Doctoral Dissertation (Censored)

博士論文 （要約）

Endocrinological studies on the estrogenic regulation of follicle stimulating hormone using a model teleost, medaka (*Oryzias latipes*)

（モデル生物メダカ（*Oryzias latipes*）を用いたエストロジェンによる濾胞刺激ホルモン制御に関する内分泌学的研究）

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Abstract

It is well known that the negative feedback systems play an indispensable role in the regulation of hypothalamus-pituitary-gonadal (HPG) axis, which regulates the gonadal functions, and prevents the waste of energy resources for survival and/or reproduction.

In vertebrates, gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) released from the pituitary stimulate the gonadal maturation. In recent years, accumulating body of evidence using various vertebrate species revealed the basic common principles and differences of the functions of each gonadotropin for gonadal maturation. As the common principle in females, FSH is necessary for the maturation of ovarian follicle throughout the folliculogenesis, while LH triggers the ovulation, which is the final step in folliculogenesis. In addition to this fundamental principle, it has also been shown that mammals show mammalian-specific pulsatile secretion of LH for the late folliculogenesis, which is strongly suggested to be acquired during evolution of mammals.

From these backgrounds, it follows that the understanding of the regulatory mechanisms of FSH but not LH will lead us to understand the general mechanisms of the negative feedback regulation of reproduction in vertebrates. In spite of the significance of FSH for reproduction, regulatory mechanism of FSH has not yet been understood in vertebrates probably because LH has relatively significant role in mammals including human. In the present study, I aimed to understand the

general regulatory mechanisms of FSH by utilizing non-mammalian model organism, medaka (*Oryzias latipes*). Medaka is one of the non-mammalian model organisms in which the genome editing tools are easily applied. Furthermore, despite its small size, medaka is amenable to surgical operation such as ovariectomy (OVX). In the previous study using medaka, it has been reported that the expression of FSH mRNA (*fshb*) is negatively regulated by estrogens, which is the most potent sex steroid hormone and well known for their role in the regulation of reproductive cycle in females. Therefore, it is important to understand how estrogens negatively regulate the production of FSH via their receptor(s) (estrogen receptor, ER).

In Chapter 1, to identify the most probable ER subtype that mediates the FSH negative feedback regulation, I generated knockout medaka of each ER (Esr1, Esr2a, and Esr2b) and analyzed their phenotype with special attention to reproductive functions in female medaka. First, the comparative analyses of the fertility and the expression level of *fshb* among each ER KO females revealed that *esr2a*$^{-/-}$ females show complete infertility and significantly high expression of *fshb* in the pituitary. Thus, it is suggested that Esr2a is necessary for fertility and estrogenic regulation of FSH in female medaka. Next, to analyze the causal relationship between the infertility and the high expression of *fshb*, I analyzed the morphology of the ovary, which is the organ to produce estrogens, in *esr2a*$^{-/-}$ females. Surprisingly, *esr2a*$^{-/-}$ females showed normally ovulated eggs in

their ovarian cavity, although they do not show oviposition. Moreover, it was also demonstrated that *esr2a*$^{-/-}$ females show normal sexual behavior without spawning. These results suggest that *esr2a*$^{-/-}$ females have some difficulties in the process of oviposition. Finally, I analyzed the histology of the oviduct in *esr2a*$^{-/-}$ females and revealed that *esr2a*$^{-/-}$ females show oviduct atresia. Therefore, I concluded that the complete infertility of *esr2a*$^{-/-}$ is caused by malformation of the oviduct. On the other hand, high expression level of *fshb* in *esr2a*$^{-/-}$ females also suggested the possibility that Esr2a may play a role in the estrogenic regulation of FSH, which is independent of oviduct atresia. From these results, I concluded that *esr2a*$^{-/-}$ female medaka may be a suitable model to study estrogenic negative feedback regulation of FSH.

In Chapter 2, I measured blood concentration of 17β-Estradiol (E2), which is the most potent and abundant estrogen in females. The concentration of E2 was 4-13 ng/ml in females and approximately 1 ng/ml in males. To elucidate the period required for the removal of endogenous E2 after OVX, I analyzed the chronological changes in the concentration of E2 after OVX. One or more days after OVX females show low E2 level comparable to the male. Finally, I compared three methods of E2 administration (feeding, water exposure, and intraperitoneal implantation), which had been used in previous studies. I used male medaka for examination of exogenous E2 administration because I demonstrated that intact male medaka show much lower E2 level than

that of sexually mature females and rather closer to that of OVX females. The medaka receiving a feeding administration of E2 showed transient increase in blood E2 concentration four hours after administration, and exogenous E2 drastically decreased within 12 to 24 hours. In contrast, the medaka that were exposed to water containing E2 or implanted with silicon block containing E2 showed unphysiologically high level of blood E2, which lasted for 12 to 24 hours after E2 administration. These results revealed that the feeding administration of E2 is the best method to mimic the endogenous E2 fluctuation in female medaka at least under the present conditions.

In Chapter 3, I performed physiological analyses aiming at the elucidation of the role of Esr2a, which was identified in Chapter 1 as the most probable candidate of ER involved in the estrogenic regulation of FSH, using *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ medaka. First, I applied feeding E2 administration to the OVX females of *esr2a*$^{+/-}$ or *esr2a*$^{-/-}$ and demonstrated that E2 administration successfully suppresses the expression of *fshb* in *esr2a*$^{+/-}$ females, while it failed to do so in *esr2a*$^{-/-}$ females. These results clearly revealed that Esr2a plays a crucial role in the estrogenic regulation of FSH. Next, I analyzed the site of action of *fshb* suppression induced by E2. To elucidate whether this estrogenic suppression of FSH requires the hypothalamic signal/s, I analyzed the effects of E2 for the regulation of *fshb* in the isolated pituitary. As a result, the isolated pituitary incubated with E2 for 20 hours showed significantly low expression of *fshb*. Moreover, I revealed that the isolated

pituitary incubated with E2 in *esr2a*$^{+/-}$ females shows significantly lower expression of *fshb* compared with control group, while *esr2a*$^{-/-}$ not. Therefore, I concluded that Esr2a expressed in the pituitary plays an indispensable role in the E2-induced down-regulation of *fshb* expression.

In the present study, I have clearly demonstrated for the first time that one of the specific ER, Esr2a, expressed in the pituitary plays an indispensable role in the E2-induced down-regulation of *fshb* expression, although the cellular site of action in the pituitary still remained uncertain. The present findings will pave the way to understand the general mechanisms of the estrogenic negative feedback regulation of FSH.

Table of contents

List of abbreviations

ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
Cy3	Cyanine 3
DIG	digoxigenin
DPO	Day of post OVX
E2	17β-Estradiol
ELISA	Enzyme-Linked Immunosorbent Assay
ER	estrogen receptor
ESR1/Esr1	estrogen receptor α
Esr2a	estrogen receptor β1
Esr2b	estrogen receptor β2
FL	fluorescein
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
gRNA	guide RNA
GSI	gonadosomatic index
HE	hematoxylin and eosin
HPG	hypothalamus-pituitary-gonadal
HRP	horseradish peroxidase
ISH	*in situ* hybridization
KO	knockout
LH	luteinizing hormone
LHR	LH receptor
NBT	nitro blue tetrazolium chloride
OVX	ovariectomy
PBS	phosphate buffered saline
PFA	paraformaldehyde
PMSG	pregnant mare serum gonadotropin
Ptger4b	prostaglandin E2 receptor 4b
PV	post-vitellogenic phase
qPCR	quantitative PCR
rLH	recombinant LH

rps13	*ribosomal protein s13*
RT-PCR	reverse transcription PCR
SSC	saline sodium citrate buffer
WT	wild type
ZT	Zeitgeber time

General Introduction

Generally, the negative feedback regulation underlies numerous biological processes to prevent possible homeostatic failures. This regulatory mechanism is not only indispensable for the survival of the organisms but also important for the reproductive regulation. Usually, the energy resource of the organism is limited, and it necessitates efficient distribution of the energy resource to their own survival or their offspring. The negative feedback mechanism in the reproductive regulation probably plays a crucial role in this efficient distribution of a nutrition.

In vertebrates, there is a general agreement that the hypothalamus-pituitary-gonadal axis is important for reproductive regulation, and there are two types of hormones in the pituitary that are involved therein: follicle stimulating hormone (FSH) and luteinizing hormone (LH). These two gonadotropins are considered to be important for the gonadal maturation. It has been reported that the fully-matured ovaries produce estrogens, and the estrogens reduce the secretion of gonadotropin(s) from the pituitary (1, 2), which is called as the estrogenic negative feedback regulation of reproduction. To my knowledge, such negative feedback regulation of reproduction is widely observed in various vertebrate species (3-7). This estrogenic feedback regulation has been intensively studied in mammals. It has been generally accepted that hypothalamic gonadotropin releasing hormone (GnRH) neurons stimulate the expression and secretion of two types of gonadotropins from the pituitary (8, 9). Especially, in females, FSH stimulates the oocyte maturation in relatively early stages of folliculogenesis. In the later stages of folliculogenesis, it

has been reported that LH released in a pulsatile manner plays an essential role. Mature follicles release estrogens into the bloodstream so that the estrogens can work as the signal of follicle maturation, and the estrogens suppress the production and release of gonadotropins in a negative feedback manner. In addition to this negative feedback system, positive feedback system also exists. When the follicles reach full maturation, they release high dose of estrogens, which enhance the secretion of LH (LH surge) and triggers ovulation. Although the negative and positive feedback regulations are considered to be dependent on distinct hypothalamic areas in mice, the both are mediated by estrogen receptor (ER).

In the previous studies using rodents (10, 11), it was demonstrated that one of the subtypes of ER, Esr1, expressed in the kisspeptin neurons in the ARC and AVPV mediates the pulsatile and surge-like LH secretion, respectively. However, accumulating body of evidence strongly suggests that this kisspeptin-mediated feedback system only applies to mammals, because kisspeptin is not considered to be involved in the regulation of gonadotropin release in non-mammalian vertebrates (12-14). In fact, it has been reported that birds lack kisspeptin and kisspeptin receptor genes. Nevertheless, they can produce their offsprings without kisspeptin.

Furthermore, in non-mammalian vertebrates, not only the mechanism of negative feedback regulation in the reproductive system but also the role of each gonadotropin in the regulation of ovaries are substantially different from that of mammals. In recent years, gene knockout studies

using teleosts clearly revealed that the signal transduction cascade that is mediated by FSH and FSH receptor plays an indispensable role in folliculogenesis, whereas the cascade mediated by LH and LH receptor only triggers a series of ovulatory processes（15-18）. Therefore, it is strongly suggested that both the regulatory mechanism of LH in the reproductive system and the contribution of LH to the late folliculogenesis is specific to mammalian species.

So far, because of apparent dominant functions in mammals of LH in late folliculogenesis compared to FSH, the regulatory mechanism of FSH by estrogens is widely unknown in mammals (19). However, considering the significance of FSH in folliculogenesis in non-mammalian vertebrates as described above, it follows that the elucidation of the regulatory mechanism of FSH in non-mammalian species may give important clues to understanding the general mechanisms of folliculogenesis in vertebrates. Similarly, the elucidation in non-mammalian species of the mechanism for negative feedback regulation of FSH mediated by estrogens may also give important clues to understanding the general mechanisms of negative feedback regulation of FSH in vertebrates, which have not been well understood in any species.

In the present thesis, I aimed at understanding the general regulatory mechanisms of FSH by utilizing a non-mammalian model organism, medaka (*Oryzias latipes*). Medaka possesses various advantages as a model organism: small, easy to breed and apply the gene editing tools such as CRSPR/Cas9 systems. Moreover, despite its small size, medaka is amenable to surgical operation

such as ovariectomy (OVX), which is necessary for the manipulation of endogenous estrogens. Medaka spawns regularly every day, and we can easily analyze the reproductive cycles in females. Because medaka is a seasonal breeder, we can also switch their reproductive states between breeding and non-breeding only by changing the light period. Therefore, medaka is an advantageous model organism for the analysis of reproductive system in non-mammalian vertebrates, and I used it to understand the general mechanism of the negative feedback regulation of FSH.

In Chapter 1, to identify the most probable ER subtype for the estrogenic regulation of FSH, I generated the ER knockout (KO) medaka and analyzed their phenotypes to analyze the functional role of three ER subtypes, Esr1, Esr2a, and Esr2b, in reproduction of female medaka. Among the three subtypes of ER KO medaka, I found that Esr2a is the most probable candidate that is involved in the negative feedback regulation of FSH, because quantitative RCR analysis revealed that *esr2a*$^{-/-}$ females show significantly high expression of FSH mRNA (*fshb*).

In Chapter 2, I analyzed the blood 17β-Estradiol (E2, the most potent and abundant estrogens) concentration because there were very few reports about endogenous E2 levels in medaka. I found that female medaka show diurnal changes in E2 concentration and males show constantly low level of E2 compared to females. Next, I examined for the first time whether the surgical ablation of ovary (OVX) successfully removes endogenous E2 in female medaka. One or more days after

OVX females show significantly low E2 comparable to the males. Moreover, I examined the best method of E2 administration for mimicking diurnal changes of E2 in female medaka. Among the three methods, feeding administration, water exposure, and intraperitoneal implantation, I demonstrated that feeding administration of E2 is most likely the best method, because the blood concentration of E2 showed instantaneous increase and then gradual decrease after feeding, and it is easy to mimic the physiological diurnal fluctuation of E2 in intact females by multiple administration.

In Chapter 3, I experimentally demonstrated that Esr2a is important for the negative feedback regulation of FSH and analyzed the site of action of this negative feedback regulation. First, I analyzed the expression level of *fshb* of OVX females and found that significantly higher level of *fshb* than the intact females can be reached 60 hours or more after OVX. Second, I applied the feeding administration of E2 and demonstrated that E2 suppresses the expression level of *fshb* 72 hours after OVX. Finally, by applying this OVX+E2 protocol to the *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ females, I demonstrated for the first time that Esr2a is important for the suppression of *fshb* induced by E2 administration. Further analyses aiming at elucidating the site of action of the estrogenic down-regulation of *fshb* revealed that Esr2a expressed in the pituitary plays an indispensable role in this estrogenic regulation.

Chapter 1

Gene knockout analysis reveals essentiality of estrogen receptor β1 (Esr2a) for female reproduction in medaka

Abstract

In vertebrates, sex steroids play crucial roles in multiple systems related to reproduction. In females, estrogens and their receptor estrogen receptor (ER or Esr) play indispensable roles in the negative sex steroid feedback regulation of pituitary gonadotropin secretion, which prevents excessive development of ovarian follicles. However, the mechanism of this feedback regulation of a gonadotropin, follicle stimulating hormone (FSH), which is essential for folliculogenesis throughout vertebrates, is poorly understood.

In Chapter 1, I generated knockouts of nuclear estrogen receptors by CRISPR/Cas9 system in a model teleost medaka, which is suitable for the study of endocrine control and behavioral assays, and analyzed the fertility, sexual behavior, and expression level of gonadotropin mRNA in each knockout line. Among the estrogen receptor knockout medaka, *esr2a*$^{-/-}$ females showed significantly high expression of FSH mRNA (*fshb*). Thus, it is suggested that Esr2a may be involved in the negative feedback regulation of FSH production. In addition to this, I found that the knockout of each gene, *esr1* and *esr2b,* did not affect the fertility, folliculogenesis, and the expression of gonadotropin genes in females. Furthermore, I also found that *esr2a*$^{-/-}$ females showed oviduct atresia, which causes complete infertility. Interestingly, *esr2a*$^{-/-}$ females showed apparently normal sexual behavior but failed in oviposition in response to male courtship because of the oviduct atresia. This failure in oviposition indicates that physical readiness and motivation

of sexual behavior is independently controlled.

Introduction

Coordinated regulation of gonadal functions and sexual behavior by neural and endocrine systems is essential for reproduction. In female vertebrates, a group of sex steroid hormones, estrogens, which are mainly produced by ovarian granulosa cells, are suggested to play crucial roles in multiple systems (11, 20-24).

One of the most important roles of estrogens is the feedback regulation of gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) secretion from the pituitary. During the folliculogenesis in female vertebrates, estrogens released from mature gonads suppress the secretion of gonadotropins from pituitary in a negative feedback manner, to prevent excessive development of ovarian follicles. In addition to such neuroendocrine regulation, estrogens are suggested to be involved in a wide variety of sex-related phenomena, such as sexual differentiation, sexual behavior, and development of female-specific organs.

Majority of estrogenic actions are suggested to be mediated by nuclear estrogen receptors (ERs), which belong to the steroid hormone receptor family (25). In vertebrates, two subtypes of ER, Esr1, also referred to as ERα, and Esr2, also referred to as ERβ (26-28), have been generally conserved (29-31).

In mammals, it has been strongly suggested that Esr1 expressed in hypothalamic kisspeptin neurons plays essential roles in this feedback regulation of folliculogenesis by changing GnRH

release from hypophysiotropic GnRH neurons and pulsatile release of LH from the pituitary (11, 21, 22, 32, 33). However, growing body of evidence strongly suggests that the kisspeptin-mediated gonadotropin release may be a mammalian-specific mechanism (12-14). Therefore, the role of estrogen receptors in the steroid feedback in mammals cannot be applied to that of vertebrates in general, and the general mechanisms of steroid feedback regulation by estrogens in vertebrates are still unknown.

A previous report (34) has tried to clarify the function of ERs in a model teleost zebrafish by knockout (KO) technology. However, because of their sexual plasticity, ER KO zebrafish easily underwent sex change to males, and female functions could not be analyzed. On the other hand, the sex is strictly determined by the sex determination gene, *dmy* in medaka (*Oryzias latipes*) (35), which led us to successfully analyze the contribution of each ER subtype to the female reproductive functions. It has been reported that teleosts possess three ERs. Esr1 and Esr2 were duplicated in the early gnathostome lineage, and Esr2 was further duplicated in the third-round whole genome duplication event in teleosts to give rise to Esr2a and Esr2b. They are considered to share roles of their common ancestor Esr2 after this sub-functionalization (36-38).

In the present chapter, I established ER KO lines for each ER subtype by using CRISPR/Cas9 and analyzed their phenotypes from several viewpoints including fertility, ovarian morphology, and expression of gonadotropin genes in females of each ER KO line.

Materials and methods

Animals

The animals were maintained and used in accordance with guiding principle for the Use and Care of Experimental Animals of the University of Tokyo. Male and female wild-type d-rR medaka and all the ER KO medaka were maintained under a 14-hour light and 10-hour dark photoperiod (light on and off time was 08:00 to 22:00 or 09:00 to 23:00) at a water temperature of 27°C. The fish were fed two to four times a day with live brine shrimp and flake food. All experiments were conducted in accordance with the protocols approved by the animal care and use committee of Graduate School of Science, the University of Tokyo (Permission number, 15-3).

Generation of ER KO medaka lines by CRISPR/Cas9

I generated ER KO medaka using CRISPR/Cas9. Mixture of Cas9 mRNA, tracer RNA, CRISPR RNA, GFP mRNA diluted with PBS and 0.02% phenol red (final concentrations: Cas9 mRNA and tracer RNA, 100 ng/μl; CRISPR RNA, 50 ng/μl; GFP mRNA; 5 ng/μl) was injected into the cytoplasm of one- or two-cell-stage embryos (F0). Guide RNA (gRNA) for *esr1* was transcribed by using a gRNA expression vector constructed from DR274 (Addgene, Cambridge,

MA). CRISPR RNA and tracer RNA for *esr2a* or *esr2b* were synthesized by a commercial company (FASMAC, Atsugi, Japan). The injected embryos were intercrossed for the identification of the germ line founders with mutations in each target locus. Their offsprings were then outcrossed for the identification of the individual founder fish. Heterozygous transgene carriers in the F1 generation were identified by the melting curve and/or sequence analyses using primers described in the primer list (Table 1-1). To obtain homozygous transgenic offspring, the carriers were crossed with each other.

Comparison of the number of eggs spawned

Female medaka (age: three to four months) of WT and *esr1*$^{+/-}$, *esr1*$^{-/-}$, *esr2a*$^{+/-}$, *esr2a*$^{-/-}$, *esr2b*$^{+/-}$, *esr2b*$^{-/-}$ were mated with WT male medaka as one to one male-female pair (Day 1). After habituation, the number of eggs spawned was counted from Day 17 to Day 22.

Gonadosomatic index (GSI) calculation

For the analysis of GSI of each KO line (age: three to four months), I used females that had been separated from males from the previous night of the sacrifice to avoid egg release (spawning). After confirming that they did not spawn, I dissected the ovary and measured the ovarian weight at noon. The GSI was calculated as the ovary weight/body weight x 100 (%).

Histological analysis of ovaries

Female medaka (age: three to four months) were deeply anesthetized by MS-222, and ovary samples were then treated with a Bouin's fixative at room temperature for one to three days. After fixation, each sample was dehydrated with ethanol or methanol, cleared with xylene and embedded in paraffin. Sections of 8-9μm thickness were stained with hematoxylin and eosin (HE). The sections were observed under an upright microscope (DM5000B; Leica Microsystems, Wetzlar, Germany). Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany).

Analysis of sexual behavior of *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ females

Four to five months old female medaka of *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ were mated with sexually mature male medaka one by one. Movies were taken using raspberry pi and raspberry pi camera V2 (Raspberry Pi Foundation, Cambridge, UK) from ten minutes before light on to one hour after light on time, during which spawning of medaka usually occurred. I analyzed the sexual behavior for nine days.

Analysis of gonadotropin expression in the pituitary

Female medaka of *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ (age: three to four months) were deeply anesthetized and their pituitaries were collected for quantitative PCR (qPCR). Total RNA was extracted from the pituitaries using the NucleoSpin RNA plus (TaKaRa, Shiga, Japan) according to the manufacture's instruction. Total RNA samples were then reverse transcribed by PrimeScript^{TM} RT Master Mix (TaKaRa). For qPCR, the cDNA was amplified by KAPA SYBR FAST qPCR kit (Nippon Genetics, Tokyo, Japan) with AriaMX Realtime PCR System (Agilent Technologies, Santa Clara, CA). The temperature profile of the reaction was 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds. The PCR products were verified using melting curve analysis. The data were normalized by a housekeeping gene, *ribosomal protein s13* (*rps13*). The primer pairs used in the real-time PCR are listed in the primer list (Table 1-1).

Histological analysis of oviducts

Female medaka were deeply anesthetized by MS-222, and whole body samples were then treated with a decalcifying solution containing 10% formic acid and 70% ethanol or methanol. Then, decalcified whole bodies were dehydrated with ethanol or methanol, cleared with xylene and embedded in paraffin. Sections of 8-9 μm thickness were stained with HE. The sections were

observed under an upright microscope (DM5000B; Leica Microsystems, Wetzlar, Germany). Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany).

Gross anatomical examination of ovaries

WT, *esr2a*$^{+/-}$, and *esr2a*$^{-/-}$ female medaka were mated with WT male medaka as one or two females to one male. After habituation, I observed their ovarian morphology and took pictures with a digital camera (α-7S; SONY, Tokyo, Japan) attached to a stereoscopic microscope (SZX12; OLYMPUS, Tokyo, Japan).

***In vitro* follicle culture and PMSG treatment**

I used the technique of *in vitro* follicle culture for medaka, which has been established previously (39). To avoid in vivo LH effects, preovulatory follicles of four-months-old WT or five to six-months-old *esr2a* KO heterozygotes and homozygotes female medaka were isolated from female ovaries by using forceps 18h before ovulation. The isolated follicles were incubated at 25°C for 12h in a 35mm culture dish containing M199 medium (2ml per dish) (Sigma-Aldrich, St. Louis, MO) and 50-100μM gentamycin. I used the incubation time used in the previous study that analyzed expression of prostaglandin E2 receptor 4b (*ptger4b*), which plays essential roles in

ovulation and is induced by recombinant LH (rLH) (40-42). Here, I used pregnant mare serum gonadotropin (PMSG), which act as LH in medaka (39), in place of rLH, because it is easily available and the concentration can be strictly defined. The follicles from three to four fish ovaries were pooled and divided into several groups. Half of the pooled follicles were incubated with 50IU of PMSG (PMSG+) and the remaining half were incubated with a control medium containing the same volume of solvent (PMSG-). After incubation, total RNA was extracted from the follicle. The total RNA was reverse transcribed for real-time PCR analysis. Protocols of RNA extraction and quantitative PCR (qPCR) were the same as described above for the pituitary analysis.

Statistics

The number of spawned eggs, and GSI of WT and ER KO were analyzed by Steel's test, a nonparametric multiple comparison with WT control. The qPCR data were expressed as mean ± SEM. The expression levels of *fshb* or *lhb* were analyzed by Tukey-Kramer test or t-test. The expression levels of *ptger4b* and *lhr* were analyzed by Welch-Aspin test or Tukey-Kramer test.

Results

Esr2a$^{-/-}$ female medaka show complete infertility

The designed CRISPR guide RNAs successfully cleaved targeted sites of each gene and induced frameshifts for each gene. After incross and/or outcross with wild type (WT), sequence analysis was performed in the targeted genes of each ER KO medaka (Fig. 1-1). I deduced the sequences of amino acid in each ER KO medaka (Fig. 1-2, 1-3, and 1-4). The domain structures of medaka's ERs have been predicted in the previous report (43), and I confirmed that each ER has lost its functionality. According to the fact that all homozygotic males were fertile, I analyzed only KO females of each ER KO in the following analyses. In the present study, I used offsprings of homozygous males and heterozygous females, which includes theoretically the same number of homozygotes and heterozygotes with non-biased backgrounds in a single tank.

I counted the number of eggs spawned by WT, hetero (*esr*$^{+/-}$), and homo (*esr*$^{-/-}$) KO female medaka of each ER subtype to analyze their fertility. The mean number of eggs spawned by each genotype during the six day analysis was as follows: WT, 16.0 ± 1.4; *esr1*$^{+/-}$, 7.1 ± 2.3; *esr1*$^{-/-}$, 7 ± 1.4; *esr2a*$^{+/-}$, 11.6 ± 2.1; *esr2a*$^{-/-}$, 0; *esr2b*$^{+/-}$, 10.3 ± 2.7; *esr2b*$^{-/-}$, 4.61 ± 2.3 (Fig. 1-5A, the number of eggs spawned in each day is shown in Fig. 1-5B-H). I found that *esr2a*$^{-/-}$ female medaka were completely infertile (*: $P<0.05$, compared with WT), while the others could spawn more or less normally. In agreement with the description in a recent review article (44), some of the adult

females of *esr2a*$^{-/-}$ showed abnormal bodily swelling. This abnormal swelling is probably because of the failure in oviposition due to the defective oviduct formation, which caused the accumulation of eggs.

Next, I analyzed gonadosomatic index (GSI) to assess the maturity of the ovaries. There was no significant difference among them (Fig. 1-6A; WT, 15.9 ± 0.89; *esr1*$^{+/-}$, 10.6 ± 0.92, P = 0.094; *esr1*$^{-/-}$, 11.5 ± 2.4, P = 0.5; *esr2a*$^{+/-}$, 16.6 ± 1.3, P = 1; *esr2a*$^{-/-}$, 16.2 ± 5, P = 0.7; *esr2b*$^{+/-}$, 11.9 ± 1.2, P = 0.18; *esr2b*$^{-/-}$, 11.8 ± 0.97, P = 0.12; compared with WT). For further analysis of the phenotype of each KO line, the ovaries of ER KO medaka were sectioned and were histologically examined by hematoxylin and eosin (HE) staining (Fig. 1-6B). As predicted by their GSI, ovaries of each ER KO line of medaka fully developed to show follicles of post-vitellogenic phase (PV). Thus, all three subtypes of ERs were shown to be dispensable for folliculogenesis.

I next examined their sexual behavior to analyze the cause of the infertility of *esr2a*$^{-/-}$ females. Interestingly, *esr2a*$^{-/-}$ females showed normal acceptance behavior in response to the male courtship and showed crossing with the approaching males (Fig. 1-7, n=4, also see Supplementary Movie 1 and 2 in Kayo et al., 2019; https://doi.org/10.1038/s41598-019-45373-y (45)). The courtship behavior of medaka consists of the following four steps. First, the male swims underneath the female. Second, the male swims with a characteristic movement called “quick circle” in front of the female (Supplementary Movie 1 (*esr2a*$^{-/-}$), 00:05-00:07; Supplementary

Movie 2 (*esr2a*$^{+/-}$), immediately after the start of movie 2). Third, the male wraps around the female's body using male's dorsal and anal fins (Supplementary Movie 1, 00:15-01:03; Supplementary Movie 2, 00:19-01:12), and finally, the male leaves the female (Supplementary Movie 1, 01:04; Supplementary Movie 2, 01:13). Although *esr2a*$^{-/-}$ females showed all of those steps of normal courtship behavior, they failed to perform oviposition (Table 1-2).

Esr2a$^{-/-}$ females show significantly high expression of _fshb_

To analyze gonadotropin (*fshb* and *lhb*) expression in the pituitary of KO females in each ER subtype, I performed qPCR analysis. Among the experimental group including hetero and homo zygotes of *esr1, esr2a or esr2b* KO lines and wild type, *esr2a*$^{-/-}$ females showed significantly higher *fshb* expression than the others except *esr1*$^{-/-}$ (Fig. 1-8A, a VS b; $P<0.05$, a VS bb; $P<0.01$). I also analyzed the expression level of *lhb*, and revealed that there was no significant difference among each ER KO line and WT (Fig. 1-8B).

Esr2a$^{-/-}$ females show atretic oviduct

To elucidate the reason for the failure of oviposition in spite of the normal sexual behavior of *esr2a*$^{-/-}$ females, I examined histological sections of the oviduct in *esr2a*$^{+/-}$ and *esr2*a$^{-/-}$ females. Adult *esr2a*$^{+/-}$ females (age: four to five months), which spawn every day, showed oviduct with

complete opening (Fig. 1-9Aa, Ab). In contrast, *esr2a*$^{-/-}$ females of the same age showed atretic oviduct (Fig. 1-9Ac, Ad, asterisks). In these medaka, a structure that was strongly stained by eosin was observed in the oviduct (Fig. 1-9Ac, Ad, asterisk), which has not been described in a previous study using wild type medaka during the development of oviduct (46). Although it could not be determined whether this structure appears transiently in the normal development of the oviduct, I did not observe such structure at the same stage of *esr2a*$^{+/-}$ medaka.

I analyzed the gross morphology of ovaries of randomly selected adult female medaka (age: four to five months) (Fig. 1-9B). The percentage of females that possessed ovulated eggs with normal morphology in *esr2a*$^{-/-}$ at the age of four to five months were analyzed (Fig. 1-9Bb, Bd). For comparison, I used *esr2a*$^{+/-}$ of the same age (Fig. 1-9Ba, Bc). Among the examined *esr2a*$^{-/-}$ females, three out of four young adult females showed ovulated eggs with normal morphology (Fig. 1-9Bb, arrowheads; Fig. 1-9Bd, black sector), and only one showed anovulation (Fig. 1-9Bd; white sector). Although the representative picture of an *esr2a*$^{+/-}$ ovary (Fig. 1-9Ba) does not contain ovulated eggs, it is considered to be because of the fact that *esr2a*$^{+/-}$ females spawned normally to give rise to fertilized eggs. Therefore, they were judged to be able to ovulate.

These results of behavioral and histological analyses clearly indicate that the infertility of *esr2a*$^{-/-}$ is due to the oviduct atresia. Eventually, older females (age: six to eight months) of *esr2a*$^{-/-}$ showed complete atresia of oviduct (Fig. 1-9Ca-d) and degenerated eggs in their ovary

(Fig. 1-9Da-e), while those of *esr2a*$^{+/-}$ or WT showed normal oviduct and eggs. This phenotype suggests that ovulated eggs, which could not be oviposited because of the oviduct atresia and accumulated in the ovary, have degenerated. Interestingly, at this stage, the structure strongly stained by eosin was not observed. It is a very interesting future topic to know the identity and properties of this structure, such as the timing and the reason of its formation and disappearance.

Although the mechanism how Esr2a mediates oviduct formation or maintenance is unknown, the results obtained in the present study indicate that Esr2a is essential for the functional oviduct. Thus, these established *esr2a*$^{-/-}$ female medaka may be useful for the future detailed analyses of the mechanism of the oviduct formation.

Effect of pregnant mare serum gonadotropin (PMSG) on the expression of prostaglandin E2 subtype 4 receptor (*ptger4b*) in the preovulatory follicle

Although estrogenic regulation of LH expression was normal in *esr2a*$^{-/-}$, it is important to elucidate if *esr2a*$^{-/-}$ can undergo normal process of final oocyte maturation and ovulation in response to LH. Here, I used *in vitro* follicle culture experiment, which has been established by Ogiwara et al. (39). According to the previous reports, it has been proposed that *ptger4b* expression is induced by LH-primed signal cascade in medaka preovulatory follicles (40-42). In the present study, I used PMSG, which acts as an LH receptor agonist in medaka ovary (39).

PMSG treatment in preovulatory follicles of WT female medaka successfully induced *ptger4b* expression in my experimental conditions (Fig. 1-10A, $P<0.05$, Welch-Aspin test). There was no significant difference in LH receptor (*lhr*) expression regardless of the presence of PMSG (Fig. 1-10B, Welch-Aspin test). I then applied the same protocol to preovulatory follicles of *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ female medaka and revealed that *ptger4b* expression was increased by PMSG in the both (Fig. 1-10C, D, $P<0.05$; Welch-Aspin test). There was no significant difference in *lhr* expression regardless of the expression of *esr2a* (Fig. 1-10E, Tukey-Kramer test).

Discussion

In the present chapter, I analyzed the functional roles of each ER subtype in the female reproduction by using ER KO lines of medaka generated by CRISPR/Cas9. Here, I demonstrated that Esr2a is one of the probable candidates that play a major role in the negative feedback regulation of FSH production because *esr2a*$^{-/-}$ females show high expression of *fshb*. Moreover, it is suggested that Esr2a is essential for oviduct formation, which is a secondary sexual characteristic of females and is critical for oviposition.

Esr2a$^{-/-}$ female is a useful model organism for the analysis of negative feedback regulation in FSH production

To analyze the contribution of each ER for gonadotropins expression, I compared the relative

expression levels of gonadotropin among ER knockout females (Fig. 1-8) and revealed that *esr2a*$^{-/-}$ females show significantly high expression of *fshb*. As described in the previous study (47), estrogens play a role in the suppression of *fshb* expression, and thus it is suggested that the dysfunction of Esr2a may cause malfunction of the negative feedback regulation of FSH induced by estrogens. In the present chapter, I found that the knockout females of *esr1* did not show any significant dysfunction in several female reproductive phenotypes that were examined (34, 48, 49). As described above, Esr1 is essential for the regulatory mechanisms of negative feedback in reproductive systems. Here, I demonstrated for the first time that Esr2a but not Esr1 may be involved in these regulatory mechanisms and characterized *esr2a*$^{-/-}$ females as a useful model animal to elucidate the estrogenic regulation of FSH.

Esr2a is indispensable for female fertility by mediating oviduct formation in medaka

In the present chapter, we revealed that *esr2a*$^{-/-}$ adult females showed atretic oviduct (Fig. 1-9Ac, Ad, Cc, Cd). This phenotype that *esr2a*$^{-/-}$ females produce no fertilized eggs is directly caused by this failure in oviduct formation. Previous studies using medaka suggested that estrogens are involved in the development of female-specific genital morphology (46, 50). The present study is consistent with this, and here I clearly identified the responsible ER subtype for the first time. Interestingly, the ovarian cavity whose formation is also dependent on estrogens normally

developed in *esr2a*$^{-/-}$ females (Fig. 1-9Cc, Cd). These results suggest that Esr2a is essential for the formation of oviduct but not for that of ovarian cavity. Furthermore, in spite of this physical misformation, *esr2a*$^{-/-}$ females displayed normal sexual behavior in response to male courtship (Fig. 1-7) in the presence of normally ovulated eggs in their ovary (Fig. 1-9Bb, Bd, Db, De). These facts strongly suggest that the neural circuits for sexual behavior are formed independently from the peripheral readiness for oviposition. The knockout models can be useful for future studies of the mechanism of sexual behavior.

Esr2a$^{-/-}$ females show normal LH responses and ovulation

Female KO of each ER subtype appeared to show normal folliculogenesis. As folliculogenesis is regulated by FSH in medaka (18), it is evident that the FSH responses in the ovary are not disrupted by KO of any single ER subtype. In addition, LH responses of *esr1*$^{-/-}$ and *esr2b*$^{-/-}$ are considered normal, because these KO females could ovulate. On the other hand, in *esr2a*$^{-/-}$, morphological observation demonstrated that most of relatively old females showed ovulated eggs with abnormal morphology, although I found a few exceptions showing normally ovulated eggs in their ovaries (Fig. 1-9D). To examine the normality of the ovarian signaling pathway from LH reception to ovulation in the *esr2a*$^{-/-}$, I analyzed LH response of preovulatory follicles in the *esr2a*$^{-/-}$ females by *in vitro* follicle culture followed by qPCR. According to the previous study,

expression levels of *ptger4b* were increased by rLH treatment in cultured medaka follicles (39). Here, I demonstrated that PMSG also increases these genes and act as an agonist of LH receptor by *in vitro* follicle culture (Fig. 1-10). In the present experimental design, *ptger4b* expression in the preovulatory follicles of *esr2a*$^{-/-}$ showed an increase by PMSG, which was comparable to that of WT or *esr2a*$^{+/-}$ (Fig. 1-10A, C, D). It is also consistent with another result of the present chapter that there was no significant difference in the expression of *lhr* in the preovulatory follicles between *esr2a*$^{+/-}$ or *esr2a*$^{-/-}$ (Fig. 1-10E). Actually, morphological analysis of ovaries in *esr2a*$^{-/-}$ females showed that many of four to five months old females have normally ovulated eggs (Fig. 1-9B) while many of older, six to seven months old females, showed only abnormal morphology (Fig. 1-9D). These results suggest that there is functional LH receptor in the preovulatory follicles and signaling pathway of LH can act normally in the absence of Esr2a. Therefore, Esr2a is not essential for oocyte maturation and ovulation. After ovulation, oviduct atresia prevents oviposition and old ovulated eggs in the ovary came to show abnormal morphology in old females. Taken together, I revealed that the pathway from final oocyte maturation induced by LH to ovulation functions normally in *esr2a*$^{-/-}$ females.

Conclusions

In the present chapter, I revealed the functional contributions of ERs for reproductive regulation

in female medaka. Moreover, I realized that *esr2a*$^{-/-}$ medaka may be suitable for further analysis of estrogenic regulation of FSH. In combination with the recent advancement of understanding of the central regulation of reproduction in teleosts, these ER KO lines should give us insights into the understanding of the evolution of regulatory mechanisms of reproduction in vertebrates.

Figure legends

Fig. 1-1 A. Schematic diagram of targeted genes. Arrowheads indicate targeted region of CRISPR/Cas9. Each box and line indicate an exon and intron, respectively. Dotted lines indicate frameshift region. The triangles pointing to the right indicate the predicted stop codon. According to the prediction by Ensembl, Esr2a and Esr2b can be transcribed as three splice variants for each, and the representative ones are shown here. B. Alignment of genomic DNA sequences of targeted region of CRISPR/Cas9 in each ER. Red rectangles indicate deleted site of sequence: *esr1*, 7 base pair (bp) deletion (Δ7); *esr2a*, Δ2; *esr2b*, Δ17. Although most of *esr2a*$^{-/-}$ lines of medaka showed sequence described as knockout (KO) (1), a few *esr2a*$^{-/-}$ medaka showed that described as KO (2). Because all *esr2a*$^{-/-}$ females were infertile, I regarded them as the same KO line.

Fig. 1-2 Alignment of deduced amino acid sequences of wild type (WT) and CRISPR/Cas9 KO

esr1 gene. Arrowhead indicates the start-point of the frameshift mutation in the amino acid sequences of Esr1. DNA binding domain and ligand binding domain are indicated in red and blue shaded region, respectively. *; termination of the amino acid sequences induced by the stop codon

Fig. 1-3 Alignment of deduced amino acid sequences of wild type (WT) and CRISPR/Cas9 KO *esr2a* gene. Arrowhead indicates the start-point of the frameshift mutation in the amino acid sequences of Esr2a. DNA binding domain and Ligand binding domain are indicated in red and blue shaded region, respectively. *; termination of the amino acid sequences induced by the stop codon

Fig. 1-4 Alignment of deduced amino acid sequences of wild type (WT) and CRISPR/Cas9 KO *esr2b* gene. Arrowhead indicates the start-point of the frameshift mutation in the amino acid sequences of Esr2b. DNA binding domain and Ligand binding domain are indicated in red and blue shaded region, respectively. *; termination of the amino acid sequences induced by the stop codon

Fig. 1-5 A. Mean number of fertilized eggs spawned by WT and ER KO females. Among ER homo knockouts, *esr2a*$^{-/-}$ females did not show spawning (*, $P < 0.05$, compared with WT),

whereas the females of *esr1*$^{-/-}$ and *esr2b*$^{-/-}$ did. B-H. The number of eggs spawned in the Day 17-22. WT (B), *esr1*$^{+/-}$ (C), *esr1*$^{-/-}$ (D), *esr2a*$^{+/-}$ (E), *esr2a*$^{-/-}$ (F), *esr2b*$^{+/-}$ (G), *esr2b*$^{-/-}$ (H). Inverted triangle and whisker indicate the mean number of eggs spawned and ± SEM, respectively.

Fig. 1-6 A. Comparison of GSI between WT and each ER KO line of females. B. Histological analysis of ovaries by HE staining. Females of each ER KO (age: three to four months old) showed fully developed follicles in the ovary. PV; post-vitellogenic follicle, O; ovulated egg, arrowheads; PV follicle layer composed of granulosa and theca cells.

Fig. 1-7 *Esr2a*$^{-/-}$ females showed sexual behavior without oviposition. Photos are snapshots of the movies (Supplemental Movie 1 and 2). Both *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ females showed sexual behavior. However, *esr2a*$^{-/-}$ females did not show oviposition during and after the spawning acts.

Fig. 1-8 Expression levels of *fshb* (A) and *lhb* (B) in the pituitary of each ER KO female medaka. A. The expression levels of *fshb* in *esr2a*$^{-/-}$ females showed significantly higher than the others except *esr1*$^{-/-}$ females (a VS b: $P < 0.05$, a VS bb: $P < 0.01$). B. The expression levels of *lhb* showed no significant difference among each type of ER KO and WT females. Relative expression levels, normalized by the average expression of WT are represented in the graph (mean

± SEM).

Fig. 1-9 Histological analysis revealed the reason of infertility of *esr2a*$^{-/-}$ females. A. Representative sections of oviduct in *esr2a*$^{+/-}$ (sagittal: Aa, coronal: Ab) and *esr2a*$^{-/-}$ (sagittal: Ac, coronal: Ad) (age: four to five months). Aa, Ab. *Esr2a*$^{+/-}$ showed oviduct with complete opening. Ac, Ad. *Esr2a*$^{-/-}$ females showed atretic oviduct (asterisk). I examined four females of each age and genotype. Two out of four were sectioned in the sagittal plane and the others in the coronal plane. OD: oviduct, OC: ovarian cavity. B. Representative photos of the ovaries in *esr2a*$^{+/-}$ (Ba) and *esr2a*$^{-/-}$ (Bb) (age: four to five months). Three out of four females of *esr2a*$^{+/-}$ showed normal ovarian morphology and oviposition, which occur after the ovulation. Three out of four females of *esr2a*$^{-/-}$ also showed normal ovulated eggs (Bb, arrow heads). Note that *esr2a*$^{+/-}$ females showed normal ovulation and oviposition and thus they did not contain ovulated eggs in the picture “Ba”. Bc, Bd. Percentage of the females that possessed ovulated eggs with normal morphology in the ovary of *esr2a*$^{+/-}$ (Bc) and *esr2a*$^{-/-}$ (Bd). Black sectors indicate the percentage of females that showed ovulated eggs with normal morphology. White sectors indicate the percentage of females that showed no ovulated eggs. C. Representative sections of oviduct in *esr2a*$^{+/-}$ (sagittal: Ca, coronal: Cb) and *esr2a*$^{-/-}$ (sagittal: Cc, coronal: Cd) (age: seven to eight months). Ca, Cb. *Esr2a*$^{+/-}$ showed oviduct with complete opening. Cc, Cd. *Esr2a*$^{-/-}$ females showed atretic oviduct (asterisk).

D. Representative photos of the ovaries in WT (Da) and *esr2a*$^{-/-}$ (Db, Dc) (age: six to seven months). Four out of five females of WT showed normal ovarian morphology and ovulated eggs (Da, enclosed by dotted black line), which occur after the ovulation. One out of five females of *esr2a*$^{-/-}$ also showed normal ovulated eggs (Db, arrow heads) while four out of five females of *esr2a*$^{-/-}$ showed only degenerated eggs (Dc, enclosed by dotted white line). Dd, De. Percentage of the females that possessed ovulated eggs with normal morphology in the ovary of WT (Dd) and *esr2a*$^{-/-}$ (De). Black sectors indicate the percentage of females that showed ovulated eggs with normal morphology. Gray sectors indicate the percentage of females that showed eggs with abnormal morphology only. White sectors indicate the percentage of females that showed no ovulated eggs.

Fig. 1-10 Esr2a does not mediate LH responses in preovulatory follicles. A. *Ptger4b* expression is induced by LH treatment (PMSG (+)) in the preovulatory follicles of WT (*: $P<0.05$). B. Expression of *lhr* in preovulatory follicles of WT with (PMSG (+)) or without (PMSG (-)) PMSG treatment. There was no significant difference between them. C, D. Expression of *ptger4b* in preovulatory follicles of *esr2a*$^{+/-}$ (C) and *esr2a*$^{-/-}$ (D). PMSG successfully induced *ptger4b* expression regardless of their genotypes (*: $P<0.05$). E. Expression of *lhr* in preovulatory follicles of *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$. There were no significant differences among comparisons of their

genotypes or the presence of PMSG (see Materials and methods for statistics).

Table 1-1 The list of PCR primers

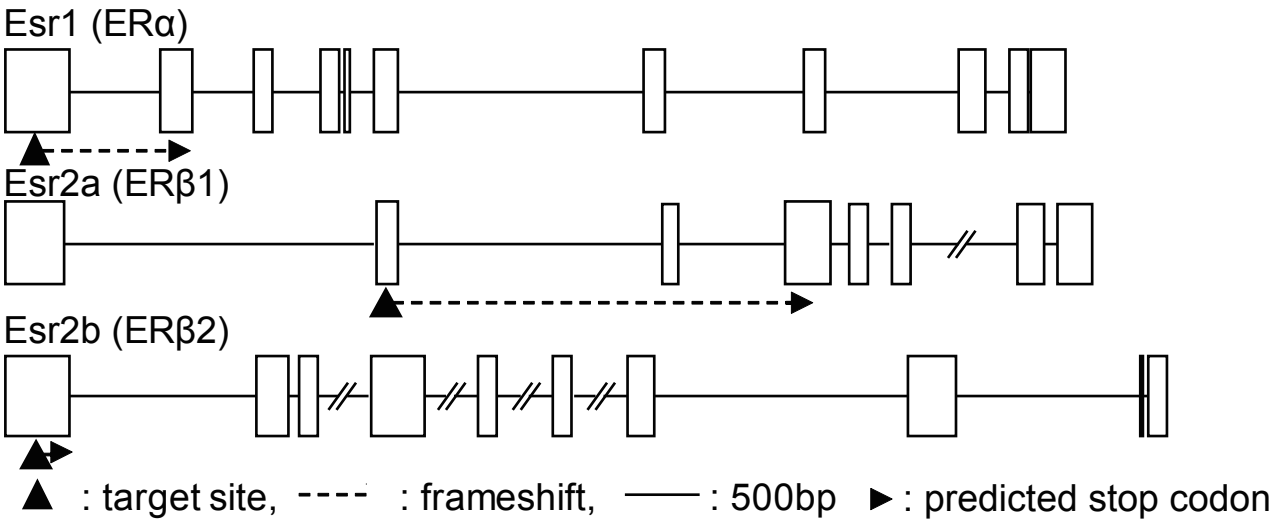
Pimer name	Gene	Primer sequence (5'-3')	Ensembl gene ID/Genbank accession number
Sequence			
era ex2 seq se2	*esr1*	AGGGACGTACGACTATGCCG	ENSORLG00000014514
era ex2 seq as2	*esr1*	GTGGCTGGGTGGATGCATAA	
erb1 ex2 seq se2	*esr2a*	TCCCAGGAGAGTGAAGAGGC	ENSORLG00000017721
erb1 ex2 seq as2	*esr2a*	CCTCTTGAAGAAGGCCTTGC	
b2 pcr se1	*esr2b*	CCTCCCCTGGGCTGAATAATG	ENSORLG00000018012
b2 pcr as1	*esr2b*	GAGGAGGGACTGTCAGTCAG	
qPCR			
rps13 F	ribosomal protein subunit 13 (*rps13*)	GTGTTCCCACTTGGCTCAAGC	ENSORLG00000001289
rps13 R	*rps13*	CACCAATTTGAGAGGGAGTGAGAC	
qPCR LHb F	*lhb*	TGCCTTACCAAGGACCCCTTGATG	ENSORLG00000003553
qPCR LHb R	*lhb*	AGGGTATGTGACTGACGGATCCAC	
qPCR FSHb F	*fshb*	TGGAGATCTACAGGCGTCGGTAC	ENSORLG00000029237
qPCR FSHb R	*fshb*	AGCTCTCCACAGGGATGCTG	
EP4bSS	*ptger4b*	CAGATGGTGATCCTGCTCAT	AB563504
EP4bAS	*ptger4b*	GCCAGGAGGTCTTCATTGAT	
LHrSS	*lhr*	TCCTCTCCCTCCACAGTCTG	AB526238
LHrAS	*lhr*	GATGGTCAAAAACTCCAGCC	
RT-PCR			
esr2a se3	*esr2a*	CTGTGCCTCAGAACCGTGGATCTTA	ENSORLG00000017721
esr2a as4	*esr2a*	GTTTAGCTGAGACGAGTGTCGAAC	

Table 1-2 The number of female medaka that showed sexual behavior and spawning in *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$

	showed sexual behavior	showed spawning
esr2a$^{+/-}$	4/4	4/4
esr2a$^{-/-}$	4/4	0/4

Fig. 1-1

A


Esr1 (ERα)
Esr2a (ERβ1)
Esr2b (ERβ2)
: target site, ---- : frameshift, —— : 500bp ► : predicted stop codon

B

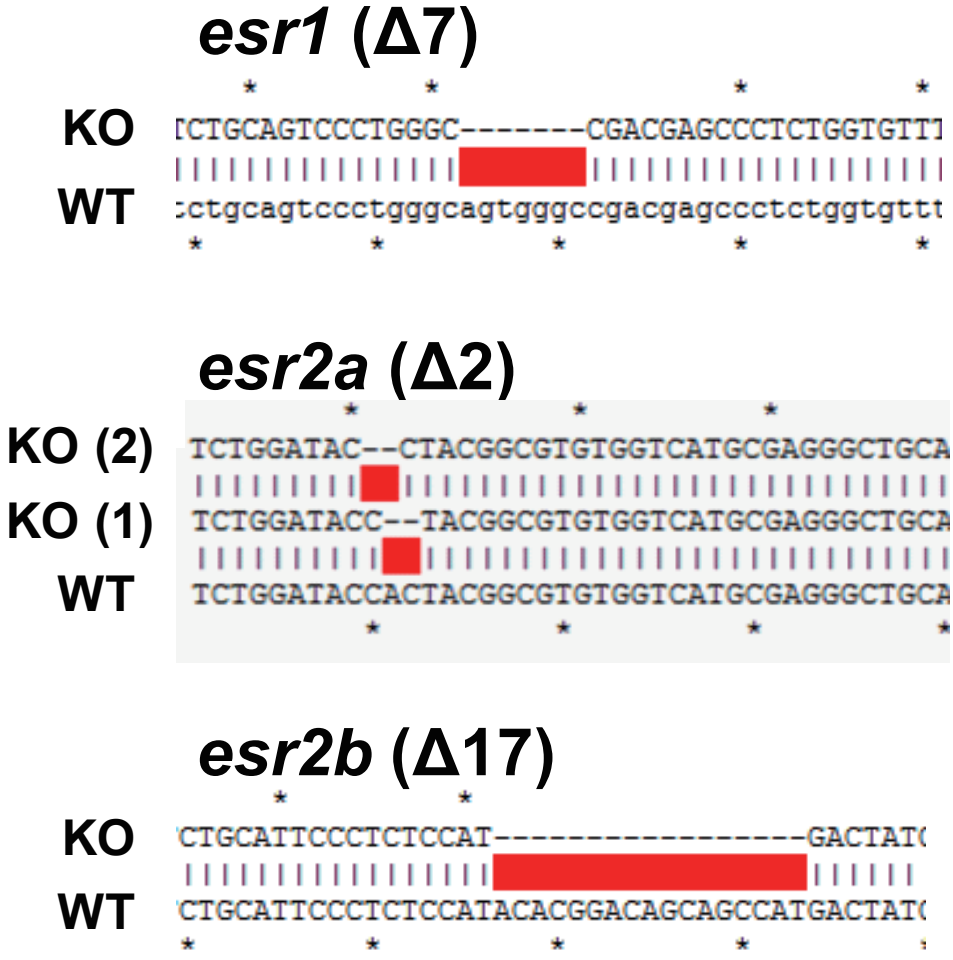

esr1 (Δ7)
KO
WT
esr2a (Δ2)
KO (2)
KO (1)
WT
esr2b (Δ17)
KO
WT

Fig. 1-2

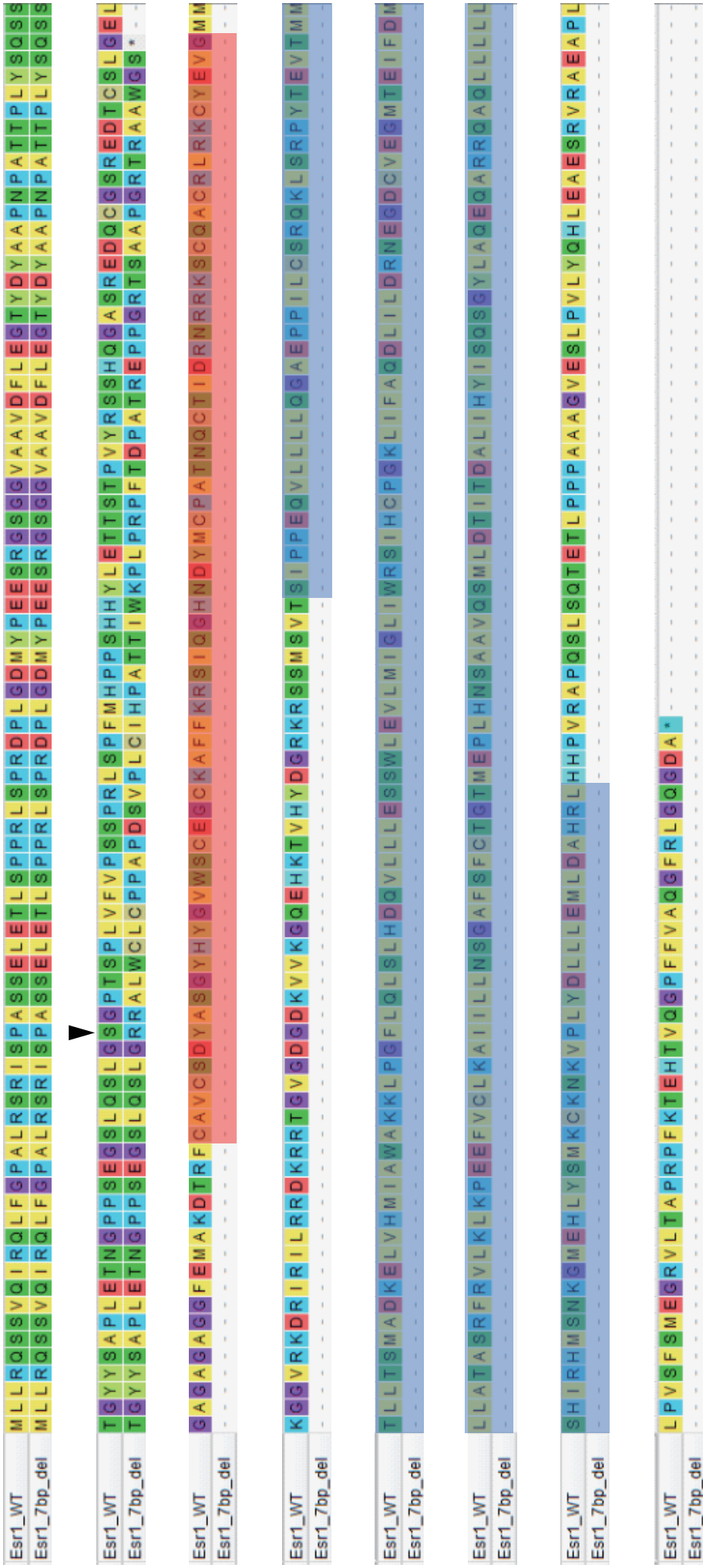


Fig. 1-3

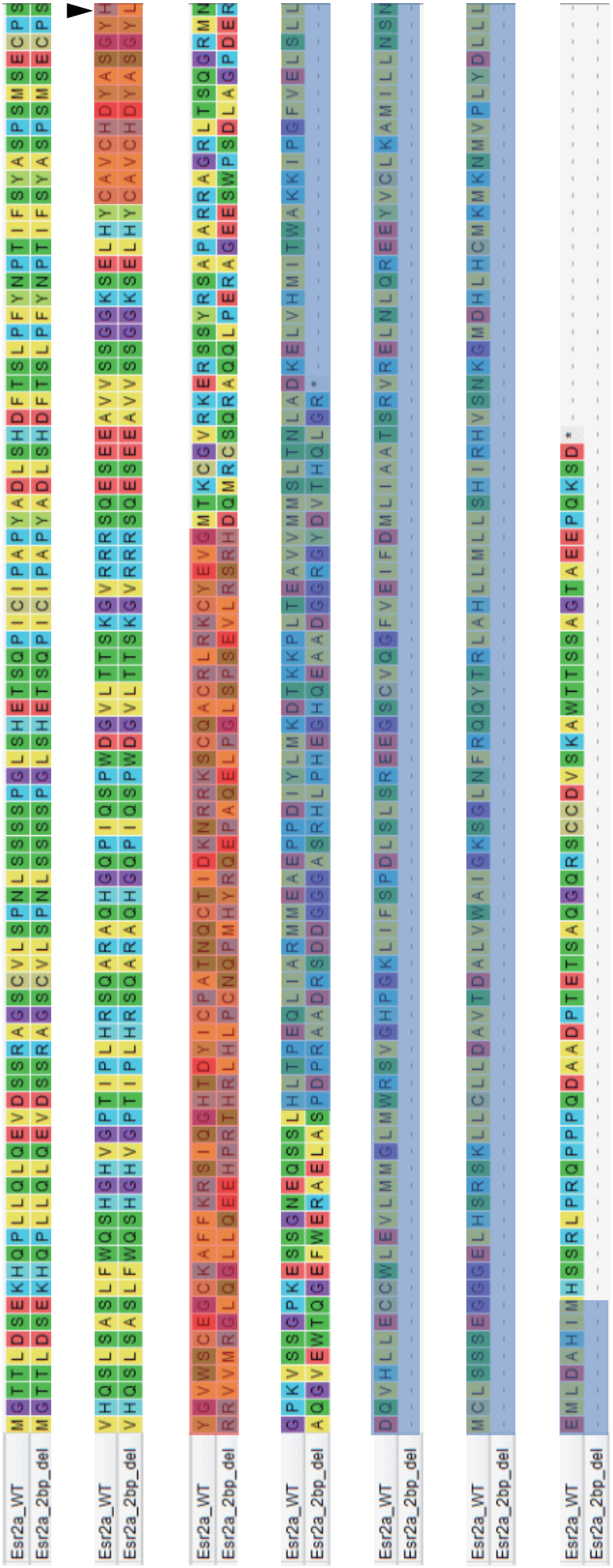


Fig. 1-4

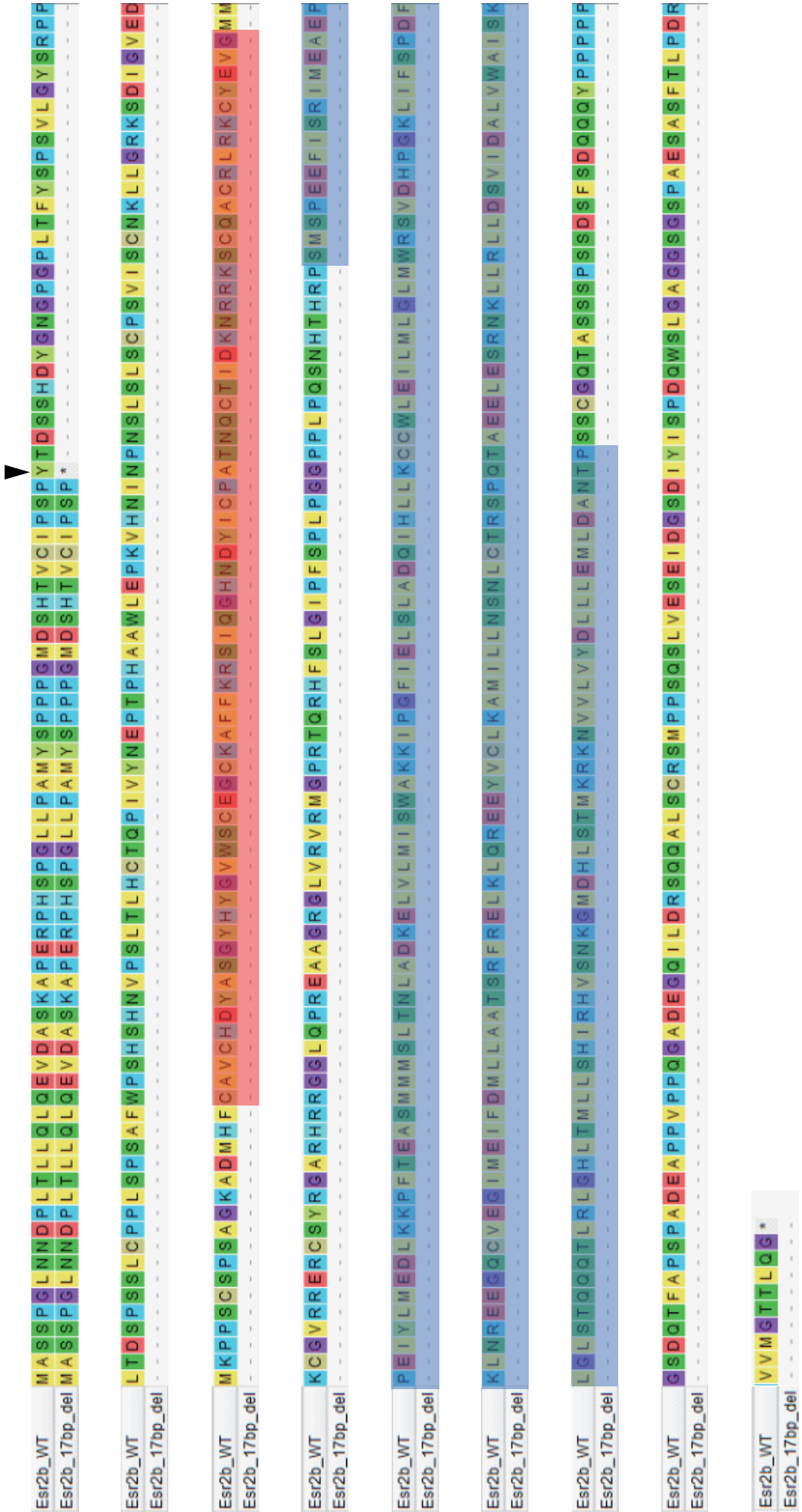


Fig. 1-5

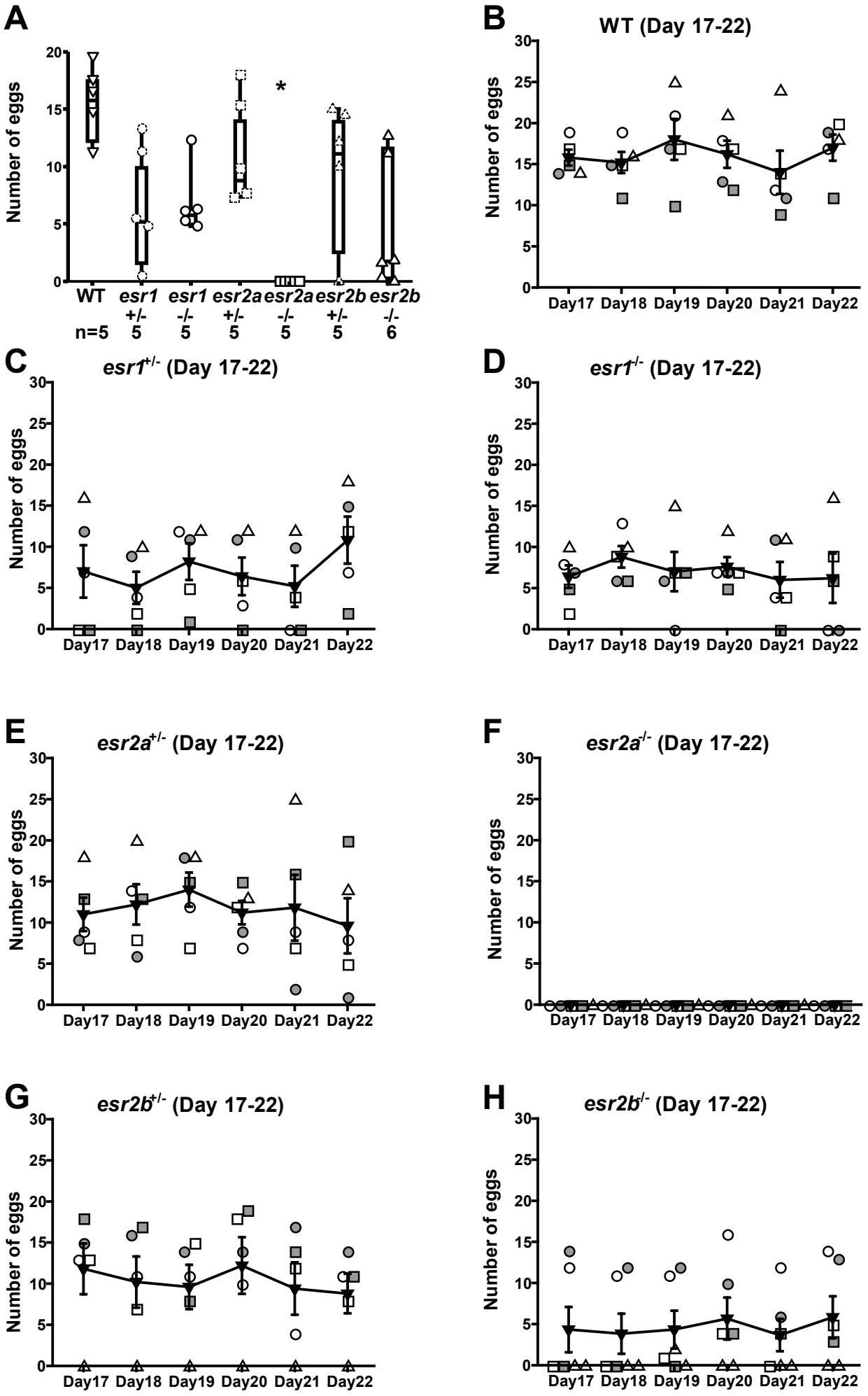
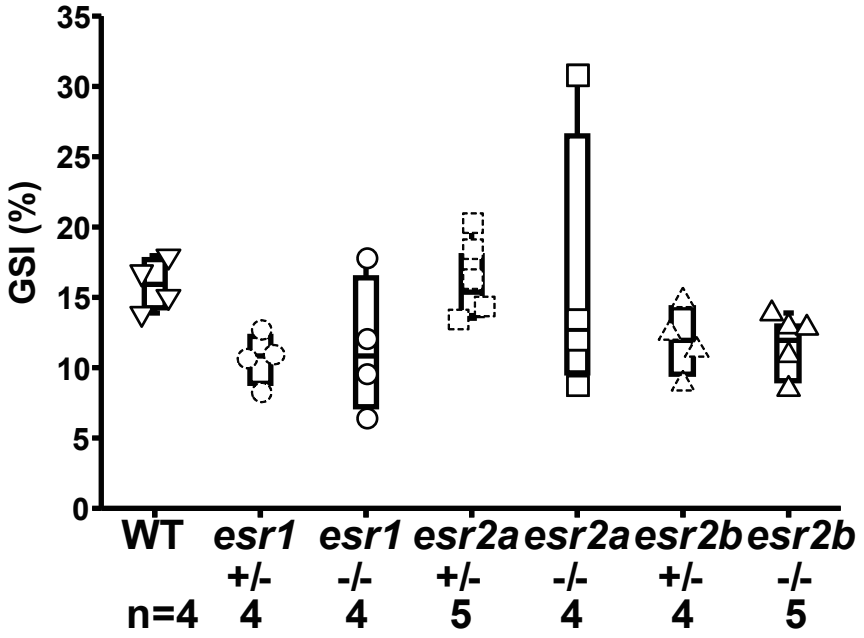

A
Number of eggs
WT
esr1 +/-
esr1 -/-
esr2a +/-
esr2a -/-
esr2b +/-
esr2b -/-
n=5 5 5 5 5 5 6
*
B
WT (Day 17-22)
C
esr1+/- (Day 17-22)
D
esr1-/- (Day 17-22)
E
esr2a+/- (Day 17-22)
F
esr2a-/- (Day 17-22)
G
esr2b+/- (Day 17-22)
H
esr2b-/- (Day 17-22)
Day17 Day18 Day19 Day20 Day21 Day22

Fig. 1-6

A


GSI (%)
35
30
25
20
15
10
5
0
WT
esr1 +/-
esr1 -/-
esr2a +/-
esr2a -/-
esr2b +/-
esr2b -/-
n=4
4
4
5
4
4
5

B

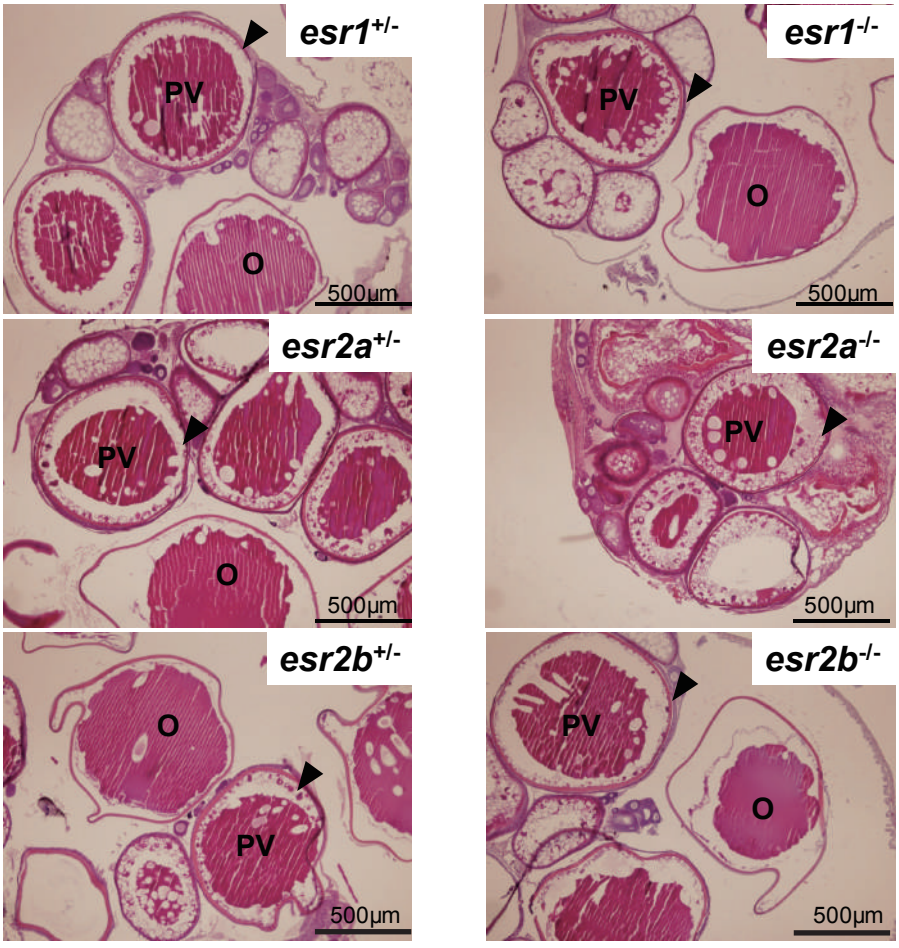

esr1+/-
esr1-/-
esr2a+/-
esr2a-/-
esr2b+/-
esr2b-/-
PV
O
500μm

Fig. 1-7

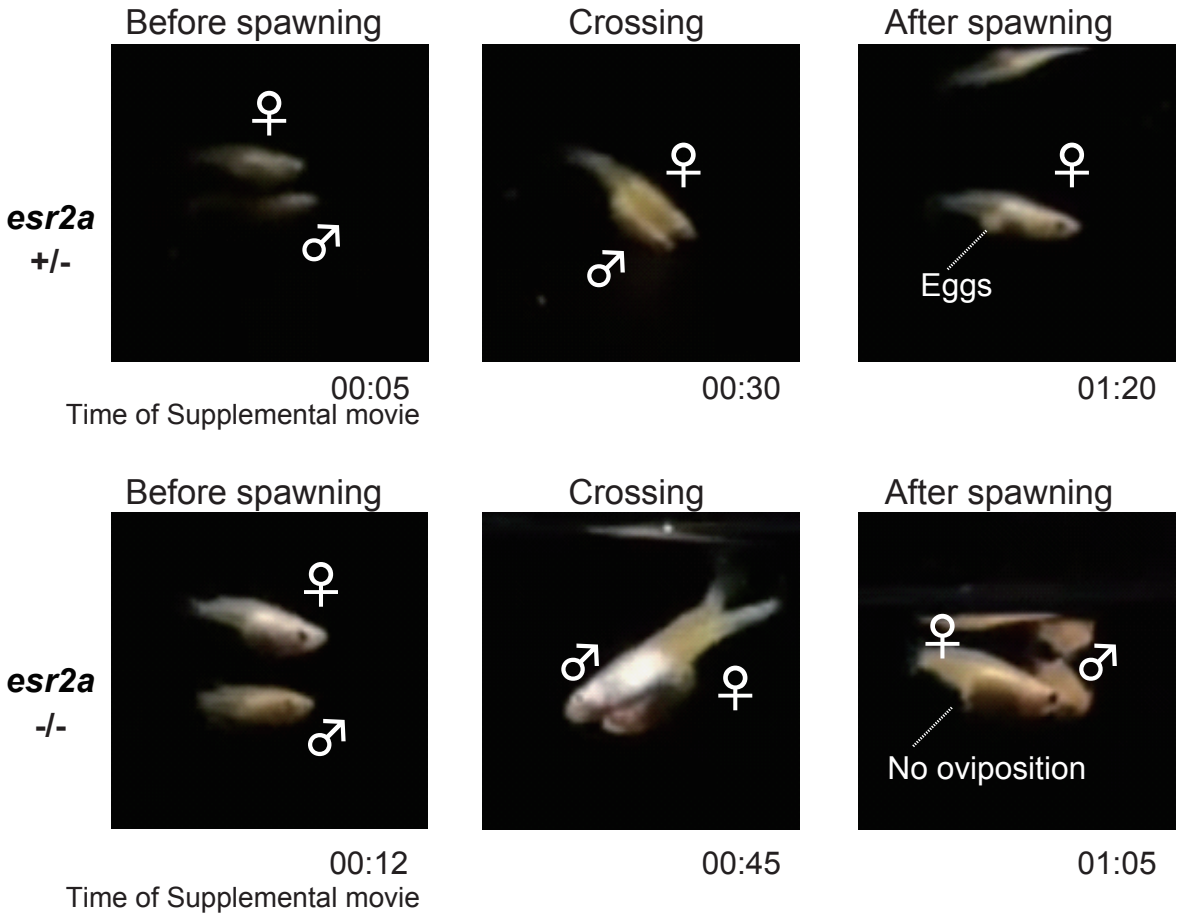

Before spawning
Crossing
After spawning
esr2a
+/-
♀
♂
Eggs
00:05
00:30
01:20
Time of Supplemental movie
Before spawning
Crossing
After spawning
esr2a
-/-
♀
♂
No oviposition
00:12
00:45
01:05
Time of Supplemental movie

Fig. 1-8

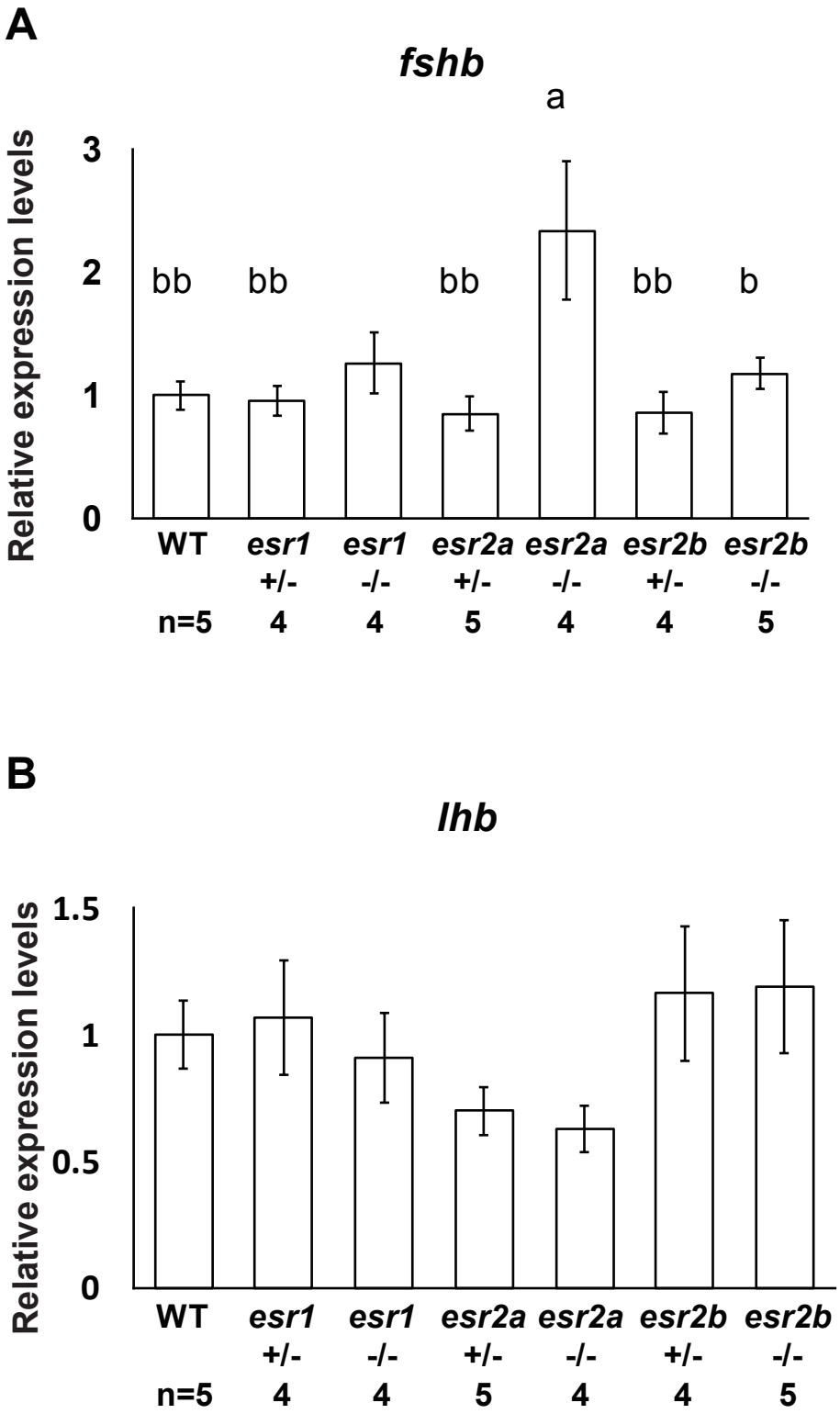

A
fshb
a
bb
bb
bb
bb
b
Relative expression levels
3
2
1
0
WT
esr1 +/-
esr1 -/-
esr2a +/-
esr2a -/-
esr2b +/-
esr2b -/-
n=5
4
4
5
4
4
5
B
lhb
Relative expression levels
1.5
1
0.5
0
WT
esr1 +/-
esr1 -/-
esr2a +/-
esr2a -/-
esr2b +/-
esr2b -/-
n=5
4
4
5
4
4
5

Fig. 1-9

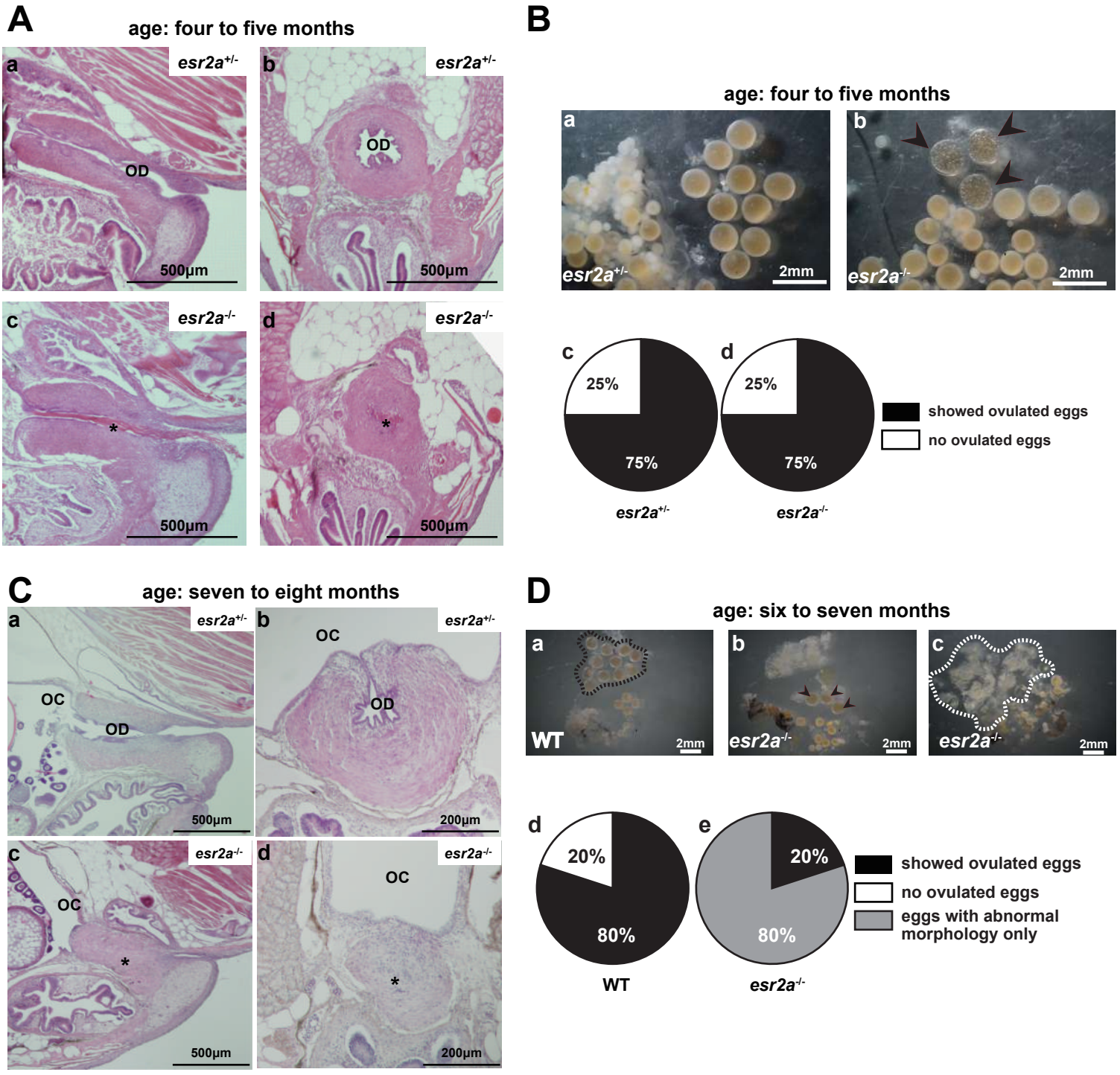
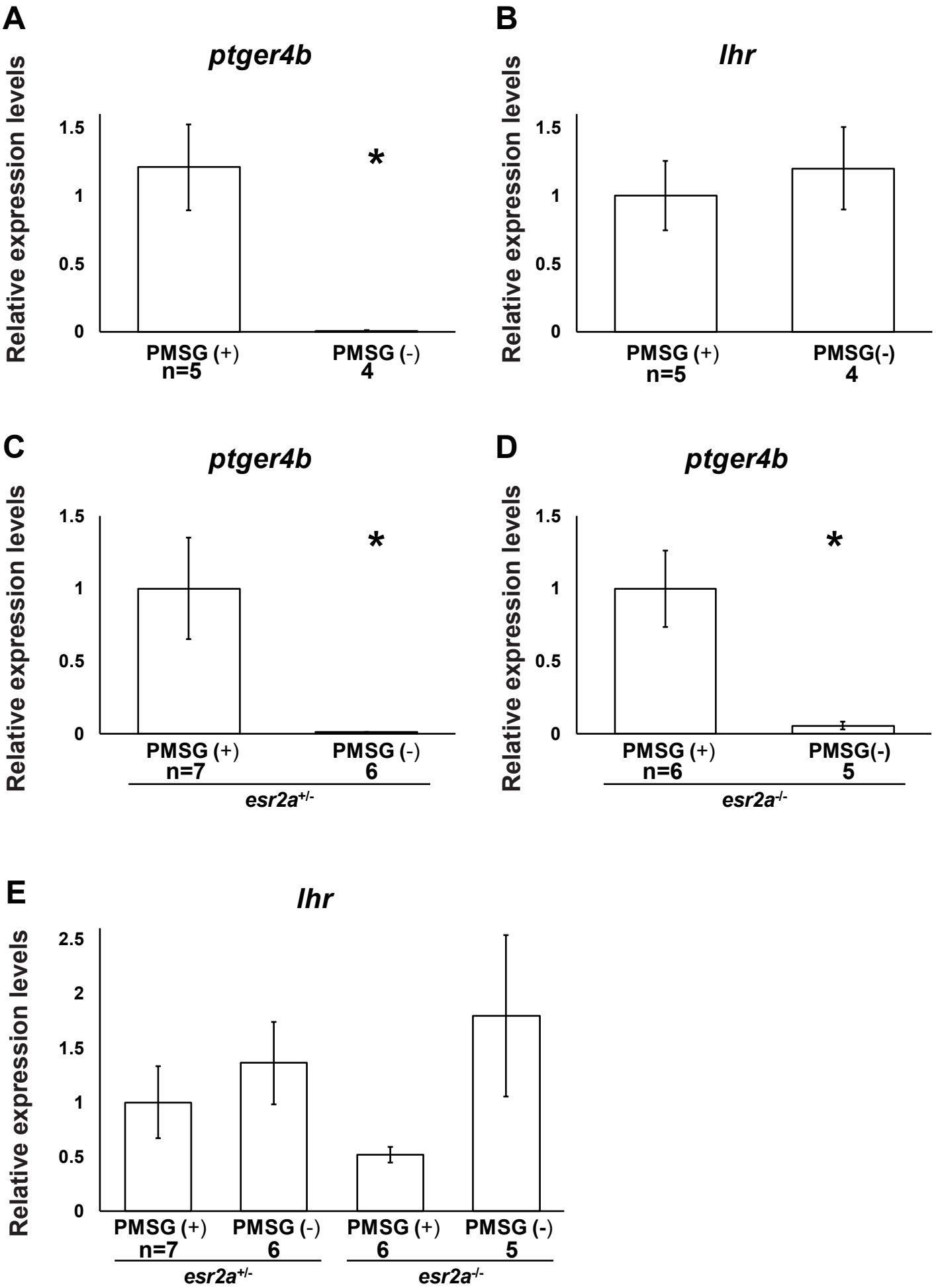

A
age: four to five months
a
esr2a+/-
OD
500μm
b
esr2a+/-
OD
500μm
c
esr2a-/-
*
500μm
d
esr2a-/-
*
500μm
B
age: four to five months
a
esr2a+/-
2mm
b
esr2a-/-
2mm
c
25%
75%
esr2a+/-
d
25%
75%
esr2a-/-
showed ovulated eggs
no ovulated eggs
C
age: seven to eight months
a
esr2a+/-
OC
OD
500μm
b
esr2a+/-
OC
OD
200μm
c
esr2a-/-
OC
*
500μm
d
esr2a-/-
OC
*
200μm
D
age: six to seven months
a
WT
2mm
b
esr2a-/-
2mm
c
esr2a-/-
2mm
d
20%
80%
WT
e
20%
80%
esr2a-/-
showed ovulated eggs
no ovulated eggs
eggs with abnormal morphology only

Fig. 1-10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Chapter 2

Examination of methods for manipulating serum 17β-Estradiol (E2) levels by analysis of blood E2 concentration in medaka

Abstract

It is widely known that reproduction in vertebrates is regulated by the hypothalamus-pituitary-gonadal (HPG) axis. Although the mechanism of the HPG axis regulation has been well documented in mammals, it cannot be always applied to that in non-mammalian species, which is a great disadvantage in understanding reproduction of vertebrates in general. Recently, transgenic and genome editing tools have rapidly been developed in small teleosts, and thus these species are expected to be useful for the understanding of general mechanism of reproduction in vertebrates. 17β-Estradiol (E2), which is one of the major sex steroid hormones in female vertebrates, plays crucial roles in the formation of sexual dimorphism and the HPG axis regulation. In spite of the importance of E2 in reproductive regulation, only a few studies have analyzed blood E2 levels in small teleosts like medaka, which are easily amenable to genetic manipulation. In the present chapter, I analyzed blood E2 concentration in medaka and demonstrated that female medaka show diurnal changes in blood E2 concentration. I then examined the best method for manipulating the circulating E2. First, I found that ovariectomy (OVX) drastically removes endogenous E2 in a day in female medaka. I examined different methods for E2 administration and revealed that feeding administration of E2-containing food is the most convenient and physiological method for mimicking the diurnal E2 changes of female medaka. On the other hand, the medaka exposed to E2-containing water showed high blood E2 concentrations, which exceeds

those of environmental water, suggesting that E2 may cause bioconcentration.

Introduction

Reproduction is one of the most important biological functions of the animals. However, the regulatory mechanisms of reproduction, including the control of hormones and behavior, vary among species. It has been demonstrated that there are many differences in their reproductive systems not only in terms of apparent traits such as morphology or behavior but also in the underlying mechanisms of gonadal control.

In vertebrates, it has been generally accepted that reproduction is controlled by the hypothalamus-pituitary-gonadal (HPG) axis. However, in contrast to the wealth of knowledge in mammals, the mechanism of HPG axis regulation in non-mammalian species is not well understood. In addition to the traditional studies of the analyses of serum hormone concentration and hormone injection using larger teleosts (51-56), the rapid development of genome editing tools and studies using knockout (KO) teleosts have provided important information on the essentiality of each hormone. These studies have suggested the differences in the reproductive endocrine systems between teleosts and mammals and have given us insights into the evolution of reproductive systems by providing the theoretical scheme of ancestral vertebrates (13, 14, 16-18, 34, 48, 57, 58). However, to date, one of the biggest limitations is that none of the species studied are appropriate for both types of analyses: macroscopic methods and genetic modification. Because it is difficult to apply a genetic approach to relatively large teleosts due to their body size

and long life cycle, I believe that the application of traditional approaches such as the measurement of the serum hormone levels and the administration of hormones to the small teleosts is much easier than the application of genetic manipulation to larger fish (59).

Among small model teleosts, medaka have many advantages for the study of reproductive regulations: they are seasonal breeders and spawn regularly every day and are amenable to surgeries such as ovariectomy (OVX), and there is accumulated knowledge of their reproductive regulations (60). Furthermore, they are also amenable to transgenic techniques and genome editing tools. Therefore, medaka should be a good model organism for the analysis of teleost reproduction, if we can understand their hormonal changes and precisely mimic these changes through hormonal manipulations. It is well known that 17β-Estradiol (E2) is one of the major sex steroid hormones in vertebrates and plays crucial roles in reproduction especially in females. As the ovarian follicles mature, E2 is released to the general circulation and modifies the expression and release of gonadotropin from the pituitary, which is known as steroid feedback regulation. In medaka, a previous report demonstrated that E2 regulates the expression of gonadotropins, follicle stimulating hormone (FSH, folliculogenesis), and luteinizing hormone (LH, ovulation) (45, 47), which are indispensable for their fertility (18). In spite of the importance of E2 in their reproduction, there are only a few studies that analyzed blood E2 level in medaka, because their small size has made it difficult to collect their blood. Some reports have analyzed the blood E2

concentration in intact males or females by enzyme-linked immunosorbent assay (ELISA), but the results were inconsistent with each other (61, 62). Moreover, despite many reports using OVX and/or E2-administrated medaka (18, 47, 63-68), no study has analyzed the actual blood concentrations of E2 after such treatments.

In the present chapter, I analyzed the blood E2 concentrations in intact, OVX, and E2-administrated medaka. To avoid the contamination of tissue fluid, I collected the blood from the bulbus arteriosus. After the analysis of the physiological levels of E2 in medaka at specific time-of-day, I examined the time course of decrease in the blood E2 concentration after OVX. Next, I compared three methods of exogenous E2 administration used in the previous studies for medaka to know which is the most convenient method for mimicking the physiological diurnal changes in E2 levels in female medaka.

Materials and methods

Animals

Medaka (d-rR strain or himedaka, which is considered to be the origin of d-rR strain and are easily available from local dealers) were maintained under a 14-hour light and 10-hour dark photoperiod (lights on at 08:00 and off at 22:00 or 09:00 on 23:00 off) at a water temperature of 27°C. I used adult male and female of d-rR strain (age: seven months, body weight, male:

0.258±0.02 g, n=8; female: 0.259±0.009 g, n=33, most of them show spawning) for the analysis of diurnal changes in E2. The fish were fed two to four times a day with live brine shrimp and flake food. All of the fish maintenance and the experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee of the University of Tokyo (permission number 15-3 and 18-3).

Ovariectomy (OVX)

Ovariectomy was performed as previously described (63). Briefly, sexually mature d-rR female medaka that showed spawning and/or vitellogenic follicles in their ovary (age: nine months, body weight, 0.297±0.007 g, n=29) were anesthetized with 0.02% MS222 (Sigma-Aldrich, St. Louis, MO) and their ovaries were excised via intraperitoneal operation. At the end of the operation, the incision was sutured with a nylon thread. OVX was performed at Zeitgeber time (ZT) 1 to ZT3. Blood sampling was performed 24, 48, 72, and 96 hours after the OVX operation. Blood samples of intact females were also collected at the same time as the OVX sampling (ZT1 to ZT3).

Methods and dose of E2 administration

Since I needed to use as many male medaka as possible for this experiment, I used adult male fish of himedaka strain purchased from a local dealer because of their easy availability (body

weight, 0.291±0.005 g, n=66). In the present study, I examined three methods of E2 administration that have already been reported in the previous studies in medaka; Feeding administration(18, 47, 63), Water exposure (64, 65, 67, 68), Intraperitoneal implantation (66) .

In the present study, I first preliminarily examined blood E2 concentration and found that male medaka show low E2 level comparable to that of OVX females. Therefore, I used male medaka as a convenient model for the examination of the increase in E2 level after exogenous E2 administration.

Feeding administration: According to the previous study that has successfully reinstated the physiological gonadotropin expression level by exogenous administration of E2 to OVX female (47), E2 dose of 100 ng/individual was administered to the male medaka by feeding food pellets containing such amount of E2. I prepared food pellet containing approximately 25ng E2/pellet. To do so, I first measured the weight of a scoopful of food for fish (Kyorin, Hyogo, Japan) whose diameter is 0.6-1.0mm and counted the number of pellets contained in it. After repeating this process three times, I estimated that 35mg food contains approximately 100 pellets of food. I adjusted 1mg/ml E2 stock dissolved in 100% ethanol and dispensed 2.5µl of stock to 100µl 70% ethanol (vehicle). This vehicle with 2.5µg E2 was dispersed throughout 35mg food pellets (approximately 100pellets) to make the final concentration of 25 ng/pellet. Ethanol was completely evaporated at 42℃. Control food was made in the same way by dispersing vehicle

without E2. I fed each fish with four pellet. To confirm that each fish has completely eaten up four pellets, I separated each fish in a small tank (length (L) 75*width (W) 55*height (H) 30 (mm)) with 100ml water in it.

Water exposure: According to the previous studies to examine effects of E2 administration in *in vitro* culture of medaka pituitary (69, 70), I used 10nM (2.72 ng/ml) E2 administration for the present analysis. Three tanks (L250*W175*H130 (mm)) were prepared for the analysis, and each tank contained seven or eight male medaka in 2L water in it. I dispensed 5.44μl of E2 stock (1 mg/ml in 100% ethanol) for the preparation of 10nM E2 tank or same amount of 100% ethanol for the control tank, with final ethanol concentrations of 2.16 ppm (2.16 mg/kg).

Intraperitoneal implantation: I used silicon block containing E2 for intraperitoneal implantation (silicon: mixture of a base compound, KE-106, Shin-Etsu Chemical, Tokyo, Japan and a curing agent CAT-RG, Shin-Etsu Chemical). For the determination of the amount of E2 administration, I referred to the previous studies using goldfish (71, 72) and a subsequent study that proportionally adjusted the dose to medaka (66). Because the goldfish implanted with E2 silicon block in a previous study showed serum E2 concentration higher than the physiological level (71), I used lower dose of E2 (approximately 25μg in L1*W1*H1 (mm)). I implanted a silicon block containing 25μg of E2 to the abdomen of male medaka. For the control experiment, I implanted a silicon block without E2.

After the steroid treatment, the medaka were anesthetized, and their blood was collected for enzyme-linked immunosorbent assay (ELISA).

Blood sampling

Medaka were anesthetized, and their heart was exposed by dissection. Blood was collected from the bulbus arteriosus using a borosilicate glass pipette made from capillaries with 1.5-mm outer diameters (GD-1.5; Narishige, Tokyo, Japan) that were coated with heparin sodium salt (Wako, Osaka, Japan). While the exit of the bulbus arteriosus was pinched with tweezers, blood circulation ran into the capillary. After collecting, I measured the volume using a 1-μl microcapillary, Microcap® (Drummond, Broomall, PA). In cases where the collected blood volume was less than 1 μl, I estimated the collected blood volume by dividing the length for which the blood occupied the micropipette and the total length of the micropipette (collected blood volume = X mm/32 mm*1 μl).

ELISA

E2 in the blood samples were extracted by using dichloromethane (Wako, Osaka, Japan). For an accurate calibration of E2 concentration in spite of possible loss during steroids extraction process, I also performed this extraction process to the standard samples included in the kits. The

extracted E2 was measured by ELISA according to the manufacturer's instructions (Estradiol ELISA kit, Cayman Chemical, Ann Arbor, MI or High Sensitivity Salivary 17β-Estradiol Enzyme Immunoassay Kit, Salimetrics LLC, Carlsbad, CA; because the Estradiol ELISA kit made by Cayman Chemical has been discontinued during the research period, I used the latter kit made by Salimetrics LLC). The absorbance in each well was measured at 450 nm using an MTP-300 microplate reader (Corona Electric, Hitachinaka, Japan). I calculated the concentration of the samples using a 4-parameter non-linear regression curve fit by a software (https://elisaanalysis.com). I confirmed that R^2 values of all assays were over 0.99 for the control dilution series of E2.

Statistics

The blood E2 concentrations of female d-rR strain were analyzed by Tukey-Kramer's test. The blood E2 concentrations of OVX females (d-rR strain) were analyzed by Dunnett's test (compared with the intact females). E2 or vehicle-administrated male medaka (himedaka strain) were also analyzed by Dunnett's test (compared with the intact males). The comparison of d-rR strain males and himedaka males was analyzed by Student's t-test.

Results

Blood E2 levels in d-rR male and d-rR female medaka

I measured the concentrations of blood E2 in sexually mature female medaka every four hours starting from the beginning of the light period (09:00, Zeitgeber time [ZT] 0/24, 12.45±3.0 ng/ml, n=6; ZT4, 4.44±1.0 ng/ml, n=5; ZT8, 5.36±1.6 ng/ml, n=5; ZT12, 13.44±1.8 ng/ml, n=5; ZT16, 7.06±2.2 ng/ml, n=5; ZT20, 8.53±2.1 ng/ml, n=7), (Fig. 2-1). Among all the time points, there were marginally significant differences in ZT4 and ZT12 females. It should be noted that male blood E2 concentration was measured at three points: ZT4, 12, and 22 (n=2-3). Because in males, no apparent difference in E2 concentration was observed among each time point (ZT4 male, 0.92±0.19 ng/ml, n=3; ZT12 male, 1.67±0.2 ng/ml, n=2; ZT22 male, 1.25±0.06 ng/ml, n=3), I pooled the data for males regardless of their sampling time (Fig. 2-1, gray bar, 1.23±0.1 ng/ml, n=8). Diurnal changes in the blood E2 concentration of females were statistically analyzed by a Tukey-Kramer's test, which is a multiple comparison analysis. Although there were no significant differences among the female blood E2 concentrations, I found the same pattern of diurnal changes as those reported in the previous study (61), in which females showed relatively low E2 in the light period, while high in the dark period.

Time-course of decrease in blood E2 after OVX d-rR female medaka

To analyze the time course of decrease in endogenous blood E2 after OVX, I measured the concentration of blood E2 in OVX d-rR females (Fig. 2-2). All OVX females showed significantly low E2 concentration compared to the intact females (intact female (ZT1-3), 7.04±2.4 ng/ml, n=5; Day of post OVX (DPO)1, 0.52±0.04, n=5; DPO2, 0.47±0.2 ng/ml; DPO3, 0.31±0.04 ng/ml; DPO4, 0.36±0.05 ng/ml, n=5; Dunnet's test, compared with intact). The E2 levels of OVX females were comparable to those of males, and thus it was suggested that OVX drastically removes the ovarian E2 in one day (24 hours). In this experiment, I collected the blood in ZT0 to ZT4. Intact females showed near the midpoint level of E2 concentration (7.04±2.4 ng/ml) between ZT0/24 (12.45±3.0 ng/ml) to ZT4 (4.44±1.0 ng/ml), which may be because of this sampling conditions.

Examination of a method for administration of E2

To evaluate the methods of E2 administration used in the previous studies, I analyzed the blood E2 concentration of medaka after administration of E2. In the present study, I demonstrated that male medaka show lower E2 level than that of intact females. Therefore, I administrated E2 to the male medaka and examined the effects of E2 administration by the following three methods: Feeding administration, Water exposure, and Intraperitoneal implantation (see Materials and

methods). Since I needed to use as many male medaka as possible for this experiment, I used adult male of himedaka strain purchased from a local dealer because of their easy availability. I confirmed that blood E2 levels were nearly equal to those of d-rR strain males (male d-rR strain medaka, 1.23±0.1 ng/ml, n=8; male himedaka; 0.74±0.4 ng/ml, n=5, no significant difference, P=0.3, Student's t-test), which is much lower than the intact females. Next I analyzed the effects of the vehicle on the blood E2 concentration and confirmed that each vehicle treatment does not influence the blood E2 concentration (intact, 0.60±0.2 ng/ml, n=4; normal food, 0.46±0.1 ng/ml, n=4; water with vehicle, 0.48±0.09 ng/ml, n=4; silicon block; 0.94±0.4 ng/ml, n=4; no significant difference, Dunnet' test, compared with intact male) (Fig. 2-3A).

All three groups of E2-treated medaka showed a transient increase in blood E2 levels four hours after administration (intact, 0.74±0.4 ng/ml, n=5; 4h after feeding, 33.41±19.3 ng/ml, n=4; 12h after feeding, 5.08±1.6 ng/ml, n=5; 24h after feeding, 2.24±0.9 ng/ml, n=5; 4h after exposure, 100.09±33.4 ng/ml, n=5; 12h after exposure, 57.68±14.9 ng/ml, n=5; 24h after exposure, 24.57±3.0 ng/ml, n=5; 4h after implantation, 91.28±20.82 ng/ml, n=5; 12h after implantation, 58.98±13.8 ng/ml, n=5; 24h after implantation, 26.20±5.4 ng/ml, n=5), (Fig. 2-3B). The medaka undergoing a feeding administration of E2 showed concentrations of blood E2 relatively close to the highest diurnal peak of d-rR females (Fig. 2-1) four hours after the administration, and exogenous E2 returned to the low comparable to the male level within 12 to 24 hours. In contrast,

the medaka that were exposed to water containing E2 or were implanted with silicon block containing E2 showed unphysiologically high concentrations of blood E2, which lasted for 12 to 24 hours after E2 administration.

Discussion

In the present chapter, I measured blood E2 concentration in intact medaka, after OVX, and following E2 administration. Because medaka are a model teleost that have advantages for the analysis of reproductive control including their amenability to genetic modifications as described above, the results of this study will be helpful for future studies in the analysis of the mechanism of reproductive regulation in teleosts. In the present study, I analyzed the E2 concentration of whole blood instead of that of serum, because the individual blood volume was so small in medaka. It should be noted that serum E2 concentrations can be estimated to be approximately 1.3-1.7 fold higher than the whole blood E2 concentrations in this study, because the fish show approximately 20-40 percent of blood hematocrit (73-77).

Diurnal changes in blood E2 concentration in d-rR strain medaka

The female medaka showed relatively low E2 blood concentrations at ZT4 and ZT8, and these values were high at ZT12 and ZT0/24, which suggests that E2 level may become higher in the

evening to dawn under natural conditions (Fig. 2-1). The existence of diurnal E2 changes in medaka is consistent with the previous report (61). In addition to the analysis of females, I analyzed the blood E2 level of male medaka (Fig. 2-1, gray bar). The males showed much lower E2 concentrations compared to those of females. Therefore, the concentration of E2 in males can be regarded as a model of low E2 fish. One of the previous studies using medaka (62) showed extremely higher value of physiological E2 concentration (plasma E2, males, 225-239 ng/ml; females, 363-401 ng/ml) than the previous report (serum E2, females, approximately 4-20 ng/ml) (61). Although it is unknown why the value of E2 are substantially different among these studies, my present results (whole blood E2, males, 0.46-1.23 ng/ml; females 4-13 ng/ml) supports the latter study (61).

One of the most important functions of E2 is to alter the expression level of gonadotropins in the pituitary and to play crucial roles in the feedback regulation of gonadotropin release. In medaka, to prevent excessive maturation of ovarian follicles, endogenous E2 suppresses the expression of FSH (*fshb*) during folliculogenesis (45), while endogenous E2 positively regulates the expression of LH (*lhb*), which is indispensable for ovulation (47). In the previous report in medaka, it was demonstrated that both *fshb* and *lhb* mRNA expression also exhibit diurnal changes (69). In accordance with changes in the firing activity of GnRH1 neurons, which facilitates the release of GnRH peptide (69), the alteration of GnRH1 release in the pituitary may

have caused the expressional changes in *fshb* and *lhb* mRNA. However, in addition to this factor, my present study suggests a possibility that diurnal changes in estrogens may also cause diurnal changes in the expression of *fshb* and *lhb*. Thus, in intact females, complicated feedback interaction between gonadotropin and sex steroids may occur even in such daily spawners.

In a previous report using *in vitro* ovarian follicle culture, preovulatory follicles isolated from the ovary 15 hours prior to ovulation (ZT9), which should be ovulated next day *in vivo*, spontaneously start the final oocyte maturation and were ovulated in the medium, while the preovulatory follicles 19 hours before ovulation (ZT5) did not exhibit such spontaneous ovulation (39). Their results clearly demonstrated that LH has already been released from the pituitary between 19 hours (ZT5) to 15 hours (ZT9) prior to ovulation. Since I have shown here that the blood E2 concentration in females increase from ZT8 to ZT12, it is possible that this increase may induce the GnRH release and LH expression and/or release for the ovulation next day. Although the time duration of the latency of LH surge after the increase of serum E2 remains unclear, the diurnal changes in E2 may be involved in the diurnal changes in gonadotropin expression and/or release.

On the other hand, in mammals, it has been reported that constant E2 administration induces diurnal oscillation of GnRH and LH release, with more GnRH/LH released in the PM (78). These results suggest that the presence of E2 is important for the regulation of GnRH and LH, but

fluctuation of E2 is not required for the diurnal oscillation of GnRH/LH release in mammals. For clarifying the generation mechanism of this gonadotropin fluctuation, analysis of GnRH/LH release in medaka with constant blood E2 levels (OVX+E2) may give us deeper insights into the relationship between E2 and GnRH/LH.

Analysis of E2 concentration in OVX medaka

I demonstrated that OVX operation drastically reduced the blood E2 levels in female medaka (Fig. 2-2). The blood E2 levels one day after OVX were significantly low and were comparable to those in males. Thus, I showed that one day is sufficient for the clearance of endogenous E2 in females.

In previous reports, it took approximately 24 hours to metabolize the administrated E2 in rodents and humans (79, 80). Similarly, almost all of the medaka 24 hours after OVX (DPO1) showed low concentrations of E2 (0.52±0.04 ng/ml) in the present study. Thus, one day after OVX can be considered as a complete OVX model and I suppose that this information will be useful for the future analyses using OVX medaka.

Examination of the method of E2 administration

The average blood E2 concentration in medaka four hours after E2 feeding (33.41±19.3 ng/ml)

showed values approximately 2.5 times higher than that of the highest concentration of the day in intact females, at ZT12 (13.44±1.8 ng/ml) (Fig. 2-3B). However, it should be noted that four hours after E2 feeding their blood E2 concentration showed a large variance. In the previous E2 administration study using rodents, the peak value of blood E2 concentration was detected immediately after the E2 administration (79). Thus, it may be possible that the actual peak of E2 concentration may exist between zero and four hours after E2 feeding. Interestingly, my present feeding administration experiment suggested that extrinsic E2 is cleared between 12 and 24 hours. These results suggest a possibility that the feeding administration of E2 twice a day can best mimic the diurnal changes of E2 in female medaka.

On the other hand, the blood E2 concentration of the medaka implanted with an E2-containing silicon block showed levels much higher than the physiological levels and remained high even 24 hours after the administration under the present conditions. As expected, the reduction of blood E2 levels was slower than that of the feeding administration, probably because the release of E2 from the silicon block was slow. It may seem to be possible to mimic physiological concentrations of E2 using lower-dose silicon block implantation. However, the female medaka show diurnal changes of endogenous E2, and thus it may be difficult to mimic these changes by intraperitoneal implantation, because the silicon implantation twice or more may cause severe physical damage to the experimental fish.

To my surprise, after four hours of exposure to the water containing 10nM (2.72 ng/ml) E2, the blood concentrations of E2 rose to 100±33.4 ng/ml, which is much higher than environmental E2 concentrations. These results suggest that something in the body of medaka may have adsorbed and accumulated the E2. According to the previous study, stickleback exposed to E2-containing water also showed extremely high plasma E2 concentrations (20-100 ng/ml) within the first six hours of exposure, which is about 50-fold greater than that of the physiological levels (81). Therefore, adsorption of E2 from the environmental water, which can be regarded as bioconcentration, may occur in teleosts in general. At present, it is not possible to draw a conclusion about the mechanism of bioconcentration, but it may be due to the sex steroid binding proteins and/or other hydrophobic substances in the blood. The results of the present study and the previous ones strongly suggest that we should administrate drugs carefully, especially when using the water-exposure method. Interestingly, after the drastic increase, blood E2 concentration decreased rather rapidly. So far, I cannot conclude if it is due to the depletion of E2 in the surrounding water or increase in clearance rate by medaka, it may be an interesting future study topic. In the present study, for the examination of the effects of E2 administration, it should be noted that I used gonado-intact male medaka as E2-free fish instead of OVX medaka. Therefore, it may be possible that there are some unexpected side effects of exogenous E2 to elevate the E2 synthesis abnormally in male medaka. Further studies using gonadectomized medaka may help understand

the mechanism of this elevation.

In the present chapter, I analyzed the blood E2 concentration of medaka to know the physiological E2 level. I demonstrated the diurnal changes in female blood E2 concentration, which shows relatively low in the light period and increases in the dark period. Moreover, I revealed that male medaka constantly show lower E2 level than those of females. I demonstrated that OVX drastically decreases endogenous E2 comparable to the male level in one day. The comparison of the methods of E2 administration suggests that the feeding administration of E2 is the most convenient and reliable method for mimicking the physiological fluctuation of blood E2 concentrations, which also enables multiple administrations per day. On the other hand, in water-exposure E2 administration, the blood E2 concentrations can exceed those of environmental water. Thus, we should note the effects of the bioconcentration of E2 and carefully consider the actual blood E2 level of experimental fish. These findings may help future analyses using OVX and/or E2 administration with small teleost fish in combination with genetic modifications.

Figure legends

Fig. 2-1 Blood E2 concentration of d-rR strain medaka. Gray and white bars indicate blood E2 concentrations in males and females, respectively. In females, the blood was collected every four

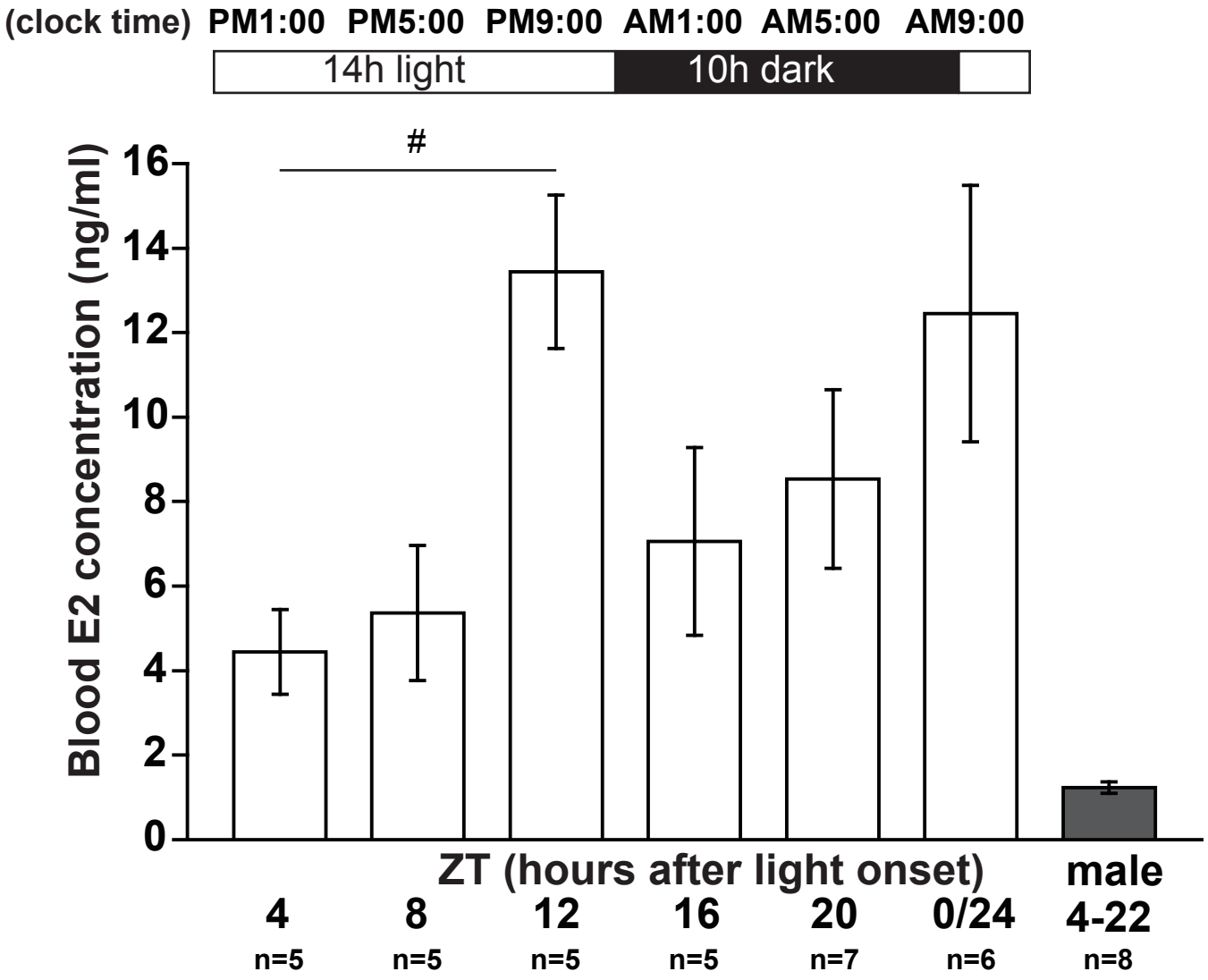
hours. ZT0/24 indicates “light on” time. Blood E2 concentration of females was constantly higher than that of males. There was no significant difference among the female blood E2 (Tukey-Kramer’s test, #: $P < 0.1$). Data are shown as mean ± SEM (ZT4, n=5; ZT8, n=5; ZT12, n=5; ZT16, n=5; ZT20, n=7; ZT0/24, n=6; male, n=8).

Fig. 2-2 Most of endogenous E2 has been cleared in one day after OVX in female medaka. Time changes in blood E2 concentration after OVX. Later than one day after OVX (DPO1-4), blood E2 concentrations were as low as the average for males (Dunnett’s test, compared with intact, **: $P < 0.01$, ***: $P < 0.001$; see Fig. 2-1, gray bar). DPO: days post OVX. Data are shown as mean ± SEM (n=5).

Fig. 2-3 Examination of the methods for E2 administration. A. The analysis of vehicle effects on the blood E2 concentration. There were no effect on the blood E2 concentration by each vehicle administration (Dunnett’s test, compared with intact, n. s.: no significant difference). The data are shown as mean ± SEM (n=4). B. The time-course of changes in blood E2 concentration, 4, 12, and 24 hours after three methods of E2 treatments in males. The medaka receiving a feeding administration of E2 (gray bars) showed a concentrations of blood E2 relatively close to the highest diurnal peak in d-rR females. The medaka that were exposed to water containing 10nM

E2 (stippled bars) or intraperitoneally implanted with silicon block containing 25μg E2 (shaded bars) showed unphysiologically high concentrations of blood E2, and it remained high for 24 hours after administration (Dunnett's test, compared with intact, **: $P < 0.01$, ***: $P < 0.001$, #: $P < 0.1$). Note that the half-life of E2 is shorter in feeding and longer in silicon implantation and water exposure. The data are shown as mean ± SEM (intact, n=5; 4h after feeding, n=4; 12h after feeding, n=5; 24h after feeding, n=5; 4h after exposure, n=5; 12h after exposure, n=5; 24h after exposure, n=5; 4h after implantation, n=5; 12h after implantation, n=5; 24h after implantation, n=5).

Fig. 2-1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14h light
10h dark
#
Blood E2 concentration (ng/ml)
0
2
4
6
8
10
12
14
16
ZT (hours after light onset)
male
4
8
12
16
20
0/24
4-22
n=5
n=5
n=5
n=5
n=7
n=6
n=8

Fig. 2-2

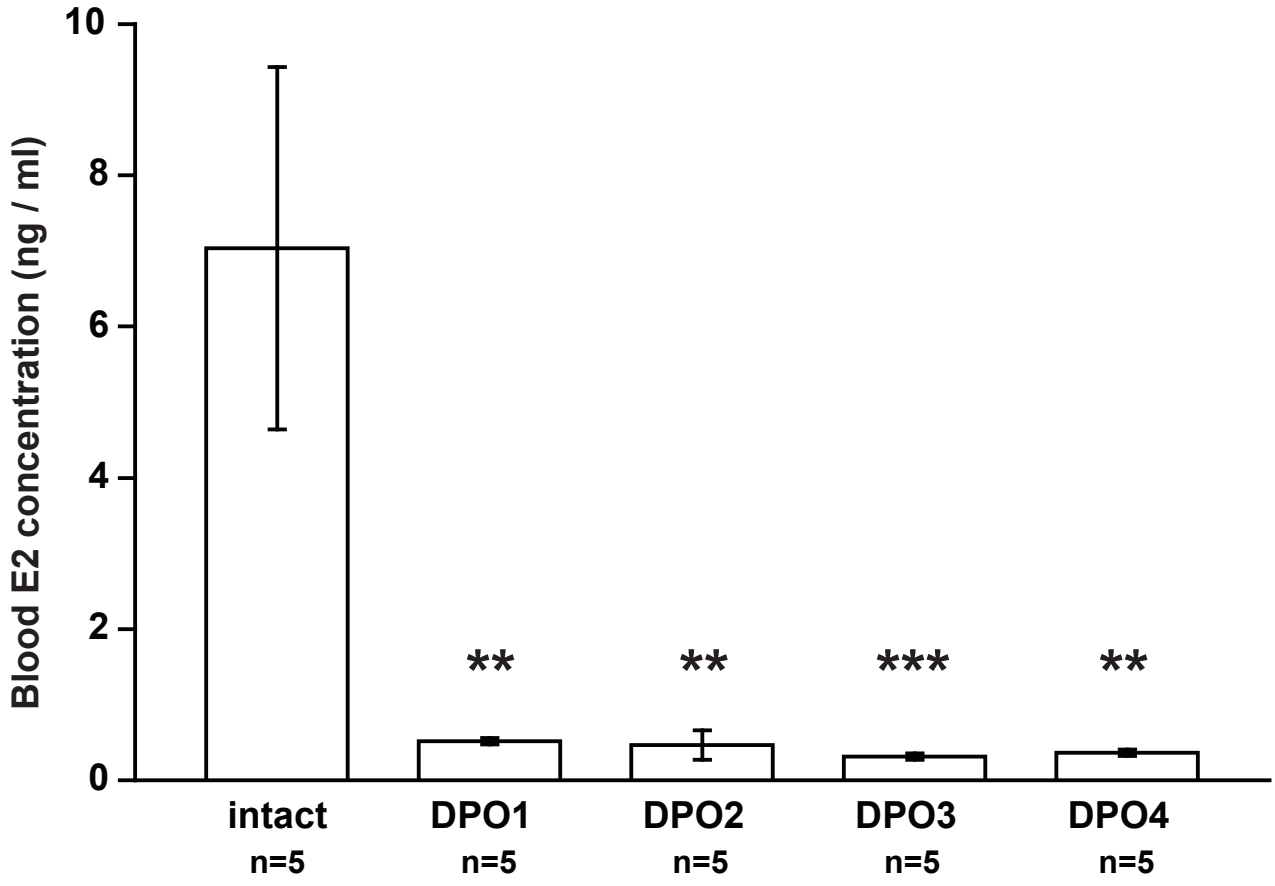
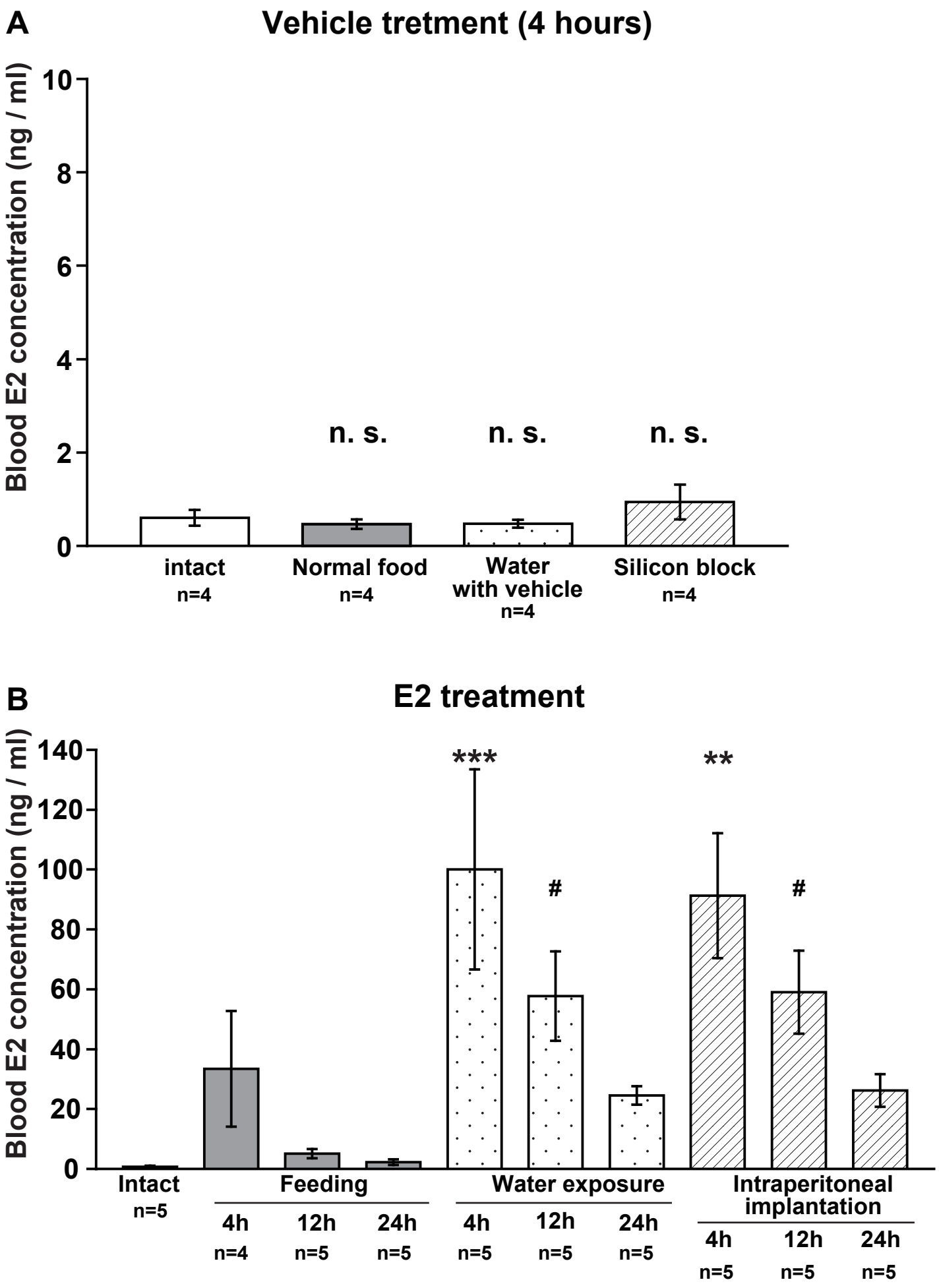

Blood E2 concentration (ng / ml)
10
8
6
4
2
0
**
**
***
**
intact
n=5
DPO1
n=5
DPO2
n=5
DPO3
n=5
DPO4
n=5

Fig. 2-3


A
Vehicle tretment (4 hours)
Blood E2 concentration (ng / ml)
10
8
6
4
2
0
n. s.
n. s.
n. s.
intact
n=4
Normal food
n=4
Water
with vehicle
n=4
Silicon block
n=4
B
E2 treatment
Blood E2 concentration (ng / ml)
140
120
100
80
60
40
20
0
***
**
#
#
Intact
n=5
Feeding
4h
n=4
12h
n=5
24h
n=5
Water exposure
4h
n=5
12h
n=5
24h
n=5
Intraperitoneal
implantation
4h
n=5
12h
n=5
24h
n=5

Chapter 3

本章については 5 年以内に雑誌などで刊行予定のため、非公開

General Discussion

In the present thesis, I aimed to understand the mechanisms of estrogenic regulation on FSH in vertebrates using medaka as a model organism. First, I analyzed the functional role of ERs in reproduction of female medaka by a loss of function analysis. From the analysis of female knockout medaka for each nuclear estrogen receptor, ones that genetically lost the functional Esr2a showed significantly higher expression of *fshb*, which suggests that Esr2a may be involved in the negative feedback regulation of FSH expression and release (Chapter 1). Next, as the concentration of endogenous and artificially administrated estrogens were poorly understood in medaka, I analyzed the blood E2 concentration and examined the best method for manipulating the circulating E2 in female medaka. From these analyses, I demonstrated that serum E2 shows diurnal changes and that feeding administration of E2 is most likely the best method to mimic the diurnal changes of E2 in female medaka (Chapter 2). Finally, to analyze the functional role of Esr2a in estrogenic regulation of FSH, I optimized the feeding administration of E2 and applied it to *esr2a*$^{-/-}$ females. From these analyses, I demonstrated that one of the ER subtypes, Esr2a, expressed in the pituitary plays an important role in estrogenic regulation of FSH (Chapter 3).

Although it is still unclear whether estrogens directly suppress the expression of *fshb* on the FSH cells, future histological and physiological analyses will give us great insights into the understanding of general regulatory mechanism of FSH, which is an indispensable gonadotropin for the ovarian follicle maturation in all vertebrates.

ER knockout analysis revealed the contribution of each estrogen receptor subtype to reproduction in female medaka

In Chapter 1, I examined which is the most potent subtype of ER for the estrogenic regulation of FSH by analyzing the phenotype of ER KO medaka. The qPCR analysis demonstrated that the *esr2a*$^{-/-}$ females show significantly higher expression of *fshb*, and thus it was suggested that Esr2a may be involved in the negative feedback regulation of FSH. Although it is well known that estrogens suppress the expression/secretion of FSH, it had not been well documented which ER subtype plays a role in this estrogenic regulation even in mammals. Thus, it is the first report that identified the contribution of a specific ER receptor (Esr2a) to the negative feedback regulation of FSH regulated. However, in spite of the high expression of *fshb*, I found that such incomplete feedback regulation of FSH does not disrupt either the folliculogenesis or the ovulation in female medaka. In fact, *esr2a*$^{-/-}$ females at the age of four to five months showed normally ovulated eggs in their ovary.

In addition to these findings described above, I also found the other important functions of each ER subtype for reproduction. First, histological analysis using *esr2a*$^{-/-}$ females revealed that they show oviduct atresia, suggesting that Esr2a plays an essential role in oviduct development in female medaka. However, because *esr2a*$^{-/-}$ females show normal ovarian morphology and

functions as described above, it is suggested that the oviduct atresia in *esr2a*$^{-/-}$ females is independent of the dysfunction of the negative feedback regulation of FSH expression. Second, some of the *esr2b*$^{-/-}$ females did not show spawning in the period of analysis. From my observation, *esr2b*$^{-/-}$ females show some failure in their sexual behavior even though they had ovulated eggs in their ovaries (data not shown). Therefore, it is also an interesting future topic to study the mechanism how Esr2b is involved in female sexual behavior. In the present analysis, unlike *esr2a* and *esr2b* knockouts, I did not observe critical defect in the fertility in *esr1*$^{-/-}$ females, although they showed relatively few number of eggs spawned compared to WT females. It is substantially different from the situation in mammals.

From the loss of function analyses regarding reproductive functions using ER KO medaka, I revealed the contribution of each ER subtype for female reproduction as their gene functions and then decided to focus on the analysis of the functional role of Esr2a in the negative feedback regulation of FSH in the following chapters.

Analyses of physiological blood E2 concentration and effects of exogenously administrated E2 on it in medaka provided important information for experimental approaches toward understanding the central regulation of reproduction

In Chapter 2, I analyzed the physiological blood E2 concentration and examined the methods to

experimentally control it in medaka. In the previous two reports, the results of endogenous E2 level in adult medaka showed substantial discrepancies (serum E2 in females, approximately 4-20 ng/ml (61); plasma E2 in males, 225-239 ng/ml; females, 363-401 ng/ml (62)). In this chapter, to know the physiological estrogen levels in medaka more precisely, I re-analyzed the concentration of E2. The whole blood E2 concentration was 4-13 ng/ml in females and 0.46-1.23 ng/ml in males. Although the reason of this substantial difference among these studies is unclear, I demonstrated that the physiological E2 level is closer to the former study. These results will help us to understand the precise level of endogenous E2 concentration, and I regard them as the normal level of physiological blood E2 concentration in medaka under the experimental conditions. Next, I proved for the first time that females show low E2 comparable to the males one or more days after OVX. Finally, the examination of the method for E2 administration used in the previous studies (63, 64, 66) suggested a possibility that feeding administration of E2 twice a day can mimic the diurnal changes of E2 in female medaka. Interestingly, I found that the medaka exposed to the water containing 10nM (2.72 ng/ml) E2 for 4 hours showed extraordinarily high concentration of blood E2 (100±33.4 ng/ml). These results may suggest that a kind of bioconcentration, in which some factors in the body of medaka have adsorbed and accumulated E2. Until now, although several studies have performed exogenous administration of E2 on medaka, none of them examined actual blood E2 concentrations after the administration. Here in

this chapter, I revealed the best method for the experimental manipulation of the blood E2 concentration.

Although a number of studies have analyzed physiological E2 concentrations and the effects of administrated E2 on them in teleosts (goldfish (55, 72), rainbow trout (51), lampreys (52), tilapia (53), catfish (54), and sablefish (56)), these studies used relatively large teleosts, to which it is difficult to apply the genome editing tools. On the other hand, because the generation time for medaka is short, it is easier to use the gene editing tools. I took advantage of this and developed a method to analyze the E2 effect on the regulation of FSH by combining the gene editing tools and the experimental manipulation of blood E2 levels by OVX+E2.

Esr2a expressed in the pituitary play an indispensable role in the down-regulation of FSH induced by estrogens

In Chapter 3, I experimentally demonstrated the importance of Esr2a in the estrogenic regulation of FSH using *esr2a* knockout female medaka generated in Chapter 1 by applying the methodology established in Chapter 2. Moreover, I revealed that the Esr2a expressed in the pituitary plays an essential role in estrogenic regulation of *fshb* expression.

By using the feeding administration of E2 to OVX females for three days, I confirmed that this method successfully suppresses the increased expression of *fshb* after OVX. As described in

Chapter 1, *esr2a*$^{-/-}$ females show significantly high expression of *fshb*, and thus it is suggested that the Esr2a may be involved in the down-regulation of *fshb* expression. To verify this possible involvement, I applied the OVX+E2 protocol to *esr2a*$^{-/-}$ females. As a result, I revealed that E2 did not suppress the expression of *fshb* in *esr2a*$^{-/-}$ OVX females. Based on these analyses, I experimentally demonstrated that the reason of high expression of *fshb* in *esr2a*$^{-/-}$ females is due to the dysfunction of the negative feedback regulation via Esr2a.

Next, to examine whether hypothalamic signals are required for the estrogenic down-regulation of *fshb*, I analyzed the E2 effects on the expression of *fshb* in the isolated pituitary. As previously suggested (Zempo doctoral thesis, 2015), isolated pituitary exposed to E2-containing medium for 20 hours showed significantly low expression of *fshb* compared to the control group, and thus it is suggested that estrogens directly regulate the *fshb* expression. Finally, I examined the same protocol of pituitary incubation using the pituitaries of *esr2a*$^{+/-}$ and *esr2a*$^{-/-}$ females and demonstrated that the pituitary of *esr2a*$^{-/-}$ females did not show this E2-dependent down-regulation of *fshb*. From these results, I demonstrated that the Esr2a expressed in the pituitary plays a crucial role in the negative feedback regulation of *fshb* expression.

Although mammalian studies have suggested that estrogens directly act on the pituitary and suppress the FSH production, my thesis is the first report that identified specific ER for the negative feedback regulation of FSH at the pituitary level. Furthermore, by the analysis of Esr2a

in teleosts, I demonstrated for the first time the functional contribution of Esr2, but not Esr1, for the HPG axis regulation in vertebrates.

I also confirmed the expression of *esr2a* in the pituitary by RT-PCR analysis. Therefore, it is the reasonable to assume that the pituitary is directly regulated by estrogens via Esr2a. However, it is still unclear whether estrogens directly suppress the expression of *fshb* on the FSH cells. As described in detail in Chapter 3, I assume that it is difficult to detect the signal of *esr2a* in the pituitary by ISH. Therefore, for future analyses, I need to perform another histological approach to understand the precise localization of *esr2a* in the pituitary. For example, establishment and analysis of the GFP knock-in medaka whose GFP expression is controlled under intrinsic *esr2a* enhancer may give us an answer. In addition to this histological analysis, it may be possible to examine the functional role of Esr2a in the suppression of *fshb* expression at the cellular level in the pituitary by the gain of function analysis using *esr2a*$^{-/-}$ female medaka in which *esr2a* is expressed specifically in FSH cells (FSH cell-specific Esr2a-rescued line).

Conclusions

In the present thesis, I aimed to understand the general mechanism of negative feedback regulation of FSH using medaka as a model organism and revealed for the first time that Esr2a expressed in the pituitary plays an essential role in the estrogenic negative feedback regulation of

FSH in vertebrates (Fig. 4-1). In addition to these, I also found that *esr2a*$^{-/-}$ female medaka, which is the dysfunction model of FSH negative feedback regulation, show normal folliculogenesis and ovulation. From these findings, I propose that the negative feedback regulation of FSH is dispensable for the normal folliculogenesis. Therefore, at least in medaka, it is considered that the negative feedback regulation of FSH may contribute to their fitness by efficiently distributing their nutrition to their survival or their offspring rather than to the regulation of folliculogenesis.

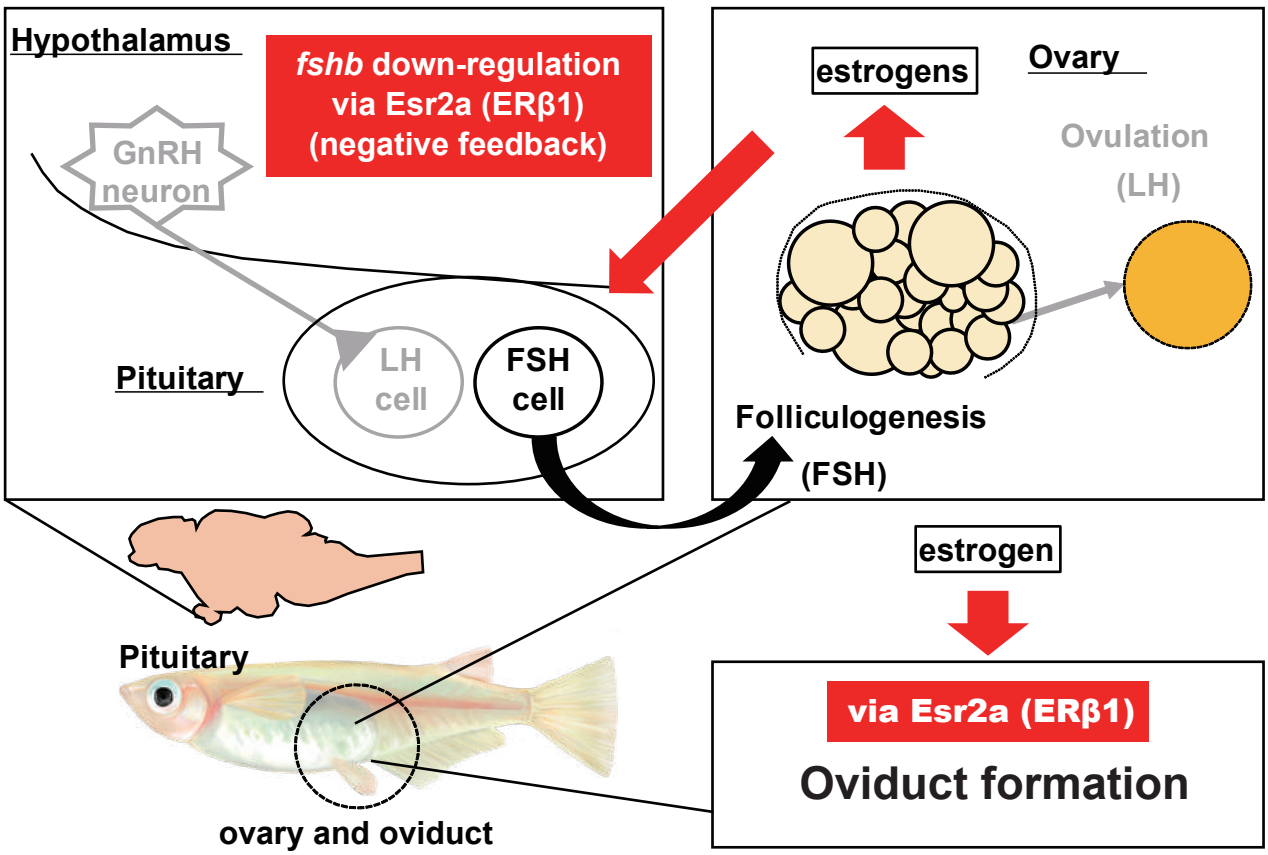
Furthermore, I established KO medaka lines for each type of ER, analyzed the physiological blood concentration of E2, and examined the best method of E2 administration to experimentally manipulate the E2 level in medaka. I believe that these efforts have made medaka an excellent model organism: easy to apply the gene editing tools, manipulate the blood level of estrogens, and analyze the regulatory mechanisms of reproduction in non-mammalian vertebrates.

Although the commonalities of this estrogenic regulation of FSH in vertebrates still remain to be addressed, I propose that the present thesis will go a long way towards the understanding of the general mechanism of the estrogen feedback regulation of FSH, which has been overlooked by studies only using mammals.

Figure legend

Fig. 4-1 Schematic illustration showing the roles of Esr2a in reproduction in female medaka. I demonstrated that Esr2a contributes to the two important processes of reproduction, the down-regulation of *fshb* and the formation of the oviduct morphology.

Fig. 4-1


Hypothalamus
fshb down-regulation via Esr2a (ERβ1) (negative feedback)
GnRH neuron
Pituitary
LH cell
FSH cell
estrogens
Ovary
Ovulation (LH)
Folliculogenesis (FSH)
estrogen
Pituitary
via Esr2a (ERβ1)
Oviduct formation
ovary and oviduct

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