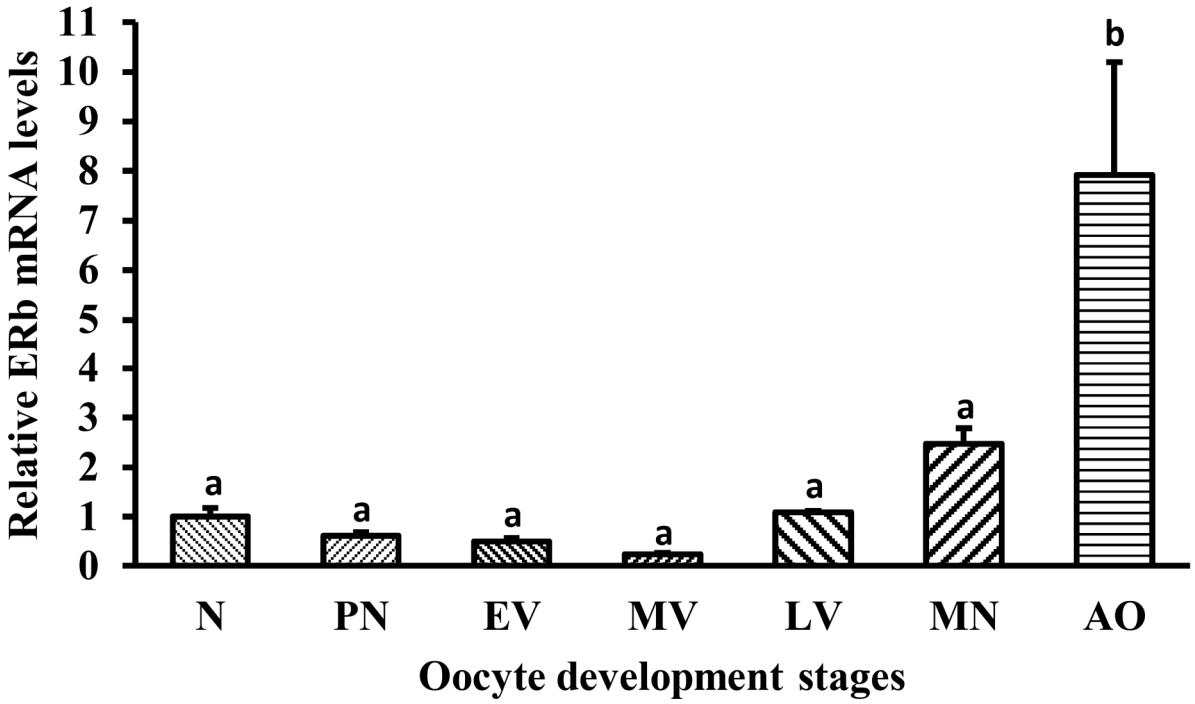

Fig 18. Gonad *ERb* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *ERb* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The *ERb* transcript levels were not significantly fluctuated during pre-vitellogenic, vitellogenic and final oocyte maturation, but then significantly increased in AO stage.

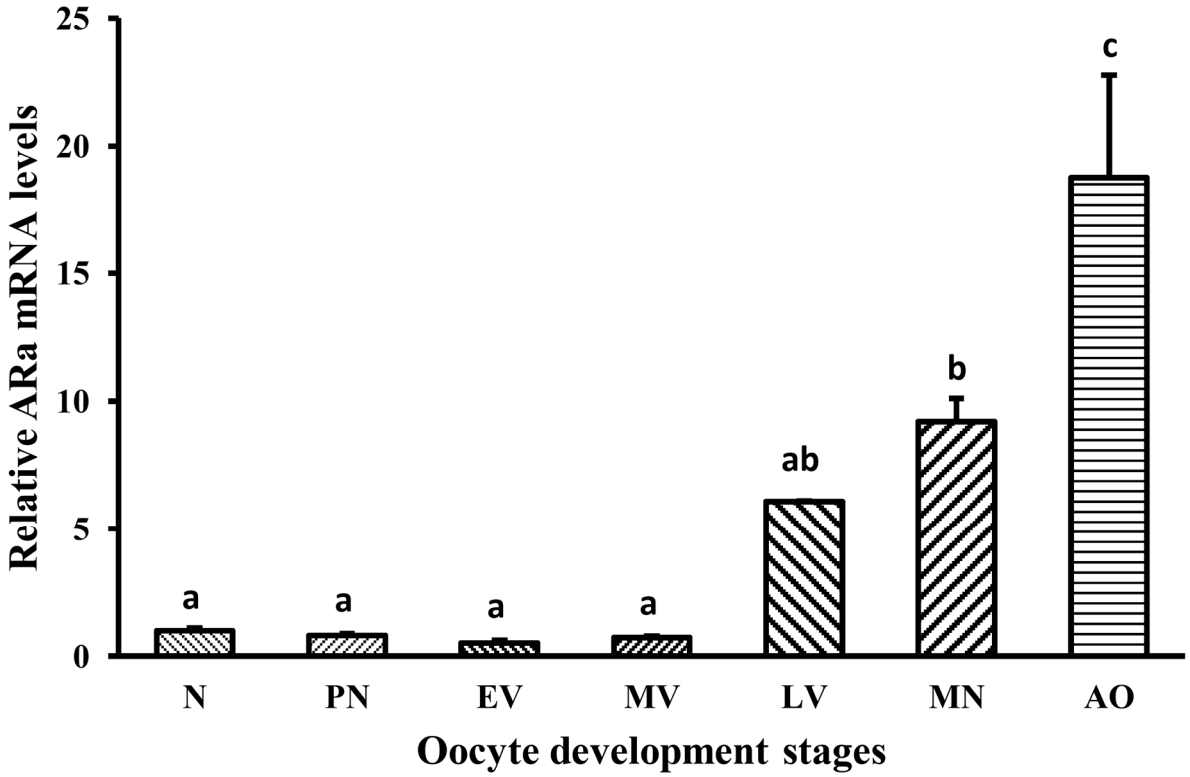

Fig 19. Gonad *ARa* transcription levels in female Japanese eels during different gonadal development stages.

The expression analysis of *ARa* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

Ovarian *ARa* transcript levels were not significantly vary up to the MV stage. But, after the LV stage it was gradually and significantly increased until AO stage.

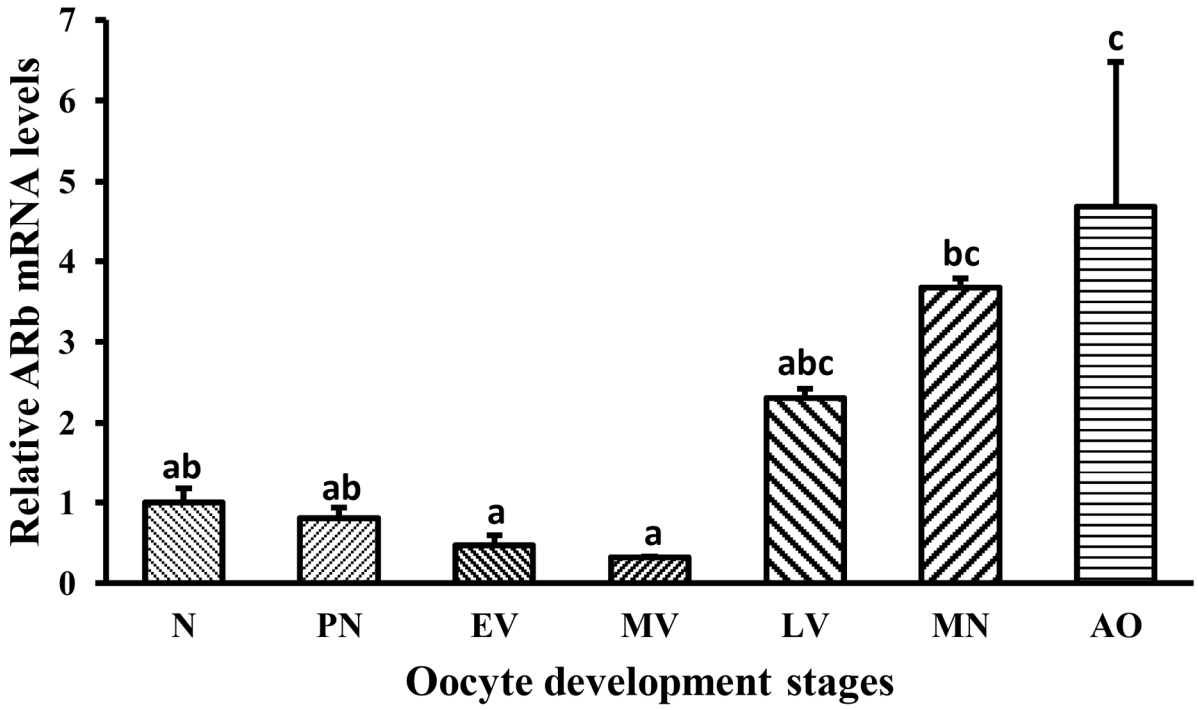

Fig 20. **Gonad *ARb* transcription levels in female Japanese eels during different gonadal development stages.**

The expression analysis of *ARb* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The gonadal *ARb* transcriptional levels were slightly but not significantly fluctuate during pre vitellogenic and vitellogenic stages in oocyte development. But, after the LV stage it was gradually and (not significantly) increased until AO stage.

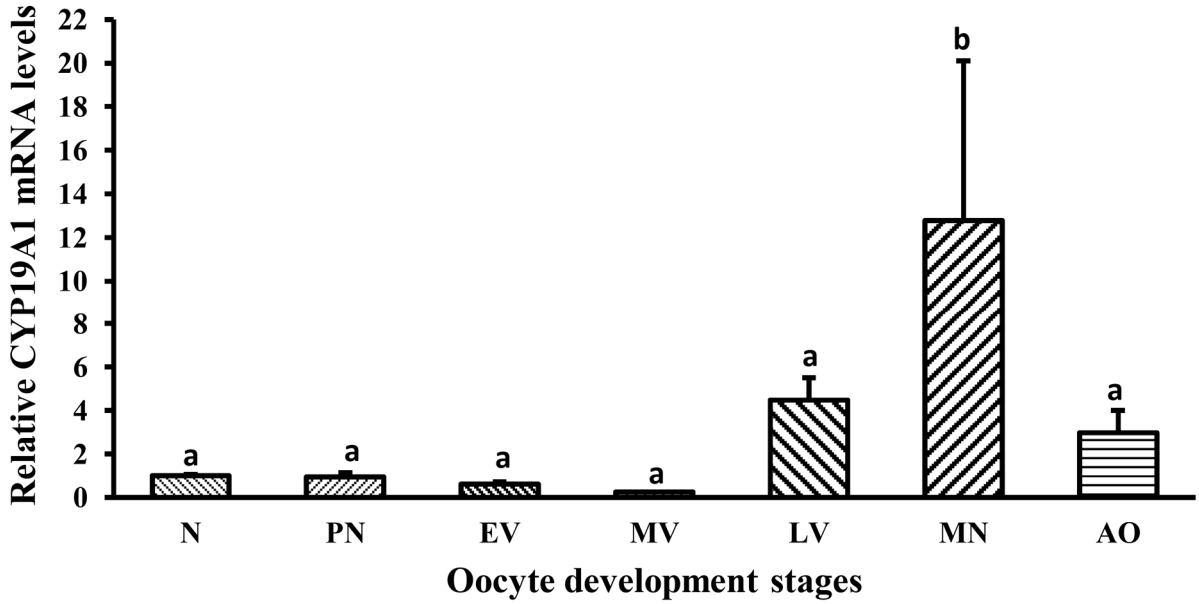

Fig 21. Gonad *CYP19A1* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *CYP19A1* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

During oocyte development, *CYP19A1* was significantly elevate in MN stage compare to the other all development stages.

3.1.4. Involvement of the Liver

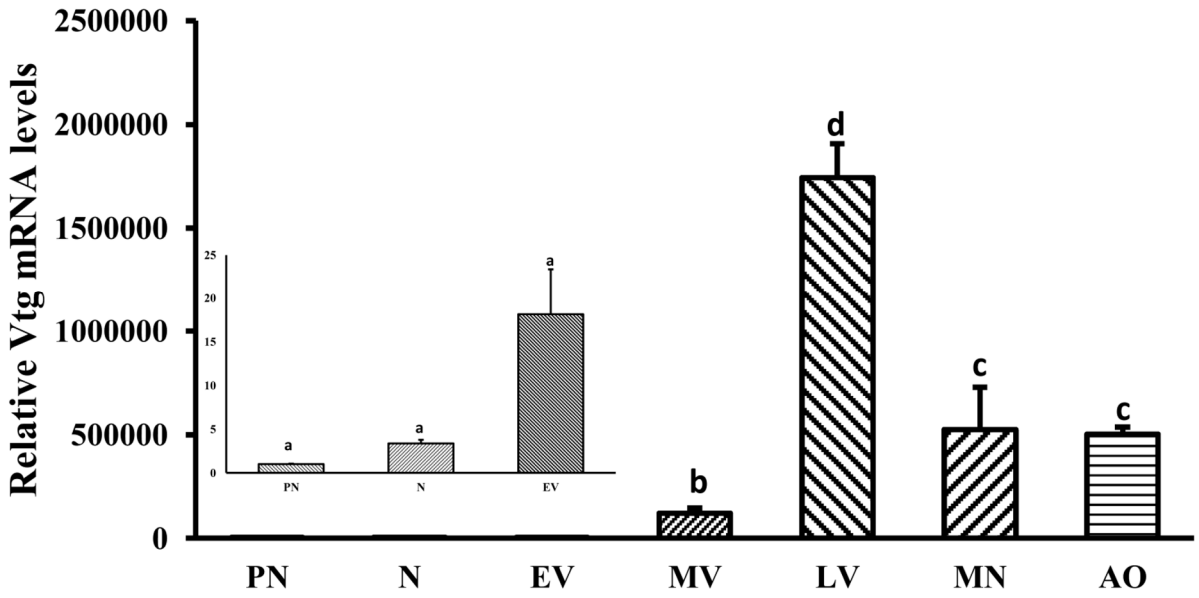

Fig 22. Liver *Vtg* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *Vtg* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c,d) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

Comparatively very low *Vtg* expression was observed during pre-vitellogenic stage. However, during vitellogenesis stage *Vtg* transcript levels were significantly increased and it was robustly increased during LV stage. After that, expression was significantly decreased and stabled until ovulation.

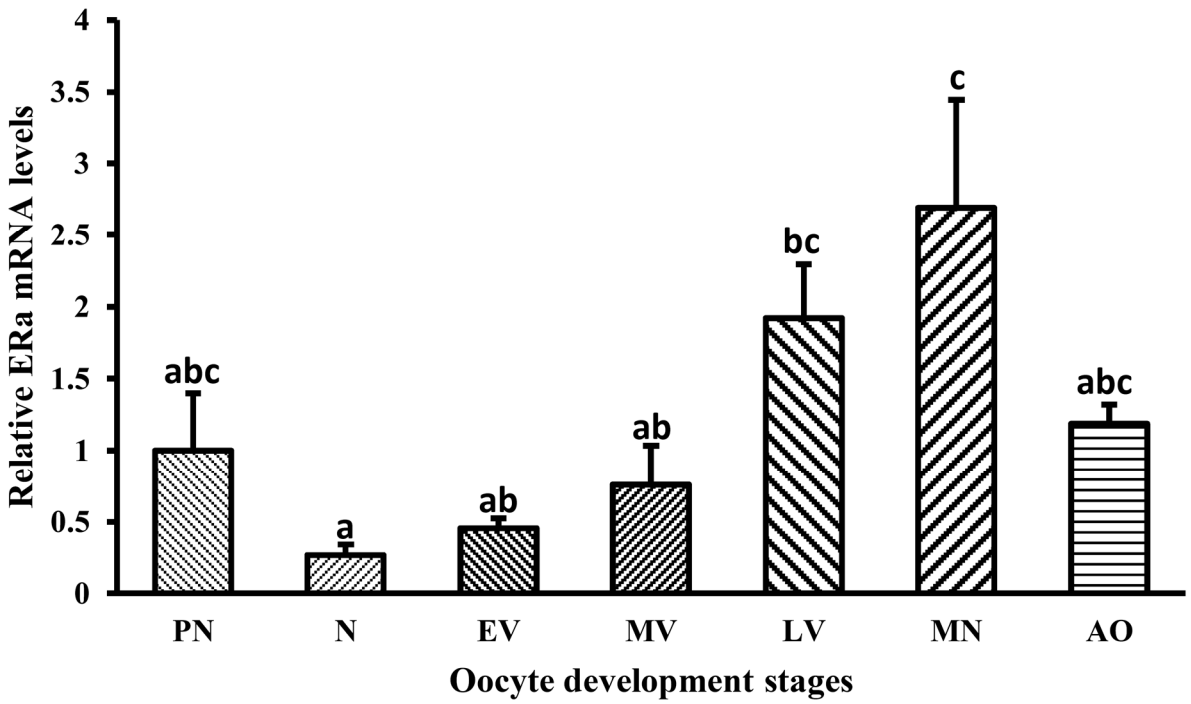

Fig 23. Liver *ERa* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *ERa* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

Lowest *ERa* transcript level was expressed in N stage and then it was gradually increased during vitellogenesis. Highest *ERa* expression was observed in MN stage. Then the expression was reduced.

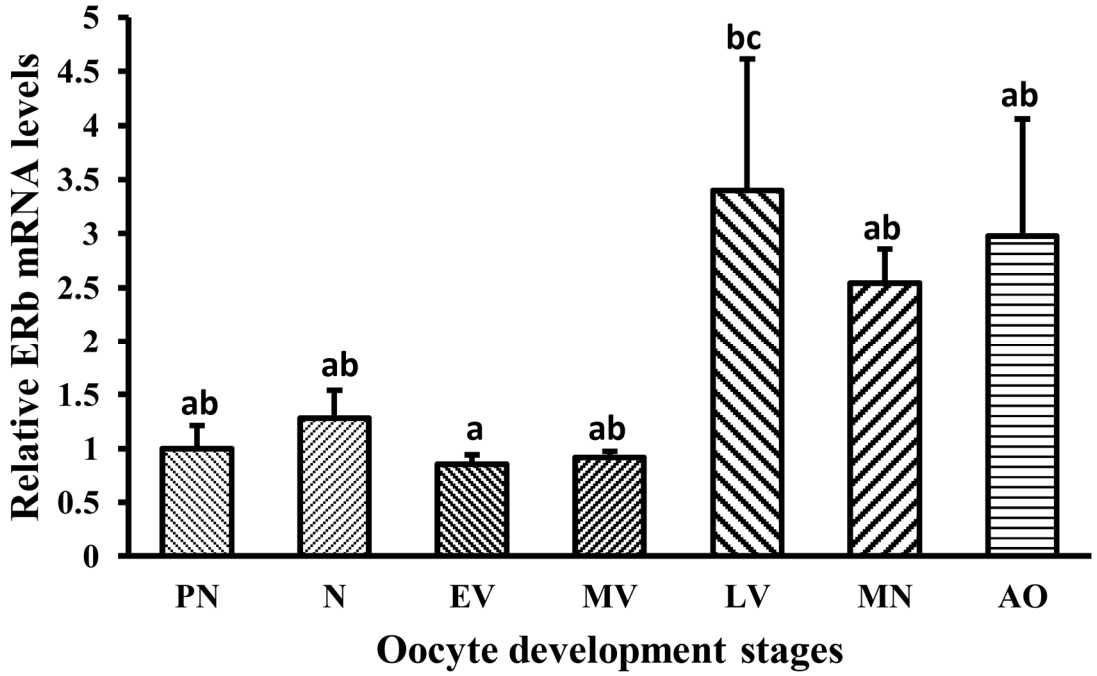

Fig 24. Liver *ERb* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *ERb* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages (PN=4, N=4, EV=5, MV=5, LV=3, MN=3 and AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

There was no significant variation observed in *ERb* along the gonadal maturation stages in liver of Japanese eel. But comparatively higher expression was observed in LV, MN and AO stages compared to the other development stages.

3.1.5 Involvement of serum

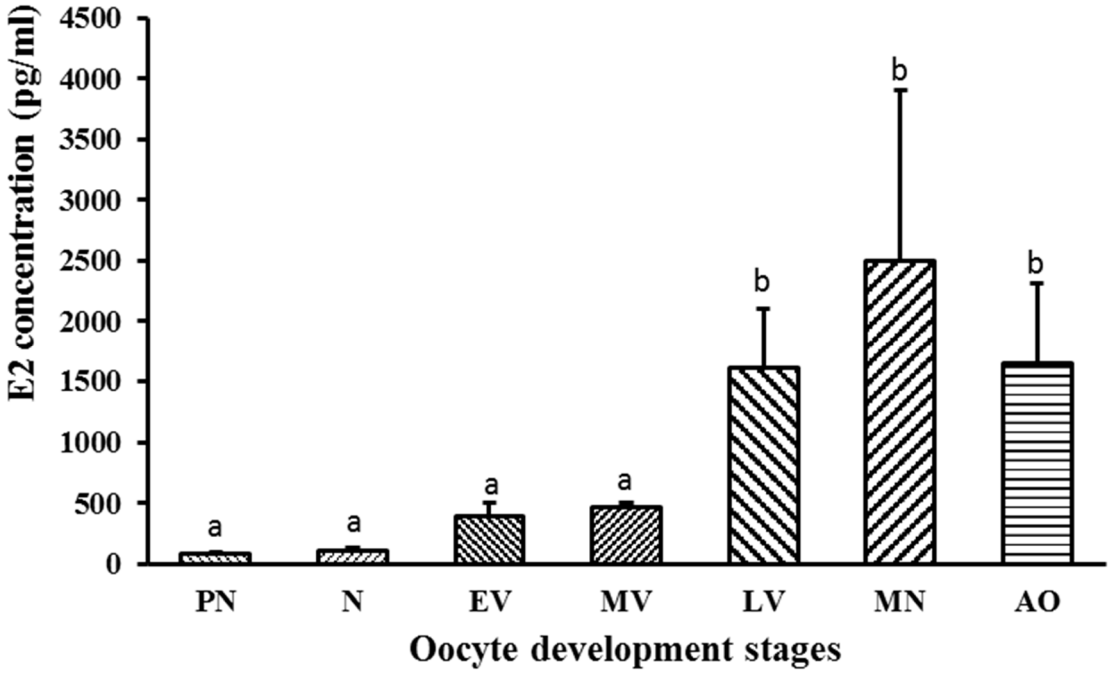

Fig. 25: Serum E2 concentration in female Japanese eels during different gonadal development stages

The concentration of serum E2 was determined by ELISA method according to the manufacture's protocol. The vertical bars represent the standard error (SD) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The serum E2 concentration was significantly increased after the LV stage and it was retained until end of the experiment.

3.2. PART 2: Involvement of kisspeptin (Kiss1) and its receptor (Kiss1r) into BPG axis sex related genes during oocyte development in Japanese eel.

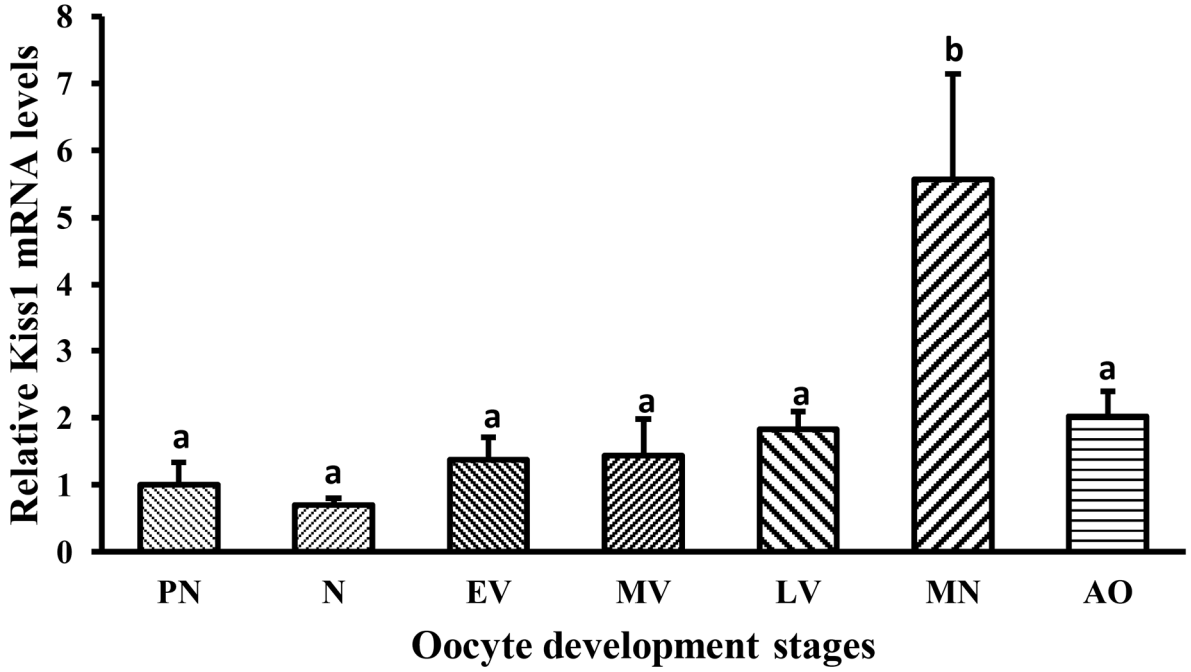

Fig 26. Brain *Kiss1* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *Kiss1* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

The brain *Kiss1* transcription was significantly increased in MN stage compared to the other development stages. There were no significant differences could be seen in among all other stages.

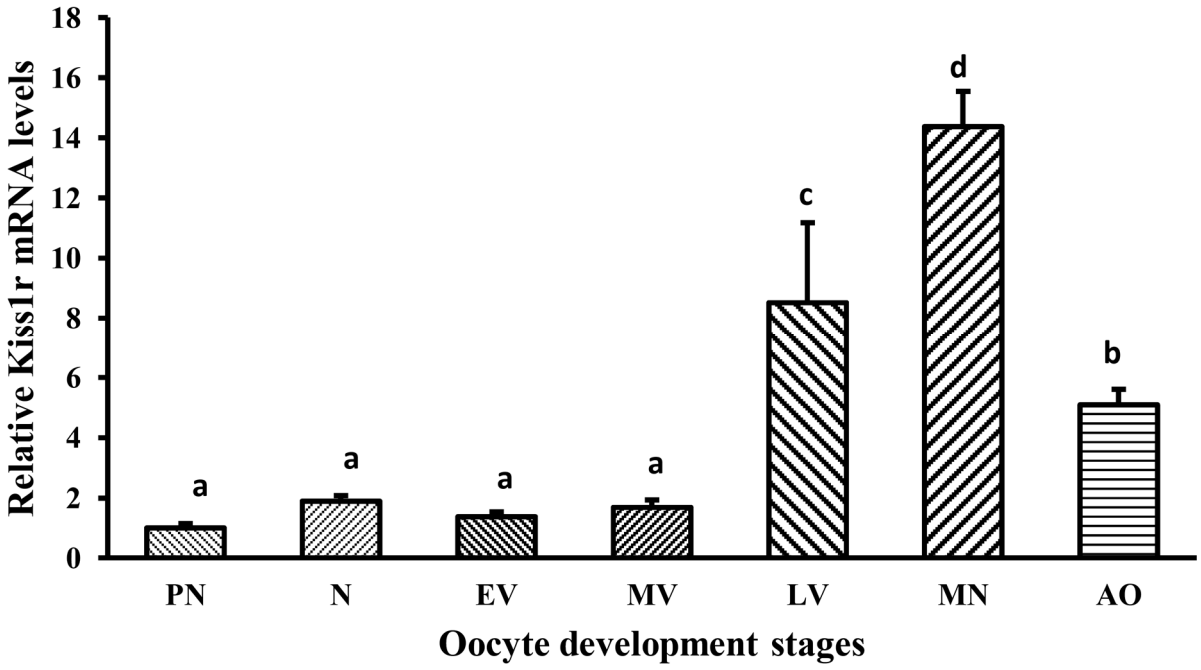

Fig 27. Brain *Kiss1r* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *Kiss1r* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c,d) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

Gonadal *Kiss1r* transcriptional level was significantly elevated in late phase of the gonadal development (LV, Mn and AO) compare to the other stages. Within late phase, *Kiss1r* expression was significantly high in the MN stage when compare to the LV and AO.

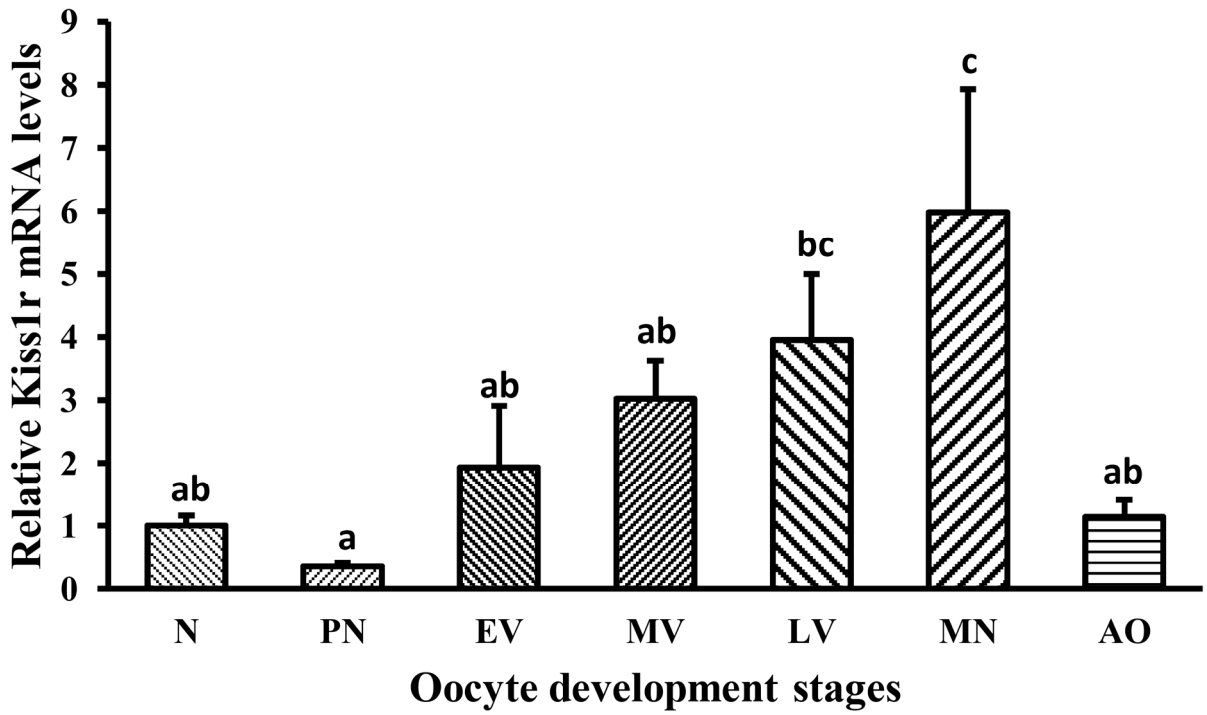

Fig 28. Gonad *Kiss1r* transcriptional levels in female Japanese eels during different gonadal development stages.

The expression analysis of *Kiss1r* was determined by SYBR green quantitative real time PCR. Livak $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels using *Anguilla japonica EF-1a* as internal control gene. The vertical bars represent the standard error (SE) of each development stages (such as perinucleolar (PN=4), nucleolar (N=4), early-vitellogenic stage (EV=5), mid-vitellogenic stage (MV=5), late-vitellogenic stage (LV=3), migratory nucleus stage (MN=3) and after ovulation (AO=5). Letters (a,b,c) in above the vertical bars represent the significant difference in between each oocyte development stages ($p < 0.05$).

In gonad, *Kiss1r* transcription level was lowest in the PN stage and after that it was gradually increase up to MN stage. Thereafter, expression level of *Kiss1r* significantly decreased in AO stage compare to the MN.

4. Discussion

The eel is a physiologically interesting model organism since it has a unique life cycle. The eel has a long period of juvenile growth before sexual maturation. Even at the beginning of a reproductive migration to the sea, its gonad is still immature. Natural spawning of freshwater has not been identified yet due to their oceanic spawning migration. Furthermore, if the reproductive migration is prevented, for instance by keeping the animal in the culturing conditions, sexual maturation never be occured. Moreover, the eel is one of the most primitive teleost species and therefore it is a good animal model for study of sex related gene actions and its regulation in the context of comparative biology.

In the present study we measured the mRNA levels of key endocrine genes in immature and artificially matured adult population of female Japanese eels. Several differences have been observed alone the BPG axis with relevant to the histological observations.

4.1. Differential regulation of *GnRH* and *GnRHr* during oocyte development in Japanese eel.

In vertebrates, including teleosts, reproductive processes are regulated by the precise coordination of neuroendocrine hormones, acting through the brain-pituitary-gonad (BPG) axis. A neurohormone, gonadotropin-releasing hormone (GnRH), plays a central role by stimulating the synthesis and release of the pituitary gonadotropins (FSH & LH). There are three commonly originated GnRH ligands (GnRH1, GnRH2, GnRH3) can be identified in vertebrates (Fernald and White, 1999). GnRH1 is specially regulate the reproduction via gonadotropin release (White et al., 2002; Amano et al., 2004) and also GnRH2 specially involve in sex behavior (Barnett et al., 2006). In addition, the actions of GnRH are mediated through binding to the membrane receptors (GnRHr). In previous studies (Selvaraj et al.,

2012) showed that GnRH1, GnRH2 and GnRH3 peptide levels were significantly increased during late phase of the oocyte development in the brain of the scombroid fish. Similarly, Nyuji et al., (2013) also investigated the involvement of *GnRH1* in the spawning of the jack mackerel (Trachurus *japonicus*). In addition, highest *GnRH1* expression was noted in grey mullet *(Mugil cephalus*), pejerrey (*Odontesthes bonariensis*), and grass pufferfish (*Takifugu niphobles*) during the spawning period (Nocillado et al., 2007; Guilgur et al., 2009; Shahjahan et al., 2010). Moreover, gold fish *GnRH2* is known to regulate the spawning behavior (Volkoff et al., 2009) and nest building behavior of dwarf gourami (Yamamoto et al., 1997). In addition, GnRH2 protein content and transcriptional levels were increased in turbot *(Scophthalmus maximus*) and goldfish during induced ovulation respectively. However, Dufour et al. (1993) has been investigated the brain GnRH1 levels implicate the regulation of pituitary gonadotropin rather than GnRH2 in European eel (*Aanguilla anguilla*).In addition to that Holland et al. (2001) investigated the GnRH1 and GnRH2 become its peaked level during the spawning time of the male and female striped bass.

In our study, *GnRH1* transcript level was significantly and robustly elevated in MN stage compare to the other all developmental stages (Fig.7) and GnRH2 also highly but not significantly elevated in the late phase of the oocyte development (LV, MN) (Fig8). Taken together, these results leads to suggest that the GnRH1 and GnRH2 are involve in final oocyte maturation and spawning behavior of the Japanese eel.

4.2. Differential involvement of gonadotrophin (GTHs) subunit and their receptors during ovarian development

Gonadotropins (FSHβ & LHβ) are pituitary glycoprotein hormones that regulate development of the gonads in vertebrates. In generally FSH mediates vitellogenesis and spermatogenesis, and LH regulates oocyte maturation and spermiation. However, most physiological studies on

teleost GTH have been conducted on LH, and FSH in fishes has not been fully elucidated. Each GTH is a heterodimer and composed of noncovalently linked alpha and beta subunits. The alpha subunit is identical, whereas the beta subunit is structurally distinct and confers specific hormonal functions (Pierce and Parsons, 1981).

According to the previous study of Perez et al. (2011) *FSHβ* mRNA was increased gradually along the oocyte development stages (PV to MV) in different temperature treated Japanese eels. Also, Huang et al. (2009) has been observed an increasement of *FSHβ* from PV to MV in marble eel (*Anguilla marmorata*) after treating with CPE plus LHRHa and hCG. Gomez et al. (1999), Suzuki et al. (1988) and Prat et al. (1996) also investigate the similar pattern during vitellogenesis in salmonids. Degani et al. (2003) also investigate the contribution of *FSHβ* during gametogenesis and vitellogenesis in European eel. Similar results were obtained in the current study, the *FSHβ* transcripts were increased during vitellogenesis and significantly elevated in MV stage and then significantly and gradually decreased in LV to AO stage. Comparatively very low *FSHβ* transcript levels could be found in final oocyte maturation and ovulation (Fig.12). Collectively, these results suggested that FSHβ regulates gametogenesis and vitellogenesis in Japanese eel.

However, in contrast to the our study, previous studies of (Okumura et al., 2001) investigate the highest FSHβ levels in the pre-vitellogenic stage and then gradually decrease during oocyte maturation of artificially induced Japanese eels. Similar results were obtained by Jeng et al. (2007) with significantly higher *FSHβ* transcriptional levels were observed in control compared to the SPH injected Japanese eels. They were suggested that, these phenomena may be due to stimulation of vitellogenesis when acclimatization of fresh water eels into sea waters prior to the SPE injection.

However, in current study there was no any correlation with brain GnRH1 or GnRH2 with the pituitary *FSHβ* transcripts during vitellogenesis in Japanese eel. Similar to our finding, Burger et al. (2004) indicate the lower importance of GnRH on FSH regulation in mammals.

In previous studies on Northern blot analysis reported that LHb could only be detected at late vitellogenic and maturation stage of Japanese eel and European eel (Nagae et al., 1996; Yoshiura et al., 1999; Degani et al., 2003). In addition to that, significant elevation of *LHb*transcript levels were detected during LV and final oocyte maturation in eels (Aroua et al., 2005; Jeng et al., 2007) similar to the other teleost (Noaksson et al., 2001; Lubzens et al., 2010).

Similar to those observations, in our study *LHb* mRNA expression was significantly increase during LV, MN and AO stages than other all stages and comparatively highest expression was shown in MN stage. However pre-vitellogenic, EV and MV stages *LHb* expression was extremely low with compare to the final development stages (Fig. 13.). These findings were collectively suggested that involvement of *LHb* during oocyte maturation and ovulation in Japanese eels. In order to that differential expression of *FSHb* and *LHb* suggest that their differential involvement during vitellogenesis and final maturation in Japanese eel.

Furthermore, brain *GnRH1* and *LHb*transcripts were synchronizely fluctuate along the oocyte development and significantly increased during oocyte maturation (MN). These findings suggested that direct involvement of brain *GnRH1* to release the pituitary *LHb* during final oocyte maturation and ovulation. Previous studies also suggested that GnRH controls the LH secretion in mammal (Gharib et al., 1990), eels (Montero et al., 1995) and other teleosts (Holland et al., 2001). In addition to that brain *GnRH2* transcript level also elevated at the late stage (LV & MN) of the gonadal development, suggested the involvement of final maturation in Japanese eel.

In generally FSH and LH act through binding to their specific receptors (FSHr and LHr) in gonads to induce steroidogenesis and gametogenesis of vertebrates. However, after the chronic administration of SPE, the gonadal *FSHr* transcripts were significantly increased in the MN stage when compare to all other developing stages (Fig 15). Atlantic cod (Mittelholzer et al., 2009) and Channel catfish (Kumar et al., 2001) provide somewhat similar finding to our observations gonadal *FSHr* . But their *FSHr* expression was increased after the ovulation. In addition supporting our observations, FSH expression detected in the pituitary reduced the *FSHr* in gonads of mice (Takase and Tsutsui, 1997). Nevertheless, our findings were in contrast with *FSHr* expression of some other teleost: FSHr was increased with ovarian growth of Salmon (Miwa et al., 1994), Tilapia (Hirai et al., 2001) channel catfish (Kumar et al., 2001) and Japanese eel (Jeng et al., 2007). However, there was no any correlation between pituitary *FSHβ* with gonadal *FSHr* during gonadal development interpreted using the results in our study of Japanese eel. Therefore, this phenomena may be due to the low specificity of FSHr with homologous recombinant hormones (FSH) (Yan et al., 1992; So et al., 2005) and also due to the capability of FSHr to bind with both FSH and LH ligands (Yan et al., 1992). Therefore, according to our results we can suggest that FSHr may bind with LH during oocyte maturation and whereby induce the final oocyte maturation of artificially maturated Japanese eels.

The significant induction of *LHr* transcripts in the ovary in MN stage can be corresponded with the final maturation of the Japanese eel (Fig.16). Nevertheless, *LHβ* and *LHr* transcript level shows the synchronized fluctuation along the oocyte development in Japanese eel. Both gens transcript levels were significantly increased during final oocyte maturation. Similar to the current work, there are several teleostean *LHr* expressions reached its peak level at the full grown stage including Zebrafish (Kwok et al., 2005), Channel catfish (Kumar et al., 2001), Atlantic cod (Mittelholzer et al., 2009), Atlantic halibut (Kobayashi et al., 2008). In

addition, Yan et al (1992) investigated that LHr is highly specific for LH. According to the homologous assays of Yan et al. (1992), So et al. (2005) and Vischer et al. (2003) exclusively LH can activate both FSHr and LHr in different teleost. Therefore, these finding collectively shows that *FSHr* and *LHr* collectively binds with *LHb* and stimulate the oocyte maturation in SPE injected Japanese eels.

4.3. A single type *CYP19A1* gene expression in BPG axis along gonadal development in the Japanese eel.

The CYP19 is mainly distributed in the brain, pituitary and gonad (Simpson et al., 1994) and main function is to convert the aromatizable androgens to eatrogens (Diotel et al., 2010). In contrast to the mammals, teleost possess two CYP19 genes differently expressed in ovary (CYP19A1a) and brain (CYP19A1b). However, interestingly only gonad type CYP19A1 could found from the ovary and brain in the eels (Jeng et al., 2012) that similar to the mammals.

In our study, brain *CYP19A1* transcript levels were significantly increased during MN and AO stages in SPE treated female eel (Fig.11). Supporting to this observation, higher *CYP19A1* expression was detected in brain during final oocyte maturation stage of several other teleosts including, Channel catfish (Kazeto and Trant, 2005), Black porgy (Lee et al., 2000), Rainbow trout (Menuet et al., 2003), Zebrafish (Goto-Kazeto et al., 2004). Moreover, our results are similar to the previous finding of Japanese eel (Jeng et al., 2012). In addition to that CYP19A1 expressed area in the hypothalamus of the brain is also a major site of the expression of GnRH1 (MacLusky and Naftolin, 1981; Khan et al., 1999; Vosges et al., 2010). In our study brain *GnRH1* and *CYP19A1* transcripts were synchronously increased during the final oocyte maturation. Supporting to our observation, *CYP19A2* was increased *GnRH1* during final stage of oocyte maturation in the brain of the channel catfish (Kazeto and Trant,

2005) and Atlantic croaker (Khan et al., 1999). Considering these findings together, the pre-ovulatory induction of the *CYP19A1* gene expression in the Japanese eel brain, could be an important agent in the regulation of *GnRH1* in the Japanese eel.

Furthermore, significant induction of pituitary *CYP19A1* can be seen in LV and MN stages in the Japanese eel (Fig.14.). Results similar to those obtained in the current work were found in the previous studies of, goldfish (Gelinas et al., 1998) and rainbow trout (Menuet et al., 2003). Jeng et al. (2012) also investigate the clear *CYP19A1* transcript level up regulation (not significantly) in pituitary during the late stage of gonadal development in the Japanese eel. In addition to that, relatively similar pattern of *LHβ* transcript fluctuation could be observed during the late stages of oocyte development in eels. Furthermore, *in vivo* (Kumar and Trant, 2004) and *in vitro* (Kazeto and Trant, 2005) results suggest that CYP19A2 in the pituitary correlated with the expression of *LHβ* gene regulation in channel catfish. Therefore, collectively our findings suggest that pituitary *CYP19A1* regulate the *LHβ* subunit gene expression in the late phase of the oocyte development in SPE treated Japanese eel.

The gonadal aromatase (CYP19) activity is critical in female gonadal differentiation and development in fish as other vertebrates (Jeng et al., 2005) and also changes in serum E2 is related to changes in follicular CYP19A activity (Ijiri et al.,1995). In commonly gonadal *CYP19A1A* transcript levels and enzymatic activity in ovarian follicles are significantly increased during vitellogenesis: as instance, rainbow trout (Tanaka et al., 1992), tilapia (Chang et al., 1997), red seabream (*Pagrus major*: Gen et al. (2001), Catfish (Aggarwal et al., 2014) and Atlantic croaker (*Micropogonias undulatus*: Nunez et al., 2006). But our finding is in contrast with the above investigations. In our study, there was no significant differences observed in *CYP19A1* transcript levels between previtellogenic, vitellogenic and AO stages but *CYP19A1* transcript levels were robustly and significantly increased during MN in ovary of eels (Fig.22). Supporting our observation aromatase activity is low in early to mid

vitellogenic stage New Zealand long finned eels (Lokman and Young., 1995). Jeng et al. (2005) also investigated the increased ovarian aromatase activity in late stage of vitellogenesis but not in the yellow or silver eels. In addition to that Northern blot analysis of Ijiri et al. (2003), showed the presence of low aromatase transcripts in the pre to late-vitellogenic ovary and comparatively higher levels were observed in the MN stage in Japanese eels.(Okumura et al., 2001; Ijiri et al., 2003) hypothesized that this phenomena may due to the abnormal maturation in final oocyte stage. But, according to our observations, liver Vtg transcript level was drastically decreased (more than 2/3transcripts) during the MN stage with compare to the LV stage. Therefore, extra vitellogenesis cannot be happened during MN stage in artificially induced Japanese eels. Therefore, we suggest that this phenomenon may be due to any other exogenous stimulation on gonad to induce *CYP19A1* transcript during final oocyte maturation (may be repeated injection of SPE). Therefore, we speculate that failure to decrease the aromatase activity after the LV may be influenced to suspend the ovulation of Japanese eels.

4.4. Differential contribution of steroid receptor subtypes during artificially induced ovarian maturation in Japanese eel

In the present study we observed the differently expressed estradiol receptor subtypes along the gonadal development in artificially matured eels. The relative expression of the *ERa* was significantly increased in the brain and ovary of the MN and AO eels (Fig.9, 17). *ERβ* transcript level was significantly elevated in AO stage of the gonad (Fig.18) Similar to the *ERa*, there was no significant variation could be seen in *ERb* during vitellogenesis and MN stages in gonad but significantly elevated after ovulation. Therefore, further researches are

required to understand the involvement of gonadal ERs, after the final oocyte maturation in Japanese eel.

ERs activate the E2 by binding the intracellular estrogen receptors. Supporting this observation, brain *ERa* was detected with relation to the brain aromatase activity in other vertebrates such as mammals (Martinez-Cerdeno et al., 2006), Japanese eel (Jeng et al., 2012), rainbow trout (Salbert et al., 1991) and Pejerrey fish (Strobl-Mazzulla et al., 2008). As same as the gonad, The *ERa* expression was co-exist with the *CYP19A1* expression. Moreover brain ERa stimulates the kisspeptine release in teleost (Franceschini et al., 2006; Roa et al., 2008) . Therefore, these results indicate the regulation of *CYP19A1* by *ERa* in eel.

When we compare the ARs along the BPG axis, brain *ARa* was significantly increased in LV and AO stage when compare with other stages (Fig.10). There was no any significant differences could be seen in gonadal *ARa* transcripts during pre-vitellogenic, vitellogenic and stages of the eels but after ovulation, it was significantly elivated (Fig.19). Recent study of Jeng et al., (2012) showed *ARa* mRNA significantly increased in the brain and ovary at the late phase of oocyte development in Japanese eel. However according to the Tosaka et al. (2010) highest gonadal *ARa* transcript was observed in control Japanese eels and slightly increased during EV stage and then decreased up to AO.

When we concern the gonad, *ARb* was not changed during pre-vitellogenic and vitelogenic stages and slightly elevated during MN and AO (Fig.19). In recently Jeng et al. (2012) investigated the significant upregulation in gonadal *ARb* transcript during late phase of the oocyte development in Japanese eel. In contrast to the our study, higher ARb transcript levels were expressed in immature ovary of Japanese eel (Ikeuchi et al., 1999) and short finned eel (Lokman et al., 2007). The biological significance of two different ARs is unclear in BPG

axis during oogenesis in teleost. Therefore, further researches are required to understand the clear involvement of ARs during oocyte development in Japanese eel.

4.5. Liver dependent vitellogenesis during oocyte maturation.

Oil droplet accumulation is important for energy generation and membrane biosynthesis in embryonic and larval development stages. Oil deposition is initiated in the pre-vitellogenic stage and continues up to oocyte maturation in teleost (Okumura et al., 2001). Vitellogenin is regulated by E2 which produced in the maturing female ovarian follicles.

In the current study, comparatively very low *Vtg* expression was observed in un-injected (pre-vitellogenic) females. But after the SPE injection *Vtg* mRNA expression was started to increase and it was robustly increased in LV stage and then significantly decreased in MN stage (Fig.23). Supporting to our observation, Okumura et al. (2002) obtained the similar *Vtg* transcript fluctuation during vitellogenesis in artificially induced Japanese eels. As well as Nelson and Habibi (2013) demonstrated the similar liver *Vtg* expression using the fish model. In contrast to our study, several eel studies have investigated that the serum Vtg increased in the EV and decreased during MV and LV stages (Okumura et al., 2001) (Okumura et al., 2001 and Chiba et al., 1994). This reason may be due to different techniques which used for analyze the target expression.

As similar to the *Vtg*, transcript levels of ERs also significantly increased in LV stage but not significantly decreased during LV stage (Fig.24 & 25). Generally Vtg are regulated by binding E2-ERs complex to the estrogen responsive elements at the promoter site of the Vtg (Wahli, 1988). In mammals the main functional contributor of the vitellogenesis is ERa than ERb (Leanos-Castaneda and Van Der Kraak, 2007). Similar to our study, in teleosts ERa induced the liver estradiol to stimulate the Vtg synthesis for staring the vitellogenin synthesis in liver (Nelson and Habibi, 2013). However, teleost ERb subtype has higher affinity for

estradol than ERa. When estradol tends to rise, it can act via ERb to increase both vitellogenesis and ERa expression (Nelson and Habibi, 2013). Increased estroiol in gold fish act via the ERb to increase the ERa which in turn increase the vitellogenin during their breeding season (Sohn et al., 1999). According to above findings, we can suggest upregulated ERa and ERb stimulated the Vtg expression in liver during vitellogenesis in artificially matured Japanese eels. However, further experiments are required to investigate the reason for continuous elevation of ERs in MN stage.

4.6. Serum E2 involvement during oocyte development

The present study serum E2 level highest in oocyte maturation stage (Fig.25.) Our findings were more similar to the previous studies of artificially matured Japanese eels (Ijiri et al., 1995; Sato et al., 2000b), New Zealand long finned eel (Lokman et al., 2001) and European eel (Chiba et al., 1994). However anguillid steroid profiling levels were rather different than several naturally maturing teleosts (Lokman et al., 2001).

According to the previous study of Smith et al. (2005) and Navarro et al. (2004), confirm that estradiol regulate the expression of Kiss1r as well as Kiss1 mRNA. Furthermore Li et al. (2007) investigated the E2 increase the activity of Kiss1 gene promoter region coupling with luciferase receptor with the presence of ERa. In our study brain ERa expression and serum E2 concentration synchronously elevated with the Kiss1 transcripts. Therefore, we also can suggest the increased serum E2 levels positively regulate the Kiss1 expression in artificially matured Japanese eels.

4.7. Role of the Kisspeptine1 and Kiss1r during oocyte development in artificially matured Japanese eel

Kisspeptine is encoded by Kiss1 gene and has an ability to activate the G-protein couple receptor (GPR54/Kiss1r) to secrete gonadotropins by stimulating GnRHs. Previous studies confirm that Kisspeptine directly activates GnRH via Kiss1r to stimulate the gonadotropins secretion (Felip et al., 2009; Plant and Ramaswamy, 2009; Pasquier et al., 2011). Previous study of Pasquier et al. (2011) also suggested the up regulated *Kiss1r* controls the GnRH in European eels. Another findings of Herbison et al. (2010), Parhar et al. (2004) and Colledge (2009) recognized the *kiss1r* is expressed in hypothalamus where the GnRH neurons are present. Therefore, these findings hints on the involvement of Kisspeptin on GnRH to stimulate the secretion of gonadotropins. On the other hand, positive feedback of E2 and *ERa* mediate *Kiss1* to upregulate the GnRH (Smith et al., 2006; Adachi et al., 2007) and release the LH from pituitary (Smith et al., 2006; Adachi et al., 2007; Robertson et al., 2009). However, comparatively there are few studies on teleostean kiss1. Biran et al. (2008) demonstrated that highest brain *Kiss1* transcripts are expressed during mature eggs stage in zebrafish. *In vivo* study of goldfish confirmed that short term incubation of Kisspeptin-10 induced the LH secretion from pituitary cells (Yang et al., 2010). In addition Felip et al. (2009) showed that sea bass Kiss1 and Kiss2 peptides can involve in gonadotropin secretion. Furthermore, *Kiss* (*Kiss1* & *Kiss2*) and GnRH (*GnRH1,GnRH2* & *GnRH3*) transcript levels were significantly increased in brain during final oocyte maturation and ovulatory period in scromboid fish (Selvaraj et al., 2012). In contrast to above all studies Pasquier et al. (2011) demonstrated the direct inhibitory action of Kisspeptin on pituitary LH.

To the best of our knowledge, we are the first group cloned the partial sequence (120bp) of *Kiss1* from family anguillidae. In addition to that we have examined the specific expression

of *Kiss1* in the brain and *Kiss1r* in brain & gonad during the gonadal maturation in Japanese eel. According to our finding, *Kiss1* and *Kiss1r* showed comparatively similar mRNA transcript fluctuation pattern during the experimental period. *Kiss1*and *Kiss1r* were significantly increased in the MN stage in brain and gonad compared to the other all development stages (Fig.26, 27&28). Meanwhile, *GnRH1* also increased during MN stage in brain suggested that involvement of *Kiss* on the *GnRH1* secretion and where by stimulate the LH surge in artificially matured Japanese eels. Similar to our finding Clarkson et al. (2008) also investigated the involvement of Kisspeptin and Kiss1r on GnRH/LH surge in pre-ovulatory mouse. Meanwhile, according to our finding brain ERa and serum E2 also highly elevated during final oocyte maturation. Our data support previous work showing estradiol enhanced the kisspeptin action on GnRH neuron activation in mice (Quaytman and Sharfstein, 1990) Moreover according to Franceschini et al. (2006)ERa and Kisspeptin co-express in same area in ovine brain. These findings suggested that positive feedback of estradiol stimulate to increase the *ERa* expression and whereby increase the *Kiss1* and *Kiss1r* expression. In addition, co-expressed Kiss1/Kiss1r complex may be involve to stimulate the expression of LH surge *via* GnRH1 during final oocyte maturation. Collectively we can suggest above mentioned pathway may play important role in local control of final oocyte maturation and ovulation by autocrine/paracrine mechanisms in SPE traded female Japanese eels (Fig.29).

5. Conclusion

In conclusion, this study provides the first evidence of kisspeptine partial sequence in anguillidae family and shows that there is a high degree of involvement during final oocyte maturation in SPE treated female Japanese eels.

As we explained above, *ERa, Kiss1, Kiss1r, CYP19A1* and *GnRH1* mRNA transcript levels were simultaneously and significantly increased in the MN stage in the brain of Japanese eel. In addition to that serum E2 level also drastically increase during this period. These findings suggest that positive feedback of increased serum E2 level increases the *ERa* in the brain and these *ERa* stimulates the secretion of *Kiss1* in the brain. Increased *Kiss1* and *Kiss1r* stimulate the *GnRH1* expression in the brain. Moreover, increased *CYP19A1* also positively stimulate the *GnRH1* secretion. These upregulated *GnRH1* give a positive feedback to pituitary to secrete LH in order to initiate the final oocyte maturation and ovulation in the gonad by stimulating the ovarian LHr. Pituitary *CYP19A1* also provide the positive feedback to increase the secretion of LH from the pituitary. According to these findings, collectively illustrate the main key steps of final oocyte maturation in Japanese eel as the first such report (Fig.29).

Our findings on gonadal *CYP19A1* and liver vitellogenin provide new logic on final oocyte maturation in contrast to the previous assumptions. According to our results, extra vitellogenesis cannot be occured during MN stage. Therefore, abnormal maturation cannot be happened during final oocyte maturation in artificially induced Japanese eels. Therefore, we speculate that increment of *CYP19A1 transcription* may aroused due to any other exogenous stimulation on gonad during final oocyte maturation (may be repeated injection of SPE). Therefore, we suggest that the failure to decrease the aromatase activity after the LV may be influenced to suspend the final oocyte maturation and ovulation of Japanese eels.

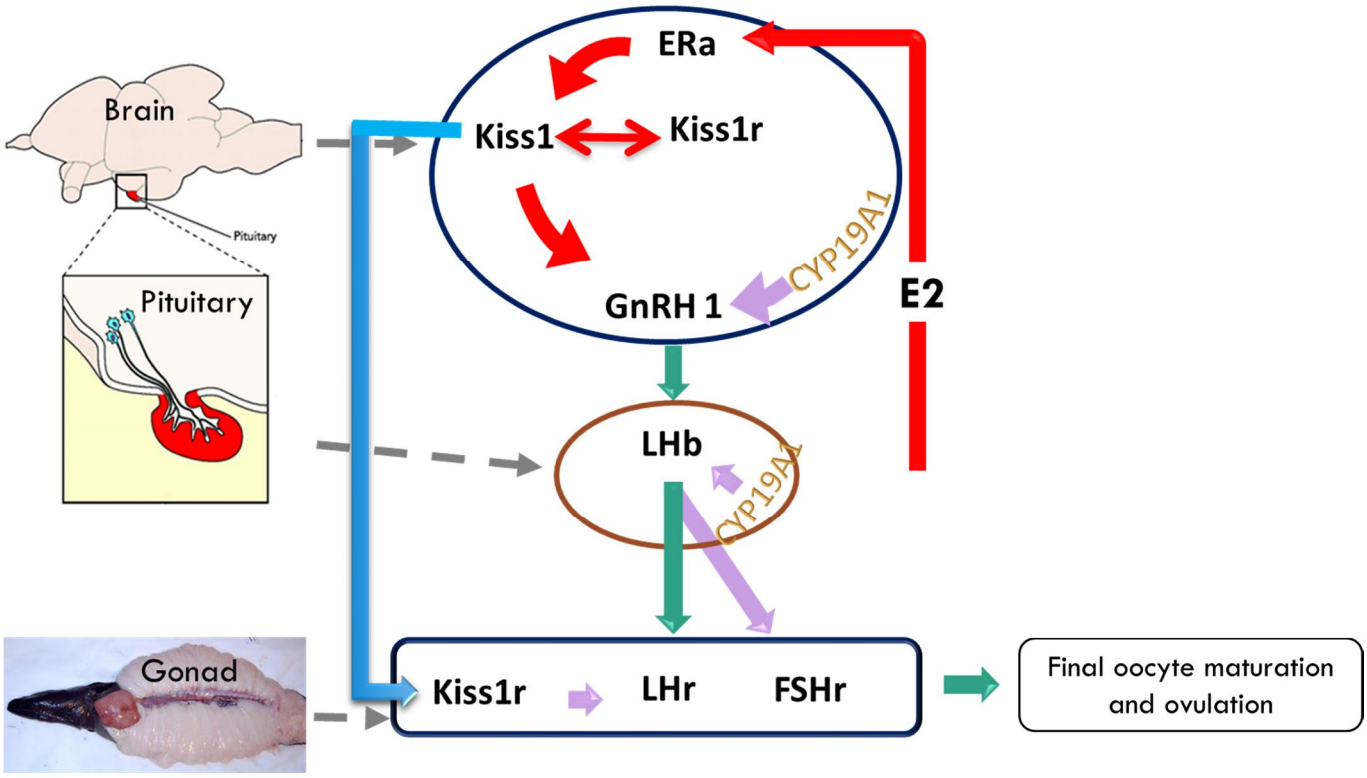

Fig.29. Schematic representation of the key steps of final oocyte maturation and ovulation of the artificially matured Japanese eel, *Anguilla japonica*. Red arrows indicate the novel finding of this experiment on anguilids.

6. References

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