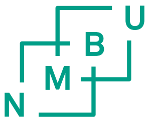

Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
Thesis 2019:6

Medaka *(Oryzias latipes)* gonadotropins: Production and developmental profiles, pharmacological characterization of their receptors, and establishment of a novel transgenic line tg(*fshb*:DsRed2)

Medaka *(Oryzias latipes)* gonadotropiner: Produksjons- og utviklingsprofiler, farmakologisk karakterisering av deres reseptorer og etablering av en ny transgen linje tg(*fshb*:DsRed2)

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Philosophiae Doctor (PhD) Thesis

Susann Burow

Department of Basic Sciences and Aquatic Medicine
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Norwegian University of Life Sciences

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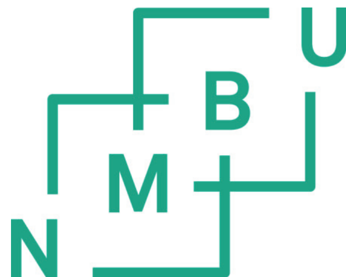

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Abstract

The gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), produced in gonadotrope cells of the pituitary, possess key roles in the endocrine control of vertebrate puberty and reproduction as part of the brain–pituitary–gonad (BPG) axis. To get a broader understanding of the specific functional roles of gonadotropins in fish, particularly Fsh for which information is scarce, I first generated recombinant medaka gonadotropins Fshβ, Lhβ, Fshβα, and Lhβα produced by *Pichia pastoris*, and specific antibodies against Fshβ and Lhβ raised in rabbits. These tools were used to develop and validate immunofluorescence (IF) protocols for the localization of Fshβ and Lhβ producing cells, and also enzyme-linked immunosorbent assays (ELISAs) for the quantification of Lh and Fsh at the single pituitary level. For gonadotrope cell localization, I compared IF using the generated antibodies against Fshβ and Lhβ, with fluorescent *in situ* hybridization (FISH) using probes complementary to *fshb* and *lhb* in adult medaka. The results highlighted cells expressing Lhβ and *lhb* to be located in the ventral part of the pituitary, and Fshβ and *fshb* expressing cells mainly to be present from the ventral to the dorsal part, being more widely distributed than the *lhb* expressing cells. Lh and Fsh protein content in male medaka pituitaries during pubertal development was compared to mRNA expression levels of *fshb* and *lhb* using qPCR. The findings highlighted that both Lh and Fsh protein amounts increased during pubertal development. Correspondingly, *lhb* mRNA levels increased significantly with maturation, while Fsh receptor (*fshr)* and Lh receptor (*lhr)* mRNA levels in testes were also demonstrated to increase. A pharmacological characterization revealed each gonadotropin activating its own cognate receptor, as well as partial ligand promiscuity and cross-species reactivity between medaka and Nile tilapia ligands and receptors. To provide a sensitive method for tracking *fshb* expression during development, and enabling studies on the functional roles of Fsh, I developed a medaka transgenic line tg(*fshb*:DsRed2) expressing red fluorescent protein under control of the endogenous *fshb* promoter. Interestingly, the first sign of DsRed2 expression was observed at 8 dpf (days post-fertilization), suggesting a functional role for *fshb* during early development. The Fsh cell number increased gradually with development. Together with our previously generated tg(*lhb*:hrGfpII) line, this line will provide a valuable tool for studies on gonadotropin regulation. Altogether, this thesis has contributed in generating key tools by developing IF-, FISH-, and ELISA-protocols, and a medaka *fshb* transgenic line, which will enable further studies on the

localization and quantification of Fsh and Lh at both mRNA and protein level, and hereby advance our understanding of gonadotropin functions in fish.

Sammendrag

De to gonadotropinene follikkelstimulerende hormon (Fsh) og luteiniserende hormon (Lh) blir produsert i hypofysens gonadotrope celler og besitter viktige roller i den endokrine kontrollen av pubertet og reproduksjon hos vertebrater som en del av hjerne-hypofyse-gonade (BPG) aksen. For å øke forståelsen for de spesifikke funksjonelle rollene til gonadotropiner i fisk, spesielt gjelder dette Fsh der informasjon er mangelfull, har jeg i min PhD-avhandling laget rekombinante gonadotropiner Fshβ, Lhβ, Fshβα og Lhβα fra japansk risfisk, eller medaka (*Oryzias latipes*). De fire proteinene ble produsert i celler av gjærsoppen *Pichia pastoris*. Spesifikke antistoffer mot Fshβ og Lhβ ble produsert i kaniner, og validert ved hjelp av Western blot og immunfluorescens (IF) hvor Fshβ- og Lhβ-produserende celler i hypofyse til medaka ble lokalisert. For lokalisering av gonadotrope celler sammenlignet jeg IF ved bruk av de produserte antistoffene mot Fshβ og Lhβ, med fluorescerende *in situ* hybridisering (FISH) ved hjelp av prober komplementære til *fshb* og *lhb*. Resultatene viste at celler som uttrykker Lhβ og *lhb* er lokalisert i den ventrale delen av hypofysen, mens Fshβ og *fshb* er uttrykt i celler som er lokalisert fra ventral til dorsal side, mer spredt enn de *lhb*-uttrykkende cellene. Antistoffer og rekombinante proteiner ble videre brukt til å validere enzyme-linked immunosorbent assays (ELISAs) for kvantifisering av Fsh og Lh i enkelthypofyser. Innholdet av Fsh- og Lh-protein i hypofyser fra hannmedaka under pubertetsutviklingen ble sammenlignet med mRNA-nivåer av *fshb* og *lhb* ved bruk av qPCR. Resultatene viste at nivå av både Fsh- og Lh-protein økte gjennom pubertet. Tilsvarende økte *lhb* mRNA-nivåer signifikant med modning, mens *fshb* viste liten endring. Genuttrykket av reseptorene til Fsh og Lh (henholdsvis *fshr* og *lhr*) ble undersøkt i testikkelvev og disse økte også gjennom pubertetsutviklingen. En farmakologisk karakterisering avdekket at hvert gonadotropin aktiverte sin egen reseptor, i tillegg til å utvise delvis ligand-promiskuitet til den andre gonadotropinreseptoren. Videre ble det avdekket interartsreaktivitet mellom medaka og tilapia (*Oreochromis niloticus*) sine ligander og reseptorer. For å få en sensitiv metode som kan detektere uttrykket av *fshb* under utviklingen, samt muliggjøre studier av Fsh sine funksjoner, utviklet jeg en transgen linje for medaka, tg(*fshb*:DsRed2), der rødt fluorescerende protein ble produsert under kontroll av den endogene *fshb*-promotoren. Interessant nok var det første tegnet på DsRed2 uttrykk observert 8 dager etter befruktning, noe som tyder på en funksjonell rolle for *fshb* under tidlig utvikling. Antall Fsh-produserende celler økte gradvis med utviklingen. I kombinasjon med vår tidligere genererte linje, tg(*lhb*:hrGfpII), vil denne linjen gi et verdifullt verktøy for studier av

gonadotropinregulering i medaka. Samlet har denne oppgaven bidratt til å videreutvikle nøkkelverktøy, som IF-, FISH- og ELISA-protokoller, samt en medaka *fshb* transgen linje, som muliggjør videre studier på lokalisering og kvantifisering av Fsh og Lh både på mRNA- og proteinnivå, og dermed bedre vår forståelse av gonadotropinfunksjonen i fisk.

Zusammenfassung

Die Gonadotropine, das follikelstimulierende Hormon (Fsh) und luteinisierende Hormon (Lh), die in den Gonadotropin produzierenden Zellen der Hypophyse erzeugt werden, spielen als Teil der Gehirn-Hypophysen-Gonaden-Achse (BPG) eine Schlüsselrolle bei der endokrinen Regulierung von Pubertät und Fortpflanzung in Wirbeltieren. Um ein breiteres Verständnis der spezifischen funktionellen Rolle von Gonadotropinen in Fischen, insbesondere von Fsh, für das es bisher kaum Informationen gibt, zu erhalten, wurden zunächst rekombinante Medaka Gonadotropine Fshβ, Lhβ, Fshβα und Lhβα in *Pichia pastoris* sowie spezifische Antikörper gegen Fshβ und Lhβ in Kaninchen hergestellt. Diese wurden zur Entwicklung und Validierung von Immunfluoreszenz (IF) Protokollen zur Lokalisierung von Fshβ- und Lhβ-produzierenden Zellen sowie von enzyme-linked immunosorbent assays (ELISAs) zur Quantifizierung von Lh und Fsh Konzentrationen in individuellen Hypophysen, verwendet. Zur Lokalisierung der Gonadotropin produzierenden Zellen in adulten Medaka wurden mit Fshβ und Lhβ Antikörpern generierte Fluoreszenz-*in-situ*-Hybridisierung (FISH) Daten mit denen von *fshb* und *lhb* komplementären Proben verglichen. Lhβ und *lhb* exprimierende Zellen befanden sich im ventralen Teil der Hypophyse, während Fshβ and *fshb* exprimierende Zellen hauptsächlich vom ventralen zum dorsalen nachgewiesen wurden. Diese waren dabei stärker verbreitet als *lhb* exprimierende Zellen. Der Proteingehalt von Lh und Fsh in der Hypophyse von männlichen Medaka während der Pubertätsentwicklung wurde mit den mRNA-Expressionsniveaus von *fshb* und *lhb* mittels qPCR verglichen. Insgesamt zeigten die Ergebnisse, dass sowohl die Lh- als auch die Fsh-Proteinmengen während der Pubertätsentwicklung anstiegen. Entsprechend erhöhten sich auch die *lhb*-mRNA-Level mit fortschreitender Entwicklung signifikant. Weiterhin wurde beobachtet, dass die mRNA-Level von Fsh-Rezeptor (*fshr*) und Lh-Rezeptor (*lhr*) in den männlichen Keimdrüsen anstiegen. Eine pharmakologische Charakterisierung ergab, dass jedes Gonadotropin seinen eigenen Rezeptor aktivierte, wobei teilweise Kreuzaktivierung zwischen den Gonadotropinen und Rezeptoren von Medaka und Tilapia auftrat. Im Zuge der Entwicklung einer sensitiven Methode zur Untersuchung der Expressionsmuster von *fshb* während der Entwicklung, die zudem Studien zur funktionellen Rolle von Fsh ermöglicht, wurde eine transgene Medaka Linie tg(*fshb*: DsRed2) hergestellt, die unter der Kontrolle des endogenen *fshb*-Promotors rot fluoreszierendes Protein exprimiert. Interessanterweise wurde die erste DsRed2-Expression bereits im Alter von 8 dpf (days post-fertilization) beobachtet, was auf eine bisher noch ungeklärte funktionelle Rolle dieser frühen *fshb*-

Expression hindeutet. Die Anzahl der Fsh produzierenden Zellen stieg mit fortschreitender Entwicklung allmählich an. Zusammen mit der zuvor generierten tg(*lhb*:hrGfpII) Linie ist die neu etablierte transgene Medaka Linie ein wertvolles Instrument für weitergehende Untersuchungen zur Regulierung von Gonadotropinen. Die vorliegende Arbeit hat durch die Weiterentwicklung von Schlüsselmethoden wie IF-, FISH- und ELISA-Protokollen, sowie der Etablierung einer Medaka *fshb*-Transgenlinie dazu beigetragen, weitergehende Studien zur Lokalisierung und Quantifizierung von Fsh und Lh, sowohl auf mRNA- als auch auf Proteinebene, zu ermöglichen.

Abbreviations

This thesis applies the nomenclature recommendations from MGI (http://www.informatics.jax.org/mgihome/nomen/gene.shtml) and ZFIN (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions).

Mammalian genes are italicized, first letter uppercase (e.g. *Fshb*); fish genes are italicized, all letters lowercase (*fshb*); mammalian (or unspecified) proteins are non-italicized, all letters uppercase (FSH); and fish proteins are non-italicized, first letter uppercase (Fsh).

11-KT	11-ketotestosterone
AC	adenylate cyclase
AH	adenohypophysis
BPG	brain–pituitary–gonad
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CG	chorionic gonadotropin
cGMP	cyclic guanosine monophosphate
Cq	quantification cycle
CV	coefficient of variation
dpf	days post-fertilization
DSB	DNA double-strand breaks
E_2	17β-estradiol
ECD	extracellular domain
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescent *in situ* hybridization
FSH	follicle-stimulating hormone
fshb:DsRed2	transgenic line expressing Red fluorescent protein (DsRed2) under the control of the endogenous *fshb* gene promoter
FSHR	follicle-stimulating hormone receptor
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GnIH	gonadotropin inhibitory hormone
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
IF	immunofluorescence
ISH	(non-fluorescent) *in situ* hybridization
LH	luteinizing hormone
LHR	luteinizing hormone receptor

mdFshβ	recombinant medaka Fshβ
mdFshβα	recombinant medaka Fshβα
mdLhβ	recombinant medaka Lhβ
mdLhβα	recombinant medaka Lhβα
MPE	medaka pituitary extract
mRNA	messenger ribonucleic acid
NH	neurohypophysis
NPY	neuropeptide Y
PD	*pars distalis*
PI	*pars intermedia*
PN	*pars nervosa*
PPD	*proximal pars distalis*
qPCR	quantitave polymerase chain reaction
RFP	red fluorescent protein
RIA	radioimmunoassay
SC	spermatocytes
SPA	spermatogonia type A
SPB	spermatogonia type B
SSC	spermatogonial stem cell
ST	spermatids
SZ	spermatozoa
T	testosterone
TMD	transmembrane domain
TPE	tilapia pituitary extract
TSA	tyramide signal amplification
TSH	thyroid-stimulating hormone

Species nomenclature

Throughout this thesis, the common name of a species is followed by the scientific name upon its first use. Only the common name will be given in consecutive use.

Scientific name	**Common name**
Acipenser gueldenstaedtii	Russian sturgeon
Anguilla anguilla	European eel
Anguilla japonica	Japanese eel
Brachymystax lenok	Manchurian trout
Carassius auratus	goldfish
Cyprinus carpio	common carp
Danio rerio	zebrafish
Dicentrarchus labrax	European sea bass
Epinephelus coioides	orange-spotted grouper
Fundulus heteroclitus	mummichog
Hippoglossus hippoglossus	Atlantic halibut
Macaca fascicularis	cynomolgus monkey
Mugil cephalus	grey mullet
Oncorhynchus keta	chum salmon
Oncorhynchus kisutch	coho salmon
Oncorhynchus mykiss	rainbow trout
Oncorhynchus nerka	sockeye salmon
Oncorhynchus rhodurus	amago salmon
Oreochromis niloticus	Nile tilapia
Oryzias latipes	Japanese medaka or ricefish
Solea senegalensis	Senegalese sole
Sparus aurata	gilt-head seabream

List of papers

Paper I a

Medaka Follicle-stimulating hormone (Fsh) and Luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression levels

Susann Burow, Romain Fontaine, Kristine von Krogh, Ian Mayer, Rasoul Nourizadeh-Lillabadi, Lian Hollander-Cohen, Yaron Cohen, Michal Shpilman, Berta Levavi-Sivan, Finn-Arne Weltzien

General and Comparative Endocrinology 272, 93-108 (2019)

Paper I b

Establishment of specific enzyme-linked immunosorbent assay (ELISA) for measuring Fsh and Lh levels in medaka, using recombinant gonadotropins

Susann Burow, Romain Fontaine, Kristine von Krogh, Ian Mayer, Rasoul Nourizadeh-Lillabadi, Lian Hollander-Cohen, Yaron Cohen, Michal Shpilman, Berta Levavi-Sivan, Finn-Arne Weltzien

MethodsX (submitted)

Paper I c

Data on Western blot and ELISA analysis of medaka (*Oryzias latipes*) follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) using recombinant proteins expressed with *Pichia pastoris*

Susann Burow, Romain Fontaine, Kristine von Krogh, Ian Mayer, Rasoul Nourizadeh-Lillabadi, Lian Hollander-Cohen, Yaron Cohen, Michal Shpilman, Berta Levavi-Sivan, Finn-Arne Weltzien

Data in Brief 22, 1057-1063 (2019)

Paper II

Pharmacological characterization of gonadotropin receptors (Fshr and Lhr) in medaka, *Oryzias latipes*

Susann Burow, Rasoul Nourizadeh-Lillabadi, Kristine von Krogh, Gersende Maugars, Lian Hollander-Cohen, Michal Shpilman, Ishwar Atre, Finn-Arne Weltzien, Berta Levavi-Sivan

General and Comparative Endocrinology (under revision)

Paper III

Spatial and temporal expression of *fshb* during early development in medaka (*Oryzias latipes*) using a novel transgenic line tg(*fshb*:DsRed2)

Susann Burow, Romain Fontaine, Rasoul Nourizadeh-Lillabadi, Finn-Arne Weltzien

Manuscript

1. Introduction

1.1 General background

With nearly 30,000 species, teleosts form approximately 98 % of all ray-finned fishes (Actinopterygii) (Nelson et al., 2016) and almost half of all extant vertebrates (Ravi and Venkatesh, 2018). Teleosts exhibit a remarkable level of morphological, physiological and behavioral diversity. They are an important food source, and aquaculture accounts for as much as 50 % of the world's fish food supply (Bostock et al., 2010).

The physiology and developmental programs of teleosts resemble that of mammals in many aspects, and additionally they possess a similar gene repertoire (Ravi and Venkatesh, 2018). Small-sized teleosts, such as Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), have become powerful research models of choice due to various reasons, such as their sequenced genome, small size, external development, transparent embryos, ease of breeding and maintenance, high fecundity, short generation times, and low maintenance cost (Dooley and Zon, 2000; Kirchmaier et al., 2015).

An improved understanding of teleost reproduction and the physiological mechanisms regulating pubertal development is of interest, not only from a fundamental perspective but also for aquaculture and fish conservation. The occurrence of early sexual maturation is an ongoing constraint in aquaculture and requires advances in control of puberty and sexual maturation. A better understanding of pubertal development will also be beneficial to conservation programs that relies on fry production for stocking purposes. In addition, research is crucial for the general understanding of vertebrate sexual maturation and puberty due to the similarity between key elements of the brain-pituitary-gonad (BPG) axis in all vertebrates. The BPG axis is functional and active during early development before entering a quiet phase until it becomes reactivated at puberty. This reactivation eventually results in gonad maturation and reproductive capacity (Weltzien et al., 2004; Zohar et al., 2010; Christensen et al., 2012). The main aim of this thesis is to improve our understanding of the functions and regulation of the pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), both crucial for gonad development, because details of their differential regulation mechanisms of synthesis and release are not well understood (Padmanabhan and McNeilly, 2001; Sharma et al., 2012).

1.2 The brain-pituitary-gonad (BPG) axis

In all vertebrates, reproductive competence is acquired through the developmental process of puberty (Weltzien et al., 2004), and is dependent on the coordinated actions of different hormones related with the BPG axis (Norris, 1997; Schulz et al., 2000; Weltzien et al., 2004) (BPG-axis figure 1). Puberty is defined as the first acquisition of the ability to reproduce in all mammalian and non-mammalian vertebrates, as well as in invertebrates (reviewed by Dufour et al., 2000). Puberty in female teleosts is marked by the onset of vitellogenesis (Patiño and Sullivan, 2002), and in males by the onset of spermatogenesis (Schulz and Miura, 2002; Schulz et al., 2010). The mechanisms that regulate the timing of the onset of puberty are not yet fully understood in any vertebrate. Current evidence indicates that the timing of pubertal development is under the control of a number of internal and external factors, including genetic factors, energy status (Thorpe, 1989; Bromage et al., 2001), environmental stimuli, notably photoperiod (Bromage et al., 2001), temperature and in some species even hydrostatic pressure (Sébert et al., 2007). In addition, in some species social interactions (Resink et al., 1989) can be an important cue for the start of sexual maturation (Dufour et al., 2000; Weltzien et al., 2004). There is also evidence that a functional coordination of a network of key genes and their transcriptional regulators are involved in the control of the onset of puberty (reviewed by Ojeda et al., 2003, 2006). However, despite what the ultimate factors may be for the initiation of puberty, the integrated signal coming from those factors is expressed in the secretion of gonadotropin-releasing hormone (GnRH). As such, the release of GnRH, leading in turn to the release of the pituitary gonadotropins, is the vital step in the initiation of sexual maturation in all vertebrates (Sisk and Foster, 2004).

In the brain, the environmental factors mentioned above affect and regulate various neurotransmitters (e.g. kisspeptin, melatonin, leptin, gamma-aminobutyric acid (GABA), neuropeptide Y (NPY), and others) (Trudeau et al., 2000; Yaron et al., 2003; Oakley et al., 2009; Chang et al., 2009; Nakane and Oka, 2010; Navarro and Tena-Sempere, 2012)) that produce an integrated output in the forebrain. This leads mainly to the release of the stimulatory Gnrh and the inhibitory dopamine (Dufour et al., 2010). Furthermore, gonadotropin inhibitory hormone (GnIH) can decrease gonadotropin release and inhibit gonadal development in mammals and birds (Tsutsui et al., 2009), however the effects of Gnih in teleost fish are conflicting. In addition, a growing number of neurotransmitters and neurohormones are shown to have a direct stimulatory effect on the pituitary, indicating roles in the differential regulation of Fsh and Lh.

In the pituitary, the activation of Gnrh receptors on the gonadotrope cells results in the synthesis and release of pituitary gonadotropins Fsh and Lh into the blood stream (Oakley et al., 2009). The action of Fsh and Lh necessitates binding to specific receptors that are embedded in the plasma membrane of target cells of the gonads.

In the gonads, Fsh and Lh activate one or more second-messenger pathways resulting in gametogenesis and steroidogenesis, the latter mostly leading to 17β-estradiol (E_2) synthesis in females and 11-ketotestosterone (11-KT) synthesis in males (Levavi-Sivan et al., 2010), in addition to the synthesis of non-steroid factors (e.g. growth factors, activins, inhibins). Depending on the maturational stage, 11-KT and E_2 apply positive or negative feedbacks at the pituitary and brain levels (Nocillado and Elizur, 2008), enabling regulatory cross-talk between central and peripheral members of the BPG axis (Dufour and Rousseau, 2007).

Although there are species differences and much is yet to be resolved regarding regulatory mechanisms, the two gonadotropic hormones have different functions in fish gonadal maturation. Fsh seems to be more important during the early stages of gametogenesis, spermatogonial proliferation in males, and vitellogenesis in females, while Lh seems more involved in processes leading to final gametogenesis, oocyte maturation and ovulation in females, and spermiogenesis and spermiation in males (Schwartz, 1974; Weltzien et al., 2004; Taranger et al., 2010; Schulz et al., 2010). How the BPG axis gains full physiological capability during puberty has not been entirely investigated in any vertebrate, but in both teleosts and mammals evidence indicates that the activation of the GnRH system is a key event.

1.3 GnRH and GnRH receptors in vertebrates

GnRH is the main neurohormone controlling the production and release of pituitary gonadotropins in all vertebrates (Schally et al., 1971; Sherwood et al., 1997). Mammalian GnRH was first characterized in 1971 (Matsuo et al., 1971), and approximately ten years later the first non-mammalian GnRH variant was discovered in chicken (King and Millar, 1982; Miyamoto et al., 1982) and one year later the first teleost Gnrh was identified in chum salmon (*Oncorhynchus keta*) (Sherwood et al., 1983). Extensive research has resulted in numerous Gnrh variants isolated from teleosts, including medaka (Okubo et al., 2000). It has been revealed that members of all vertebrate classes have at least two functional molecular forms of GnRH within each single species, and many teleosts possess three forms (reviewed in Kah et al., 2007).

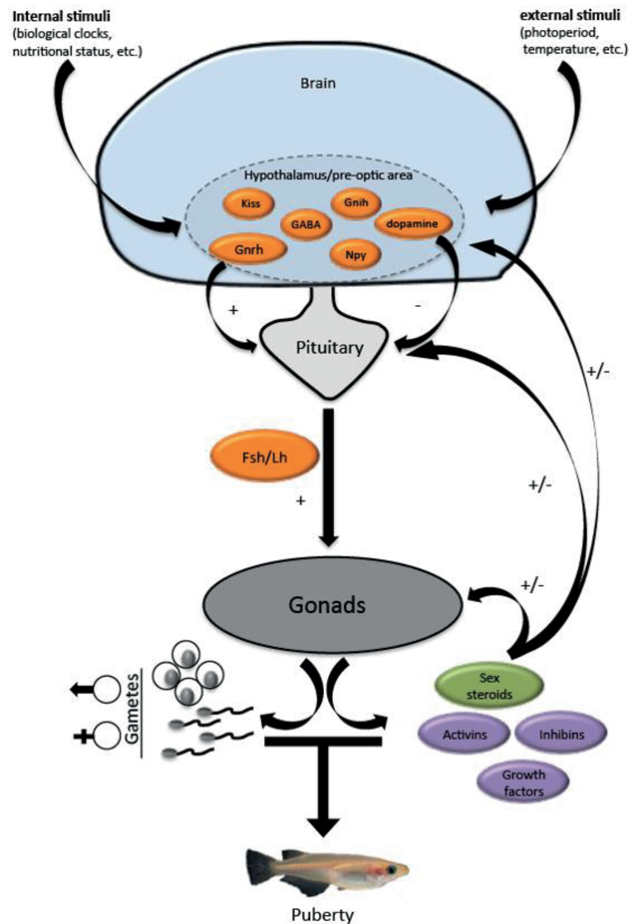

Figure 1. Brain-pituitary-gonad (BPG) axis, demonstrating the cascade of major hormones that are involved in the regulation of reproductive function. Stimulatory effect is indicated by a plus (+) sign, inhibitory effect is indicated by a negative (-) sign. Stimulatory and inhibitory signals produce an integrated output, which can result in the release of stimulatory gonadotropin-releasing hormone (Gnrh). Binding of Gnrh to specific receptors on gonadotrope cells in the pituitary leads to the secretion of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). After release into the blood stream, Fsh and Lh are transported by the circulation to the gonads where they bind to their respective receptors initiating the production of egg and sperm (gametogenesis) and synthesis and secretion of steroid hormones (steroidogenesis). Dopamine can either bind to receptors on the gonadotropes or on the cells producing Gnrh, leading to inhibition of Fsh and/or Lh release. Sex steroids 11-ketotestosterone, testosterone and 17β-estradiol can both have stimulatory or inhibitory effects on the production of gonadotropins, and can influence all levels of the BPG axis by different feedback mechanisms. GABA - gamma-aminobutyric acid; Gnih - gonadotropin inhibitory hormone; Npy - neuropeptide Y.

Initially, the different forms of GnRH were named after the first organism from which they were characterized, but this terminology became confusing since different GnRH variants could be found within the same species, and the same GnRH was found across species. Based on phylogenetic analysis and expression sites, White et al. (1995) and Fernald and White (1999) reported that GnRHs are branched into three paralogous groups termed GnRH1, GnRH2, and GnRH3. The hypothalamic GnRH1 or GnRH3 is the hypophysiotrophic isoform, dependent on the number of GnRH variants present (reviewed by Sherwood and Wu, 2005). GnRH2 is indicated to possess neuromodulatory functions related to reproductive behavior (Yamamoto et al., 1997; Uchida et al., 2005). Teleost fish express either two or three Gnrh variants that are derived from distinct genes (Lethimonier et al., 2004).

In mammals, GnRH is released into the median eminence portal vessels in a pulsatile manner (Carmel et al., 1976). While FSH is released both tonically and episodically, LH seems mainly to be released episodically (Padmanabhan, 1998), regulated through GnRH pulse frequency modulation (Haisenleder et al., 1991). Other factors, such as activin, inhibin, and follistatin seem to have an important role in both mammals (Ling et al., 1990; Ying et al., 1988) and teleosts (Aroua et al., 2012). However, how these factors differentially regulate Fsh and Lh, and whether Gnrh is also released in pulses in teleost fish remains unclear.

In teleost fish, various neurotransmitters modulate Gnrh secretion from the preoptic-hypothalamic region. Dopamine has been shown to inhibit the reproductive process, including the onset of puberty, in some fish species (grey mullet (*Mugil cephalus*), Aizen et al., 2005; European eel (*Anguilla anguilla*), Vidal et al., 2004). Dopamine blocks the synthesis and release of Gnrh (reviewed by Dufour et al., 2005), downregulates the synthesis of Gnrh receptors (de Leeuw et al., 1989; Levavi-Sivan et al., 2004), and inhibits basal and Gnrh-induced gonadotropin secretion from the pituitary (reviewed by Yaron et al., 2003; Dufour et al., 2005). Kisspeptin, a member of the RF amide peptide family, has been shown to possess a main role in controlling the onset of puberty in mammals (Colledge, 2004; Murphy, 2005; Seminara, 2005; Smith et al., 2005). It's been observed to act via Gpr54 receptor, a G protein-coupled receptor, directly on Gnrh neurons to induce Gnrh release during puberty, which stimulates the pituitary generation of Fsh and Lh (Seminara et al., 2003; de Roux et al., 2003). In medaka, kisspeptins might be involved in endocrine regulations besides the gonadotropin release, as kisspeptin neurons were revealed to control various neural systems, such as systems expressing neuropeptide Yb, cholecystokinin,

isotocin, vasotocin, and neuropeptide B (Nakajo et al., 2018). Notably, recent knockout studies in medaka suggest strongly that kisspeptins are dispensable for reproduction in teleosts (Nakajo et al., 2018). This is in contrast to earlier *in vivo* and *in vitro* studies demonstrating that Kisspeptin administration could variably enhance Lh release, depending on form and species (Espigares et al., 2015; Zmora et al., 2015). The central role of Gpr54 in the regulation of puberty was demonstrated in various studies (reviewed by Seminara, 2005; Murphy, 2005; Dungan et al., 2006), revealing that knockout of Gpr54 causes a blockade of Gnrh release (Messager et al., 2005).

The GnRH receptors (GnRHRs) belong to the rhodopsin family of G-protein coupled receptors (Strader et al., 1995). In general, the GnRH receptors are classified in two groups, type I and type II, each of which is separated into subgroups A and B (Hildahl et al., 2011). In teleosts, all Gnrhrs belong to either type IA or type IIB, and possess the highest affinity for Gnrh2 in *in vitro* binding studies (Illing et al., 1999; Okubo et al., 2001; Okubo et al., 2003; Servili et al., 2010). The establishment of molecular methods resulted in the discovery of Gnrh receptors from various different teleost species, for instance medaka (Okubo et al., 2001) and European eel (Penaranda et al., 2013). In teleosts there are up to five different Gnrh receptors, but knowledge of the relationship between ligand and receptor is still missing (Kah et al., 2007).

1.4 The pituitary

The pituitary gland is a vertebrate invention and a key endocrine organ producing and secreting six to eight hormones, from specific cell types, which are involved in different physiological processes, such as reproduction, growth and homeostasis (Weltzien et al., 2014). As in mammals, the teleost pituitary is located posterior to the optic chiasm below the hypothalamus surrounded by a bony chamber, the Turkish saddle or *sella turcica* (Frisen, 1967). The pituitary is divided into two main compartments, the neurohypophysis (NH) (or posterior pituitary), comprising the *pars nervosa* (PN), and the adenohypophysis (AH) (or anterior pituitary), composed of the *pars distalis* (PD) and *pars intermedia* (PI) (Wingstrand, 1966). The PD is further structured into the *rostral pars distalis* (RPD) and *proximal pars distalis* (PPD) (figure 2). The NH in mammals is located posterior to the AH, whereas in fish it is often situated dorsal to the AH (Norris, 2007).

There are some important differences between mammalian and piscine pituitary morphology. In mammals, hypothalamic neurohormones are released to the median eminence portal system. However, teleosts lack a portal system and instead, the anterior pituitary endocrine

cells are directly innervated by hypothalamic axons (Ball, 1981). In addition, many teleosts have a compartmentalized distribution of endocrine pituitary cell types, while this organization is restricted to the embryonic stage in mammals (Weltzien et al., 2004; Pogoda and Hammerschmidt, 2007). Furthermore, Fsh and Lh in teleosts are mainly generated and released from different pituitary cell types (Nozaki et al., 1990; Naito et al., 1991; Naito et al., 1993; Kanda et al., 2011; Golan et al., 2016), while in mammals there is mainly one pituitary cell type that secretes both gonadotropins. However, fish gonadotropes that synthesize both hormones have also been shown (García et al., 2002; Kasper et al., 2006; Pandolfi et al., 2006), as well as mammalian gonadotropes that produce only one hormone (Pope et al., 2006). The gonadotropes in mammals are distributed throughout the ventral portion of the PD, whereas in teleosts, both gonadotropes expressing *fshb* and *lhb* are located mainly in the ventral portion of the PPD, with some cells present in the PI of the pituitary gland (Weltzien et al., 2003). In teleosts, the gonadotropes (Fsh and Lh producing cells), thyrotropes (thyroid-stimulating hormone producing cells) and somatotropes (growth hormone producing cells) are situated in the PPD, while the lactotropes (prolactin producing cells) and adrenocorticotropes (adrenocorticotropic hormone producing cells) are located in the RPD. The melanotropes (melanocyte-stimulating hormone producing cells) and somatolactotropes (somatolactin producing cells) are located in the PI (Weltzien et al., 2004) (figure 2).

1.5 Gonadotropins

Both gonadotropins FSH and LH together with thyroid-stimulating hormone (TSH) and the placenta-specific chorionic gonadotropin (CG), belong to a large family of glycoprotein hormones (Pierce and Parsons, 1981). FSH and LH stimulate growth and development of the vertebrate gonads through gonadal biosynthesis of steroid hormones and growth factors (Weltzien et al., 2004). As in mammals, teleost Fsh and Lh have been isolated and characterized in a number of species (Suzuki et al., 1988; Swanson et al., 1989; Yaron et al., 2003; Weltzien et al., 2003). They were initially termed GtH I and GtH II, respectively, due to the unclear relationship with the corresponding tetrapod hormones. The structural and biological characteristics (Prat et al., 1996) of these fish gonadotropins, together with their phylogenetic relationships (Li and Ford, 1998), caused their classification as the homologous piscine counterparts of the mammalian FSH and LH (Swanson, 2000).

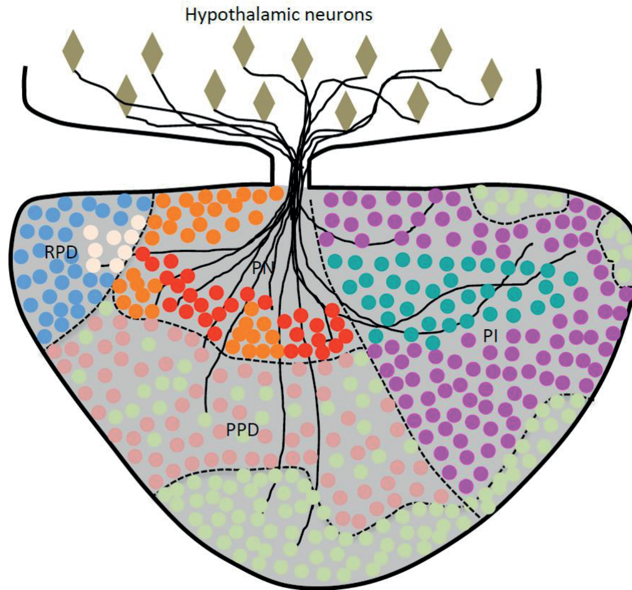

Figure. 2. Sagittal view of medaka pituitary. Schematic representation of the distribution of cell types. Endocrine cells in the anterior pituitary are directly innervated by hypothalamic axons. There are two gonadotrope cell types in teleosts producing primarily Fsh or Lh. Light green areas are dominated by Lh-cells, whereas light green and rose areas contain both gonadotrope cell types (dominated by Fsh-cells). RPD, *rostral pars distalis*; PPD, *proximal pars distalis*; PI, *pars intermedia*; PN, *pars nervosa*; Blue, lactotropes; White, corticotropes; Orange, thyrotropes; Red, somatotropes; Rose, Fsh-producing gonadotropes; Light green, Lh-producing gonadotropes; Dark green, somatolactotropes; Purple, melanotropes.

The α-subunit (GPα), which is common to Fsh and Lh, is non-covalently associated to a hormone-specific β-subunit, which defines the biological activity and specificity of the hormone. The α-subunit is the most conserved at the amino acid level among fish species (Li and Ford, 1998) (medaka and Nile tilapia (*Oreochromis niloticus*) gonadotropin β-subunit and α-subunit models in figure 3). The C-terminal region of the α-subunit and the N-terminal region of the β subunit of the gonadotropins were demonstrated to be important for receptor binding (Chen et al., 1992; Huang et al., 1993). Both subunits comprise in their structure a cysteine knot, stabilized by intramolecular disulphide bonds, and generally two *N*-linked glycosylation sites. All subunits Fshβ, Lhβ, and the α-subunit are encoded by distinct genes (Fiddes and Talmadge, 1984) (Yaron et al., 2003). Oligosaccharide formations of glycoprotein hormones have a crucial role in many of the molecules

functional characteristics (Ulloa-Aguirre et al., 1999), in addition, they are considered to constitute 30 % of the molecular weight (Weltzien et al., 2004).

The availability of molecular cloning techniques enabled isolation of genes encoding for the gonadotropin subunits from 56 fish species representing at least 14 teleost orders (Hollander-Cohen et al., 2017). Previously, the main limitation involved in isolating native gonadotropins was mainly the large number of pituitaries needed, and the low amount of protein isolated. The inconvenience of native protein purification, combined with the considerable increase in the number of isolated cDNAs encoding fish gonadotropin, enabled the production of species-specific recombinant fish gonadotropins through cDNA expression in heterologous systems (Levavi-Sivan et al., 2010). Chapter 3.2 elaborates both the current state of assays to quantify endogenous hormones, and different expression systems available for production of recombinant gonadotropins.

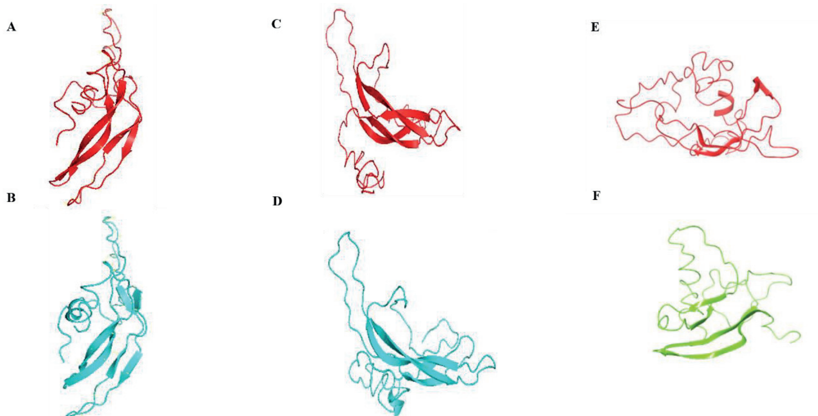

Figure 3. Medaka and Nile tilapia gonadotropin β-subunit and α-subunit models. Structural three-dimensional model of medaka Fshβ (A), Nile tilapia Fshβ (B), medaka Lhβ (C), Nile tilapia Lhβ (D), medaka α subunit (E), and Nile tilapia α subunit (F). The gonadotropin subunits are shown in ribbon representation. A high structural similarity was observed between the predicted models for the β subunits of both Fsh and Lh. The β sheet domains were highly conserved between medaka Fshβ (A) and Nile tilapia Fshβ (B), as well as between medaka Lhβ (C) and Nile tilapia Lhβ (D). The α subunit region, which is common for both the hormones within the species, displayed a varying homology between medaka (E) and Nile tilapia (F). (Models included in Paper II).

Specific pituitary cell types are defined by differential expression of transcription factors during early embryonic development (Pogoda and Hammerschmidt, 2009). However, knowledge is limited regarding the early development of pituitary gonadotropes producing Fsh and Lh (Hildahl et al., 2012; Weltzien et al., 2014). Both *Fshb* and *Lhb* gonadotropin subunit expression can be observed from embryonic developmental stages in mammals (Asa et al., 1988; Brooks et al., 1992; Japon et al., 1994; Pope et al., 2006; Szarek et al., 2008) and fish (Nica et al., 2006; Chen and Ge, 2012; Hildahl et al., 2012). Nevertheless, details concerning the function of this early expression are hardly known (Weltzien et al., 2014). The ontogeny of pituitary cell types has been studied in various fish species, demonstrating that gonadotropes appear after the other pituitary cell types, first occuring at hatching or post-hatch, similar to the situation in mammals (Weltzien et al., 2014). However, the *fshb* and *lhb* ontogeny during fish larval development remains to be fully investigated.

The generation of a medaka tg(*lhb*:hrGfpII) transgenic line (Hildahl et al., 2012) enabled the study of *lhb* expression during embryonic development, suggesting *lhb* expressing cells to be localized in the developing gut from an early stage (32 hours post-fertilization (hpf)), a long time before the first expression within the pituitary (2 weeks post-fertilization (wpf)). Hildahl et al. (2012) indicate a function during gut development, possibly involved in early steroidogenesis or osmoregulation. There are suggestions of very early expression of medaka *fshb* as well, however the functional implication is not clear (Hildahl et al., 2012). In conclusion, both ontogeny and functions of early *fshb* and *lhb* expression during embryonic and larval development remain to be elucidated.

1.6 Gonadotropin receptors

FSH and LH act on somatic cells in the gonads via activation of their cognate receptors, follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR), which are G-protein-coupled receptors from the family of rhodopsin-like receptors (family A) (Gether, 2000). The specific membrane-bound gonadotropin receptors were first demonstrated in fish in the early 1970s from binding studies using mammalian gonadotropins (human chorionic gonadotropin, hCG) and partly purified hypophysial glycoprotein hormones (Breton et al., 1973). As in mammals (rat, Heckert and Griswold, 1991; cynomolgus monkey (*Macaca fascicularis*), Dankbar et al., 1995), the teleost Fshr in the testis was identified on Sertoli cells, and the Lhr on Leydig cells, in

coho salmon (*Oncorhynchus kisutch*) (Yan et al., 1992; Miwa et al., 1994). The Fshr in the ovary was observed on the thecal cells, the granulosa cells, and in interstitial connective tissue, whereas the Lhr expression was restricted to the granulosa cells (rat, Camp et al., 1991; coho salmon, Yan et al., 1992; Miwa et al., 1994). Previous studies on medaka gonadotropin receptors (Ogiwara et al., 2013) and receptors of other teleost species (Levavi-Sivan et al., 2010; Nagahama and Yamashita, 2008; Yaron et al., 2003; Kwok et al., 2005) indicate that Fshr is absent in the large preovulatory follicles, which are designated to ovulate. This in agreement with the hypothesis that Fsh/Fshr determines the early phases of gametogenesis, such as vitellogenesis. However, the expression of Fshr and Lhr during puberty in male fish remains to be elucidated.

A two-receptor model for teleost gonadotropins was indicated by the mid-1990s (Miwa et al., 1994). The presence of two distinct gonadotropin receptors in a single fish species was confirmed by molecular cloning of two distinct receptor cDNA in amago salmon (*Oncorhynchus rhodurus*) (Oba et al., 1999a,b), and thereafter in several other species. Currently, a study based on genome analysis reported the presence of a single *fshr* but duplicated *lhr* genes in several teleost species, the latter probably caused by a local duplication event that occurred early in the actinopterygian lineage (Maugars and Dufour et al., 2015).

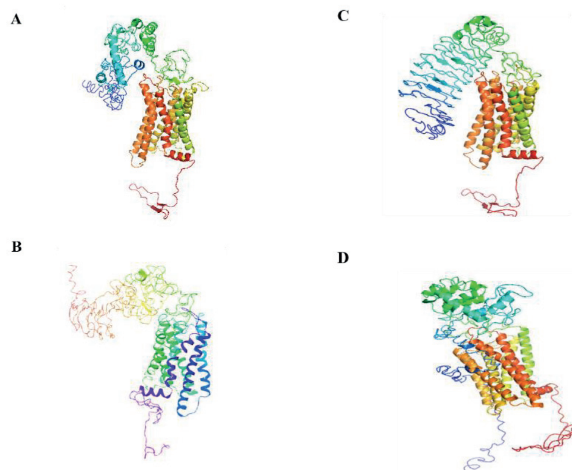

Figure 4. Medaka and Nile tilapia gonadotropin receptor models. Structural three-dimensional model of medaka Fshr (A), Nile tilapia Fshr (B), medaka Lhr (C), and Nile tilapia Lhr (D). The gonadotropin receptors are shown in ribbon representation. The receptors are structurally similar comparing medaka Fshr (A) and Nile tilapia Fshr (B), as well as medaka Lhr (C) and Nile tilapia Lhr (D). (Models included in Paper II).

FSHR and LHR comprise a subfamily of glycoprotein hormone receptors, which generally contains an N-terminal large extracellular domain (ECD), joined to a seven transmembrane domain (TMD) and an intracellular C-terminal domain coupled to a G protein (Ji, Grossmann and Ji, 1998; Vassart et al., 2004) (medaka and Nile tilapia gonadotropin receptor models in figure 4). Receptor activation results from specific hormone binding, which promotes the rearrangement of the transmembrane domain from an inactive to an active conformational state that is connected to the activation mainly via the Gs/cAMP/PKA pathway (Ji et al., 1998; Vassart et al., 2004).

In contrast to mammals, where FSHR and LHR are highly specific for their cognate hormones (Costagliola et al., 2005), studies in teleosts indicate that the specificities of the piscine Fshr and Lhr are less apparent. This was first demonstrated in coho salmon, where it was reported that Fshr, located on both theca and granulosa cells, did not distinguish between Fsh and Lh binding, whereas Lhr, present on granulosa cells, bound only Lh (Miwa et al., 1994; Yan et al., 1992). Various functional studies on teleost gonadotropin receptors using transfected mammalian cell lines reported on the pharmacological characterization of cloned piscine gonadotropin receptors, revealing a more general pattern. Teleost Lhrs seem to be specific for Lh, similar to their mammalian counterparts, whereas Fshrs possess a broader but still limited functional selectivity for both gonadotropins that may depend on the fish species or taxa (Aizen et al., 2012; Cahoreau 2015).

1.7 Gonads

Testis

Fish reproductive organs include the testes and ovaries. In most fish species, the gonads are a paired organ that can be partially or totally fused. The male gonad, the testis, performs the two major functions, spermatogenesis and steroidogenesis/growth factor production (medaka immature testes in figure 5 A, maturing testes in figure 5 B). Dependent on the formation of the germinal compartment, the testis is structured either in anastomosing tubules or branching tubules (Grier, 1981; Grier, 1993; Schulz and Miura, 2002). Based on the distribution of spermatogonia in the germinal compartment, both testis types can further be classified into unrestricted (spermatogonia occur anywhere along the length of the tubule or lobule; e.g. zebrafish,) or restricted types (spermatogonia are restricted to testis periphery; e.g. medaka) (Selman and Wallace, 1986; Grier, 1993). The germinal compartments comprise of spermatogenic cysts, which

are formed by one germ cell or isogenic clones of developing germ cells at the same developmental stage enclosed by one or several Sertoli cells (Billard et al., 1982; Pudney, 1995). In the interstitium, different somatic cells are present including Leydig cells. Leydig cells, which produce steroid hormones, are crucial for the regulation of spermatogenesis and development of secondary sexual characters and sexual behavior (Weltzien et al., 2004). Spermatogenesis occurs in the spermatogenic cysts or spermatocysts.

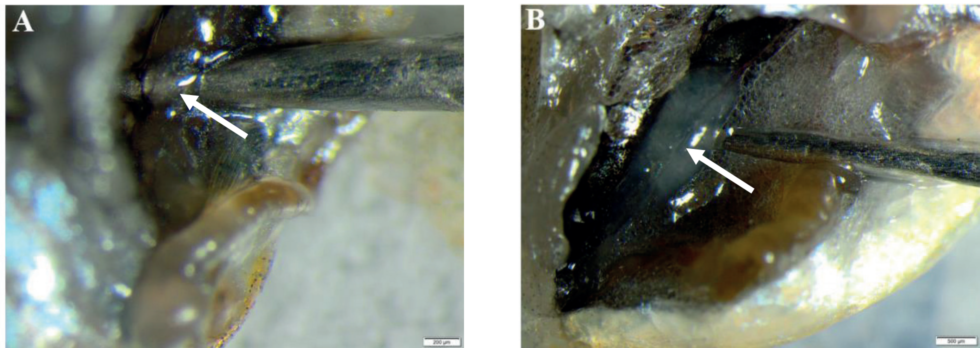

Figure 5. Medaka testes from A) immature fish with transparent, thin, and threadlike appearance (scale bar 200 µm) and B) maturing fish with white, milky, and large apparition (scale bar 500 µm; white arrow indicates testes).

Spermatogenesis

The development of male germ cells, from spermatogonial stem cells to mature spermatozoa, is called spermatogenesis. The onset of male puberty is marked by initiation of spermatogenesis, and depends on the close contact between the germ cells and Sertoli cells (Miura et al., 1996). The three major processes of spermatogenesis start by spermatogonial stem cells undergoing mitotic asynchronous proliferation, resulting in one undifferentiated spermatogonia type A (SPA) cell and one daughter stem cell. The undifferentiated SPA then divides further, generating differentiated SPA's. Continuing synchronous division generates spermatogonia type B (SPB) cells that after a number of generations will enter meiosis and produce spermatocytes (SC) (Loir, 1999; Ando et al., 2000). Then, primary spermatocytes (SC) divide in the first meiotic division to become secondary spermatocytes, which through the second meiotic division become haploid spermatids (ST). Thirdly, spermiogenesis occurs when spermatids differentiate into flagellated spermatozoa (SZ). Finally, the rupture between Sertoli cells and spermatozoa leads to spermatozoa being

released into the sperm duct, a process called spermiation. The major regulators of spermatogenesis are Fsh and the androgens (Weltzien et al., 2004). Fsh is crucial for proliferation of Sertoli cells and their generation of growth factors (Lejeune et al., 1996). Leydig cells produce androgens, mainly in response to stimulation by Lh. Furthermore, various paracrine and autocrine factors (e.g. Pescovitz et al., 1994; Miura et al., 1997; Schlatt et al., 1997) probably fine-tune the general regulation by the gonadotropins (Weltzien et al., 2004).

In this thesis, germ cells were grouped into five categories and the maturational stage of the testes defined according to the most advanced germ cell type present in the tissue: stage I (SPA; immature, figure 6A), stage II (SPA, SPB; early maturing), stage III (SPA, SPB, SC; maturing), stage IV (SPA, SPB, SC, ST; late maturing), stage V (SPA, SPB, SC, ST, SZ; mature, figure 6B).

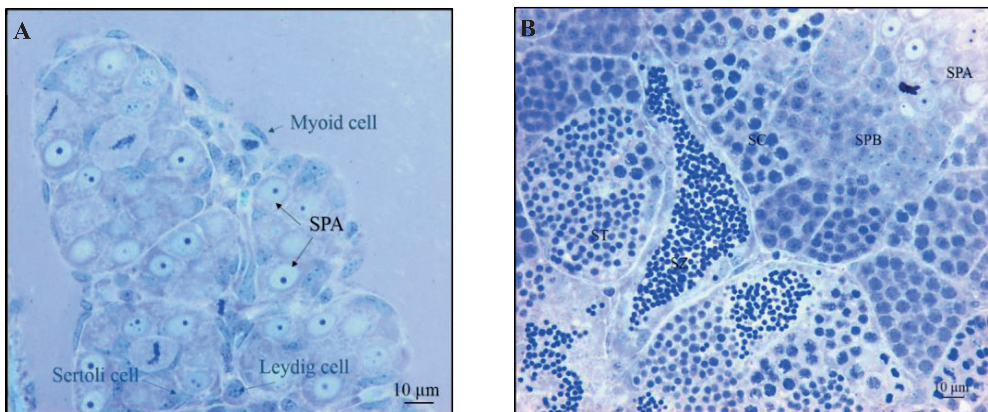

Figure 6. Testis histology sections from (A) sexually immature and (B) mature medaka. A) Section from sexually immature fish with spermatogonia type A (SPA) as the only germ cell present. B) Testis section from sexually mature medaka with all germ cell stages present (SPA; spermatogonia type B (SPB); spermatocytes (SC); spermatids (ST); spermatozoa (SZ)). Sagittal section, 3 µm thickness, stained with Toluidine Blue O (scale bar 10 µm). (Image included in Paper I).

Steroidogenesis

The vertebrate testis produces steroids in the process of steroidogenesis. An increased transport of cholesterol into Leydig cell mitochondria is the basis for the steroidogenic effect of Lh. The process is facilitated by the steroidogenic acute regulatory protein (StAR; Stocco and Clark, 1996).

Cholesterol is converted to pregnenolone, the precursor for all vertebrate sex steroid hormones. 11-KT (and to a lesser extent testosterone (T)) is considered to be the main androgen in teleost fish (Scott et al., 1980; Borg, 1994), while T is the main mammalian androgen (Hall, 1994). 11-KT was first described from plasma of male sockeye salmon (*Oncorhynchus nerka*) (Idler et al., 1960) and is unique to teleosts. The main reason for the difference in bioactivity between T and 11-KT is that testosterone can be aromatized into estrogen by aromatase, while 11-KT cannot. The existence of two different androgen receptors in fish, which possess different hormone binding characteristics (Ikeuchi et al., 1999; Todo et al., 1999), may also explain the differences in bioactivity between T and 11-KT.

1.8 Tools of reproductive physiology

An important tool when studying gonadotropin regulation and function is the quantification of hormone levels in blood and pituitary by immunoassays. In the following, immunoassays that have traditionally been used in fish, as well as difficulties of quantifying gonadotropin levels, will be discussed.

Different types of assays have been generated to measure gonadotropins in various species during recent decades. These methods can be grouped into assays using antigen-antibody recognition that reveal the molecule number or their mass (e.g. immunoassays) and methods that measure a response of a biological system after stimulation with Fsh or Lh (e.g. *in vivo* and *in vitro* bioassays) (Mazón et al., 2015). Traditionally, the assays used to determine gonadotropin levels in fish have been either radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Even though the sensitivities of ELISA and RIA for the measurement of gonadotropins seem to be in the same order of magnitude, ELISA is the first choice due to its practical advantages compared to the RIA (Yom-Din et al., 2016). For a small number of fish species, homologous immunoassays for Fsh have been established, including chum salmon (Suzuki et al., 1988), coho salmon (Swanson et al., 1989), rainbow trout (*Oncorhynchus mykiss*) (Prat et al., 1996), Nile tilapia (Aizen et al., 2007a), mummichog (*Fundulus heteroclitus*) (Shimizu et al., 2012), European sea bass (*Dicentrarchus labrax*) (Molés et al., 2012), Senegalese sole (*Solea senegalensis*) (Chauvigné et al., 2016), Russian sturgeon (*Acipenser gueldenstaedtii*) (Yom-Din et al., 2016), and common carp (*Cyprinus carpio*) (Hollander-Cohen et al., 2017). Assays to quantify Lh are available for a larger

number of species, leading to better understanding of Lh than Fsh physiology in fish (Yom-Din et al., 2016).

The availability of fish gonadotropins has been dependent on the extraction and purification of native gonadotropin subunits from fish pituitaries. The purification of native gonadotropins is costly and time-consuming, requires large amounts of biological material and has not been successful in various cases (Kamei et al., 2003; Vischer et al., 2003). It provides only low amounts of gonadotropin, particularly the Fsh whose concentration in fish pituitary is very limited (e.g. Weltzien et al., 2003; Aizen et al., 2007a). Within the past two decades, the number of isolated and characterized cDNAs encoding for fish gonadotropin subunits in various fish species has increased considerably due to molecular tools. This development enabled the generation of species-specific recombinant fish gonadotropins by expression of their cDNAs in heterologous systems (Yom-Din et al., 2016). Recombinant gonadotropins have various advantages compared to native purified hormones since they can be continually produced without being dependent on fish, allowing their continuous availability. Further, cross-contamination with other related glycoproteins is avoided (Levavi-Sivan et al., 2010).

A substantial challenge in the production of recombinant proteins and downstream usage in quantitative methods, such as ELISA, is a reliable determination of the exact mass of the standard hormone. This is especially the case when using newly developed ELISAs as shown in the present work (Paper I). Currently, the most accurate way to determine the concentration of recombinant proteins was found to be an ELISA specific for the 6-His residues contained in the protein sequence. This is different to a formerly used spectrophotometrical method which measured the absorbance originating from the intrinsic chromophores tryptophan, tyrosine and cysteine components (Hollander-Cohen et al., 2017). The differences in assessed concentrations using either the 6-His ELISA or spectrophotometry methods can be caused by high quantities of glycosylation inserted by the yeast, or by other biochemical characteristics of the gonadotropins, such as low quantities of tyrosine or tryptophan that can lead to incorrect results (Noble and Bailey, 2009).

When generating complete gonadotropins, a correct assembly, folding and glycosylation of both subunits is crucial for the production of biologically active hormones (Levavi-Sivan et al., 2010). The glycosylation degree is crucial for intracellular folding, secretion, clearance from the blood, and binding and signaling at the target-cell level (Ulloa-Aguirre et al., 2001, 2003). The

glycosylation degree of the recombinant gonadotropins has been investigated in some cases and indicated that the carbohydrate variations on the mature protein occur exclusively through *N*-linked glycosylation, not through *O*-linked glycosylation in Japanese eel (*Anguilla japonica)* (Kamei et al., 2003), Nile tilapia (Kasuto and Levavi-Sivan, 2005), and Manchurian trout (*Brachymystax lenok*) (Ko et al., 2007). Compared to this, mammalian glycoprotein hormones carry *O*-linked and *N*-linked oligosaccharides. *N*-linked oligosaccharide chains were revealed to possess a minor role in receptor binding of glycoprotein hormones yet are crucial for bioactivity, whereas *O*-linked oligosaccharide chains were shown to play a minor role in receptor binding and signal transduction yet are important for *in vivo* half-life and bioactivity (Fares, 2006).

1.9 Model species medaka

The Japanese medaka or ricefish (figure 7) is a small egg-laying fresh water fish that is native to East Asian countries, such as Japan, Taiwan, Korea, and China. The ricefishes, including medaka, belong to the family Adrianichthyidae, one of six families of the order Beloniformes. While medaka occurs in fresh or brackish water, the majority of the 264 species belonging to the order Beloniformes is marine. This indicates that the common ancestors of medaka and relatives were marine fish, and some species adapted to freshwater (Inoue and Takei, 2003). Medaka shares the same advantages of other small model fishes such as zebrafish, and is recognized as excellent model species for vertebrate research. Since it is oviparous, like most teleosts, embryonic development occurs externally and embryos are completely transparent during most of the embryonic development, providing an excellent model for microinjection studies (Kinoshita et al., 2009). Further, medaka is a powerful research model because of its rapid development and short generation time, sequenced genome, and availability of advanced forward and reverse genetic techniques (Kinoshita et al., 2009). Medaka is one of the few teleosts possessing the male heterogametic (XX-XY) sex-determining system (*dmy* gene in male), enabling genetic sex determination already from fertilization (Aida, 1921).

Figure 7. Oviparous adult medaka female (right) and male (left), female with transparent embryos.

2. Aims of the study

The overall aim of my PhD thesis has been to generate key tools that enable localization and quantification of Fsh and Lh at both mRNA and protein level in the model species medaka. These tools are necessary in order to get a broader understanding of the specific functional roles of gonadotropins in fish, particularly Fsh, for which information is scarce. The following sub-goals were identified:

- To generate recombinant medaka gonadotropins Fshβ (mdFshβ), Lhβ (mdLhβ), Fshβα (mdFshβα), and Lhβα (mdLhβα), to produce specific antibodies against medaka Fshβ and Lhβ, to develop and validate immunofluorescence (IF) protocols for localization of Fshβ and Lhβ producing cells, and to establish enzyme-linked immunosorbent assays (ELISAs) for the quantification of Fsh and Lh at the single pituitary level (Paper I)
- To characterize the localization of gonadotrope cells by comparing IF using the generated antibodies against Fshβ and Lhβ, with fluorescent *in situ* hybridization (FISH) using probes complementary to *fshb* and *lhb* in adult medaka (Paper I)
- To evaluate a profile of pituitary Lh and Fsh protein content comparing juveniles and adults using ELISA and to compare with a developmental profile of mRNA expression levels of *lhb* and *fshb* by qPCR in male medaka (Paper I)
- To pharmacologically characterize the Fsh receptor (Fshr) and Lh receptor (Lhr) in medaka, and to study intra- and interspecies ligand specificity and cross-reactivity of gonadotropins comparing receptors and ligands from medaka and tilapia, the latter in light of comparative amino acid sequence alignment and three-dimensional models (Paper II)
- To determine the developmental gene expression profile of medaka *fshr* and *lhr* in the testes (Paper II), as compared to the corresponding profile of pituitary Fsh and Lh protein levels in both juvenile and adult male medaka (Paper I)
- To develop a novel medaka transgenic line where the expression of red fluorescent protein is under control of the endogenous *fshb* promotor [tg(*fshb*:DsRed2)] to provide a sensitive method tracking *fshb* expression during development, and enabling studies on the functional roles and regulation of Fsh (Paper III)
- To identify spatial and temporal expression of *fshb* during early development in medaka using the novel medaka transgenic line tg(*fshb*:DsRed2) (Paper III)

3. Consideration of methods

The focus of this thesis was on the development and validation of protocols of methods for the quantification and localization of Fsh and Lh at both mRNA and protein level, in addition to Fsh and Lh receptor pharmacology. The methodologies that were used to answer these aims are discussed in the following sections. They comprise: production and quantification of recombinant gonadotropins, production and validation of specific polyclonal antibodies for mdFshβ and mdLhβ, competitive enzyme-linked immunosorbent assay (ELISA), quantitative real-time PCR (qPCR), fluorescence *in situ* hybridization (FISH), immunofluorescence (IF), luciferase reporter gene assay, and transgenesis in medaka.

3.1 Animals

In this thesis, all fish used were obtained from the NMBU medaka facility. Husbandry protocols for fish maintenance (described in **Paper I**) and all experimental procedures were in conformity with standard operational procedures at the NMBU (Norwegian University of Life Sciences, Oslo, Norway) medaka facility, the Norwegian Animal Health Authority, and the Norwegian Animal Welfare Act (https://www.regjeringen.no/en/dokumenter/animal-welfare-act/id571188/).

3.2 Production and quantification of recombinant gonadotropins

The availability of fish gonadotropins has traditionally been dependent on the extraction and purification of the native hormones from fish pituitaries, but due to the increasing number of isolated cDNAs coding for fish gonadotropin subunits, advanced molecular engineering now provides the technology to generate recombinant gonadotropins from isolated cDNAs. Recombinant gonadotropins possess several advantages over native purified hormones, for instance their continuous availability (Levavi-Sivan et al., 2010). The applications of recombinant gonadotropins are diverse, such as basic investigations on their structure or biochemical characteristics, or their use as antigens for the development of specific antibodies (Muasher et al., 2006; Vegetti and Alagna, 2006).

For the generation of the whole dimer, cDNAs encoding for both subunits have been expressed together. This has been performed using different strategies, for instance by co-transfecting two expression plasmids with each comprising one of the subunit cDNAs (Kobayashi

et al., 2006; Vischer and Bogerd, 2003), or by transfecting one expression plasmid containing both cDNAs under the control of different promoters (Cui et al., 2007; Kamei et al., 2003; So et al., 2005; Zmora et al., 2007). Another possibility is the construction of a fusion cDNA consisting of the sequence of both subunits in a single open reading frame, which leads to a single protein instead of a dimer, known as single-chain gonadotropins (e.g. Kasuto and Levavi-Sivan, 2005; Aizen et al., 2007a; Ko et al., 2007; Yom-Din et al., 2016; Hollander-Cohen et al., 2017). The subunits in the single-chain gonadotropins are commonly linked by a spacer, which consists of different amino acids, such as histidine tags (Aizen et al., 2007a; Kasuto and Levavi-Sivan, 2005; Ko et al., 2007; Morita et al., 2004), synthetic DNAs encoding groups of amino acids (Aizen et al., 2007a; Kasuto and Levavi-Sivan, 2005; Ko et al., 2007) or the carboxy-terminal peptide from the chorionic gonadotropin (Morita et al., 2004). These spacers enable a correct folding of the subunits, and all of them produced functionally active hormones. In **Paper I**, the genes contained a "linker" sequence comprising six amino acids (three Gly-Ser pairs) between the β- and α-chains to assist in subunit chimerization (Aizen et al., 2017), as well as a six-His tail (His^6) that was placed at the end of the β subunit to enable purification of the recombinant proteins. Challenges and critical elements when quantifying gonadotropins are elaborated in chapter 1.8 of the Introduction.

Various expression systems exist for the generation of recombinant proteins including prokaryotic systems, such as *Escherichia coli* (*E. coli*), as well as eukaryotic organisms like yeast, or mammalian or plant cells (Levavi-Sivan et al., 2010). The choice of an expression system depends mainly on the properties of the protein to be produced, the required yield, and the need of further purification. The production cost is usually a major factor when a continuous generation of a recombinant protein is required (Reyes-Ruiz and Barrera-Saldaña, 2006; Sethuraman and Stadheim, 2006). Furthermore, the ability of the organism to resemble the protein post-translational modifications in the target organism should be considered, as well as growth rate, scalability, or glycosylation. A comparison of the protein production efficiency of all those systems is difficult due to purification or concentration of the gonadotropins in some cases (Levavi-Sivan et al., 2010). In the following, various expression systems for the production of recombinant proteins commonly used in the field will be discussed.

Pichia pastoris (*P. pastoris*) has been developed as an expression system for the high-level production of recombinant proteins (Hollenberg and Gellissen, 1997) and has been successfully used to generate recombinant proteins of both farm animals (Gifre et al., 2017) and fish (Levavi-

Sivan et al., 2010, 2008). To date, recombinant human chorionic gonadotropin (hCG; Sen Gupta and Dighe, 1999), ovine Fsh (Fidler et al., 1998), bovine Fsh (Samaddar et al., 1997), porcine Fsh (Richard et al., 1998), Japanese eel Fsh (Kamei et al., 2003), Nile tilapia Fsh (Aizen et al., 2007a) and Lh (Kasuto and Levavi-Sivan, 2005), zebrafish Fsh (Yu et al., 2010), Russian sturgeon Fsh and Lh (Yom-Din et al., 2016), and common carp Fsh and Lh (Hollander-Cohen et al., 2017) have been produced. In **Paper I**, *P. pastoris* has been used as expression system to produce medaka Fsh an Lh because the system possesses advantages of both bacterial and mammalian expression systems (Cereghino and Cregg, 2000). It can be cultivated at high cell densities and high expression level in inexpensive serum-free, chemically defined medium (Cregg, 2007). Furthermore, *P. pastoris* has the capacity to carry out post-translational modification events that resemble to those of vertebrate cells (Cereghino and Cregg, 2000). Passage of the expressed protein through the secretory pathway enables proteolytic maturation, glycosylation and disulfide-bond formation to arise (Hollenberg and Gellissen, 1997; Hamilton et al., 2006). *P. pastoris* has only a few disadvantages, for instance that the use of methanol as an inducer has a certain hazard, and that the glycosylation is still different from mammalian cells.

Various systems have been used for the generation of fish gonadotropins, such as cultured insect cells employing baculovirus-based systems (Cui et al., 2007; Meiri et al., 2000) or plasmids (Kazeto et al., 2008; Zmora et al., 2007), silkworm larvae (Ko et al., 2007; Kobayashi et al., 2006), the mammalian cell line CHO-K1 (Choi et al., 2005; So et al., 2005), the soil amoeba (Vischer et al., 2003), transgenic rainbow trout embryos (Morita et al., 2004), and the yeast *P. pastoris* (Aizen et al., 2007a, b; Burow et al., in press); Hollander-Cohen et al., 2017; Kamei et al., 2003; Kasuto and Levavi-Sivan, 2005; Yom-Din et al., 2016). All of these systems have been successful in producing recombinant hormones, albeit showing certain strengths or weaknesses that are summarized in Table 1). The bacterial expression system usually enables high yield, however the expressed proteins might not have appropriate structure (Langley et al., 1987). In contrast, high yielding and suitable forms for expressed proteins have been performed in baculovirus expression system using insect cells (Cui et al., 2007). This system has been well used for the functional expression of recombinant proteins in eukaryotes, however it is an expensive expression system (Luckow and Summers, 1988). The expression system utilizing a mammalian cell line also allows the suitable forms of expressed proteins, but the system has higher costs and is usually low yielding

(Kamei et al., 2003). Less expensive is the use of soil amoeba, silkworm larvae, and fish embryos, since they do not necessitate costly and sophisticated growth media (Levavi-Sivan et al., 2010).

In **Paper I**, recombinant medaka Fshβ (mdFshβ), medaka Lhβ (mdLhβ), medaka Fshβα (mdFshβα), and medaka Lhβα (mdLhβα) were produced using *P. pastoris*, following a procedure described by Kasuto and Levavi-Sivan (2005) and Yom-Din et al. (2016). mdFshβ and mdLhβ were further used to produce and validate polyclonal antibodies in rabbits, anti-mdFshβ and anti-mdLhβ, which were utilized as primary antibodies for the ELISA profile of pituitary Fsh and Lh levels. Recombinant mdFshβα and mdLhβα were used for ELISA standard curves.

Table 1. Commonly used protein expression systems to express recombinant proteins. Comparison of different expression systems used for expression of recombinant gonadotropins showing strengths/advantages (+) and weaknesses/disadvantages (-) of each system, including applications.

Expression system	Bacterial expression systems	*Pichia pastoris* expression system	Baculovirus based expression systems	Mammalian expression systems
High-level expression / High yield	+	+ (even though may not produce high yield compared to the baculovirus-based methods)	+ especially intracellular protein	+/- modest
Low cost of media / Fermentor costs	+	+	- expensive medium, need a lot of virus	- expensive medium
Fast growth rate	+	+	-	-
Simple culture conditions	+	+	-	- complex growth conditions
Fast expression	+	+	-	-
Scalability	+	+	+/- modest	+/- modest
Post-translational modifications	- few	+ extensive	+ extensive	+ full
Expression of large proteins	- difficulties expressing large proteins (> 50 kDa)	+ can express large proteins (> 50 kDa)	+ can express large proteins (> 50 kDa)	+ can express large proteins (> 50 kDa)
Glycosylation / Signal peptide removal	-	+	+ (mostly)	+ produces authentic proteins
+ More advantages	easy to manipulate easy to form sulfide bonds	for secretory protein or intracellular expression protein secretion efficient, allows simple purification N-terminal glycosylation superior to *Saccharomyces cerevisiae* no endotoxin chaperonins help folding can handle S-S rich proteins	chaperonins help folding effective cell folding glycosylation similar to mammalian cells relatively easy enzymatic de-glycosylation (beneficial for protein structure assays) no endotoxin	suspension culture characteristics of the cells can be produced on a large scale effective protein folding suitable for secreting proteins no endotoxin chaperonins help folding
- More disadvantages	eukaryotic proteins sometimes toxic cannot handle S-S rich proteins easy to form inclusion bodies ineffective *in vitro* folding different codon usage	use of methanol as inducer has certain hazard glycosylation but different from mammalian cells	cell culture sustainable only 4-5 days set-up time consuming secretion pathway of the pro-peptide inefficient glycosylation but different from mammalian cells virus infection can lead to cell lysis and potential expression of protein degradation	set-up time consuming cell culture sustainable for limited period of time selection takes time
Applications	production of purified protein (structure, enzyme, drug discovery) drug protein production	production of purified protein (structure, enzyme, drug discovery)	production of purified protein (structure, enzyme, drug discovery)	production of purified protein (structure, enzyme, drug discovery) drug protein production cell-based studies
References	Langley et al. (1987) Recombinant-DNA-derived bovine growth hormone; Chung et al. (2015), giant grouper (*Epinephelus lanceolatus*) growth hormone	Aizen et al. (2007a, b) in Nile tilapia (*Oreochromis niloticus*); Burow et al. (2018, in press) in medaka; Hollander-Cohen et al. (2017) in common carp (*Cyprinus carpio*); Kamei et al. (2006b, 2003) in Japanese eel (*Anguilla japonica*); Kasuto and Levavi-Sivan (2005) in Nile tilapia (*Oreochromis niloticus*); Levavi-Sivan et al. (2008), in Nile tilapia (*Oreochromis niloticus*); Yom-Din et al. (2016) in Russian sturgeon (*Acipenser gueldenstaedtii*)	Cui et al. (2007) in orange-spotted grouper (*Epinephelus coioides*); Huang et al. (1991) in common carp (*Cyprinus carpio*); Kobayashi et al. (2003), Kobayashi et al. (2006), Hayakawa et al. (2008) in goldfish (*Carassius auratus*); Ko et al. (2007) in Manchurian trout (*Brachymystax lenok*); Meiri et al. (2000) in gilt-head seabream (*Sparus aurata*)	Choi et al. (2005) in Manchurian trout (*Brachymystax lenok*); So et al. (2005) in zebrafish (*Danio rerio*)

3.3 Production and validation of specific polyclonal antibodies for mdFshβ and mdLhβ

Polyclonal antibodies are defined as the total population of antibodies that are present in an animal serum. They are derived from multiple B-cell clones, which have differentiated into antibody-producing plasma cells in response to an immunogen. In contrast to a monoclonal antibody that recognizes a single epitope, a polyclonal antibody against a single molecular species of antigen commonly recognizes multiple epitopes on the target molecule, enabling multiple antibodies binding through the antigen. The choice of species is based on different parameters, most importantly on the intended amount of polyclonal antibody. For most experiments, the rabbit is the species of choice for generating polyclonal antibodies (Houen, 2015), as performed in **Paper I**. Goats, sheeps and horses have been used for the production of antitoxin sera for therapeutic applications, where large amounts of serum are needed (Houen, 2015).

Identification of antibody epitopes should be considered when generating antibodies, indicating which specific residues are directly (e.g. contact residues) or indirectly (e.g. framework determinants) involved within the antigen-antibody interaction. This information could be crucial for the identification of antibody targets, as well as off-targets. The golden standard is a structural investigation of epitope analysis, particularly of discontinuous epitopes, and when detailed information is required (Gershoni et al., 2007).

The polyclonal antisera against recombinant mdFshβ and mdLhβ described in **Paper I** were produced in rabbits , generally following a procedure reported by Aizen et al. (2007a). In order to validate the generated antibodies, the recombinant proteins and medaka pituitary extract were visualized using anti-mdFshβ, or anti-mdLhβ antisera. To verify that the plasma of the rabbit before the final injections did not react with mdFshβ and mdLhβ, a Western blot using medaka pre-immune serum against medaka pituitary extract, mdFshβ, and mdLhβ was performed.

3.4 Enzyme-linked immunosorbent assay (ELISA)

Traditionally, methods to quantify gonadotropin levels in fish have been either radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). These assays have been shown to possess similar sensitivity levels, however, ELISA is the first choice because of its practical advantages, for instance it does not rely on the use of radioisotopes (Yom-Din et al., 2016). There are relatively few fish species for which both protein content and pituitary mRNA expression of gonadotropins

can be measured, mainly because of missing valid protein assays for quantitative measurement, especially Fsh, available for fishes (Hollander-Cohen et al., 2017). Therefore, in **Paper I**, the aim was to develop and validate specific and homologous competitive ELISAs for determining Fsh and Lh protein levels in pituitaries of male medaka, using a procedure based on the methods described by Mañanós et al. (1997) and Aizen et al. (2007b).

ELISA was first described by Engvall and Perlmann (1971) as a new method to detect various proteins, including many recombinant molecules, and to identify specific antibodies following immunization (Hornbeck et al., 2015). There are different types of ELISA, such as the **direct ELISA** that detects an antigen immobilized to the plate by an antibody directly conjugated to an enzyme, for instance horseradish peroxidase (HRP). In contrast, the **indirect ELISA** detects an antigen in two steps; firstly, an unlabeled primary antibody binds to the specific antigen, and secondly, a secondary antibody conjugated to an enzyme directed against the host species of the primary antibody, is applied. The **Sandwich ELISA** necessitates matched antibody pairs (capture and detection antibodies) that each is specific for a different epitope, with the capture antibody binding the antigen that is detected in a direct or indirect ELISA configuration. The **competitive ELISA** (figure 8) is more complex, and predominantly used to measure the concentration of an antigen, by the sample antigen competing with a reference for binding to a limited amount of labeled antibody.

The central event of a competitive ELISA is a process of competitive binding between the original target antigen (sample antigen) and the "add-in antigen" (recombinant antigen). Simplified, the primary unlabeled antibody (anti-mdFshβ or anti-mdLhβ, raised in rabbit, in **Paper I**) is incubated with the sample antigen, containing the native molecule of interest (Fshβ or Lhβ from pituitary extract in **Paper I**). Antibody-antigen complexes are transferred to 96-well plates that are pre-coated with the recombinant protein, the "competing" molecule (mdFshβ or mdLhβ in **Paper I**). The more antigen was present in the sample, the less antibody will be able to bind to the antigen in the well ("competition"). Then, by washing the plate, the antibody-sample antigen complexes are removed. Further, the secondary antibody (goat anti-rabbit horseradish peroxidase (GAR-HRP) in **Paper I**) is added, which is specific to the primary antibody and conjugated with an enzyme. A colorimetric substrate (3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate) is added, which, when oxidized by HRP using hydrogen peroxide as the oxidizing agent, yields a characteristic color change, detectable by spectrophotometric method. After the acid stop solution

is applied, optical density can be read at 450 nm. Substrate hydrolysis yields a signal that is inversely proportional to antigen concentration within the sample. The standard curve is developed applying the same principle, but serial dilutions of a known antigen concentration (recombinant Fshβα or Lhβα) are used instead.

The competitive ELISA possesses various advantages over the other types, such as a high sensitivity, consistency, and the requirement for only one antibody. No sample processing is required since the antigen does not necessitate purification prior to measurement. The competitive ELISA demands that the primary antibody is highly specific to the antigen, allowing amplification of the signal resulting in higher sensitivity. Furthermore, the competitive ELISA possesses advantages over the competitive RIA, since the radioisotope labeling of a RIA is a laborious procedure, and is implementing problems, such as antigen denaturation by the labeling and low stability of the labeled antigens (Govoroun et al., 1998). Other immunoassays that allow the detection of specific proteins by validated specific antibodies, such as Western Blot and Dot Blot, lack the possibility to accurately determine the concentration of specific proteins in biological samples.

Several controls were included in each ELISA experiment, such as a blank sample control in order to confirm that the antibody is highly specific to the antigen. In addition, a positive control was implemented, consisting of a known concentration of the recombinant protein; the positive control indicates if the procedure is optimized and working. To check for non-specific binding and false positive results, each plate contained a negative control sample in order to validate the results. A control subcategory were spiked samples with known amount of standard that has been added to the matrix used for the ELISA. Spiked controls indicate assay performance by calculating percent recovery from the ELISA readout.

Furthermore, the ELISA was validated using medaka pituitary extracts. Displacement curves for pituitary samples were achieved by serial dilutions of samples and compared to the standard curve for either mdFshβα or mdLhβα. Assay sensitivity was revealed, which is defined as the lowest dose of Fsh or Lh capable of reducing the optical density more than the mean plus two standard deviations of the zero dose of Fsh or Lh [B0 - 2SD]. It was calculated by adding the mean of the blank to two times the standard deviation of the blank. In addition, intra-assay coefficient of variation (CV) was calculated by assaying six replicates of one of the standard

concentrations (1.56 ng/ml, **Paper I**) on the same assay plate. Inter-assay CV was determined by assaying the same sample five times in different plates.

In **Paper I**, specific and homologous competitive ELISAs were established for quantifying Fsh and Lh protein levels in pituitaries of male medaka, which were used to quantify pituitary Fsh and Lh levels in both juvenile and adult male medaka. Further details of the ELISA protocol establishment can be found in **Paper I b** (Establishment of specific enzyme-linked immunosorbent assay (ELISA) for measuring Fsh and Lh levels in medaka (*Oryzias latipes*), using recombinant gonadotropins; MethodsX article, submitted).

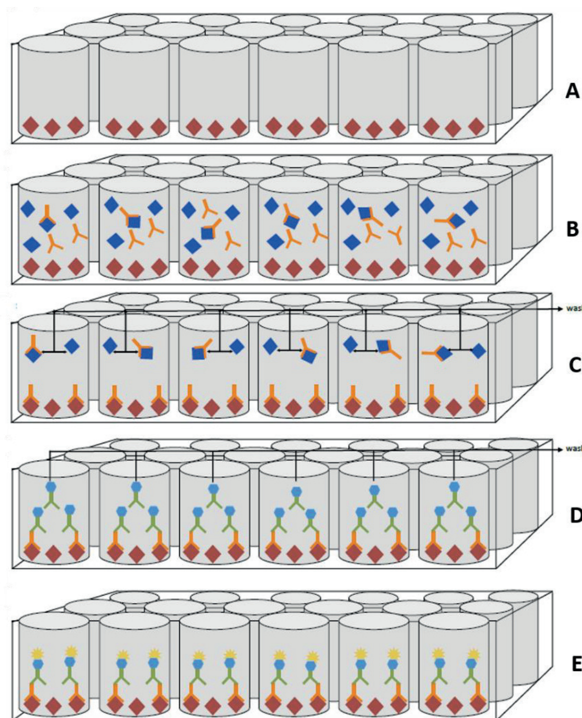

Figure 8. Principle of competitive ELISA. A) Coating with immobilized antigen (recombinant mdFshβ or mdLhβ). B) Incubating of primary antibody (anti-mdFshβ or anti-mdLhβ) with sample antigen (Fshβ or Lhβ from pituitary extract), Adding of antibody-antigen complexes to the antigen coated plate. C) Washing (unbound antibodies are removed), Indirect competitive binding (the more antigen in the sample, the less antibody will be able to bind to the antigen in the well). D) Adding of labeled secondary antibody that is specific to the primary antibody. E) Adding of Substrate (TMB), signal detection and quantification.

3.5 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) is currently the method of choice for biased quantification of gene expression levels. This method possesses various advantages compared to other gene expression techniques, such as northern blot or RNase protection assay. It is relatively easy to perform with high accuracy, reproducibility, and sensitivity, only requires a small sample amount for the analysis, and allows for both relative and absolute quantification (reviewed in Fraga et al., 2014).

In qPCR, the RNA template from the tissue of interest is transcribed into complementary DNA (cDNA) using a reverse transcriptase enzyme. The resulting cDNA product can be measured with gene-specific primers together with a non-specific fluorochrome in a PCR reaction, measuring the increasing amount of a specific product in real time (Bustin, 2002). Chemistry and instrumentation vary among suppliers, however, fluorescence-based qPCR is a commonly used method for determining mRNA levels of a specific gene or group of genes (Bustin, 2002). Fluorescence-based methods allow the quantification of a DNA molecule being amplified as the signal gains intensity, since the amount of DNA amplicons increases over time. The results are then normalized against one or several internal reference genes, being amplified at the same time. In **Paper I** and **II**, a combination of the three reference genes *18s*, *rpl7* and *gapdh* was used for normalization of gene expression.

There are two types of fluorescence-based methods, DNA-binding dyes (e.g. SYBR Green) and DNA-specific fluorescence quencher probes (e.g. SensiFAST, TagMan, Beacons, and Scorpions) (Bustin et al., 2009). SYBR Green is a non-specific fluorochrome binding to the minor groove of double stranded DNA which increases its fluorescence signal drastically compared to free dye in solution (Wilhelm and Pingoud, 2003). All qPCR performed in **Paper I** and **II** were achieved using the DNA-binding dye SYBR Green. This system has been shown to be convenient, cost-effective, straight forward, flexible and suitable for the amplification of any DNA molecule within a DNA sample, and does not need custom designed probes. However, some drawbacks should be considered when choosing DNA-binding dyes. Fluorescence dyes cannot discriminate between targets and off-target amplicons and primer dimers. Also, primers may require a thorough optimization, and melting curves must be analyzed to exclude off-target amplification. The exponential amplification of the target DNA is determined via the fluorescent emission of the fluorochrome (figure 9 A). There are three phases of the PCR amplification, the exponential, linear

and plateau phase (figure 9 B). The exponential phase is characterized by doubling of the amount of target at each cycle, with nearly 100 % reaction efficiency. Due to product accumulation and substrate decline, the reaction is inhibited over time, leading to the linear amplification phase. Product inhibition and substrate decline results in arresting of the reaction in the plateau phase.

The intensity of the fluorescent emission that is measured after every cycle is proportional to the number of amplicons (Farrell, 2010; Fraga et al., 2014). Dependent on the amount of the target gene mRNA, the fluorescence will reach a distinct threshold at a specific cycle of the PCR, named the quantification cycle (Cq). The Cq value provides an indication of the expression level of gene of interest, since it is defined as the cycle when fluorescent emission intensity level occurs above background levels (threshold line). The lower the Cq value, the higher the expression of that particular gene of interest (reviewed by Glover et al., 2016).

An optimized qPCR protocol and determination of primer efficiency are crucial parameters in order to generate highly reliable qPCR data. 260/280 and 260/230 ratios (representative for contamination with phenol, proteins, carbohydrates), as well as RNA integrity numbers (representative for quality of RNA) were measured to assess the quality of the RNA. In addition, qPCR primers were designed to span exon-exon boundaries, ensuring that amplification of genomic DNA was excluded. Furthermore, all RNA samples were DNase treated to remove any possible contaminating DNA in the sample. A melting curve analysis (figure 9 C) was performed after execution of each qPCR run, ensuring that a specific product was amplified in each sample analyzed, thus checking for primer dimers or unspecific product formation. Furthermore, a non-template control and inter-plate calibrators were present in triplicates in each qPCR plate.

In **Paper I**, the pituitary developmental profile of *fshb* and *lhb* gene expression levels in male medaka was compared on the one hand to the profile of Fsh and Lh pituitary protein levels in male medaka comparing juveniles and adults using the ELISA assay developed in **Paper I**. On the other hand, it was compared to the testis developmental profile of *fshr* and *lhr* gene expression achieved in **Paper II**. In addition, a tissue screen of *fshb, lhb* (**Paper I**), *fshr, and lhr* (**Paper II**) expression was performed on brain, pituitary, testis, ovary, eyes, heart, intestine, liver, gills, gallbladder, and spleen from three adult 6 month-old males or females.

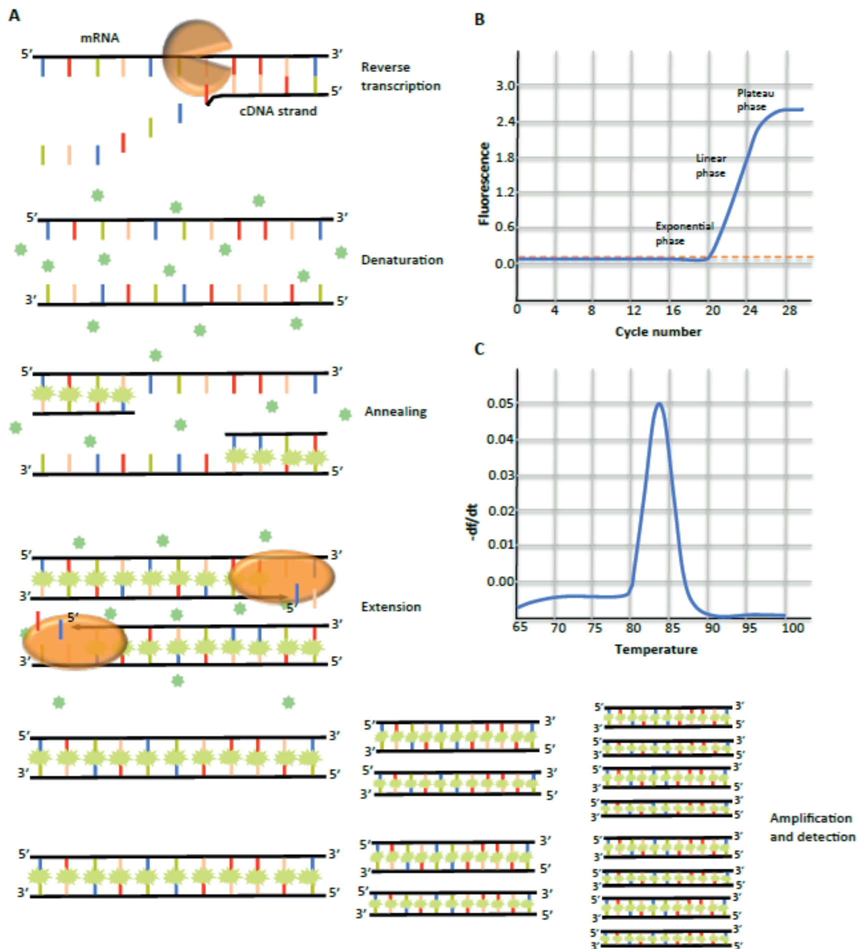

Figure 9. Schematic representation of quantitative real-time PCR (qPCR). A) Reverse transcription mediated by reverse transcriptase, complementary DNA (cDNA) synthesis. Downstream reactions comprise denaturation, annealing of specific forward and reverse primers, extension and amplification phases mediated by DNA polymerase. During extension and amplification, the SYBR Green beads (turquoise spheres) bind to the minor groove of the amplified DNA (double stranded DNA). The intensity of the fluorescence dye bound to the DNA (light green asterisks) increases proportionally to the number of copies of the amplified DNA (Fraga et al., 2014). B) Amplification curve of a qPCR reaction demonstrating the three phases exponential, linear and plateau. X axis: cycle number; Y axis: fluorescence intensity as an increase of amplified DNA. C) Melting curve showing a single peak indicating target specificity and the absence of primer dimers. X axis: temperature; Y axis: negative derivate of fluorescence over temperature (-dF/dT).

3.6 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is used to detect and localize the presence or absence of specific DNA or RNA targets in tissues or cell cultures. The technique is particularly important for determining spatial-temporal patterns of gene expression. The method is derived from the non-fluorescent *in situ* hybridization (ISH) method that was first reported in 1969 (Gall and Pardue, 1969; John et al., 1969) as a staining approach, based on complementary binding of a nucleotide probe labeled with a reporter molecule to a specific target nucleic acid sequence in cellular compartments (McNicol and Farquharson, 1997). The first FISH application was reported in 1980 for specific DNA binding using a 3′-fluorescence-labeled RNA probe (Amann and Moraru, 2012; Bauman et al., 1980). One year later, the use of amino-allyl modified bases in nucleic acid probes was demonstrated (Langer et al., 1981), which is the molecular basis for conjugations to various haptens or fluorophores.

The principle of FISH is based on the ability of complementary single-stranded DNA or RNA sequences to specifically hybridize, resulting in double stranded hybrid filaments. Fluorescence labeled target specific oligonucleotide probes, which are complementary to the target of interest, bind to the ribosomal RNA. Due to the fact that probes used in this thesis were 400-500 base pair sized, proteinase treatment was crucial, in order to permeabilize the tissue, which facilitates the entry of the probes into the cells. The enzyme concentration and treatment duration must be optimized for every tissue, in order to achieve minimal tissue damage (Bartlett, 2004). In detail, probes that are complementary to the target of interest are labelled enzymatically using modified nucleotides. Probes are denaturated, resulting in a single filament that anneals with the target of the tissue or cell culture. After several washing and detection procedures, a specific signal can be observed at the site of probe hybridization (Haaf, 2006). This technique can be used on formalin-fixed paraffin embedded tissue, frozen tissues, fresh tissues, and cells. A confocal microscope allows high quality images with low background due to spatial pinholes that block out of focus light, and sequentially captured fluorescent signal (Pawley, 2006).

Fluorescent labelling can be performed either by direct or indirect methods (reviewed by Tsuchiya, 2011). The direct, faster approach uses fluorescent reporter molecules that are directly incorporated into the probe. The disadvantage of the direct method is that probes are susceptible to photo bleaching during the preparation and hybridization steps, which can result to signal loss. The indirect approach uses antibodies coupled to fluorophores, which are used to detect the probes,

resulting in a stronger fluorescent signal (Haaf, 2006). In **Paper I** and **III**, the indirect method was used, together with tyramide signal amplification (TSA) (figure 10). TSA is based on a catalytic reporter deposit that is in close vicinity to the epitope of interest, and is commonly used due to its simplicity, high specificity, enhanced sensitivity, and compatibility with modern multi-label fluorescent microscopy (Faget and Hnasko, 2015). This technique uses a peroxidase-conjugated primary antibody that binds to the probe before adding tyramides and H_2O_2. The enzyme catalyzes the oxidative radicalization of tyramides, resulting in the deposition of fluorescent intermediates in proximity to the site of reaction, which further increases the sensitivity (Raap et al., 1995; Speel et al., 1999).

The advantage of fluorescent protocols is the possibility to use different colors due to different fluorophores (Nederlof et al., 1989; Hopmann et al., 1998), which allows simultaneous visualization of different targets that is necessary when studying co-expression of genes, as shown in **Paper I**. A challenge of the *in situ* hybridization technique are the numerous steps involved and the requirement to optimize protocols for the different tissues and probes. Crucial parameters, which effect the FISH results are tissue fixation, control of RNA degradation, slide permeabilization, hybridization, and post hybridization elements. A further challenge of the FISH method is that it necessitates experienced and highly trained personnel due to the fact that FISH as a microscopic technique is poorly standardized and requires experience for the correct interpretation of the results (Frickmann et al., 2017).

In **Paper I**, FISH was performed on free-floating parasagittal brain-pituitary sections, in order to validate polyclonal anti-mdFshβ and anti-mdLhβ. In **Paper III**, FISH was carried out for the confirmation of the transgenic line tg(*fshb*:DsRed2) using the anti-mdFshβ.

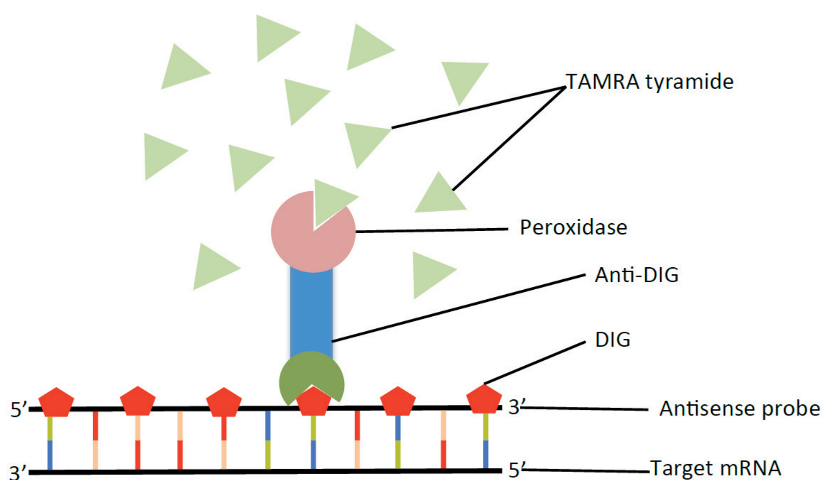

Figure 10. Schematic overview of fluorescence *in situ* hybridization (FISH) principle. The antisense probe binds to its target mRNA sequence. The antibody peroxidase complex (Anti Digoxygenin (DIG)-POD here) recognizes the probe and catalyzes the oxidative radicalization of tyramides (Tetramethylrhodamine (TAMRA) fluorophore here). This results in the deposition of fluorescent intermediates in proximity of the reaction site.

3.7 Immunofluorescence (IF)

Over 50 years ago, fluorescence-labeled antibodies were first used to localize a protein in cells (Coons, 1961). Since then, the accessibility of various antibodies and advancements in indirect labeling methods and fluorophores has resulted in the immunofluorescent localization of proteins in cells becoming a routine and a vital element in many studies. Combined with biochemical or ultrastructural studies, immunofluorescence labeling is effective (Harford and Bonifacino, 2011), due to the rapid technique and many parameters that can be measured. In comparison to biochemical studies that presume samples are uniform, immunofluorescence (IF) enables studies of individual cell differences. IF has been applied in various cell-biological studies (Munro and Pelham, 1987; Lippincott-Schwartz et al., 1990). Recently, immunolocalization of endogenous proteins is a crucial control when fluorescent chimeric proteins are being expressed, in order to investigate the dynamic behavior of the protein of interest inside living cells (Donaldson, 2015).

IF is distinguished as either direct or indirect IF, depending on whether the fluorophore is conjugated to the primary or the secondary antibody. Direct IF utilizes a single antibody that is

directed against the target of interest. The primary antibody is directly conjugated to a fluorophore. In contrast, indirect IF (figure 11) uses two antibodies; the primary antibody is unconjugated and a fluorophore-conjugated secondary antibody directed against the primary antibody is used for detection. Indirect IF, which has been used in **Paper I**, is a powerful and widely used technique, which provides information about the location of specific molecules and the structure of the cell. IF is highly specific and allows simultaneous determination of antibodies against several biochemically different antigens on one single biological substrate. Antibody molecules that are specific for a target molecule are exposed to the cell or tissue that is studied. By incubating the sample with a secondary antibody that is specific for immunoglobulin molecules and conjugated to a fluorophore, the binding sites of these molecules can be identified. This enables both a visible signal and amplification of the signal; the results are observed with a fluorescence microscope. The location can be revealed by double labeling using an antibody directed against a protein of known location. This method can be applied supplementary to immunolocalization by electron microscopy and subcellular fractionation. IF enables both the identification of the antigen distribution in the cell, and an examination of the dynamic aspects of protein movements in the cell (Donaldson, 2015). Three elements are crucial when performing IF, namely fixation, permeabilization, and confirmation of labeling specificity. The fixation and permeabilization conditions must be adjusted individually for each antibody and cell type studied (Griffiths, 1993). Various fixatives should be investigated to improve preservation of the antigen, its distribution, and the morphology of other cellular constituents. In addition, the primary antibody should be purified to ensure that it recognizes only the protein of interest (Donaldson, 2015). Crucial controls that were included in each experiment were primary antibody controls confirming specificity of the primary antibody binding to the antigen, and secondary antibody controls revealing labeling specificity to the primary antibody.

In order to characterize the localization of gonadotrope cells, IF using the generated antibodies against Fshβ and Lhβ, was compared with FISH using probes complementary to *fshb* and *lhb* in adult medaka (**Paper I**).

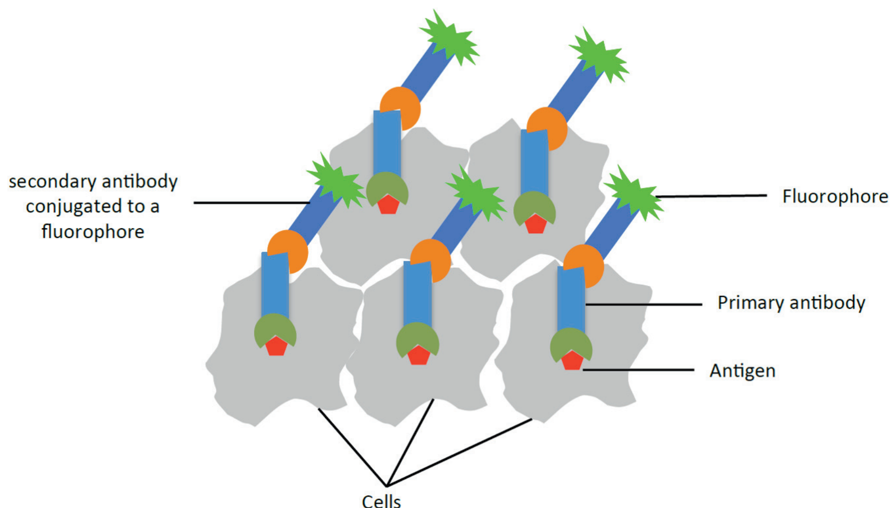

Figure 11. Scheme of indirect Immunofluorescence principle (IF). Indirect IF uses two antibodies, the unlabeled primary antibody that specifically binds the target molecule, and the secondary antibody, which carries the fluorophore and recognizes the primary antibody by binding to it. Multiple secondary antibodies can bind a single primary antibody, allowing signal amplification by increasing the number of fluorophore molecules per antigen.

3.8 Luciferase reporter gene assay

Reporter gene assays are commonly used to study G-protein-coupled receptors (GPCRs) and for the characterization of receptor functionality. The general principle of reporter gene assays is the transfection of a cell line with the receptor of interest, and a vector that consists of a specific responsive element and a reporter gene. After exposing cells to the ligand, the receptor response can be determined by the enzymatic activity of the reporter gene, for instance by luminescence, fluorescence, or colorimetry (reviewed by Dingermann et al., 2004).

Various reporter genes are available including green fluorescent protein (GFP), β-galactosidase, alkaline phosphatase, and luciferase (Azimzadeh et al., 2017). Chemiluminescent luciferase assays possess various advantages due to the wide dynamic range, robustness, and scarce endogenous luminescence, which contributed to its use in high-throughput screening (Azimzadeh et al., 2017). The generation of photons by this bioluminescent reporter is slower than fluorescent-based methods, for instance excitation of GFP, which uses a high-intensity laser to rapidly excite GFP. Due to the different mechanisms to produce photons, chemiluminescent reporters are usually

less bright compared to fluorescent proteins. However, they possess the advantage of lower background levels and improved signal sensitivity.

Commonly applied in reporter gene assays has been the bacterial luciferase enzyme from firefly (*Photinus pyralis*) (Fan and Wood, 2007), which is an enzyme that catalyzes the conversion of D-luciferin substrate to oxyluciferin, in the presence of ATP, Mg^{2+}, and O_2. Oxyluciferin emits photons to become stabilized at the basal low-energy state; the quantification of luminescence is performed in a luminometer. Bacterial luciferase is thermolabile (Baggett et al., 2004), therefore it has restricted applications as reporter gene in mammalian cells. In luciferase assays, cells are transfected with a plasmid, which expresses the GPCR of interest together with a reporter plasmid. This plasmid contains specific sequence repeats of the transcription factor's response elements within the enhance region before promoter on the 5′ of luciferase gene. After transcription factors are activated and bind to the response elements, the expression of luciferase enzyme can be determined in a luminometric assay (figure 12).

The biological action of a putative hormone depends on its ability to bind and activate its receptor on a specific tissue. Gonadotropins are glycoprotein hormones, which bind their cognate GPCRs on the surface of the cell plasma membrane (Gether, 2000). The activation of gonadotropin cognate receptors triggers the mechanism of the second messenger, mediated by cyclic adenosine or guanosine monophosphate (cAMP or cGMP); this mechanism is present in all eukaryote cells (Gether, 2000). Usually, the final outcome of the signaling pathway is modification of gene expression profiles by activation of certain transcription factors (Gutkind, 2000). Therefore, gene reporter assays that can measure quantitatively the binding of transcription factors to their specific response elements on the genome provide the possibility for GPCR deorphanization (Levoye and Jockers, 2008).

In order to gain more insight into the structure and differential specificity of medaka Fshr and Lhr, the selectivity of the activation response towards their cognate homologous hormones, and towards Nile tilapia hormones, was investigated by a luciferase reporter gene assay using the COS-7 cell line (figure 12), as describedin **Paper II**. The mammalian COS-7 cell line has been successfully used by other authors to characterize D_2 dopamine receptor from Nile tilapia pituitary (Levavi-Sivan et al., 2005). Transient transfection, cell procedures and stimulation protocols were generally performed according to Levavi-Sivan et al. (2005).

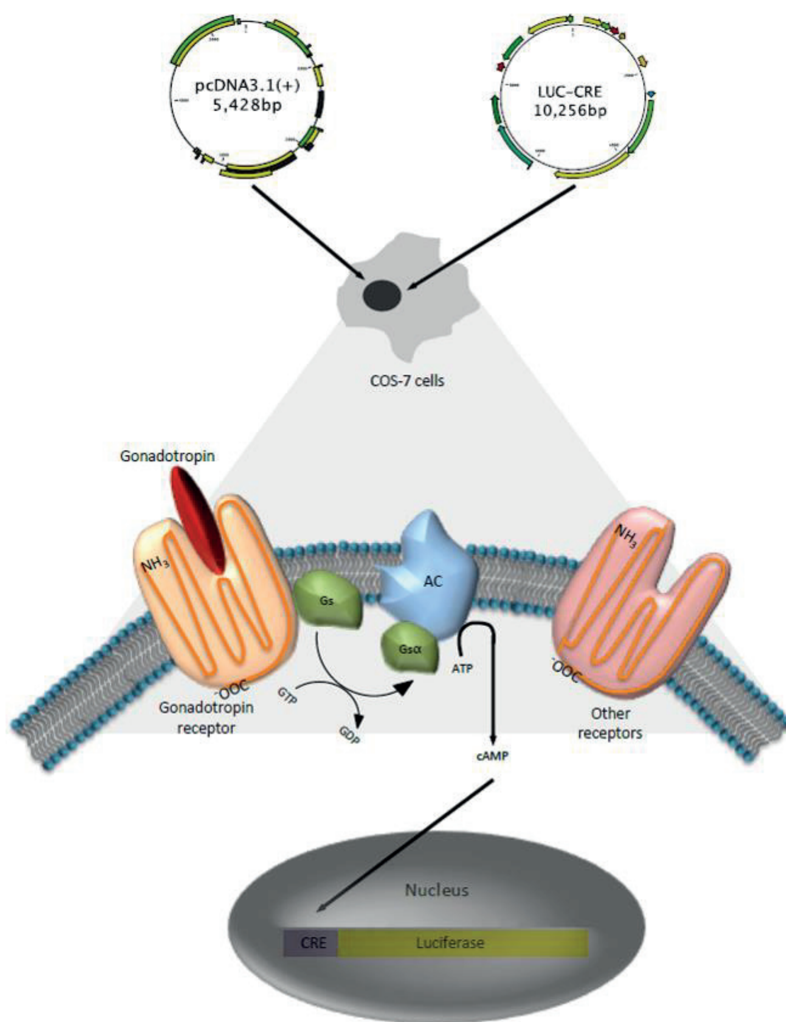

Figure 12. Schematic representation of luciferase reporter gene assay. Expression vectors pcDNA3.1(+) carrying either mdFshr, mdLhr, tiFshr, or tiLhr under the control of cytomegalovirus promoter (CMV) and LUC-CRE (Luciferase - cyclic adenosine monophosphate (cAMP) response element) carrying luciferase were co-transfected in mammalian COS-7 cells. The gonadotropin receptors expressed in COS-7 cells recognize its own cognate gonadotropin. The binding of the gonadotropin to the receptor induces a conformational change in the receptor transmembrane domain that activates the G protein (Gsα). The active Gsα then activates adenylate cyclase (AC), which increases cAMP levels. DNA responsive element CRE promotes the expression of luciferase in response to the increased levels of cAMP.

COS-7 cells were co-transfected either with mdFshr, mdLhr, tiFshr, or tiLhr and sensitive luciferase (LUC) reporter gene, which is transcriptionally regulated by cAMP response element (CRE). The activation of receptors was measured as the increase in LUC signal in response to treatment with recombinant mdFshβα, mdLhβα, tiFshβα, tiLhβα, medaka pituitary extract (MPE) or tilapia pituitary extract (TPE). Recombinant gonadotropins and receptors were obtained as described in **Paper I** and **II**.

3.9 Transgenesis in medaka

Transgenesis, which is the genetic engineering of a transgenic organism, is different from gene delivery since the former applies integration of exogenous DNA (transgene) into the host genomic DNA producing a modified gamete (Wakchaure et al., 2015; Mclean and Laight, 2000), while the latter is an insertion of a gene into a cell (Tonelli et al., 2017). Fish are excellent model organisms for genetic manipulation due to various advantages, such as a short life cycle, with sexual maturity being reached quickly (Nkhoma and Musuka, 2014). In addition, in all teleosts, there are distinct gonadotropes producing Lh and Fsh, thus providing a good model organism to characterize gonadotropin regulation (Kanda et al., 2011). The Japanese medaka that has been used to establish the transgenic line tg(*fshb*:DsRed2) (**Paper III)**, provides an excellent model for microinjection studies due to rapid development in transparent eggs (Kinoshita et al., 2009).

The most commonly used method is microinjection of DNA into the pronuclei of fertilized eggs at the one-cell stage (Cheers and Ettensohn, 2004; Pitkänen et al., 1999), which has been used in the work of **Paper III**. Microinjection necessitates thin injection pipettes with varying inner diameters, dependent on species egg size (Sulaiman, 1995). Since the chorion that surrounds medaka embryos is tough, a short, strong and wide injection needle is necessary (Porazinski et al., 2010) (microinjection setup in figure 13 A; medaka embryo microinjected into the cytoplasm at the one-cell stage in figure 13 B). Despite its frequent application in fish since the 1980s (Mclean et al., 1987; Guyomard et al., 1989), microinjection can be challenging when pronuclei of fertilized fish embryos may be difficult to identify if the eggs are opaque (Sin et al., 1997); in addition, this technique requires a well-trained manipulator.

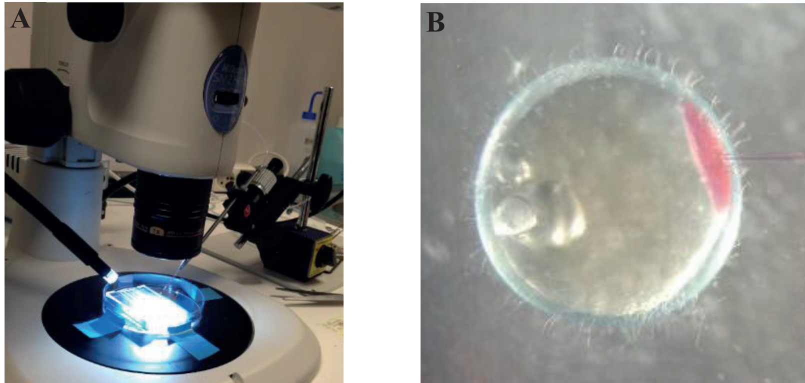

Figure 13. Microinjection technique A) Microinjection setup to microinject medaka embryos. B) Medaka embryo microinjected into the cytoplasm at the one-cell stage.

Another technique used to generate transgenic fish is electroporation that involves applying electric pulses resulting in the formation of pores in the cell membrane, enabling the incorporation of DNA sequences (Sugar and Neumann, 1984). The procedure is considered to be quick and simple, but may induce high mortality rates (Tonelli et al., 2017). Spermatogonial stem cell (SSC) modification prior to germ-cell transplantation (Brinster and Avarbock, 1994) is a technique, which involves SSC genetic manipulation and transplantation, resulting in the creation of a progeny with a modified donor genome. SSCs provide a permanent source of modified gametes, however, the obtainment of SSCs should be standardized for each species (Tonelli et al., 2017). Among the new methodologies applied to perform site-directed genomic integration of transgene, TALEN (Transcription activator-like effector nucleases) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats) are commonly used to generate knock-ins of specific genes in fish (Tonelli et al., 2017). TALENs are produced by combining artificial DNA sequences (TALE effector repeats) with the endonuclease domain of the FokI restriction enzyme. These structures function as dimers and can produce DNA double-strand breaks (DSBs) at the target site sequence, in order to insert the gene of interest. The CRISPR)/Cas system necessitates the action of Cas9 nuclease. A guide RNA (gRNA) directs Cas9 to a specific region in the genome and produces a site-specific DSB. After DSB production, homologous recombination of the knock-in (DNA cassettes of interests) will occur. Both TALEN and CRISPR/Cas can induce off-target effects (Tonelli et al., 2017).

The microinjection of plasmid DNA into the cytoplasm of one cell-stage medaka embryos provides a fast and easy technique, however, it is rather inefficient (Thermes et al., 2002). Therefore, I-SceI meganuclease was co-injected into the one-cell stage embryos together with the injection solution (**Paper III)**. This resulted in a strong enhancement of the promoter dependent expression already in F_0, and an increased transgenesis frequency due to integration at the one-cell stage (Thermes et al., 2002). In general, the microinjected DNA remains as long extrachromosomal concatamers transiently transcribed during early embryogenesis. An uneven distribution of the episomal DNA results in a mosaic expression of the transgene in F_0. The DNA becomes integrated into the genome at later cleavage stages, causing the DNA to be stably inherited only by subsets of blastomeres (Etkin and Pearman, 1987; Westerfield et al., 1992). Due to this, transgenic founder fish transmit the transgene to only a low percentage of their offspring, leading to low germline transmission rates (Collas and Aleström, 1998; Culp et al., 1991; Lin et al., 1994; Stuart et al., 1988, 1990; Tanaka and Kinoshita, 2001).

In the work described in **Paper III**, a novel transgenic line was developed, which expresses Red fluorescent protein (DsRed2) under the control of the endogenous *fshb* gene promoter (tg(*fshb*:DsRed2)). It was hypothesized that developing a transgenic line expressing DsRed2, controlled by the *fshb* promoter (3833 bp), will result in the expression of DsRed2 under conditions and stimuli similar to those controlling natural expression. The choice of this promoter was due to the aim of expressing DsRed2 in the pituitary, thus allowing to track and visualize *fshb* expression by means of DsRed2 expression in the medaka pituitary. Other promoters, such as constitutive β-actin or β-tubulin promoters would have a pleiotropic DsRed2 expression in all tissues. For instance, a medaka transgenic line (tg(*β-actin*:DsRed), in which all cells emit Rfp fluorescence under the regulation of the β-actin promoter, resulted in unprecedentedly strong and widespread transgene expression from embryonic to adult stages (Yoshinari et al. 2012).

The choice for the Red fluorescent protein DsRed2 in Paper III was due to several reasons. DsRed was shown to have a high quantum yield and to be photo stable (Baird et al., 2000). Further, DsRed forms a stable tetramer and each monomer is structurally very similar to GFP (Baird et al., 2000; Wall et al., 2000; Yarbrough et al., 2001), which makes DsRed an ideal candidate for multicolor experiments involving GFP. This is one of the reasons we have used DsRed, since initially, this thesis aimed to produce a double transgenic line with our previously developed medaka transgenic line tg(*lhb*:hrGfpII) (established by Hildahl et al., 2012). Despite advantages,

it was reported that the chromophore maturation of DsRed is slow, with a half-time of >24 h at room temperature (Baird et al., 2000; Wiehler et al., 2001), and that it has a poor solubility (Baird et al., 2000; Jakobs et al., 2000). The variant DsRed2 was shown to mature faster than DsRed1 with a half-time of ∼6.5 h (Bevis and Glick, 2002). The variants DsRed.T3 and DsRed.T4 were shown to be optimized variants due to faster maturation, but were reported to be less bright than wild-type DsRed (Bevis and Glick, 2002).

It was crucial to include controls during the development of the transgenic line tg(*fshb*:DsRed2) in **Paper III**, due to the necessity to confirm that the DsRed2 expression specifically reflects the regulation of endogenous *fshb* gene and was not ectopic. This was performed by checking for co-localization of *dsred2* with *fshb*, and by incorporating both genes under the control of the native *fshb* promoter. This ensured a tissue specific expression, as determined by the co-localization of *dsred2* and *fshb* in adult pituitaries. In order to confirm the specificity of the transgene, two separate transgenic F_0 founder fish were included. Confirmation of the tg(*fshb*:DsRed2) was achieved by means of FISH using the riboprobes developed in **Paper I**. The establishment of the novel medaka transgenic line tg(*fshb*:DsRed2) completes our previously generated medaka transgenic line tg(*lhb*:hrGfpII), both valuable tools for studies on gonadotropin regulation.

4. Results

Paper I

The major purpose of this study was to develop key tools that allow the localization and quantification of Fsh and Lh at both mRNA and protein level in the model species medaka. Recombinant medaka gonadotropins Fshβ (mdFshβ), Lhβ (mdLhβ), Fshβα (mdFshβα), and Lhβα (mdLhβα) were produced, specific antibodies against medaka Fshβ and Lhβ generated, immunofluorescence (IF) protocols developed and validated for localization of Fshβ and Lhβ producing cells, and enzyme-linked immunosorbent assays (ELISAs) established for the quantification of Lh and Fsh at the single pituitary level. The comparison of IF and fluorescent *in situ* hybridization (FISH) revealed cells expressing Lhβ and *lhb* to be located in the ventral part of the pituitary, and Fshβ and *fshb* expressing cells mainly to be present from the ventral to the dorsal part, and more widely distributed than the *lhb* expressing cells. Using the validated ELISA assays, both Lh and Fsh protein amounts in male medaka pituitaries were shown to increase during pubertal development, correspondingly to *lhb* mRNA levels that increased significantly with maturation. Taken together, the paper provides key methodologies, such as developed IF-, FISH-, and ELISA-protocols for medaka that will enable further studies on medaka Fsh and Lh at both mRNA and protein level, thereby advancing our understanding of gonadotropin function in fish.

Paper II

This paper describes the first pharmacological characterization of medaka Fshr (mdFshr) and Lhr (mdLhr), including a comparison with the corresponding receptors in tilapia. Intra-species ligand selectivity studies revealed each gonadotropin activating its own cognate receptor for both medaka and tilapia. Cross-reactivity was detected to some extent for mdFshβα activating mdLhr, and mdLhβα stimulating mdFshr. Inter-species ligand promiscuity was observed since mdLhr was activated with Nile tilapia Fshβα (tiFshβα) and Nile tilapia pituitary extract (TPE), mdFshr by stimulation with TPE, and tilapia Fshr (tiFshr) and Lhr (tiLhr) using medaka pituitary extract (MPE). Analysis of sequence alignment and three-dimensional models highlighted highly conserved beta sheet domains of both Fsh and Lh between medaka and tilapia, a common alpha subunit for both Fsh and Lh with varying homology between the two species, and a higher

structural homology and similarity of transmembrane regions of Lhr between both species, in contrast to Fshr. A tissue distribution study revealed that *fshr* and *lhr* receptor transcripts were highly expressed in ovary and testis of adult medaka. Non-gonadal expression in the brain demonstrated high *lhr* expression levels and low *fshr* expression levels. Trace amounts for *fshr* and *lhr* were detected in gills, gallbladder, intestine, spleen, pituitary, eyes (low levels of *lhr*), and for *lhr* in heart. A developmental profile indicated that both testicular *fshr* and *lhr* transcript levels increase significantly during gonadal development, accordingly to Paper I that revealed increased pituitary *lhb* mRNA levels and Lh and Fsh protein levels with maturation. In conclusion, these results help to increase our understanding of the mechanisms involved in the activation of the BPG axis during puberty in medaka.

Paper III

The major purpose of this paper was to generate a transgenic line of medaka, where expression of red fluorescent protein is controlled by the endogenous medaka *fshb* promotor [tg(*fshb*:DsRed2)], and to use this transgenic line as a tool to investigate Fsh function and regulation during sexual maturation. In contrast to mammals, separate cell types produce the two gonadotropins in teleost fish, making them excellent models for studying the development and regulation of the two hormones separately. The transgenic line was developed by microinjection into the cytoplasm of one cell-stage medaka embryos. For this purpose, a DNA fragment coding for DsRed2 was cloned into *I-SceI*-MCS-leader-Gfp-trailer plasmid replacing Gfp, and medaka *fshb* promoter was cloned subsequently into the *I-SceI*-MCS-leader-Rfp2-trailer. The line was confirmed using FISH revealing co-localization of *dsred2* and *fshb* transcripts in adult pituitary cells. Cells expressing *fshb* were localized in the median part of the pituitary (*proximal pars distalis*), for the most part distributed from the ventral to the dorsal part. Fluorescent imaging analysis detected initial DsRed2 expression in larvae at the age of 8 days post-fertilization (dpf). The number of Fsh cells increased gradually with development from embryonic to juvenile stages and further to adults, and there were no differences between sexes. Interestingly, some cells were observed in the ventral pituitary surface in juvenile fish, while cells were mostly distributed inside the pituitary in mature fish. Taken together, this line provides a sensitive method to track expression patterns of *fshb* during development in medaka, and the results suggest a functional role for this early *fshb* expression, which is not unraveled yet.

5. General findings and discussion

This PhD thesis describes the development of key technologies allowing for the localization and quantification of the gonadotropins Fsh and Lh at both mRNA and protein level, and furthermore elucidates on the pharmacological characteristics of their receptors in Japanese medaka, altogether advancing our understanding of gonadotropin functions in fish.

Although intensively studied over the past four decades, knowledge gaps still exist on our understanding of the function and regulation of the main hormones of the BPG axis, and their role in pubertal development. An important technology when studying gonadotropin regulation and function is the quantitative determination of hormone levels in the blood and pituitary by immunoassays. The scarce accessibility of quantitative assays for fish, particularly assays to determine Fsh levels (Yom-Din et al., 2016), prompted the development of homologous ELISA assays for medaka Fsh and Lh, the initial step of **Paper I**. Traditionally, methods quantifying pituitary Fsh and Lh in fish have been based on native gonadotropins from fish pituitaries (e.g. Prat et al., 1996), however, more recently recombinant gonadotropins have been used (e.g. Chauvigné et al., 2016). For the first time, single-chain recombinant gonadotropins Fshβα and Lhβα from medaka were successfully produced by *P. pastoris* in this thesis, containing a "linker" sequence between β- and α-chains (Aizen et al., 2017) assisting in chimerization, and a six-His tail (His^6) (Kasuto and Levavi-Sivan, 2005) enabling purification. One of the proposed scientific objectives of this thesis was accomplished by confirming specificity and validation of the antisera against Fshβ and Lhβ using FISH and IF in **Paper I**. This was shown by Lhβ protein labeling in the ventral part of the pituitary with near perfect co-localization with *lhb* mRNA. *fshb* mRNA and mFshβ protein were distributed over the exact same pituitary regions, with *fshb*-expressing cells located mainly from the ventral to the dorsal part, with a few cells in the posterior part. They were more widely distributed than the *lhb* expressing cells forming close cell-cell contacts, which was expected according to literature (zebrafish and tilapia; Golan et al., 2016). As reported from various other fish species belonging to distinct teleost orders (e.g. Golan et al., 2014; Weltzien et al., 2003), medaka Fsh and Lh were generally expressed in different cells.

In order to answer a further objective of this dissertation, competitive ELISA assays specific for medaka Fsh and Lh were developed using recombinant gonadotropins and antibodies against mdFshβ or mdLhβ in **Paper I**, performing comparably to available RIAs and ELISAs established for other fish species. The sensitivity of the assays for Fsh and Lh were generally high

compared to previously published fish RIAs (Bogomolnaya et al., 1989; Govoroun et al., 1998). The medaka ELISA was demonstrated to be highly reproducible with low intra- and inter-assay CVs for Fsh and Lh. The values are comparable and expected according to reported CVs of gonadotropins in other fish species (e.g. Hollander-Cohen et al., 2017). After the successful development of medaka Fsh and Lh ELISAs at the single pituitary level, **Paper I** focuses further on applying these assays during sexual maturation of male medaka, in order to obtain a profile of pituitary Lh and Fsh protein levels comparing juveniles and adults, as proposed as a further sub-goal of **Paper I**. The findings highlighted that both Lh and Fsh protein amounts increased from juvenile to adult male fish. Correspondingly, pituitary *lhb* mRNA levels increased significantly with maturation in male medaka, as shown in **Paper I**, while also testis *fshr* and *lhr* transcripts were revealed to increase during testis development in **Paper II.** The peak of *fshr* and *lhr* expression during spermatogenesis (**Paper II**) was expected corresponding to literature, since the peak has been determined in various seasonally breeding species (e.g. Baron et al., 2005; Maugars and Schmitz, 2008; Kusakabe et al., 2006). Studies on gonadotropin receptor gene expression in male fish are primarily focused on seasonal spawners, and still restricted for fish species that are daily spawners, such as zebrafish and medaka, emphasizing the value of the obtained expression data in **Paper II**.

The classification of medaka into a number of standard body length groups was employed for developmental profiles measuring *lhb*, *fshb* (**Paper I**), *fshr* and *lhr* (**Paper II**) expression, and was based on a histological analysis of testes assessing maturational stages in **Paper I**. Male medaka with body length above 19 mm, body weight above 130 mg, or age above 100 days, were shown to be sexually mature. Histology sections of medaka between 14 - 19 mm consisted of different testicular developmental stages from immature to late maturing (**Paper I**). This is probably because varying densities in the different fish tanks resulted in differences in individual food availability, as shown in earlier studies by Dibattista et al. (2006) and Auer et al. (2015). Studies determining pituitary Fsh and Lh protein levels in male fish during development are limited until today, therefore the established ELISA represents a valuable technology for future studies.

In order to provide better understanding of processes involved in the activation of the BPG axis during puberty in medaka, and to accomplish a further objective of this thesis, **Paper II** focuses on the first pharmacological and structural characterization of the interactions between

gonadotropins and gonadotropin receptors between medaka and Nile tilapia. Tissue distribution screening revealed *fshr* and *lhr* transcripts to be highly expressed in ovary and testis of adult medaka, which was expected according to the literature (e.g. Kazeto et al., 2012; Maugars and Dufour, 2015), emphasizing the importance of both *fshr* and *lhr* in the regulation of gonadal development. Further, non-gonadal expression was observed for *lhr* in brain at high levels, and for *fshr* at low levels, and trace amounts for various other non-gonadal tissues, in accordance to the literature (e.g. Maugars and Dufour, 2015). To unravel intra-species ligand promiscuity of medaka and Nile tilapia Fshr and Lhr, which was identified as a further objective of this dissertation, **Paper II** examines the intra-species ligand promiscuity of Fshr and Lhr, demonstrating that in both species, both gonadotropins activated their own cognate receptor as expected (e.g. Levavi-Sivan et al., 2010), and cross-reactivity was observed for mdFshβα activating mdLhr, and mdLhβα stimulating mdFshr. In contrast to mammals where Fshr and Lhr are suggested to be highly specific to their receptors, bioactivity of fish gonadotropins seems to be less specific due to promiscuous hormone-receptor interactions (reviewed in Levavi-Sivan et al., 2010). Some studies indicate that teleost Lhrs tend to be specific for Lh, similar to their mammalian counterparts, while Fshrs possesses a broader but still restricted functional selectivity for both gonadotropins that may depend on the fish species or taxa (Aizen et al., 2012b; Cahoreau et al., 2015). From the results of **Paper II** this does not appear to be the case in medaka, which indicated that mdFshr and mdLhr are less specific to Fsh and Lh than other fish species. Inter-species ligand promiscuity was revealed for tiFshβα and TPE activating mdLhr, TPE stimulating mdFshr, and MPE activating tiFshr and tiLhr, which was expected due to similar findings of inter-species cross-activation in literature (e.g. Aizen et al., 2012b).

In order to elucidate explanations for the divergence in the transactivation assay in **Paper II,** amino acid sequence alignment and protein structural modeling, a powerful technique to analyze and predict ligand-receptor binding and activation (e.g. Brockhoff et al., 2010), was performed, accordingly to one of this thesis' proposed objectives. An additional N-glycosylation site predicted between the cysteine residue 2 and 3 of the hinge region in tiFshr, in contrast to an additional N-glycosylation site predicted between the cysteine residue 3 and 4 of the hinge region in mdLhr was found, which could be a possible reason for divergence. This is expected from literature reporting the hinge region of the glycoprotein hormone receptors to effect hormone binding and induction of signaling pathway (Jiang et al., 2012; Kleinau et al., 2017). In addition,

structural modeling revealed the seatbelt region, which is essentially involved in determining receptor specificity, for Fshβ and Lhβ in both species to be well aligned and superpositioned. However, only medaka Fshβ showed a positive net charge for the determinant loop, which might be a cause for its slightly promiscuous nature, as suggested in this study. This conclusion is in accordance to other studies indicating net charge differences in the determinant loop to possess separated Lhr- from Fshr-/Tshr-activating properties (Campbell et al., 1997; Han et al., 1996).

Considering the thesis' objective of providing a sensitive tool to track *fshb* expression patterns during development in medaka, **Paper III** developed a medaka transgenic line, where expression of red fluorescent protein is controlled by the endogenous medaka *fshb* promotor [tg(*fshb*:DsRed2)]. Specificity of the transgenic line was verified in **Paper I** by the co-localization of *dsred2* and *fshb* transcripts in adult fish using *fshb* riboprobe, and by comparing Rfp expression patterns in offspring from two separate transgenic F_0 founder fish. *fshb* was expressed exclusively in the pituitary, with first expression appearing at 8 dpf, indicating a functional role for this early *fshb* expression. Previously, Fshβ cells in medaka were first detected at 6 dpf just after sex determination by Horie et al. (2014), who proposed that Fsh plays a role in the early stages of gonadal differentiation in this species. Cells expressing *fshb* were localized in the median part of the pituitary (*PPD*), mainly distributed from the ventral to the dorsal part, which was expected from previous studies in medaka (Horie et al., 2014) and other teleost species (e.g. Cerdà et al. 2008; Weltzien et al., 2003). DsRed2 cells were shown not to be clustered with other DsRed2 cells, contrary to studies revealing *fshb* and *lhb* expressing cells to be arranged in cell clusters (So et al., 2005; Candelma et al., 2017). Co-localization of the two mRNAs in the same gonadotrope cell was not demonstrated and accordingly to studies in other teleosts (e.g. Weltzien et al., 2014; Candelma et al., 2017) and mammals (Nakane, 1970).

Fsh and Lh are of particular importance within the BPG axis of vertebrates. Although gonadotropins have been studied in many teleosts, only a few have been investigated at both protein and mRNA levels during puberty, emphasizing the contribution of the established key tools IF, FISH, ELISA, and a medaka *fshb* transgenic line in this dissertation, which together will help to advance our understanding of Fsh and Lh functions in fish in future research. The pharmacological characterization of medaka and Nile tilapia ligands and receptors elucidated each gonadotropin activating its own cognate receptor, and partial ligand promiscuity and cross-species reactivity, suggesting future studies are needed to assess the physiological significance of the

gonadotropin receptor cross reactivity in fish. Finally, the novel medaka transgenic line tg(*fshb*:DsRed2) highlighted the first sign of DsRed2 expression at 8 dpf, indicating a functional role of *fshb* during early development that needs to be enlightened.

6. General conclusions

The results of my PhD thesis provided key technologies enabling localization and quantification of Fsh and Lh at both mRNA and protein level in the model species medaka. In addition, a pharmacological characterization of medaka Fsh and Lh receptors contributed to our understanding of processes involved in the activation of the BPG axis during puberty in medaka. The main scientific objectives proposed yielded the following answers:

- Recombinant medaka gonadotropins Fshβ (mdFshβ), Lhβ (mdLhβ), Fshβα (mdFshβα), and Lhβα (mdLhβα) were produced by *Pichia pastoris*, and specific polyclonal antisera generated against medaka Fshβ (anti-mdFshβ) and Lhβ (anti-mdLhβ) in rabbits. Immunofluorescence (IF) protocols were developed and validated for the localization of Fshβ and Lhβ producing cells. Enzyme-linked immunosorbent assays (ELISAs) were established for the quantification of Fsh and Lh at the single pituitary level.
- Comparison of IF using the generated antibodies anti-mdFshβ and anti-mdLhβ, and fluorescent *in situ* hybridization (FISH) using probes complementary to *fshb* and *lhb*, demonstrated Lhβ and *lhb* co-localized in cells in the ventral part of the pituitary. Fshβ and *fshb* expressing cells were distributed over the exact same pituitary region, mainly located from the ventral to the dorsal part, being more widely distributed than the *lhb* expressing cells in adult medaka.
- Validated ELISA assays revealed both Lh and Fsh protein levels in male medaka pituitaries to increase during pubertal development, correspondingly to *lhb* mRNA levels, which increased significantly with gonadal maturation.
- Pharmacological characterization confirmed each gonadotropin activating its own cognate receptor, as well as partial ligand promiscuity and cross-species reactivity between medaka and Nile tilapia ligands and receptors. The investigation of sequence alignment and structural modeling exposed highly conserved beta sheet domains of Fsh and Lh between medaka and tilapia, common alpha subunit for both Fsh and Lh with varying homology between the two species, and a higher structural homology and similarity of transmembrane regions of Lhr between both species, in contrast to Fshr.
- A developmental gene expression profile suggested that both testicular medaka *fshr* and *lhr* mRNA levels increase significantly during gonadal development, accordingly to the

profile of Fsh and Lh protein levels in male medaka, which determined increased pituitary *lhb* mRNA levels and Lh and Fsh protein levels with gonadal maturation.

- A novel medaka transgenic line, where expression of red fluorescent protein is controlled by the endogenous medaka *fshb* promotor [tg(*fshb*:DsRed2)] was developed and confirmed using FISH, revealing co-localization of *dsred2* and *fshb* transcripts in adult pituitary cells.
- Fluorescent imaging analysis observed initial DsRed2 expression in larvae at the age of 8 dpf. The number of Fsh cells was determined to increase gradually with development from embryonic to juvenile stages and further to adults, with no differences between sexes. Some cells were revealed in the ventral pituitary surface in juvenile fish, while cells were mainly distributed inside the pituitary in mature fish.

7. Future perspectives

Considering the lack of knowledge on differential regulation of cells expressing *fshb* and *lhb*, the establishment of our novel medaka transgenic line tg(*fshb*:DsRed2) provides a powerful tool for future studies aimed at investigating the different physiological properties of *fshb*-expressing gonadotrope cells in fish during puberty.

An important follow up study would be to reveal whether there are indications for cells migrating to or within the pituitary, or if cells seem to emerge at one place before migrating to their final location during puberty. This could be performed by tracking cells and time lapses, which will allow a distinction between moving cells and arising cells. As a continuance of this, it would be very interesting to perform investigations on changing cell shapes or sizes, as well as a three-dimensional structural model of the pituitary, which would enable examining the exact location of the cells at each stage. Furthermore, future investigations should include experiments on identifying cell type(s) expressing *fshb*, and studying the possible functions of Fsh during puberty, for instance through IF, FISH or possibly combined with gene knockout studies.

Based on the finding that *fshb* was first expressed in the medaka pituitary at 8 dpf and the indicated function in early stages of gonadal development, it would be interesting for follow up studies to examine its potential involvement in primordial germ cell development, for instance in proliferation, migration, and survival. This can be carried out by means of genome wide gene expression analysis, by exposing primary cell cultures of medaka pituitary cells to the recombinant gonadotropin and observing whether Fsh can activate its receptors in these cells. Hereby it can be analyzed which genes and pathways are activated by Fsh, and finally to understand the biological action of Fsh.

The tools established and applied during this PhD thesis can be further used for studying a range of factors contributing in the regulation of gonadotrope cells during puberty. Considering that Fsh and Lh in teleosts are mainly produced and released from different pituitary cell types, it could also be of great interest to generate a double transgenic line with our previously developed tg(*lhb*:hrGfpII) line, in order to study different spatial and temporal expression patterns of *fshb* and *lhb* during puberty. In this regard, an interesting follow up would be to unravel the physiological effects of environmental stressors, such as temperature, nutritional status (overfeeding vs. starvation vs. normal status), salinity, endocrine disruptors, or toxins on Fsh or Lh expression, secretion, or biological action, and hereby on the modulation and regulation of the BPG axis. This

could be performed electrophysiologically, combined with FISH, IF, or ELISA, by determining hormone secretion from *fshb or lhb* expressing gonadotropes after exposure to environmental stressors. Summarizing, these investigations will hopefully contribute further to a better understanding of the differential regulation of *fshb* and *lhb* expressing cells during puberty, and possibly provide the aquaculture industry with improved knowledge on how to control puberty and sexual maturation.

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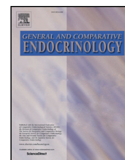

Medaka follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression levels

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ABSTRACT

The two gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) are of particular importance within the hypothalamic-pituitary-gonadal (HPG) axis of vertebrates. In the current study, we demonstrate the production and validation of Japanese medaka (*Oryzias latipes*) recombinant (md) gonadotropins Fshβ (mdFshβ), Lhβ (mdLhβ), Fshβα (mdFshβα), and Lhβα (mdLhβα) by *Pichia pastoris*, the generation of specific rabbit antibodies against their respective β subunits, and their use within the development and validation of competitive enzyme-linked immunosorbent assays (ELISAs) for quantification of medaka Fsh and Lh. mdFsh and mdLh were produced as single-chain polypeptides by linking the α subunit with mdFshβ or mdLhβ mature protein coding sequences to produce a "tethered" polypeptide with the β-chain at the N-terminal and the α-chain at the C-terminal. The specificity of the antibodies raised against mdFshβ and mdLhβ was determined by immunofluorescence (IF) for Fshβ and Lhβ on medaka pituitary tissue, while comparison with fluorescence *in situ* hybridization (FISH) for *fshb* and *lhb* mRNA was used for validation. Competitive ELISAs were developed using antibodies against mdFshβ or mdLhβ, and the tethered proteins mdFshβα or mdLhβα for standard curves. The standard curve for the Fsh ELISA ranged from 97.6 pg/ml to 50 ng/ml, and for the Lh ELISA from 12.21 pg/ml to 6.25 ng/ml. The sensitivity of the assays for Fsh and Lh was 44.7 and 70.8 pg/ml, respectively. A profile of pituitary protein levels of medaka Fsh and Lh comparing juveniles with adults showed significant increase of protein amount from juvenile group (body length from 12 mm to 16.5 mm) to adult group (body length from 21 mm to 26.5 mm) for both hormones in male medaka. Comparing these data to a developmental profile of pituitary mRNA expression of medaka *fshb* and *lhb*, the mRNA expression of *lhb* also increased during male maturation and a linear regression analysis revealed a significant increase of *lhb* expression with increased body length that proposes a linear model. However, *fshb* mRNA expression did not change significantly during male development and therefore was not correlated with body length. In summary, we have developed and validated homologous ELISA assays for medaka Fsh and Lh based on proteins produced in *P. pastoris*, assays that will be used to study the functions and regulations of Fsh and Lh in more detail.

1. Introduction

The pituitary gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) control gonad development and maturation in all vertebrates (Weltzien et al., 2004). Fsh and Lh belong to a large family of heterodimeric glycoproteins containing two non-covalently associated subunits. The common α-subunit is specifically and strongly linked to a hormone-specific β-subunit that defines the biological activity and specificity of the hormone.

Teleost fish constitutes the largest vertebrate class, and as of today, gonadotropin subunit-encoding genes have been isolated from 56 teleost species representing at least 14 orders (Hollander-Cohen et al., 2017). Fsh and Lh are produced and released from distinct pituitary cell types in teleosts (Nozaki et al., 1990; Naito et al., 1991, 1993; Kanda

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et al., 2011; Golan et al., 2016b). After their release into the blood stream, Fsh and Lh are transported to and bind to specific membrane receptors in the gonads, follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhr), together stimulating gametogenesis and steroidogenesis. Although being under intense study over the past four decades, detailed knowledge of each hormones' function and regulation is still limited. The accepted model in fish suggests that Fsh is primarily involved in early gametogenesis and vitellogenesis, whereas Lh is most important for processes leading to final oocyte maturation and ovulation in females and spermiation in males (Yaron et al., 2003; reviewed by Levavi-Sivan et al., 2010).

Recent genetic studies have advanced our understanding of gonadotropin function in fish. For instance, although gonad development was significantly delayed, and puberty was delayed in females, Fsh-deficient zebrafish (*fshb*$^{-/-}$) were still fertile in both sex. Lh-deficient zebrafish (*lhb*$^{-/-}$) on the other hand, showed normal gonadal growth, but females failed to spawn and thus infertile. Neither *fshb* nor *lhb* mutation alone seemed to influence gonadal differentiation. Nevertheless, simultaneous mutation of both genes resulted in all male population, although with delayed testis development (Zhang et al., 2015; reviewed by Trudeau, 2018). The same authors reported that Fsh may play a role in maintaining female status, probably through regulation of ovarian aromatase. In medaka, knockout of both Fsh and Lh led to infertile females, but fertile males (Takahashi et al., 2016), indicating compensatory mechanisms at least in male medaka. Furthermore, mutation of the hypophysiotropic Gnrh1 in medaka demonstrated an essential role in ovulation. Females were reported to possess well-developed ovaries, but failed to ovulate. Mutation of *gnrh1* did not affect *fshb* in females but had a minor suppressive influence on *lhb*, hence gonadal development was maintained. *Gnrh1* knockout males remained fertile and no effects on gonadotropin expression was revealed (Takahashi et al., 2016).

An important tool when investigating gonadotropin regulation and function is the quantification of hormone levels in blood and pituitary by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). ELISA and RIA assays in fish have been detected traditionally based on native gonadotropins from fish pituitaries and their specific antibodies. The purification of native gonadotropins has been shown challenging, particularly regarding Fsh, due to insufficient amounts from fish pituitaries thus hindering immunization of rabbits for antibody production and enough native proteins as standard for the RIA or ELISA. Furthermore, the purification of native gonadotropins is a labor and resource demanding process. Despite this, homologous immunoassays for Fsh have been generated for a few species, including chum salmon (*Oncorhynchus keta*) (Suzuki et al., 1988), coho salmon (*Oncorhynchus kisutch*) (Swanson et al., 1989), rainbow trout (*Oncorhynchus mykiss*) (Prat et al., 1996), Nile tilapia (*Oreochromis niloticus*) (Aizen et al., 2007a), mummichog (*Fundulus heteroclitus*) (Shimizu et al., 2012), European seabass (*Dicentrarchus labrax*) (Molés et al., 2012), Senegalese sole (*Solea senegalensis*) (Chauvigné et al., 2016), Russian sturgeon (*Acipenser gueldenstaedtii*) (Yom-Din et al., 2016), and common carp (*Cyprinus carpio*) (Hollander-Cohen et al., 2017). Quantitative assays for Lh are accessible for a larger number of species, which has led to a particular lack of understanding of Fsh physiology in fish (Yom-Din et al., 2016). As a result, recombinant gonadotropins are now being used as a substitute to native hormones (Levavi-Sivan et al., 2010). They have advantages compared to natively purified hormones since they can be continually produced, and potential cross-contamination with other related glycoproteins is minimized (Levavi-Sivan et al., 2010).

The objectives of the present work was to generate recombinant medaka Fsh (mdFsh) and Lh (mdLh) using the *Pichia pastoris* system, and furthermore to produce specific antibodies against their respective β-subunits, and their utilization in the development and validation of specific and homologous competitive immunoassays (ELISAs). The validated ELISAs were used to study the profile of Fsh and Lh content in medaka pituitary comparing juveniles and adults, and compared to the corresponding pituitary developmental profile of *fshb* and *lhb* gene expression levels.

2. Materials and methods

2.1. Animals

Japanese medaka (*Oryzias latipes*) of the dr-R strain were kept in recirculating systems with water temperature of 28 ± 1 °C and light-dark cycle of L14:D10. Embryos were incubated in embryo culture medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ (all Sigma-Aldrich, St. Louis, U.S.A.)), and kept at 26 °C until hatching and transfer to system tanks. A combination of dry feed and live brine shrimp nauplii larvae (*Artemia salina*) was used to feed the fish three meals per day. All fish were raised under the same conditions regarding temperature, photoperiod, tank size, density, and food. Handling, husbandry and use of fish were in accordance with the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences.

2.2. Production and purification of recombinant gonadotropins mdFshβ, mdLhβ, mdFshβα, and mdLhβα

Production of recombinant proteins was performed in the methylotrophic yeast *P. pastoris* expression system following a procedure described by Kasuto and Levavi-Sivan (2005) and Yom-Din et al. (2016). Synthesis of genes for medaka *fshb* (Accession Number NM_001309017.1), *lhb* (Accession Number AB541982.1), *fshba*, and *lhba* (*gpa*; Accession Number NM_001122906) was performed commercially (GenScript, New Jersey, U.S.A.) (see sequences Fig. 8). Gene expression constructs were generated (Fig. 1) and gene expression cassettes were produced for each construct with codon optimized DNA sequence according to the codon usage of the *P. pastoris* expression system. The synthetic genes were joined to form a fusion gene encoding a "tethered" polypeptide (mdFshβα, mdLhβα) in which the α chain forms the C-terminal domain and one of the β chains forms the N-terminal domain. A six-His tail was positioned at the end of the β subunit enabling purification of the recombinant protein, and a "linker" sequence containing six amino acids (three Gly-Ser pairs) (Aizen et al., 2017) was placed between the α and β chains. Synthesized DNA fragments were cloned into pPIC9K vector using EcoRI and NotI restrictions sites and confirmed by sequencing (Weizmann Institute, Rehovot, Israel). The constructs were digested with SalI and used to transform *P. pastoris* strain GS115 (Invitrogen, Carlsbad, U.S.A.) by electroporation (GenePulser, Bio-Rad, Hercules, U.S.A.). This resulted in insertion of the construct at the *AOX1* locus of *P. pastoris*, generating a His$^+$ Mut^S (slow methanol utilization) phenotype. Transformants were selected for the His$^+$ phenotype on 2% agar containing regeneration dextrose-biotin medium. The protein was expressed in a shaker flask and harvested at 72 h after methanol induction. Recombinant medaka Fshβ (mdFshβ), medaka Lhβ (mdLhβ), medaka Fshβα (mdFshβα), and medaka Lhβα (mdLhβα) were isolated by batch purification of the his-tagged proteins, and eluted fractions were dialyzed with Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, Waltham, U.S.A) according to the manufacturer's protocol. The purified proteins, tagged with a six-His tail, were detected on a Western blot as described below.

2.3. Production and validation of specific antibodies for mdFshβ and mdLhβ

Polyclonal antisera against recombinant mdFshβ and mdLhβ were raised in two different rabbits for each protein. Each rabbit received three intradermal injections of purified protein (mdFshβ; 1 mg first injection, 0.5 mg second and third injection; mdLhβ; 0.7 mg first injection, 0.4 mg second and third injection) in 0.9% NaCl and emulsified

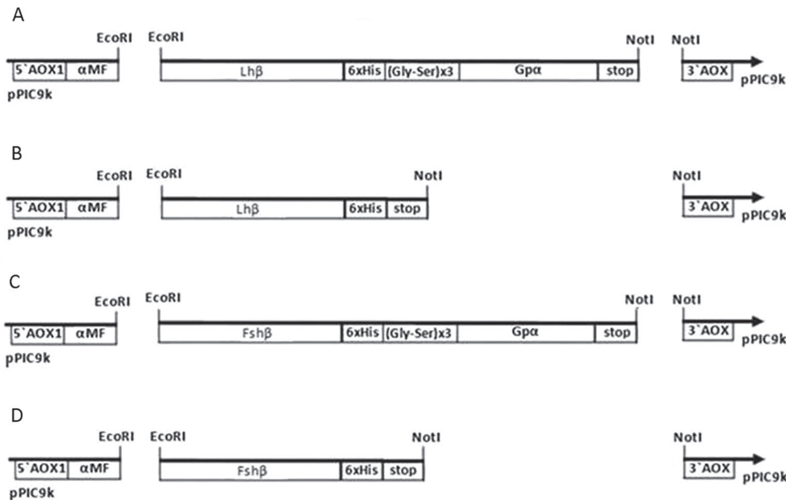

Fig. 1. Schematic overview of the synthesis of medaka *mdlhba* (A), *mdlhb* (B), *mdfshba* (C), and *mdfshb* (D). Fusion genes encoding "tethered" polypeptides in which one of the β-chains forms the *N*-terminal domain and the α-chain forms the C-terminal domain were produced for *mdlhba* and *mdfshba*. These include a "linker" sequence of six amino acids (three Gly-Ser pairs) between the β- and α-chains which assist in subunit chimerization. To enable purification of the recombinant proteins, a six-His tail (His^6) was placed at the end of the β subunit. All genes were subcloned into the *P. pastoris* expression vector pPIC9K using EcoRI and NotI restriction enzymes. pPIC9K contains the yeast α mating factor (αMF) secretion signal downstream of the *AOX1* promoter that directs the desired recombinant protein into the secretory pathway. The pPIC9K vector enables isolation of multicopy inserts by an *in vivo* method to test if increased copy number of the recombinant gene will lead to increased protein expression. Resistance to Geneticin (G418 sulfate) was utilized to screen for possible multicopy inserts.

in an equal volume of complete Freund's adjuvant (Sigma-Aldrich) at 3-week intervals, generally according to Aizen et al. (2007b). The rabbits were bled at 2 weeks after the final injection, and the serum was aliquoted and lyophilized. Recombinant mdFshβ, mdLhβ, mdFshβα, mdLhβα, and medaka pituitary extract were visualized using the antibodies against mdFshβ and mdLhβ as a validation of the antibodies. To verify that the plasma of the rabbit before the final injections did not react with mdFshβ and mdLhβ, a Western blot analysis using medaka pre-immune serum as a negative control against medaka pituitary extract, mdFshβ, and mdLhβ was performed. Western blot analysis methodology was performed as described in Section 2.4.

2.4. Western blot analysis

Recombinant proteins and pituitary extracts from medaka and tilapia were analyzed by Western blot analysis according to Yom-Din et al. (2016), using anti-His (diluted 1:2000), anti-mdFshβ, or anti-mdLhβ (both diluted 1:2000, 1:100,000, 1:600,000) antisera.

2.5. Fluorescence in situ hybridization (FISH)

For further evaluation of mdLhβ and mdFshβ antibody specificity, fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) were performed. FISH was performed on free-floating parasagittal brain-pituitary sections as described previously (Fontaine et al., 2013). Briefly, after being sacrificed with ice water, brain and pituitary from 12 unsexed 6 month-old adult fish were dissected and fixated overnight with 4% PFA at 4 °C. Tissues were then gradually dehydrated in ethanol and stored in 100% methanol until used. Tissues were rehydrated, embedded in 3% agarose and para-sagittally sectioned (60 µm sections) using a vibratome (VT1000S Leica, Wetzlar, Germany). They were then treated with proteinase K (1 µg/ml; P6556, Sigma-Aldrich) for 30 min. *fshb* riboprobe was cloned using AGAGCAGAGGAAGCAACACT and GGGGCACAGTTTCTTTATTTCAG as primers, and synthesized using PGEM-T vector (Promega, Madison, WI), whereas we used the *lhb* riboprobe previously described (Hildahl et al., 2012). *fshb* and *lhb* sense and antisense riboprobes were conjugated with digoxigenin (DIG; 11277073910; Roche, Basel, Switzerland) using SP6 or T7 RNA polymerase (Promega). Tissues were hybridized with either sense or antisense riboprobes for 18 h at 65 °C and then incubated with sheep anti-DIG conjugated with peroxidase (POD; 1:500; 11207733910; Roche) over night. Signal was revealed using TAMRA-conjugated tyramide constructed in our lab.

2.6. Immunofluorescence (IF)

IF staining was performed on free-floating sections as described by Fontaine et al (2013). Anti-Lhβ and anti-Fshβ described earlier (see Section 2.3) were used. For anti-Lhβ, the tissues labelled for *lhb* mRNA by FISH were used (see above). For anti-Fshβ, IF could not be performed after *in situ* labelling because an antigen retrieval treatment was required before the IF, destroying the labeling of the FISH. Thus, IF was performed on consecutive parasagittal sections of the one used for *fshb* FISH. Tissue sections were treated using 2 N Hydrochloric acid (HCl) for 1 h at 37 °C and then incubated with primary antibody (anti-Lhβ; 1:2000, anti-Fshβ; 1:1000) overnight at 4 °C. A secondary goat anti-rabbit antibody coupled to AlexaFluor 488 (A-11034, Thermo Fisher Scientific, Waltham, U.S.A.) at a concentration of 1:1000 was used for 4 h incubation. Control experiments included incubation without primary antibody. Tissues for anti-Lhβ were treated for nuclei staining with DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride; 32,670 Sigma) by incubation at RT for 20 min at a titer of 1:1000 and rinsing.

2.7. Imaging

Stained tissues were mounted with Vectashield H-1000 (Vector, California, U.S.A.). Images were acquired using a Zeiss LSM710 confocal microscope with 25× (LCI Plan-Neofluar 25×/0.8NA) objective. Channels were acquired sequentially to avoid signal crossover between the different filters. Images were processed using the ZEN software (Carl Zeiss AG, Oberkochen, Germany). Z-projections from confocal stacks of images were obtained using Image J software (http://rsbweb.nih.gov/ij/). Composites were assembled using Adobe Photoshop and Illustrator CS6 (Adobe Systems, San Francisco, California).

2.8. qPCR gene expression analysis of fshb and lhb – tissue screening

A tissue screen of *fshb* and *lhb* expression was performed on brain, pituitary, testis, ovary, eyes, heart, intestine, liver, gills, gallbladder, and spleen from three adult 6 month-old males or females. Total RNA was extracted using 500 µL Trizol agent (Invitrogen, Carlsbad, U.S.A.) and 100 µL chloroform (Invitrogen), or 300 µL Trizol agent and 120 µL

Table 1
qPCR primers used in the present study.

Target	Reference	Primer sequence	Accession Number	Amplicon size (nt)	Efficiency
lhb	Hildahl et al. (2012)	Forward: 5′CCACTGCCTTACCAAGGACC-3′ Reverse: 5′-AGGAAGCTCAAATGTCTTGTAG-3′	AB541982.1	100	2.00
fshb	this study	Forward: 5′-GACGGTGCTACCATGAGGAT-3′ Reverse: 5′-TCCCCACTGCAGATCTTTTC-3′	NM_001309017.1	73	2.03
18s	this study	Forward: 5′-CCTGCGGCTTAATTTGACTC-3′ Reverse: 5′-AACTAAGAACGGCCATGCAC-3′	AB105163.1	118	2.02
rpl7	this study	Forward: 5′-TGCTTTGGTGGAGAAAGCTC-3′ Reverse: 5′-TGGCAGGCTTGAAGTTCTTT-3′	NM_001104870	98	2.03
gapdh	this study	Forward: 5′-CCTCCATCTTTGATGCTGGT-3′ Reverse: 5′- ACGGTTGCTGTAGCCAAACT-3′	XM_004077972.3	75	2.01

chloroform for pituitary tissue, and resuspended in 14 µL of nuclease free water. cDNA was prepared from 25 ng to 500 ng of total RNA (brain, ovary, eyes, intestine, spleen 500 ng; testis, gills 250 ng; heart, gallbladder 100 ng; liver 50 ng; pituitary 25 ng) using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (Thermofisher scientific). qPCR primers were designed using Primer3Plus shareware (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cg) and analyzed for possible hairpin loops and primer dimer formations using Vector NTI (Life Technologies) (Table 1). One primer in each pair was targeted to an exon–exon border. PCR was performed on a LightCycler 96 (Roche, Mannheim, Germany) using SYBR Green I Master (Roche). cDNA samples were run in duplicate, using 3 µL of 10x diluted cDNA and 5 µM each of forward and reverse primer in a total volume of 10 µL. The cycling parameters were 10 min pre-incubation at 95 °C, followed by 40 cycles of 95 °C for 10 s, 60 °C for 5 s and 72 °C for 3 s, 4 s or 5 s (3 s for *fshb*, 4 s for *lhb*, *rpl7*, *gapdh* and 5 s for *18s*), followed by melting curve analysis to assess PCR product specificity. Non-template control and positive control (calibrator) were run in triplicate in all qPCR plates. To perform accurate normalization of the qPCR, the stability of three candidate reference genes was investigated using RefFinder analysis available at http://leonxie.esy.es/RefFinder/. Reference gene candidates *18s*, *rpl7* and *gapdh* were analyzed using RefFinder, resulting in the use of *18s* and *rpl7* as most stable combination of reference genes.

Relative gene expression values were calculated according to Weltzien et al. (2005) and Hodne et al. (2012).

2.9. Gonadal histology

Testicular germ cell development was analyzed to assess the maturational stage of fish sampled for developmental series (see Table 3). Testes from 36 males, grouped according to standard body length (SL), were dissected and transferred to phosphate buffered saline (PBS; Sigma-Aldrich) prior to overnight fixation in ice-cold 4% glutaraldehyde phosphate buffered solution (pH 7.2) at 4 °C. Tissues were dehydrated at RT in increasing concentrations of EtOH (70–100%), each step lasting at least 30 min. The last step (100%) was repeated trice and then replaced with approx. 5 ml of preparation solution (100 ml Technovit 7100 added 1 g of Hardener I (Heraeus Kulzer, Hanau, Germany)) and kept at slow shaking overnight. After infiltration, tissue samples were embedded in cold Histoform S (Heraeus Kulzer) added approx. 1 ml preparation solution w/50 µL Hardener II (Heraeus Kulzer) and incubated at 37 °C. Cured samples were mounted unto Histoblocs using Technovit 3040 (both from Heraeus Kulzer), before sagittal sections (3 µm) were prepared using a Leica RM2245 microtome (Leica Biosystems, Wetzlar, Germany). Sections were collected from the periphery until the middle of the testes tissue every 30 µm and placed onto microscope slides. Dried sections were stained with Toluidine Blue O (Sigma-Aldrich) and mounted with Coverquick 4000 (VWR International, Radnor, PA, USA) before histological analysis. Germ cells were identified according to Schulz et al. (2010), but within the five main germ cell stages (spermatogonia type A (SPA), spermatogonia type B (SPB), spermatocytes (SC), spermatids (ST) and spermatozoa (SZ)), no further distinctions were made (see Table 2).

2.10. Development and validation of specific ELISA for Fsh and Lh, and profile of pituitary levels of Fsh and Lh in male medaka

Specific and homologous competitive ELISAs were developed for determination of mdFsh and mdLh according to Mañanós et al. (1997) and Aizen et al. (2007b), and using antibodies against mdFshβ or mdLhβ (Section 2.3), recombinant β-subunits mdFshβ or mdLhβ (Section 2.2) to coat ELISA microplates, and recombinant mdFshβα or mdLhβα for standard curves. Briefly, microtiter plates were coated with mdFshβ (1 ng/well) or mdLhβ (0.5 ng/well). Single pituitaries were homogenized and diluted 1:2.7 with 0.1% BSA in PBST. Samples and standards were pre-incubated with primary antibodies (final dilution 1:10,000 for mdFshβ and 1:50,000 for mdLhβ in 0.1% BSA in PBST) overnight at RT. After pre-incubation, samples were distributed into the coated microtiter plates and incubated for 3 h at RT. Antigen–antibody complexes were detected using GAR-HRP antibody (Bio-Rad) at a

Table 2
Criteria for determining stage of testes development.

Maturational stages[a]	
I	Only SPA (immature)
II	SPA + SPB (early maturing)
III	SPA + SPB + SC (maturing)
IV	SPA + SPB + SC + ST (late maturing)
V	SPA + SPB + SC + ST + SZ (mature)

No quantification of relative abundance was conducted. Spermatogonia, type A (SPA).
Spermatogonia, type B (SPB); Spermatocytes (SC); Spermatids (ST); Spermatozoa (SZ).
[a] Each stage is here defined by the most advanced germ cell type present in the gonad.

Table 3
To achieve a developmental profile of Fsh and Lh (ELISA)[a] and *fshb* and *lhb* (qPCR), pituitaries from male medaka were dissected from fish structured in the six following body length groups, n = 6.

Group numbering	Body length (mm)	Maturational stages within group
1	14– < 16	I–V
2	16– < 18	I–V
3	18– < 19	I–V
3	19– < 20	V
4	20– < 22	V
5	22– < 24	V
6	24– < 26	V

[a] For ELISA analysis, juveniles had body length between 12 mm and 16.5 mm and adults between 21 mm and 26.5 mm.

1:5000 dilution, before visualization using 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Zotal, Israel). Absorbance was read at 405 nm on a Microplate Spectrophotometer (Epoch 2, Biotek, Winooski, U.S.A.). The ELISA was validated for mdFsh and mdLh using medaka pituitary extracts. Displacement curves for pituitary samples were achieved by serial dilutions of samples in 0.1% BSA in PBST and compared to the standard curve for either mdFshβα or mdLhβα. Assay sensitivity is defined as the lowest dose of Fsh or Lh capable of reducing the optical density more than the mean plus two standard deviations of the zero dose of Fsh or Lh [B_0 - 2SD]; it was calculated by adding the mean of the blank to two times the standard deviation of the blank. Intra-assay coefficient of variation (CV) was determined by assaying six replicates of one of the standard concentrations (1.56 ng/ml) on the same assay plate. Inter-assay CV was calculated by assaying the same sample five times in different plates.

2.11. Profile of pituitary levels of Fsh and Lh in male medaka comparing juveniles and adults

A profile of Fsh and Lh in male medaka pituitaries was performed using the ELISA method described above. For Fsh, pituitaries from 24 juvenile males with SL between 12 mm and 16.5 mm, and of 24 adult males between 21 mm and 25.5 mm were dissected. For the profile of Lh, pituitaries from 12 juvenile males with SL between 12 mm and 16 mm, and of 12 adult males between 22.5 mm and 26.5 mm were dissected (for both Fsh and Lh 1 pituitary in 40 µL 0.1% BSA in PBST per biological replicate was used). The distinction between juvenile and adult was based on unpublished results relating medaka testicular maturation stage to SL (see Section 2.9), and showing that males with SL below 16 mm were completely immature and males with SL above 20 mm to be fully mature.

2.12. qPCR gene expression analysis of fshb and lhb – pituitary developmental profile

A developmental profile of *fshb* and *lhb* pituitary gene expression in male medaka from juvenile to adult stage was compared to the corresponding protein levels (Section 2.10). Pituitaries from 50 males were dissected from fish grouped according to SL: group 1 (14– < 16 mm), group 2 (16– < 18 mm), group 3 (18– < 20 mm), group 4 (20– < 22 mm), group 5 (22– < 24 mm), group 6 (24– < 26 mm) (see Table 3). Total RNA was isolated from one pituitary (group 4–6), two pituitaries (group 2–3), or three pituitaries (group 1) per biological replicate. RNA extraction and qPCR analysis was performed as described in Section 2.8 for five biological replicates per group (all samples in two technical duplicates). A combination of the three reference genes *18s*, *rpl7* and *gapdh* was used for normalization of gene expression.

2.13. Statistical analysis

Data are presented as mean ± SEM. All data were tested for normal distribution (Shapiro-Wilk normality test). For sample groups that did not follow a normal distribution (Lh ELISA profile for juvenile group in Section 2.10), data were log-transformed. For ELISA data calculations, sigmoid curves were linearized using logit transformation. Correlations were calculated by Graph-Pad Prism software (version 7; GraphPad, San Diego, U.S.A.). Significance level was set to 0.05. To test for parallelism between regressions lines, logit transformation was conducted, with logit (B/Bo) = log [r/(1 − r)], where r = B/Bo, B represents the binding at each point, and Bo the maximum binding. The linear regression parameter in Prism was used to compare the slopes. Potential significance of differences in expression levels between body length groups was calculated by one-way ANOVA followed by Tukey test using the Graph-Pad Prism software, and linear regression. Linear regression analysis was performed to analyze the relation between body length and expression level for the various hormones.

3. Results

3.1. Production of recombinant proteins mdFshβ, mdLhβ, mdFshβα, mdLhβα

Among the 300 His^+ Mut^S clones, which contained sequences of mdFshβ, mdLhβ, mdFshβα, and mdLhβα in their genome at the *AOX1* locus, 10 clones for each construct were selected based on their resistance to the antibiotic G418. For each construct, the highest-secreting clone was chosen for further purification by one-step nickel affinity chromatography. The eluted fractions contained 2.78 mg recombinant mdFshβ (of which 2 mg were used for antibody production, see Section 2.3) and 1.53 mg recombinant mdLhβ (of which 1.5 mg were used for antibody production, see Section 2.3) in a first batch. In a second batch 813.1 µg recombinant mdFshβ, 201.3 µg recombinant mdLhβ, 314.6 µg recombinant mdFshβα, and 44.6 µg recombinant mdLhβα were produced (measured by 6-His-ELISA).

3.2. Validation of recombinant proteins mdFshβ, mdLhβ, mdFshβα, mdLhβα

The supernatants from *P. pastoris* transformed with pPIC9KmdFshβ, pPIC9KmdLhβ, pPIC9KmdFshβα, or pPIC9KmdLhβα were immunoreacted against the His-tag and validated by comparing the observed molecular weights with those expected according to sequence. Under reducing conditions, mdFshβ and mdFshβα were revealed as bands of 14–16 kDa and 25–30 kDa, respectively, and after deglycosylation with PNGase F as bands of 12–14 kDa and 24–25 kDa, respectively. This is in accordance with the calculated molecular weight without glycosylation residues for mdFshβ (13 kDa) and for mdFshβα (25 kDa). After SDS-PAGE under reducing conditions, mdLhβ and mdLhβα had a molecular weight of 15 kDa and 35 kDa, respectively, and after deglycosylation with PNGase F 12–14 kDa and 27–28 kDa, respectively. Again, this met the expectation for deglycosylated mdLhβ (15 kDa) and mdLhβα (28 kDa). In short, all recombinant proteins mdFshβ, mdLhβ, mdFshβα, and mdLhβα were successfully detected with His-tail antibodies, and their molecular sizes derived from Western blots were in accordance with the calculated estimates (Fig. 1 in Burow et al., 2018).

3.3. Production and validation of specific antibodies against mdFshβ and mdLhβ

The supernatants from *P. pastoris* either transformed with pPIC9KmdFshβ and pPIC9KmdFshβα, or pPIC9KmdLhβ and pPIC9KmdLhβα were immunoreacted against specific mdFshβ and mdLhβ antibodies to ensure that they could detect the correct proteins

and to verify the absence of cross-reactions. Under reducing conditions and after deglycosylation, mdFshβ and mdFshβα were revealed as bands of 12–13 kDa and 23–25 kDa, respectively. mdLhβ was detected after deglycosylation very weakly as a band of 12–13 kDa, and mdLhβα could be detected as a band of 27–29 kDa (Fig. 2 in Burow et al., 2018). The antibodies were also utilized on medaka pituitary extracts. With both antibodies, deglycosylated mdFshβ and mdLhβ could be detected in medaka pituitary extract. Using the mdFshβ antibody, bands of approximately 13 kDa were revealed for mdFshβ. When using the mdLhβ antibody, there was no clean band for mdLhβ due to very strong signals. Note that no bands were detected for mdLhβ with the mdFshβ antibody and no bands for mdFshβ using the mdLhβ antibody (Fig. 3 in Burow et al., 2018). Medaka pituitary extract, mdFshβ, and mdLhβ were immunoreacted against medaka pre-immune serum as a negative control (test bleeding). As expected, there was no specific band observed (Fig. 3C in Burow et al., 2018).

3.4. Fluorescence in situ hybridization and immunofluorescence

To further validate the antibodies, we performed fluorescence *in situ* hybridization (FISH) for *lhb* mRNA followed by immunofluorescence (IF) for Lhβ on medaka pituitary tissue (Fig. 2A–D). At low magnification, both *lhb* mRNA (cyan) and Lhβ protein (magenta) labeling were in the ventral part of the pituitary. Higher magnification of this region revealed a high degree of co-localization between *lhb* mRNA and Lhβ protein labeling. Even if *lhb* mRNA (cyan, Fig. 2B) was localized close to the nucleus and Lhβ protein was found only in the cytoplasm, they were found in the same cells. Because of the HCL treatment which was needed to unmask the epitope of mdFshβ, we were not able to do double labeling for *fshb* mRNA and Fshβ protein. Instead we performed FISH for *fshb* mRNA and IF for Fshβ on neighboring pituitary sections (Fig. 2E–G). *fshb* mRNA was expressed in cells located in the ventro-median to dorso-median part of the pituitary, with a few cells in the posterior part, and more spread than the *lhb* expressing cells. IF with the mdFshβ antibody labeled also cells located within the median part of the pituitary with a few cells in the posterior part. The very similar distribution of immunoreactive cells strongly suggests the specificity of the antibody raised against mdFshβ. When merging IF and FISH, Fsh cells are clearly in the same region of two consecutive serial para-sagittal sections (Fig. 2G).

3.5. qPCR gene expression analysis of fshb and lhb – tissue screening

Both *fshb* (Fig. 3A) and *lhb* (Fig. 3B) were highly expressed in the pituitary. *fshb* was also expressed at high levels in brain and testis, at low levels in eyes, gills, gallbladder, heart, intestine, and ovary, and at trace amounts in spleen. *fshb* was not detected in liver. *lhb* expression was observed at high levels in brain, at low levels in eyes, gallbladder, intestine, spleen and testis, and at trace amounts in gills, heart and ovary. *lhb* was not detected in liver.

3.6. Gonadal histology

Testes from 36 males were analyzed and staged according to the most advanced germ cell type present, and thereafter grouped according to SL (see Tables 2, 3, and Fig. 4). Although preliminary results from the histological analysis indicated that fish with SL between 14 and < 16 mm were all immature (stage I), completion of the analyses revealed one fish maturing (stage III) at size 14.9 mm and another fish fully mature (stage V) at 15.5 mm. Fish between 16 and < 19 mm contained all stages from immature to mature, and fish between 19 and < 26 mm were fully mature (see Table 3). Analyzing the data from body weight and age together with SL, it was determined that all fish with SL above 19 mm, body weight above 130 mg, or age above 100 days, were all at stage V. A lower limit regarding SL, weight or age at which all fish were immature could not be determined.

3.7. Development and validation of specific ELISA for Fsh and Lh, and profile of pituitary levels of Fsh and Lh in male medaka

Competitive ELISAs were developed for medaka Fsh and Lh using recombinant mdFshβα and mdLhβα as standards, recombinant mdFshβ and mdLhβ for coating, and specific primary antibodies against recombinant mdFshβ or mdLhβ. The ELISA for Fsh had a standard curve ranging from 97.6 pg/ml to 50 ng/ml, and the ELISA for Lh ranging from 12.21 pg/ml to 6.25 ng/ml, with R^2 values of 0.9 and 0.94 for medaka Fsh and Lh, respectively. The sensitivity (lower limit of detection) for Fsh was 44.7 pg/ml and for Lh 70.8 pg/ml. Intra-assay CV for the Fsh ELISA, calculated by measuring replicates of the same sample on the same plate, was 2.7% (n = 6), while inter-assay CV, determined by measuring replicates of the same sample on different plates, was estimated at 5.3% (n = 5). Intra-assay CV for the Lh ELISA 3.0% (n = 6), while inter-assay CVs for the same sample on different plates was 5.7% (n = 5). Parallelism is one of the first steps when validating an assay for use with biological samples, with dilutions of a sample plotted against the standard curve. Validation for the medaka Fsh and Lh ELISA assays was performed by testing the parallelism between the standard curves (recombinant mdFshβα and mdLhβα) and displacement curves achieved by serial dilutions of male medaka pituitary extract using different homogenization conditions (10 ceramic beads, 6 m/s; 20 ceramic beads, 4 m/s; 20 ceramic beads, 6 m/s). Serial dilutions of pituitary extract from medaka were found to parallel both Fsh (Fig. 5A) and Lh (Fig. 5B) standard curves, implying that the assay matrix that is recognized by the antibody, is compatible. The slope of the displacement curve achieved with recombinant mdFshβα was not different from that obtained with native pituitary extract Fsh, and the slope of the displacement curve obtained with recombinant mdLhβα was not different from that produced with native pituitary extract Lh. These data suggest that the recombinant gonadotropins are immunologically similar to the authentic glycoproteins present in the fish pituitary.

Pituitary levels of Fsh and Lh was determined and compared with the corresponding *fshb* and *lhb* pituitary mRNA gene expression developmental profile (see Section 3.8). The data were analyzed as a function of three independent variables (SL, body weight, and age of the fish). Pituitary Fsh levels increased significantly from juveniles to adults (Fig. 6A). Body weight (R^2 = 0,3276; Fig. 4A in Burow et al., 2018) explains the variance in the dependent variable (gonadotropin) better compared to SL (R^2 = 0,3078; Fig. 6C), and age of the fish (R^2 = 0,2499; Fig. 4B in Burow et al., 2018) using a linear trendline. Pituitary Lh levels also showed a significant increase from juveniles to adults (Fig. 6B). As for Fsh, body weight (R^2 = 0,6221; Fig. 4C in Burow et al., 2018) explains the variance in the dependent variable better compared to SL (R^2 = 0,5722; Fig. 6D), and age (R^2 = 0,524; Fig. 4D in Burow et al., 2018) using a power trendline. Comparing the R^2 of body weight, SL, and age between the Fsh and Lh profiles, it is important to note that the R^2s are higher in Lh than in Fsh, suggesting that body weight explains the variance in the dependent variable for Lh better compared to Fsh.

3.8. qPCR gene expression analysis of fshb and lhb – pituitary developmental profile

A developmental profile of *fshb* and *lhb* pituitary gene expression was performed to compare the profile with the corresponding pituitary protein levels (see Section 3.7). Regarding *lhb* expression, a significant difference of *lhb* expression was detected between group 1 and group 6 (Fig. 7A; x-axis shows average SL per group). Furthermore, a linear regression analysis detected a significant increase of *lhb* expression with increased body length with a p value < 0.05 (95% confidence interval) and R^2 of 0.925, which suggests that the data can be explained by a linear model (Fig. 7B). The consistent results by both one-way ANOVA/ Tukey and linear regression suggest a positive relation between *lhb*

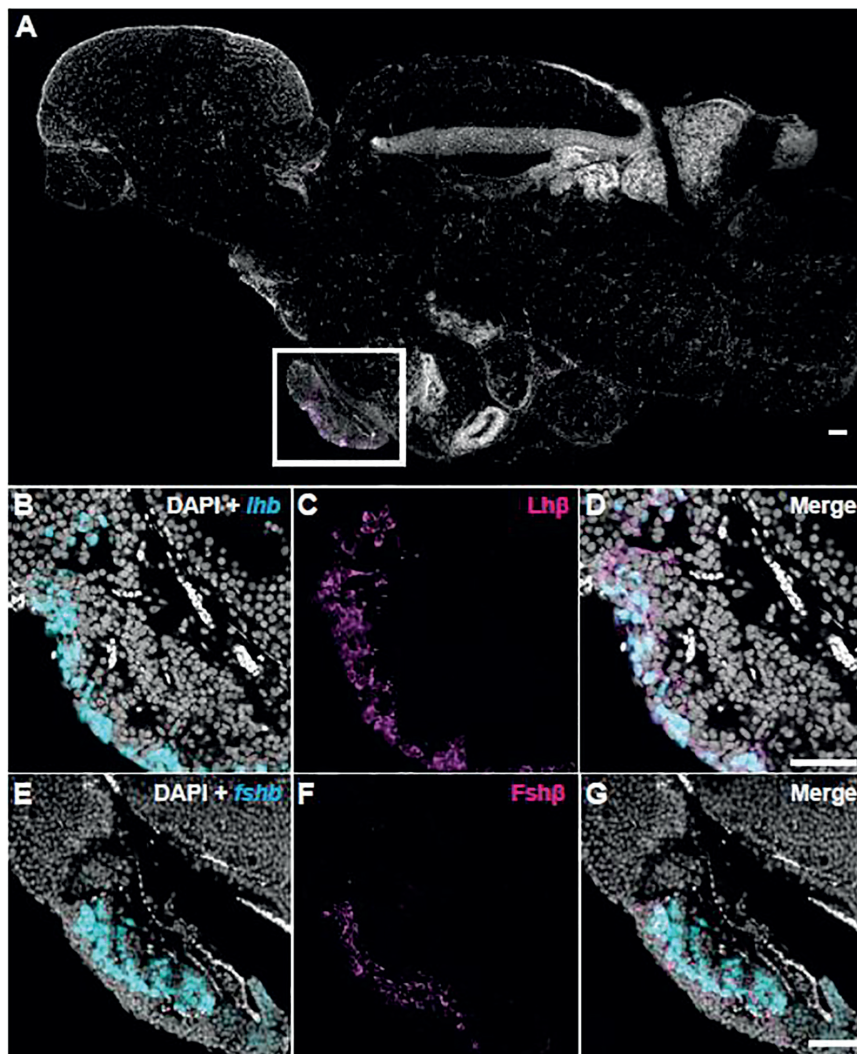

Fig. 2. Parasagittal section of brain with pituitary from 6 month-old medaka (A). Higher magnification of the pituitary representing the area from the square in (A) are presented in (B–G). Labeling of *lhb* (B) and *fshb* mRNA (E) by FISH (cyan), and Lhβ (C) and Fshβ protein (F) by IF (magenta), with DAPI counter staining (grey). (D) is a merge of (B) and (C), G is a merge of (E) and (F). FISH for *fshb* (E) and IF for Fshβ (F) was performed on adjacent pituitary sections because HCL treatment needed to unmask the epitope for IF prevented FISH analysis of the same section. Scale bars, 50 µm.

expression levels and SL. Concerning *fshb* expression, no significant difference was observed between any of the SL groups (one-way ANOVA, multiple comparison by Tukey) with a p value > 0.05 (95% confidence interval) (Fig. 7C). A linear regression analysis revealed no significant relation between the *fshb* expression and SL with a p value > 0.05 (95% confidence interval) and R^2 of 0.35 (Fig. 7D). This suggests that SL has no influence on *fshb* expression, in contrast to the situation for *lhb* expression.

4. Discussion

In the current study, we show (a) production and validation of medaka recombinant gonadotropins mdFshβ, mdLhβ, mdFshβα, and mdLhβα by *P. pastoris*; (b) generation and validation of specific antibodies against mdFshβ and mdLhβ using Western blot, FISH and IF; (c) development and validation of homologous competitive ELISA assays for mdFsh and mdLh at the single pituitary level; (d) application of the mdFsh and mdLh ELISA assays during medaka development; and (e) comparison of Fsh and Lh pituitary protein levels with pituitary *fshb* and *lhb* gene expression levels at corresponding developmental stages.

Purification of the recombinant gonadotropins from the *P. pastoris* culture medium was facilitated by a His^6-tag included in the protein sequence, which was shown not to have a negative impacts on hormone bioactivity (Kasuto and Levavi-Sivan, 2005; Aizen et al., 2007a). Western blot analysis indicated that both recombinant gonadotropins were considerably glycosylated by asparagine-linked glycans, and were thus

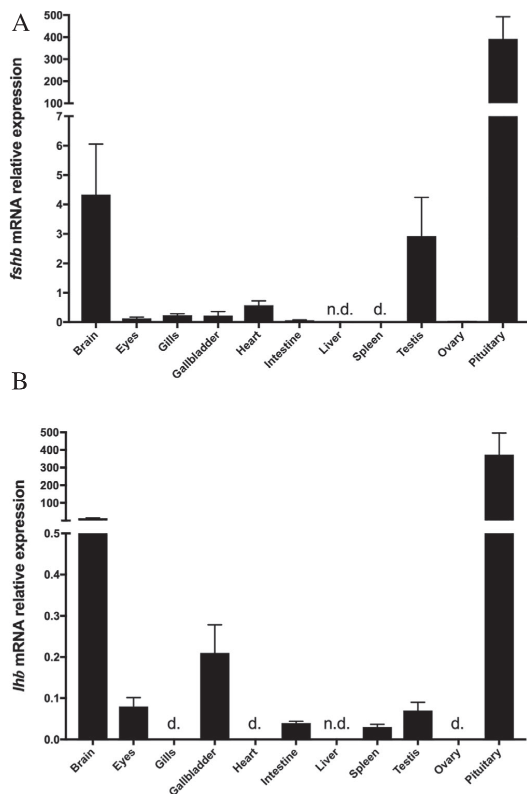

Fig. 3. Gene expression of *fshb and lhb* in various medaka tissues. (A) *fshb* relative expression, (B) *lhb* relative expression. Detectable but not quantifiable (d.), not detectable (n.d.). n = 3 per tissue.

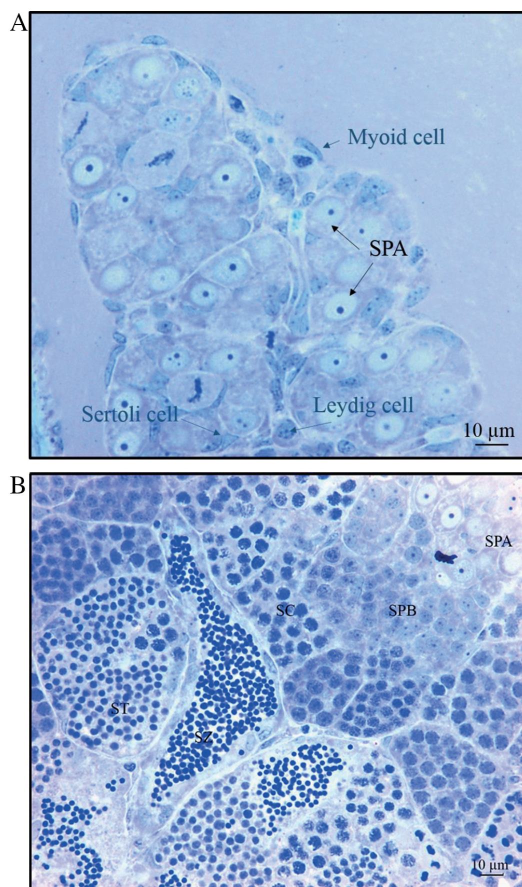

Fig. 4. Testis histology section from (A) sexually immature medaka and (B) sexually mature medaka. Section from sexually immature fish with SPA as the only germ cell present, testis section from sexually mature medaka with all germ cell stages present. Sagittal section, 3 µm thickness, stained with Toluidine Blue O. Spermatogonia type A (SPA), spermatogonia type B (SPB), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ).

deglycosylated by PNGase F treatment. Because PNGase F hydrolyzes all types of *N*-Glycan chain, all remaining carbohydrate modifications on the mature proteins were exclusively *O*-linked. This has been described earlier for other recombinant gonadotropins including Japanese eel Fsh (Kamei et al., 2003), tilapia Lh (Kasuto and Levavi-Sivan, 2005), tilapia Fsh (Aizen et al., 2007a), Russian Sturgeon Fsh and Lh (Yom-Din et al., 2016), and carp Fsh and Lh (Hollander-Cohen et al., 2017). This is in contrast to recombinant mammalian glycoprotein hormones that carry both *N*-linked and *O*-linked oligosaccharides (Fares, 2006). *N*-linked oligosaccharides were found to have a minor role in receptor binding of glycoprotein hormones but are important for bioactivity, while *O*-linked oligosaccharide chains were shown to have a minor role in receptor binding and signal transduction, yet are critical for half-life and bioactivity *in vivo* (Fares, 2006). The glycosylation moieties produced by *P. pastoris* are of the high-mannose type (Grinna and Tschopp, 1989) and thus differ from those of vertebrate cells.

The small differences in the estimated molecular mass between native and recombinant gonadotropins are presumably due to differences in the type and degree of glycosylation, since *P. pastoris* tends to implement different glycosylation moieties than eukaryotes (Cereghino and Cregg, 2000). It seems that the molecular weights of recombinant gonadotropins and their subunits are quite similar among fish species. They differ between 25 and 32 kDa for the tethered dimer and 15–25 kDa for the β subunits (Kasuto and Levavi-Sivan, 2005; Aizen et al., 2007a; Yu et al., 2010; Molés et al., 2011; Yom-Din et al., 2016). Additional bands on the Western blot could represent incomplete processing of the αMF leader sequence, strongly glycosylated proteins, or dimers and degradations of the protein. Additional bands could also be caused by PNGase F that has a slight reactivity to the 6-His antibody. Since the constructs *fshb* and *lhb* were used to transform *P. pastoris* strain, and the mdFshβ and mdLhβ proteins were used to produce antibodies in rabbits, there is the possibility that the mdFshβ and mdLhβ antibodies detect yeast residues. However, the molecular weights achieved in this study are similar to that of native proteins purified from pituitaries (14 kDa tilapia Lhβ, 35 kDa tilapia Lhβα (Kasuto and Levavi-Sivan, 2005); 17 kDa tilapia Fshβ, 33 kDa tilapia Fshβα (Aizen et al., 2007a); 33 kDa halibut Fshβα, 32 kDa halibut Lhβα (Weltzien et al., 2003b)), indicating high antibody specificity.

Concerning purification of the recombinant proteins, the yield of the heterodimers was significantly lower than the yield of the monomers, which is probably due to the simplicity of expressing an isolated subunit rather than a complex heterodimer. A similar phenomenon was reported for tilapia Fshβ and Fshβα (Aizen et al., 2007b) and Lhβ and Lhβα (Kasuto and Levavi-Sivan, 2005). The yield determined in the present study is higher than that reported for recombinant tilapia Fshβα

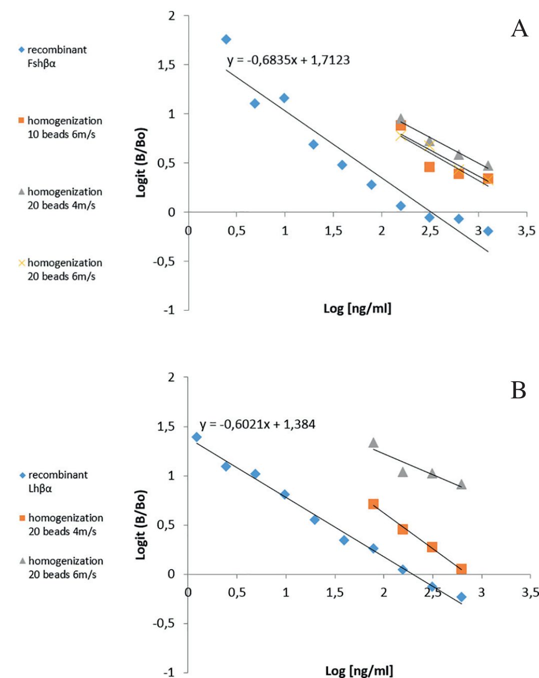

Fig. 5. Parallelism in ELISA for Fsh (A) and Lh (B) between the standard curve using recombinant mdFshβα or mdLhβα (labelled in blue) and displacement curves obtained by serial dilutions of pituitary extract from male medaka (labelled in orange, grey, yellow) using different homogenization conditions (10 ceramic beads, 6 m/s; 20 ceramic beads, 4 m/s; 20 ceramic beads, 6 m/s).

(100 μg; Aizen et al., 2007b), Lhβ (400 μg; Kasuto and Levavi-Sivan, 2005), and Lhβα (8 μg; Kasuto and Levavi-Sivan, 2005), respectively, but lower than recombinant tilapia Fshβ (6.5 mg; Aizen et al., 2007b). Single-chain recombinant gonadotropins have earlier been generated in several fish species, among them seabream (*Sparus aurata*) (Meiri et al., 2000), zebrafish (*Danio rerio*) (So et al., 2005), Nile tilapia (*Oreochromis niloticus*) (Aizen et al., 2007a; Kasuto and Levavi-Sivan, 2005), Manchurian trout (*Brachymystax lenok*) (Ko et al., 2007), channel catfish (*Ictalurus punctatus*) (Zmora et al., 2007), Japanese eel (*Anguilla japonica*) (Kamei et al., 2003; Kobayashi et al., 2010), European seabass (*Dicentrarchus labrax*) (Molés et al., 2011), Senegalese sole (Chauvigné et al., 2012), mummichog (Shimizu et al., 2012), sea lamprey (*Petromyzon marinus*) (Sower et al., 2015), greater amberjack (*Seriola dumerili*) (Nyuji et al., 2016), Russian sturgeon (Yom-Din et al., 2016), and yellowtail kingfish (*Seriola lalandi*) (Sanchís-Benlloch et al., 2017). The present study is the first attempt to produce medaka recombinant Fsh and Lh.

P. pastoris has been used in the present study and others as a heterologous expression system to produce recombinant fish gonadotropins because of its efficient secretion, high expression level, glycosylation potential, and high cell density (Levavi-Sivan et al., 2010). Various other expression systems exist for the production of recombinant proteins including prokaryotic systems as *Escherichia coli*, and eukaryotic organisms like mammalian or plant cells (Levavi-Sivan et al., 2010). The choice of an expression system depends mostly on the expected yield, the characteristics of the protein to be produced, and the need for further purification. The production cost is usually a major factor when sustained production of protein is required (Reyes-Ruiz and Barrera-Saldaña, 2006; Sethuraman and Stadheim, 2006).

Antiserum specificity against mdFshβ and mdLhβ was revealed by Western blot analysis, and further by FISH and IF. On Western blot, using medaka pre-immune serum as a negative control against medaka pituitary extract, it was confirmed that naïve rabbit plasma does not react with mdFshβ and mdLhβ. Important to mention is the lack of cross reactivity between the mdFshβ antibody and recombinant mdLhβ, and between the mdLhβ antibody and recombinant mdFshβ. In all Western blots, there were several other bands visible, this could possibly be explained by antibodies detecting glycosylation residues, which are identical between mdFshβ and mdLhβ.

Further validation of antibody specificity was achieved by FISH and IF on medaka pituitaries. Lhβ protein labeling were observed in the ventral part of the pituitary, with a near perfect co-localization with *lhb* mRNA. FISH for *fshb* mRNA and IF for Fshβ were carried out on serial pituitary sections because the HCl treatment required for unmasking the Fshβ epitopes prevents FISH. Without HCl treatment, IF for Fsh was not working. The reason why the epitopes of Fshβ necessitates unmasking could be related to the 3D configuration of the Fsh protein, or its lower quantity in the medaka pituitary. Even if co-localization cannot be stated with 100% certainty, *fshb* mRNA and mFshβ protein were distributed over the exact same pituitary region. *fshb* expressing cells were located in the median part of the pituitary, mainly distributed from the ventral to the dorsal part, with a few cells in the posterior part. They were more widely distributed than the *lhb* expressing cells. Similar results were reported for zebrafish and tilapia, since Lh cells were shown to form close cell-cell contacts and a continuous network throughout the gland, and Fsh cells to be more loosely distributed but to maintain some degree of cell-cell contact by cytoplasmic processes (Golan et al., 2016b). Medaka Fsh and Lh were generally expressed in different cells, confirming reports from several other fish species belonging to distinct teleost orders (Candelma et al., 2017; Chaube et al., 2015; Golan et al., 2014, 2016a; Weltzien et al., 2003a; Yom-Din et al., 2016).

Using the recombinant gonadotropins and mdFshβ and mdLhβ antibodies, we developed and validated ELISA assays specific for medaka Fsh and Lh. Validation included testing for parallelism between serial dilutions of medaka pituitary extract (native medaka Fsh and Lh) and the standard curves (recombinant medaka Fsh or Lh heterodimers). The ELISA assays for medaka Fsh and Lh perform comparably to available RIAs and ELISAs established for other fish species. The sensitivity of 44.7 pg/ml and 70.8 pg/ml for medaka Fsh and Lh, respectively, is generally high compared to published fish RIAs: 100 pg/ml for Nile tilapia Lh (Bogomolnaya et al., 1989), 156 pg/ml for hybrid striped bass (hybrid between *Morone chrysops* and *Morone saxatilis*) Lh (Mañanós et al., 1997), 780 pg/ml for red seabream (*Pagrus major*) Lh (Tanaka et al., 1993), 580 pg/ml for silver carp (*Hypophthalmichthys molitrix*) Lh (Kobayashi et al., 1985), 0.87 ng/ml for rainbow trout Lh and 0.15 ng/ml for rainbow trout Fsh (Govoroun et al., 1998). The sensitivity for gonadotropin ELISAs in fish include 0.65 ng/ml for European seabass Lh (Mateos et al., 2006), 0.1 ng/ml for rainbow trout Fsh (Santos et al., 2001), 0.24 pg/ml for Nile tilapia Fsh and 15.84 pg/ml for Nile tilapia Lh (Aizen et al., 2007b), 7.54 pg/ml for common carp Fsh and 32 pg/ml for common carp Lh (Hollander-Cohen et al., 2017), and 1.56 ng/ml for Russian sturgeon Fsh and 218 pg/ml for Russian sturgeon Lh (Yom-Din et al., 2016). Even though the sensitivities of ELISA and RIA assays are generally comparable, ELISA is the first choice due to its practical advantages compared to the RIA (Yom-Din et al., 2016). The reproducibility of the medaka ELISA was high, as shown by low intra-assay CVs of 2.7 and 3% for Fsh and Lh, respectively, and the low inter-assay CVs of 5.3 and 5.7% for Fsh and Lh, respectively. These values are comparable to previous studies of gonadotropins in other fish species; 7 (intra-assay) and 15% (inter-assay) for hybrid striped bass Lh ELISA (Mañanós et al., 1997), 6.3 (intra-assay) and 17.7% (inter-assay) for Russian sturgeon Fsh ELISA, and 5.9 (intra-assay) and 9.2% (inter-assay) for Russian sturgeon Lh ELISA (Yom-Din et al., 2016), 1.06 (intra-assay) and 8.66% (inter-assay) for common carp Fsh ELISA, and 7.6 (intra-

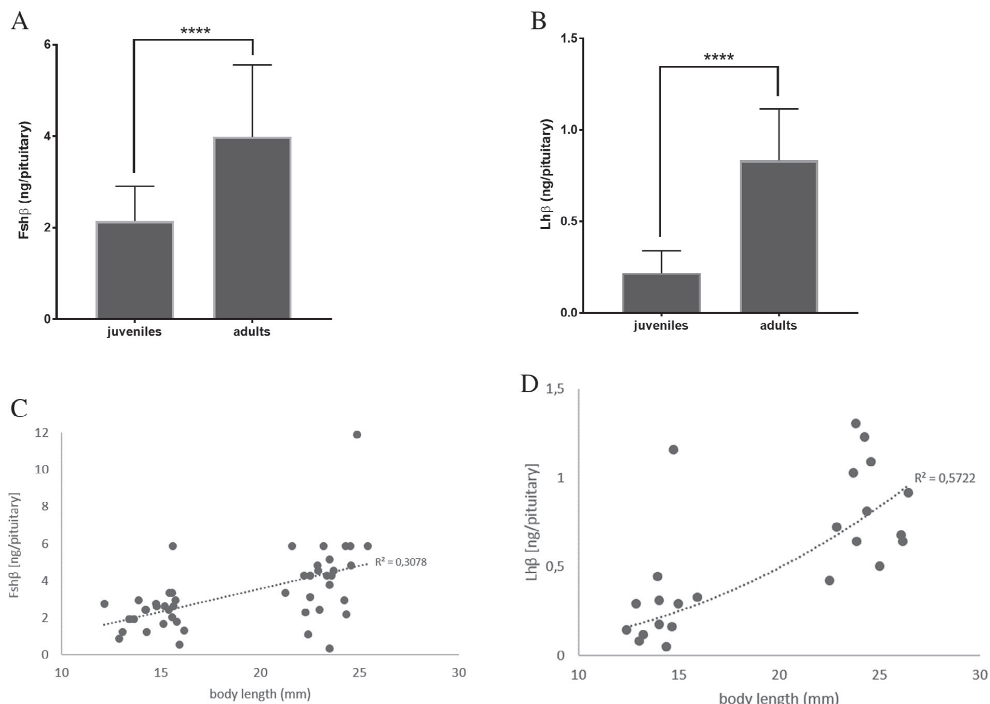

Fig. 6. Pituitary levels of Fsh (A) and Lh (B) in male medaka juveniles and adults. For juveniles, 24 fish with SL of 12–16.5 mm were sampled for quantification of Fsh, and 12 fish with SL of 12–16 mm for Lh. For adults, 24 fish with SL of 21–25.5 mm were sampled for quantification of Fsh, and 12 fish with SL of 22.5–26.5 mm for Lh. Data were analyzed using three independent variables body length (Fsh (C), linear trendline; Lh (D), power trendline), body weight (Fsh Fig. 4A in Burow et al., 2018, linear trendline; Lh Fig. 4C in Burow et al., 2018, power trendline), and age of the fish (Fsh Fig. 4B in Burow et al., 2018, linear trendline; Lh Fig. 4D in Burow et al., 2018, power trendline). **** means p ≤ 0.0001.

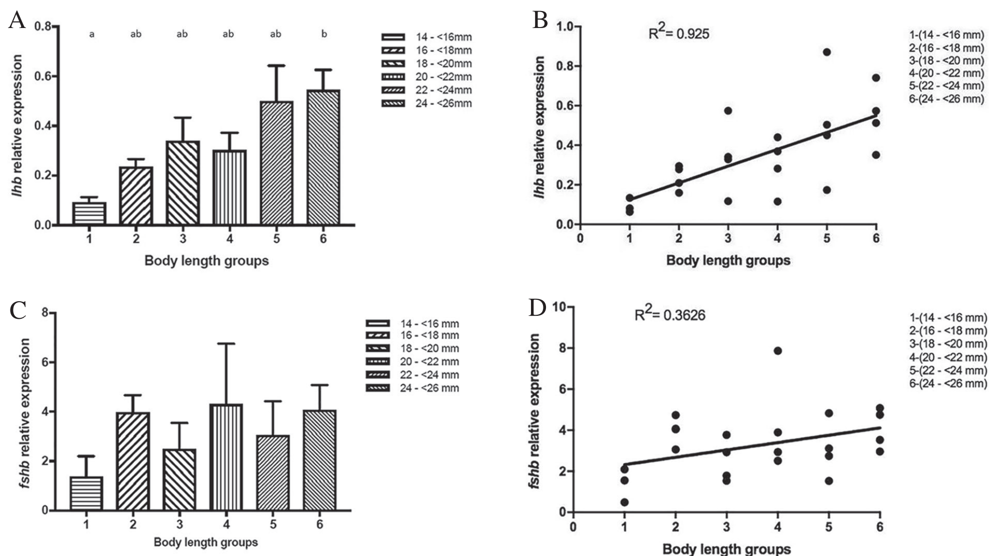

Fig. 7. Developmental profile of *fshb* and *lhb* gene expression levels from male medaka pituitaries as a function of body length, SL (14– < 16 mm; 16– < 18 mm; 18– < 20 mm; 20– < 22 mm; 22– < 24 mm; 24– < 26 mm), and presented as mean values ± SEM (A, n = 5) and (C, n = 5) or as linear regression curves based on individual pituitaries (B) and (D). Different letters indicate significant difference ($p < 0.05$).

Legend

Blue = *lhb*

Green = *fshb*

Yellow = *gpa*

Grey = 6 x Histidine or 6 x Histidine + 3 x Glycine-Serine

taa or tag = stop codon

lhb:

aggatgatttcccgggttagcagagtgatgtttctcctcatgttgagttttattctaggaacctcaactttcctctggtccctggcccctgcagcg
gccttccagctgccttactgccagccagtcaagcagaagttgtctctgcagaaggagggctgctctggctgtcatacggtggaaaccactgt
ctgcagtggccactgccttaccaaggaccccttgatgaagatacgatcaattcagtaccagaatgtgtgtacgtaccgggacttttactacaa
gacatttgagcttcctgactgcctgcctggcgtggatccgtcagtcacataccctgtggctctgagttgtcactgtggagcctgcatcatgaac
gcgtctgactgcacctttgagagcctgccaccagacttctgcgtgaaacatgattctttttattattag

fshb:

gatgcatcttagagaagagcagaggaagcaacactttcagccgagctcgcagtgacgctggagatctacaggcgtcggtactgcaccag
gcagaggatgcagctggttgtcatggcagctgcgttggtgctggcggaagtggggcaggtctccagcttttcctgtcatcccaaaaacgtca
gcatccctgtggagagctgtggcatcagcgggtgcgtccacaccaccatatgcgaaggacggtgctaccatgaggatcccaactacatca
gctatgaagaccaccctaaagaaaagatctgcagtggggactggtcctacgaagttaaattcattgagggatgtccagtgggtttcaaatatc
ctgtggccaaaagctgcgagtgcactacatgcaacacaagaaccacatactgcggccgactttctgcagacatgccgagctgttaaagtga
ccttttgtttgcctcatgtggttaaattaccctaatgtcttcagcactgaaataaagaaactgtgcccccccct

gpa:

ttttttaatacgcctcattatggggcaagcggtgttatccacccagagtccgggggggataaaggagtcctgcagaacggagggatgaagga
gaaacgttcccataacatgaaatctactcctcctatgatgggctttttgaaatcagctgaagtgtctgttctgctgatgtctattcttctttgcacag
ccgacacctactccaatctggcttcctcaaacttggactgcatggaatgccggctggagaaaaacagcatattctcaagggaaggtaaacc
ggtctaccagtgcataggatgttgcttctccagagcgtacccaacaccactgcgagctatgcagaccatgacagttccaaagaacatcactt
cagaggcaacttgctgtgttgcaaagcacagccacgagttcctctttcagacaatcgaacataccatcccagtgagaaaccatacggagtgt
cactgcagcacctgctattatcacaagatgtgagaagaggagatcagtctgtgctcagctttgcaatagagttggattcttaaaaaaaaaaaa
aaaaaaaaa

Fig. 8. Sequences for medaka *lhb, fshb, lhba*, and *fshba*. Synthesis was performed commercially (GenScript). The coding region of each DNA fragment was used with human codon optimized DNA sequence. Synthesized DNA fragments consisted of EcoRI and NotI restriction enzyme sites overhang in both 5′ and 3′ ends. Marked regions were used for synthesis and production.

assay) and 11.3% (inter-assay) for common carp Lh ELISA (Hollander-Cohen et al., 2017), 5.9 (intra-assay) and 8.3% (inter-assay) for rainbow trout Lh RIA, and 4.6% (intra-assay) and 9.8% (inter-assay) for rainbow trout Fsh RIA (Govoroun et al., 1998). Essential to mention is that methodologies for quantifying pituitary Lh, and particularly Fsh, both at the mRNA and protein level are accessible for only a few teleost species. Protein assays to determine gonadotropin levels in fish traditionally have been based usually on native gonadotropins isolated from fish pituitaries (Suzuki et al., 1988 (chum salmon; RIA, both Fsh and Lh); Swanson et al., 1989 (coho salmon; RIA, both Fsh and Lh); Prat

lhb

DNA from medaka:

cagctgccttactgccagccagtcaagcagaagttgtctctgcagaaggagggctgctctggctgtcatacggtggaaaccactgtctgca
gtggccactgccttaccaaggacccccttgatgaagatacgatcaattcagtaccagaatgtgtgtacgtaccgggacttttactacaagacatt
tgagcttcctgactgcctgcctggcgtggatccgtcagtcacataccctgtggctctgagttgtcactgtggagcctgcatcatgaacgcgtct
gactgcacctttgagagcctgccaccagacttctgcgtgaaacatgattctttttattatcatcatcatcatcatcattaa

DNA codon optimized (produced DNA which has been used):

cagttgccatactgtcagccagttaagcagaagttgtccttgcagaaagagggttgttccggttgtcacactgttgagactactgtttgttccgg
acactgtttgactaaggacccattgatgaagatcagatccatccagtaccagaacgtttgtacttacagagacttctactacaagactttcgagt
tgcctgactgtttgccaggtgttgacccatctgttacttacccagttgctttgtcctgtcactgtggtgcttgtatcatgaacgcttccgactgtact
ttcgaatccttgcctccagacttctgtgttaagcacgactctttctactaccatcaccaccaccatcactaa

Protein:

QLPYCQPVKQKLSLQKEGCSGCHTVETTVCSGHCLTKDPLMKIRSIQYQNVCTYRDFYY
KTFELPDCLPGVDPSVTYPVALSCHCGACIMNASDCTFESLPPDFCVKHDSFYYHHHHH
H*

fshb

DNA from medaka:

tcctgtcatcccaaaaacgtcagcatccctgtggagagctgtggcatcagcgggtgcgtccacaccaccatatgcgaaggacggtgctacc
atgaggatcccaactacatcagctatgaagaccaccctaaagaaaagatctgcagtggggactggtcctacgaagttaaattcattgaggga
tgtccagtgggtttcaaatatcctgtggccaaaagctgcgagtgcactacatgcaacacaagaaccacatactgcggccgactttctgcaga
catgccgagctgtcatcatcatcatcatcattaa

DNA codon optimized (produced DNA which has been used):

Fig. 8. (*continued*)

et al., 1996 (rainbow trout; RIA, both Fsh and Lh); Shimizu et al., 2012 (mummichog; ELISA, both Fsh and Lh)). More recently recombinant gonadotropins have been utilized, making homologous assays more accessible for a variety of species (e.g. Aizen et al., 2007b (Nile tilapia; ELISA, both Fsh and Lh); Chauvigné et al., 2016 (Senegalese sole; ELISA, both Fsh and Lh); Yom-Din et al., 2016 (Russian sturgeon; ELISA, both Fsh and Lh); Hollander-Cohen et al., 2017 (common carp; ELISA, both Fsh and Lh)).

The competitive ELISAs for quantification of medaka Fsh and Lh were utilized to quantify and compare pituitary Fsh and Lh levels in juvenile and adult medaka males. Both Fsh and Lh showed a highly significant increase from juvenile to adult fish. Regarding the corresponding gene expression levels, pituitary *lhb* expression also showed a significant increase from juvenile (group 1) to adult fish (group 6). In addition, we found a significant increase in *lhb* expression as a function of body length, which gave better correlation than age or body weight. For Lh protein, on the other hand, we found the best data correlation when using body weight (and not age or body length). In contrast, we found no significant difference for pituitary *fshb* expression levels between the body length groups. A linear regression analysis revealed no significant relation between *fshb* expression and body length, proposing that body length has no influence on the *fshb* expression. Studies in rainbow trout demonstrated that changes in *fshb* subunit transcript levels are well correlated with changes in Fsh

tcctgtcacccaaagaacgtttccatcccagttgagtcctgtggtatctctggttgtgttcacactactatctgtgagggtagatgttaccacgag
gacccaaactacatttcctacgaggaccacccaaaagaaaagatctgttctggtgactggtcttacgaggttaagttcatcgagggttgtcca
gttggtttcaagtacccagttgctaagtcctgtgagtgtactacttgtaacactagaactacttactgtggtagattgtccgctgacatgccatctt
gtcatcatcaccatcaccactaa

Protein:

SCHPKNVSIPVESCGISGCVHTTICEGRCYHEDPNYISYEDHPKEKICSGDWSYEVKFIEG
CPVGFKYPVAKSCECTTCNTRTTYCGRLSADMPSCHHHHHH*

lhba

DNA from medaka:

cagctgccttactgccagccagtcaagcagaagttgtctctgcagaaggagggctgctctggctgtcatacggtggaaaccactgtctgca
gtggccactgccttaccaaggacccccttgatgaagatacgatcaattcagtaccagaatgtgtgtacgtaccgggacttttactacaagacatt
tgagcttcctgactgcctgcctggcgtggatccgtcagtcacatacccctgtggctctgagttgtcactgtggagcctgcatcatgaacgcgtct
gactgcacctttgagagcctgccaccagacttctgcgtgaaacatgattctttttattatcatcatcatcatcatcatggctcgggttctggttctta
ctccaatctggcttcctcaaacttggactgcatggaatgccggctggagaaaaacagcatattctcaagggaaggtaaaccggtctaccagt
gcataggatgttgcttctccagagcgtacccaacaccactgcgagctatgcagaccatgacagttccaaagaacatcacttcagaggcaact
tgctgtgttgcaaagcacagccacgagttcctctttcagacaatcgaacataccatcccagtgagaaaccatacggagtgtcactgcagcac
ctgctattatcacaagatgtaa

DNA codon optimized (produced DNA which has been used):

cagttgccatactgtcagccagttaagcagaagttgtccttgcagaaagagggttgttccggttgtcacactgttgagactactgtttgttccgg
acactgtttgactaaggacccattgatgaagatcagatccatccagtaccagaacgtttgtacttacagagacttctactacaagactttcgagt
tgcctgactgtttgccaggtgttgacccatctgttacttacccagttgctttgtcctgtcactgtggtgcttgtatcatgaacgcttccgactgtact
ttcgaatccttgcctccagacttctgtgttaagcacgactctttctactaccatcaccaccaccatcacggttctggatctggttcttactctaactt
ggcttcctccaacttggactgtatggaatgtagattggagaagaactccatcttctccagagagggtaagccagtttaccagtgtatcggttgtt
gtttctctagagcttacccaactccattgagagctatgcagactatgactgttccaaagaacatcacttccgaggctacttgttgtgttgctaagc
actctcacgagttcttgttccagactatcgagcacactatcccagttagaaaccacactgagtgtcactgttccacttgttactaccacaagatgt
aa

Protein:

Fig. 8. (*continued*)

pituitary content (Gomez et al., 1999), which couldn't be shown in the present study for medaka males. Interestingly, higher Fsh than Lh levels were detected in this work at both mRNA expression levels and at the protein levels for male medaka. In contrast, Lh protein levels for female tilapia (7.16 µg Lh/pituitary) were shown to be higher than Fsh levels (0.45 µg Fsh/pituitary) (Aizen et al., 2007b). It should be mentioned that these data correspond to females during the reproductive phase, and they are likely to show seasonal fluctuations. In addition, *lhb* mRNA levels in the pituitary were observed to be higher than *fshb* expression for female common carp (Hollander-Cohen et al., 2017). Significant correlation was demonstrated between *lhb* and *fshb* mRNA levels, whereas no significant correlation was revealed between the pituitary protein content of the two gonadotropins for female common carp (Hollander-Cohen et al., 2017). For Russian sturgeon females, Lh

QLPYCQPVKQKLSLQKEGCSGCHTVETTVCSGHCLTKDPLMKIRSIQYQNVCTYRDFYY
KTFELPDCLPGVDPSVTYPVALSCHCGACIMNASDCTFESLPPDFCVKHDSFYYHHHHH
HGSGSGSYSNLASSNLDCMECRLEKNSIFSREGKPVYQCIGCCFSRAYPTPLRAMQTMT
VPKNITSEATCCVAKHSHEFLFQTIEHTIPVRNHTECHCSTCYYHKM*

fshba

DNA from medaka:

tcctgtcatcccaaaaacgtcagcatccctgtggagagctgtggcatcagcgggtgcgtccacaccaccatatgcgaaggacggtgctacc
atgaggatcccaactacatcagctatgaagaccaccctaaagaaaagatctgcagtggggactggtcctacgaagttaaattcattgaggga
tgtccagtgggtttcaaatatcctgtggccaaaagctgcgagtgcactacatgcaacacaagaaccacatactgcggccgactttctgcaga
catgccgagctgtcatcatcatcatcatcatggctcgggttctggttcttactccaatctggcttcctcaaacttggactgcatggaatgccggct
ggagaaaaacagcatattctcaagggaaggtaaaccggtctaccagtgcataggatgttgcttctccagagcgtacccaacaccactgcga
gctatgcagaccatgacagttccaaagaacatcacttcagaggcaacttgctgtgttgcaaagcacagccacgagttcctctttcagacaatc
gaacataccatcccagtgagaaaccatacggagtgtcactgcagcacctgctattatcacaagatgtaa

DNA codon optimized (produced DNA which has been used):

tcctgtcacccaaagaacgtttccatcccagttgagtcctgtggtatctctggttgtgttcacactactatctgtgagggtagatgttaccacgag
gacccaaactacatttcctacgaggaccacccaaaagaaaagatctgttctggtgactggtcttacgaggttaagttcatcgagggttgtcca
gttggtttcaagtacccagttgctaagtcctgtgagtgtactacttgtaacactagaactacttactgtggtagattgtccgctgacatgccatctt
gtcatcatcatcaccatcacggttctggttccggttcttactctaacttggcttcctccaacttggactgtatggaatgtagattggagaagaact
ccatcttctccagagagggtaagccagtttaccagtgtatcggttgttgtttctctagagcttacccaactccattgagagctatgcagactatga
ctgttccaaagaacatcacttccgaggctacttgttgtgttgctaagcactctcacgagttcttgttccagactatcgagcacactatcccagtta
gaaaccacactgagtgtcactgttccacttgttactaccacaagatgtaa

Protein:

SCHPKNVSIPVESCGISGCVHTTICEGRCYHEDPNYISYEDHPKEKICSGDWSYEVKFIEG
CPVGFKYPVAKSCECTTCNTRTTYCGRLSADMPSCHHHHHHGSGSGSYSNLASSNLDC
MECRLEKNSIFSREGKPVYQCIGCCFSRAYPTPLRAMQTMTVPKNITSEATCCVAKHSHE
FLFQTIEHTIPVRNHTECHCSTCYYHKM*

Fig. 8. (*continued*)

protein levels (63 mg Lh/pituitary) were detected to be higher than Fsh levels (18 mg Fsh/pituitary) as well. Studies investigating pituitary Fsh and Lh protein levels in male fish during development are quite limited until today, therefore the established ELISA shown in this study represents a valuable tool for future studies.

Regarding testis histology, we show that medaka with body length above 19 mm, body weight above 130 mg, or age above 100 days, are fully mature. It's important to note that for future studies even smaller specimens (SL < 14 mm) should be included because preliminary results in this work did not agree with the final results, as fully mature specimens were found down to an SL of 15.5 mm. The fact that some fish were immature and some others mature at the same size (14– < 19 mm) is probably due to different densities in fish tanks and therefore different availability of food; biological variation could be a possible factor as well. Fish often live in variable environments in which conditions such as food availability change profoundly and are significantly correlated with growth, metabolism and (or) behavioral traits (Killen et al., 2011; Priyadarshana et al., 2006; Auer et al., 2016). For triploid carp, it was shown that food availability significantly influences physiological, behavioral and ecological processes in these fish by changing the trade-off between metabolism and growth (Liu and Fu, 2017). Furthermore, Liu and Fu (2017) declared that the relations between metabolism, behavior and growth might be species specific. Typically, fish show high food consumption and therefore faster growth

rates when food availability is high (Dibattista et al., 2006; Auer et al., 2015).

In summary, the herein presented development and validation of homologous competitive ELISAs for medaka Fsh and Lh, thus enabling quantification of biologically more relevant protein levels, will significantly improve the value of future studies of gonadotropin physiology in this important fish model.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.006.

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Ib

Establishment of specific enzyme-linked immunosorbent assay (ELISA) for measuring Fsh and Lh levels in medaka (*Oryzias latipes*), using recombinant gonadotropins

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Abstract

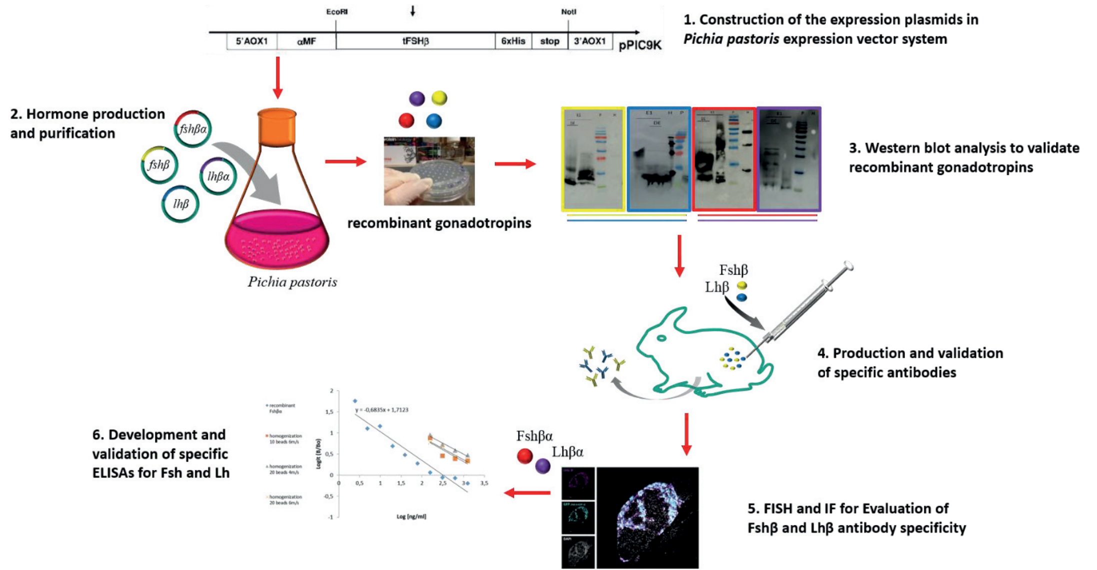
The paucity of information on understanding the regulatory mechanisms that are involved in the control of piscine Fsh and Lh synthesis, secretion, and function, prompted the present work. Part of the problem is related to the molecular heterogeneity and the unavailability of Fsh and Lh assays for quantifying gonadotropins, in particular assays regarding the measurement of Fsh, and such assays are available today for only a few teleost species. The present study reports the development and validation of competitive ELISAs for quantitative determination of medaka Fsh and Lh by first producing medaka recombinant (md) gonadotropins mdFshβ, mdLhβ, mdFshβα, and mdLhβα by *Pichia pastoris,* generating specific antibodies against their respective β subunits, and their use within the development of ELISAs.

The advantages of this protocol include:

- The reproducibility of the ELISA demonstrated was relatively high, as shown by reasonably low intra- (Fsh 2.7 %, Lh 3 %) and interassay CVs (Fsh 5.3 %, Lh 5.7 %).
- The high degree of parallelism between serial dilutions of the recombinant and native pituitary-derived Fsh and Lh, may be a sign of similar structures and immunologically similarity.
- Two new competitive ELISAs for the quantification of medaka Fsh and Lh were established for the first time.

Keywords: Competitive enzyme-linked immunosorbent assay; Follicle-stimulating hormone; Gonadotropin quantification; Luteinizing hormone; *Oryzias latipes*; Specific antibodies

Graphical Abstract



Specifications table

Subject Area	Biochemistry, Genetics and Molecular Biology
More specific subject area	Quantification of gonadotropin levels in medaka
Method name	Specific enzyme-linked immunosorbent assay (ELISA) for determining Fsh and Lh levels in medaka
Name and reference of original method	Mañanós, E.L., Swanson, P., Stubblefield, J., Zohar, Y., Purification of gonadotropin II from a teleost fish, the hybrid striped bass, and development of a specific enzyme-linked immunosorbent assay, Gen. Comp. Endocrinol. 108 (1997) 209-222. [1]
Resource availability	The information about all reagents, hardware and software is included in the present article.

Method details

Background

The gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play essential roles within the brain-pituitary-gonadal (BPG) axis by controlling gonad development and maturation in all vertebrates [2]. Detailed knowledge of function and regulation of these hormones is still limited. The accepted model in fish suggests that Fsh is important for early stage of gametogenesis, spermatogonial proliferation, and vitellogenesis in female, whereas Lh is mostly involved in processes leading to final gametogenesis, oocyte maturation and ovulation in females and spermiogenesis and spermiation in males [3,4]. When studying gonadotropin regulation and function, an important tool is the quantification of hormone levels in blood and pituitary by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). ELISA and RIA assays to determine gonadotropin levels in fish traditionally have been based on native gonadotropins from fish pituitaries and their specific antibodies. The purification of native gonadotropins has been shown challenging, and recombinant gonadotropins are now being used to substitute native hormones. They have various advantages compared to natively purified hormones because they can be continually produced, and potential cross-contamination with other related glycoproteins is minimized [4]. Based on the abovementioned considerations, the objective of this study was to develop and validate homologous competitive ELISAs for medaka (*Oryzias latipes*) Fsh and Lh using recombinant gonadotropins, thus enabling quantification of biologically more relevant protein levels, which will considerably advance the value of future studies of gonadotropin physiology in this important fish model.

Animals

Japanese medaka (*Oryzias latipes*) of the dr-R strain were kept and bred in our fish facility in re-circulating systems under a photoperiod of L14:D10 and water temperature of 28 ± 1 °C. Embryos were incubated in embryo culture medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ (all Sigma-Aldrich, St. Louis, U.S.A.)), and kept at 26 °C until hatching and transfer to system tanks. The fish were fed three meals per day with a combination of dry feed and live brine shrimp nauplii larvae (*Artemia salina*). All fish were raised under the same conditions regarding to temperature, photoperiod, food, tank size, and density. Handling, husbandry and all

experimental procedures of fish were in compliance with the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences. In addition, the work described has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments and Uniform Requirements for manuscripts submitted to Biomedical journals, and informed consent was obtained for experimentation with animal subjects.

Production and purification of recombinant gonadotropins mdFshβ, mdLhβ, mdFshβα, and mdLhβα

Using the methylotrophic yeast *P. pastoris* expression system recombinant proteins were produced generally according to Kasuto and Levavi-Sivan [5] and Yom-Din et al. [6]. Synthesis of genes for medaka *fshb* (Accession Number NM_001309017.1), *lhb* (Accession Number AB541982.1), *fshba*, and *lhba* (*gpa*; Accession Number NM_001122906) was outsourced to GenScript, New Jersey, U.S.A. (see sequences in Burow et al. [7]). Gene expression constructs were produced (see Figure 1 in Burow et al. [7]) and gene expression cassettes were generated with *P. pastoris* codon optimized DNA sequence. The synthetic genes were joined to form a fusion gene that encodes a "tethered" polypeptide (mdFshβα, mdLhβα) in which one of the β chains forms the N-terminal domain and the α chain forms the C-terminal domain. A "linker" sequence containing six amino acids (three Gly-Ser pairs) [8] was positioned between the β and α chains to assist in chimerization of the subunits. The Gly-Ser linker sequence is minimally hydrophobic and can thus maximize the possibility for chimerized subunits folding into their native conformational structure enabling independent interaction with their receptors [5]. A six-His tail was placed at the end of the β subunit enabling purification of the recombinant protein. Synthesized DNA fragments were cloned into pPIC9K vector using EcoRI and NotI restrictions sites and confirmed by sequencing (Weizmann Institute, Rehovot, Israel). The pPIC9K plasmid contained the yeast α mating factor (αMF) secretion signal that directs the recombinant protein into the secretory pathway. The constructs were digested with SalI and used to transform *P. pastoris* strain GS115 (Invitrogen, Carlsbad, U.S.A.) by electroporation (GenePulser, Bio-Rad, Hercules, U.S.A.). This resulted in insertion of the construct at the *AOX1* locus of *P. pastoris*, generating a His^{+} Mut^{S} (slow methanol utilization) phenotype. Transformants were selected for the His^{+} phenotype on 2% agar containing regeneration dextrose-biotin medium (1 M sorbitol, 1.34 % yeast nitrogen base without amino

acids, 4 x 10^{-5} % biotin, and 0.005 % of L-glutamic acid, L-methionine, and L-leucine (all from Sigma-Aldrich), and 0.005 % of L-lysine, and L-isoleucine (all from Biological Industries, Kibbutz Beit-Haemek, Israel)). An additional selection for antibiotic geneticin G418 (Gibco-BRL, Carlsbad, U.S.A.) resistance was performed on 2% agar containing 1% yeast extract (Becton, Dickinson and Company, Franklin Lakes, U.S.A.), 2% peptone (Becton, Dickinson and Company), 2% dextrose medium (Sigma-Aldrich) and the antibiotic G418 (1 mg/ml) (Gibco-BRL). Ten His^+ Mut^S clones from each construct were cultivated with shaking for 24h (growth phase) in 1.5 ml of buffered minimal glycerol (BMG) (1.34 % yeast nitrogen base without amino acids, 1 % glycerol, 4 x 10^{-5} % biotin, and 100 mM potassium phosphate buffer (all from Sigma-Aldrich) , pH 6) at 28 °C. Afterwards, cells were harvested (1500 *g*, 5 min), resuspended and cultivated for 3 days (induction phase) in 1 ml of buffered minimal methanol (BMM) (BMG, supplemented with 0.5 % methanol every 24 h). The protein was expressed in a shaker flask and harvested at 72 h after induction by methanol. Recombinant medaka Fshβ (mdFshβ), medaka Lhβ (mdLhβ), medaka Fshβα (mdFshβα), and medaka Lhβα (mdLhβα) were purified using nickel nitrilotriacetic acid agarose (Ni-NTA) (Qiagen, Hilden, Germany) according to the method described by Kasuto and Levavi-Sivan [5] and Yom-Din et al. [6] with certain modifications. Briefly, the pH of the supernatant was adjusted to 8.0 with 5 N sodium hydroxide (Sigma-Aldrich). Beads (QIAexpressionist, Qiagen) were supplemented to the medium and stirred on magnetic stirrer for 18 h at 4 °C. The purification was performed by batch purification of the his-tagged proteins by washing with 5 mM imidazole (Sigma-Aldrich) that prevents binding of unspecific proteins to the beads. The bound protein was eluted with PBS pH 4.5 containing 250 mM imidazole. The eluted fractions of mdFshβ, mdLhβ, mdFshβα, and mdLhβα were dialyzed with Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, Waltham, U.S.A) according to the manufacturer's protocols against PBS pH 7.5, meaning unwanted compounds were removed by selective and passive diffusion through a semi-permeable membrane. The purified proteins, tagged with a six-His tail, were detected on a Western blot as described below.

Production and validation of specific antibodies for mdFshβ and mdLhβ

Polyclonal antisera against recombinant mdFshβ and mdLhβ were generated generally following a procedure described by Aizen et al. [9]. Two different rabbits for each protein received three intradermal injections of purified protein (mdFshβ; 1 mg first injection, 0.5 mg second and third

injection; mdLhβ; 0.7 mg first injection, 0.4 mg second and third injection) in 0,9 % NaCl and emulsified in an equal volume of complete Freund´s adjuvant (Sigma-Aldrich) at 3-week intervals. Two weeks after the final injection, the rabbits were bled, and the serum was aliquoted and lyophilized. To validate the produced antibodies, the recombinant proteins and medaka pituitary extract were visualized using anti-mdFshβ, or anti-mdLhβ antisera. To ensure that the plasma of the rabbit before the final injections did not react with mdFshβ and mdLhβ, a Western blot using medaka pre-immune serum against medaka pituitary extract, mdFshβ, and mdLhβ was conducted as described below.

Western blot Analysis

From culture supernatants, reduced and non-reduced samples were resolved by SDS-PAGE for Western blot analysis. Recombinant mdFshβ, mdLhβ, mdFshβα, and mdLhβα were visualized using anti-His (diluted 1:2000) (QIAexpress anti-His antibodies; Qiagen), generally according to Yom-Din et al. [6], and validated by molecular weight. To validate the produced antibodies, the recombinant proteins and medaka pituitary extract were visualized using anti-mdFshβ, or anti-mdLhβ (both diluted 1:2000, 1:100000, 1:600000) antisera.

To confirm that the plasma of the rabbit before the final injections did not react with mdFshβ and mdLhβ, a Western blot using medaka pre-immune serum as a negative control against medaka pituitary extract, mdFshβ, and mdLhβ was performed. Precisely, reduced samples from culture supernatants were electrophoresed on 15 % SDS-polyacrylamide running gels with a 5 % stacking gel. The gels were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassell, Germany) and blocked with 3% BSA in Tris Buffered Saline (TBS) buffer (20 mM Tris, 150 mM NaCl) to prevent nonspecific binding of the antibodies during subsequent steps. The membranes were incubated in PBS containing 1 % nonfat milk with the antisera (dilutions 1:2000, 1:100000, 1:600000) for 1 h at room temperature (RT) and afterwards with goat anti rabbit horseradish peroxidase (GAR-HRP) conjugate (dilution 1:5000; Jackson Immuno Research Laboratories) for 1 h at RT. All membranes were washed and treated with enhanced chemiluminescence reagent (Chemiluminescence detection kit for HRP, Biological Industries) to reveal immunoreactive bands. For glycosylation analysis, prior to Western blot analysis, *N*-glycosidase F (PNGase F) was used to produce deglycosylated proteins by hydrolyzing all types of *N*-glycan chains. According to supplier recommendations (Roche Applied Science, Mannheim, Germany), 100 ng of reduced

and denatured mdFshβ, mdLhβ, mdFshβα, mdLhβα, and medaka pituitary extract were incubated for 2 h at 37 °C in the presence or absence of PNGase F.

Fluorescence *in situ* hybridization (FISH)

To further evaluate the antibody specificity of mdLhβ and mdFshβ, fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) were conducted. FISH was performed on free-floating parasagittal brain-pituitary sections, generally according to Fontaine et al. [10]. Briefly, after being sacrificed with ice water, brain and pituitary from 12 unsexed 6 month-old adult fish were dissected and fixated overnight with 4% PFA at 4°C. Tissues were then gradually dehydrated with a series of increasing concentrations of ethanol and stored in 100% methanol until used. Tissues were rehydrated, embedded in 3% agarose, para-sagittally sectioned (60 μm sections) using a vibratome (VT1000S Leica, Wetzlar, Germany), and then treated with proteinase K (1 μg/ml; P6556, Sigma-Aldrich) for 30 min. *fshb* riboprobe was cloned using AGAGCAGAGGAAGCAACACT and GGGGCACAGTTTCTTTATTTCAG as primers, and synthesized using PGEM-T vector (Promega, Madison, WI), whereas we used the *lhb* riboprobe previously described [11]. *fshb* and *lhb* sense and antisense riboprobes were conjugated with digoxigenin (DIG; 11277073910; Roche, Basel, Switzerland) using SP6 or T7 RNA polymerase (Promega). Tissues were hybridized with either sense or antisense riboprobes for 18 h at 65°C and then incubated with sheep anti-DIG conjugated with peroxidase (POD; 1:500; 11207733910; Roche) over night. Signal was revealed using TAMRA-conjugated tyramide constructed in our lab.

Immunofluorescence (IF)

IF staining was conducted on free-floating sections according to Fontaine et al. [10] using anti-Lhβ and anti-Fshβ (described earlier). For anti-Lhβ, the tissues labelled for *lhb* mRNA by FISH were used (see above). For anti-Fshβ, IF could not be carried out after *in situ* labelling due to an antigen retrieval treatment that was necessary before the IF, destroying the labeling of the FISH. Thus, IF was performed on consecutive parasagittal sections of the one used for the *fshb* FISH. Tissue sections were treated with 2N Hydrochloric acid (HCl) for 1 h at 37°C and then incubated with primary antibody (anti-Lhβ; 1:2000, anti-Fshβ; 1:1000) overnight at 4°C. A secondary goat anti-rabbit antibody coupled to AlexaFluor 488 (A-11034, Thermo Fisher Scientific, Waltham,

U.S.A.) at a concentration of 1:1000 was used for 4 h incubation. Control experiment without the primary antibody was included. Tissues for anti-Lhβ were then treated for nuclei staining with DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride; 32670 Sigma) by incubation at RT for 20 min at a titer of 1:1000 and rinsing.

Imaging

Stained tissues were mounted between slide and coverslip with the mounting medium for fluorescence Vectashield H-1000 (Vector, California, U.S.A.). Images were obtained using a Zeiss LSM710 confocal microscope with 25× (LCI Plan-Neofluar 25x/0.8 NA) objective. Channels were acquired sequentially in order to avoid signal crossover between the different filters. Images were processed using the ZEN software (Carl Zeiss AG, Oberkochen, Germany). Z-projections from confocal stacks of images were acquired using Image J software (http://rsbweb.nih.gov/ij/). Composites were assembled using Adobe Photoshop and Illustrator CS6 (Adobe Systems, San Francisco, California).

Gonadal histology

Histological analysis of testes was performed to assess the maturational stages of fish sampled for developmental series by identifying the germ cell stages (see Table 3 in Burow et al. [7]). Testes from 36 males, grouped according to standard body length (SL), were dissected and transferred to phosphate buffered saline (PBS; Sigma-Aldrich) prior to overnight fixation in ice-cold 4% glutaraldehyde phosphate buffered solution at 4 ºC. Note that fish from the smaller size groups had testicular tissue of such low volumes that, in fear of losing it later in the fixation process, it was not dissected out of the body cavity. Rather, all other organs were removed from the abdomen, and body and gonads fixated as a whole. Glutaraldehyde fixative solution was prepared fresh by mixing 28 ml 0.2 M NaH_2PO_4-H_2O with 72 ml 0.2 M Na_2HPO_4-$2H_2O$ (both from Merck Millipore, Billerica, MA, USA), adjusting the mixture to pH 7.2, and then adding 68 ml mq-H_2O and 32 ml of 25 % glutaraldehyde (Merck Millipore). Tissues were dehydrated in a series of increasing concentrations of EtOH (70-100%) at RT, each step lasting at least 30 min. The last step (100%) was repeated trice and replaced with approx. 5 ml of preparation solution (100 ml Technovit 7100 added 1 g of Hardener I (Heraeus Kulzer, Hanau, Germany)) and kept at slow shaking overnight.

All fixation steps were performed in small glass bottles. After infiltration, tissue samples were embedded in cold Histoform S (Heraeus Kulzer) added approx. 1 ml preparation solution w/50 µl Hardener II (Heraeus Kulzer) and incubated at 37 ºC. Cured samples were mounted unto Histoblocs using Technovit 3040 (both from Heraeus Kulzer), before sagittal sections (3 µm) were prepared using a Leica RM2245 microtome (Leica Biosystems, Wetzlar, Germany). Sections were collected from the periphery until the middle of the testes tissue every 30 µm and placed unto microscope slides. Dried sections were stained with Toluidine Blue O (Sigma-Aldrich) and mounted with Coverquick 4000 (VWR International, Radnor, PA, USA) before histological analysis. Germ cells were determined according to Schulz et al. (2010), within the five main germ cell stages (spermatogonia typa A (SPA), spermatogonia type B (SPB), spermatocytes (SC), spermatids (ST) and spermatozoa (SZ)), no further distinctions were made (see Table 2 in Burow et al. [7]).

Development and validation of specific ELISA for Fsh and Lh, and profile of pituitary levels of Fsh and Lh in male medaka

The recombinant gonadotropins were used to develop specific and homologous competitive ELISAs for determination of mdFsh and mdLh in the pituitary, generally according to Mañanós et al. [1]. Competitive ELISAs were developed using specific β-subunit polyclonal rabbit primary antibodies against mdFshβ or mdLhβ (described earlier), recombinant β-subunits mdFshβ or mdLhβ (described previously) to coat the ELISA microplates, and recombinant mdFshβα or mdLhβα (described earlier) for the standard curves. Briefly, ELISA microtiter plates (Nunc-Immuno™ Plates; Nunc, Denmark) were coated with 100 µl/well of 10 ng/ml (1 ng/well) mdFshβ or 5 ng/ml (0.5 ng/well) mdLhβ. The following day, plates were washed with 200 µl/well phosphate buffered saline with Tween 20 (PBST) buffer (10 mM Na_2PO_4, 2 mM KH_2PO_4 (pH 7.4), 140 mM NaCl, 3 mM KCl, and 0.05% Tween 20 (all from Sigma-Aldrich)). To reduce background by blocking the unspecific binding sites, plates were blocked for 1 h with 200 µl/well of PBST buffer containing 1% bovine serum albumin (BSA, Sigma). Single pituitaries were dissected and kept on ice until homogenization (6 m/s, 3 x 30 sec using ceramic beads, cat.no 116933050, MP Biomedicals, California, U.S.A.) using FastPrep-24 (MP Biomedicals) diluted 1:2.7 with 0.1% BSA in PBST, in sufficient volume to allow technical duplicates (125 µl/well). The homogenate was centrifuged at 10,000*g* for 5 min. Samples and standards were first pre-

incubated overnight at RT with the primary antibodies (125 µl/well) (final dilution 1:10,000 for mdFshβ and 1:50,000 for mdLhβ in 0.1% BSA in PBST) in 96-well microtest plates (Sarstedt, Nümbrecht, Germany). After pre-incubation, each sample was distributed into the wells (100 µl/well) of the coated microtiter plates and incubated for 3 h at RT. After incubation, the plates were washed with PBST. Formed antigen–antibody complexes were detected by addition of 100 µl/well of GAR-HRP (Bio-Rad) diluted 1:5000 in PBST-0.1% BSA buffer for 2 h at RT. The plates were washed again with PBST. The visualization of the presence of enzyme complexes was performed by addition of 100 µl/well of 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Zotal, Israel) diluted 1:4. The reaction was carried out in complete darkness at RT and stopped after approximately 15 min with TMB stop solution 1 M H_2SO_4 (50 µl/well). Absorbance was read at 450 nm, using a Microplate Spectrophotometer (Epoch 2, Biotek, Winooski, U.S.A.).

The ELISA was validated for medaka Lh and Fsh determinations in pituitary extract of medaka. Displacement curves for pituitary samples were achieved by serial dilutions of the sample in 0.1% BSA in PBST and comparison with the standard curve using recombinant mdFshβα or mdLhβα. For the parallelism analysis, pituitaries were collected from sexually mature male fish and homogenized using different conditions (10 ceramic beads, 6 m/s; 20 ceramic beads, 4 m/s; 20 ceramic beads, 6 m/s; all 3 x 30 sec). The homogenate was centrifuged at 10,000*g* for 5 min and the resulting supernatant was used as the pituitary extract. The sensitivity of the assay is defined as the lowest dose of Fsh or Lh capable of reducing the optical density more than the mean plus two standard deviations of the zero dose of Fsh or Lh [B_0 - 2SD]; it was calculated by adding the mean of the blank to two times the standard deviation of the blank. Intra-assay coefficient of variation (CV) was determined by assaying six replicates of one of the standard concentrations (1.56 ng/ml) on the same assay plate. Inter-assay CV was calculated by assaying the same sample five times in different plates.

Profile of pituitary levels of Fsh and Lh in male medaka comparing juveniles versus adults: A profile of Fsh and Lh in male medaka pituitaries was conducted using the ELISA method described above. To achieve a profile for Fsh, pituitaries from 24 juvenile males with SL between 12 mm and 16.5 mm, and of 24 adult males between 21 mm and 25.5 mm were dissected. For the profile of Lh pituitaries from 12 juvenile males with SL between 12 mm and 16 mm, and of 12

adult males between 22.5 mm and 26.5 mm were dissected (for both Fsh and Lh 1 pituitary in 40 µl 0.1% BSA in PBST per biological replicate was used). The distinction between juvenile and adult was based on unpublished results relating medaka testicular maturation stage to SL [7], and showing that males with SL below 16 mm were completely immature and males with SL above 20 mm to be fully mature.

Statistical analysis

Data are presented as mean ± SEM. All data were tested for normal distribution (Shapiro-Wilk normality test). For sample groups, which did not follow a normal distribution, the data were first log-transformed. For ELISA data calculations, sigmoid curves were linearized using logit transformation. Correlations were calculated by Graph-Pad Prism software (version 7; GraphPad, San Diego, U.S.A.). Significance level was set to 0.05.

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Data Article

Data on Western blot and ELISA analysis of medaka (*Oryzias latipes*) follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) using recombinant proteins expressed with *Pichia pastoris*

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A R T I C L E I N F O

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A B S T R A C T

The gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play essential roles in vertebrate reproduction. This article presents data on molecular weight validation of recombinant medaka (*Oryzias latipes*) (md) gonadotropins Fshβ (mdFshβ), Lhβ (mdLhβ), Fshβα (mdFshβα), and Lhβα (mdLhβα) generated by *Pichia pastoris*, as well as data on a validation of produced antibodies against Fshβ and Lhβ by Western blot analysis. Furthermore, the article includes data on Fsh and Lh protein levels in male medaka pituitaries using recombinant mdFshβα and mdLhβα within enzyme-linked immunosorbent assays (ELISAs), in which protein amounts were analyzed related to body weight and age of the fish. This dataset is associated with the research article entitled “Medaka Follicle-stimulating hormone (Fsh) and

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Luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression” (Burow et al., in press).
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Specifications table

Subject area	Biology
More specific subject area	Physiology, Neuroendocrinology
Type of data	Image (Western blot analysis), Graph (ELISA)
How data was acquired	Data for validation of recombinant proteins and antibodies were acquired through Western blot, data for protein levels were obtained through ELISA using microplate spectrophotometer.
Data format	Analyzed
Experimental factors	Prior to Western blot analysis, *N*-glycosidase F was used to produce deglycosylated proteins by hydrolyzing all types of *N*-glycan chains.
Experimental features	Validation of medaka recombinant proteins and antibodies, and generation of pituitary Fsh and Lh levels in male medaka was performed.
Data source location	Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 0454 Oslo, Norway.
Data accessibility	Data are presented in this article.
Related research article	Burow, S., Fontaine, R., von Krogh, K., Mayer, I., Nourizadeh-Lillabadi, R., Hollander-Cohen, L., Cohen, Y., Shpilman, M., Levavi-Sivan, B., Weltzien, F.A., Medaka Follicle-stimulating hormone (Fsh) and Luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression levels, Gen. Comp. Endocrinol. (in press) [1].

Value of the data

- The establishment of competitive ELISAs using recombinant medaka gonadotropins to quantify the content of Fsh and Lh, for the first time, extends the accessibility of quantitative methods for medaka and enables advanced functional studies on gonadotropin physiology in fish.
- The generated ELISA data determining pituitary Fsh and Lh protein levels in male fish during development in this article represent valuable data and a tool for future studies, since investigations in male fish during puberty are quite limited until today.
- The data on Fsh and Lh protein levels in male medaka pituitaries using recombinant mdFshβα and mdLhβα reveal that body weight explains the variance in the dependent variable (gonadotropin) better compared to age of the fish for Fshβ and Lhβ. In addition, body weight is indicated to explain the variance in the dependent variable for Lhβ better compared to Fshβ.
- The generation of specific antibodies against medaka Fshβ and Lhβ presented here will be a valuable tool for future experiments on gonadotropins in medaka, an important model organism in biology.

1. Data

The data on characterization of recombinant medaka (md) gonadotropins Fshβ (mdFshβ) (Fig. 1A), Lhβ (mdLhβ) (Fig. 1B), Fshβα (mdFshβα) (Fig. 1C), and Lhβα (mdLhβα) (Fig. 1D) by immunoreacting

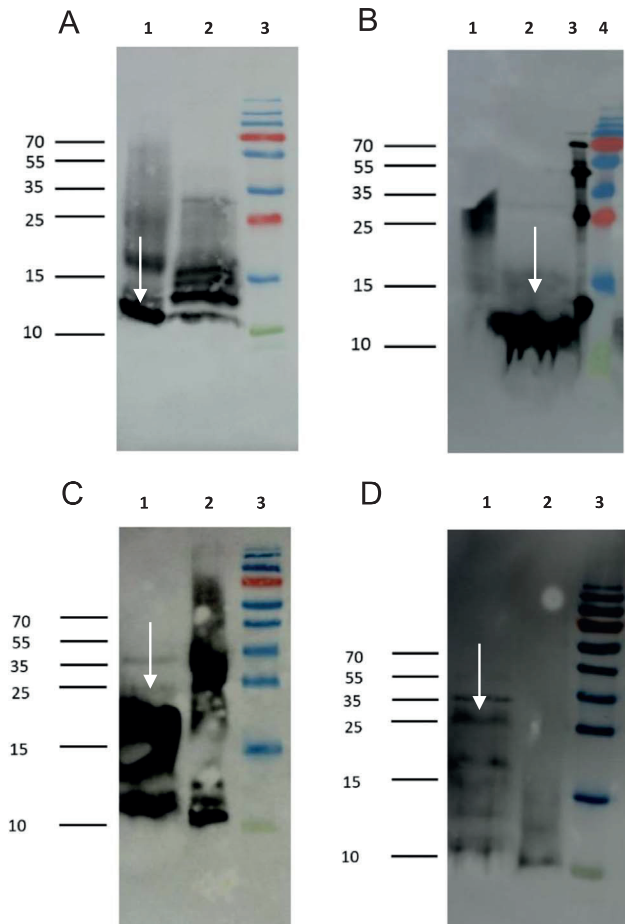

Fig. 1. Molecular weight validation of mdFshβ, mdLhβ, mdFshβα, and mdLhβα expressed with *P. pastoris* by Western blot analysis. Supernatants of transformed *P. pastoris* cultures were separated by SDS-PAGE and immunoreacted with antibodies against His. PageRuler Plus Prestained Protein Ladder lane 3 (A), lane 4 (B), lane 3 (C), lane 3 (D). His-tagged Protein Standard lane 3 (B). The Western blot confirmed the expected molecular weight of A) mdFshβ, Lane 2 represents mdFshβ; Lane 1 represents deglycosylated mdFshβ. B) mdLhβ, Lane 1 represents mdLhβ; Lane 2 represents deglycosylated mdLhβ C) mdFshβα, Lane 2 represents mdFshβα; Lane 1 represents deglycosylated mdFshβα D) mdLhβα, Lane 2 represents mdLhβα; Lane 1 represents deglycosylated mdLhβα. White arrows indicate protein bands after deglycosylation with PNGase F.

them against the His-tag demonstrated a clear validation since all recombinant proteins were successfully detected with His-tail antibodies, and their molecular sizes derived from Western blots were in accordance with the calculated estimates (according to sequence). Under reducing conditions, mdFshβ and mdFshβα were detected as bands of 14–16 kDa (Fig. 1A) and 25–30 kDa (Fig. 1C), respectively, and after deglycosylation with PNGase F as bands of 12–14 kDa (Fig. 1A) and 24–25 kDa (Fig. 1C), respectively. This is in accordance with the calculated molecular weight without glycosylation residues for mdFshβ (13 kDa) and for mdFshβα (25 kDa). Under reducing conditions, mdLhβ and mdLhβα had a molecular weight of 15 kDa (Fig. 1B) and 35 kDa (Fig. 1D), respectively, and after deglycosylation 12–14 kDa (Fig. 1B) and 27–28 kDa (Fig. 1D), respectively. Again, this was in accordance with the expectation for deglycosylated mdLhβ (15 kDa) and mdLhβα (28 kDa).

Western blot analysis of antibodies produced against medaka Fshβ and Lhβ revealed specificity and absence of cross-reactions as all recombinant proteins mdFshβ, mdLhβ, mdFshβα, and mdLhβα were detected exclusively with antibodies against either medaka Fshβ (Fig. 2A, B) or Lhβ (Fig. 2C, D). Under

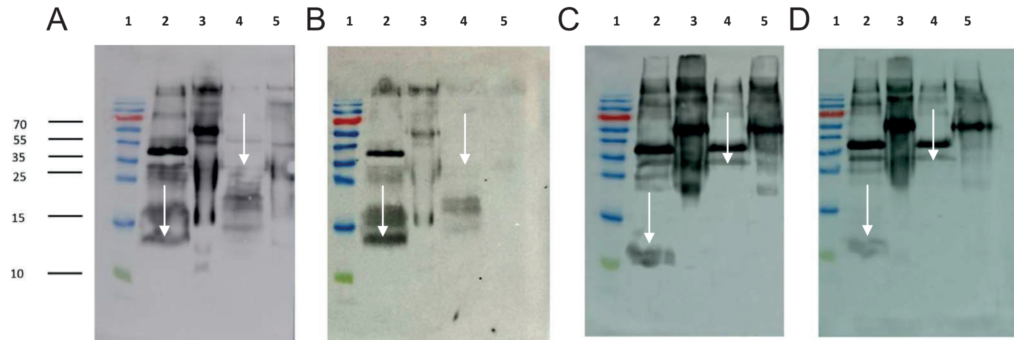

Fig. 2. Validation of recombinant proteins mdFshβ, mdLhβ, mdFshβα, and mdLhβα expressed with *P. pastoris* by Western blot analysis. Supernatants of transformed *P. pastoris* cultures were separated by SDS-PAGE and immunoreacted with antibodies against mdFshβ (2A, 2B) and mdLhβ (2C, 2D). First lane represents PageRuler Plus Prestained Protein Ladder. The Western blot confirmed that the antibodies detected the correct proteins, and verified the absence of cross-reactions. A and B) mdFshβ and mdFshβα, Antibody against mdFshβ, Dilution 1:100.000 (2A) and 1:600.000 (2B); Lane 3 represents mdFshβ, lane 5 represents mdFshβα; Lanes 2 and 4 represent deglycosylated samples of those shown in lanes 3 and 5 respectively. C and D) mdLhβ and mdLhβα, Antibody against mdLhβ, Dilution 1:100.000 (2C) and 1:600.000 (2D); Lane 3 represents mdLhβ, lane 5 represents mdLhβα; Lanes 2 and 4 represent deglycosylated samples of those shown in lanes 3 and 5 respectively. White arrows indicate protein bands after deglycosylation with PNGase F.

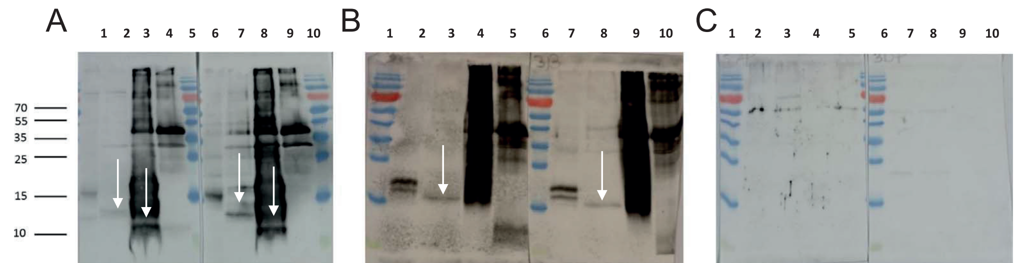

Fig. 3. Validation of the produced antibodies against mdFshβ and mdLhβ and characterization of medaka pituitary extract, mdFshβ, and mdLhβ expressed with *P. pastoris* by Western blot analysis. Supernatants of transformed *P. pastoris* cultures were separated by SDS-PAGE and immunoreacted with antibodies against mdFshβ (3A) and mdLhβ (3B) and with medaka pre-immune serum (3C). The Western blot confirmed that the antibodies detected proteins of the right size in medaka pituitaries. A) Antibody against mdFshβ, Dilution 1:2000; Lane 1 (Rabbit 1 (R1)) and 6 (Rabbit 2 (R2)) represent medaka pituitary extract, lane 2 (R1) and 7 (R2) represent medaka pituitary extract after deglycosylation; Lanes 3 (R1) and 8 (R2) represent mdFshβ after deglycosylation; Lanes 4 (R1) and 9 (R2) represent deglycosylated samples of mdLhβ. Lane 5 and 10 represent PageRuler Plus Prestained Protein Ladder. B) Antibody against mdLhβ, Dilution 1:2000; Lane 2 (R1) and 7 (R2) represent medaka pituitary extract, lane 3 (R1) and 8 (R2) represent medaka pituitary extract after deglycosylation; Lanes 4 (R1) and 9 (R2) represent mdLhβ after deglycosylation; Lanes 5 (R1) and 10 (R2) represent deglycosylated samples of mdFshβ. Lane 1 and 6 represent PageRuler Plus Prestained Protein Ladder. C) The Western blot confirmed the validation of the produced antibodies and verified that the plasma taken before the final injections did not react with mdFshβ and mdLhβ. Medaka pituitary extract, mdFshβ, and mdLhβ were immunoreacted against medaka pre-immune serum as a negative control (test bleeding). Negative control: Pre-immune serum of Rabbit 1 (3C, lane 1 to 5) and Rabbit 2 (3C, lane 6 to 10); Lane 2 (R1) and 7 (R2) represent medaka pituitary extract, lane 3 (R1) and 8 (R2) represent medaka pituitary extract after deglycosylation; Lanes 4 (R1) and 9 (R2) represent mdFshβ after deglycosylation; Lanes 5 (R1) and 10 (R2) represent deglycosylated samples of mdLhβ. Lane 1 and 6 represent PageRuler Plus Prestained Protein Ladder. White arrows indicate protein bands after deglycosylation with PNGase F.

reducing conditions and after deglycosylation, mdFshβ and mdFshβα were determined as bands of 12–13 kDa and 23–25 kDa (Fig. 2A, B), respectively. mdLhβ was revealed after deglycosylation very weakly as a band of 12–13 kDa, and mdLhβα was observed as a band of 27–29 kDa (Fig. 2C, D).

When using the antibodies on medaka pituitary extracts, native mdFshβ (Fig. 3A) and mdLhβ (Fig. 3B) could be detected. Using the mdFshβ antibody, bands of approximately 13 kDa were revealed for mdFshβ (Fig. 3A). When using the mdLhβ antibody, there was no clean band for mdLhβ due to very strong signals (Fig. 3B). No bands were revealed for mdLhβ with the mdFshβ antibody (Fig. 3A) and no

bands for mdFshβ using the mdLhβ antibody (Fig. 3B). When medaka pituitary extract, recombinant mdFshβ, or recombinant mdLhβ were immunoreacted against rabbit pre-immune serum as a negative control (test bleeding), there was no specific band observed (Fig. 3C).

Furthermore, this article provides data on Fsh and Lh protein levels in pituitaries from juvenile and adult male medaka that were obtained by enzyme-linked immunosorbent assay (ELISA). The data have been analyzed as a function of body weight (Fsh Fig. 4A, Lh Fig. 4C) and age of the fish (Fsh Fig. 4B, Lh Fig. 4D). Body weight (R^2 = 0,3276; Fig. 4A) explains the variance in the dependent variable (gonadotropin) better compared to age of the fish (R^2 = 0,2499; Fig. 4B) or body length (protein levels in relation to body length has been shown in Burow et al. [1]) for Fshβ using a linear trendline. As for Fshβ, body weight (R^2 = 0,6221; Fig. 4C) explains the variance in the dependent variable better compared to age (R^2 = 0,524; Fig. 4D) for Lhβ using a power trendline. Notably, the R^2s are higher for Lhβ than for Fshβ, indicating that body weight explains the variance in the dependent variable for Lhβ better compared to Fshβ. Since none of the R^2 is close to 1, a correlation of Fsh/Lh levels to either body weight or age of the fish is not indicated.

2. Experimental design, materials and methods

2.1. Animals

Japanese medaka (*Oryzias latipes*) of the dr-R strain were kept in re-circulating systems with light-dark cycle of L14:D10 and water temperature of 28 ± 1 °C. Embryos were incubated in embryo culture medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ (all Sigma-Aldrich, St. Louis, U.S.A.)), and kept at 26 °C until hatching and transfer to system tanks. The fish were fed three times per day with a combination of dry feed and live brine shrimp nauplii larvae (*Artemia salina*). Fish were raised under the same conditions with regard to temperature, photoperiod, food, tank size, and density. Handling, husbandry and use of fish were according to the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences. The work of the present article has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments and Uniform Requirements for manuscripts submitted to Biomedical journals, and informed consent was obtained for experimentation with animal subjects.

2.2. Production and purification of recombinant gonadotropins mdFshβ, mdLhβ, mdFshβα, and mdLhβα, generation of specific antibodies for mdFshβ and mdLhβ, and Western blot analysis

Generation of recombinant proteins was conducted using the methylotrophic yeast *Pichia pastoris* (*P. pastoris*) expression system, generally according to Kasuto and Levavi-Sivan [2] and Yom-Din et al. [3], and described in detail in Burow et al. [1]. Synthesis of genes for medaka *fshb* (Accession Number NM_001309017.1), *lhb* (Accession Number AB541982.1), *fshba*, and *lhba* (*gpa*; Accession Number NM_001122906) was outsourced to GenScript, New Jersey, U.S.A. For each construct gene expression cassettes were generated with *P. pastoris* codon optimized DNA sequence. Polyclonal antisera against recombinant mdFshβ and mdLhβ were produced following a procedure according to Aizen et al. [4], which is reported in detail in Burow et al. [1].

For molecular weight validation, the purified recombinant proteins were analyzed by Western blot analysis using anti-His (diluted 1:2000), generally according to Yom-Din et al. [3]. To validate the produced antibodies, the recombinant proteins and medaka pituitary extract were visualized using anti-mdFshβ, or anti-mdLhβ (both diluted 1:2000, 1:100000, 1:600000) antisera. To confirm that the plasma of the rabbit before the final injections did not react with mdFshβ and mdLhβ, a Western blot using medaka pre-immune serum as a negative control against medaka pituitary extract, mdFshβ, and mdLhβ was performed.

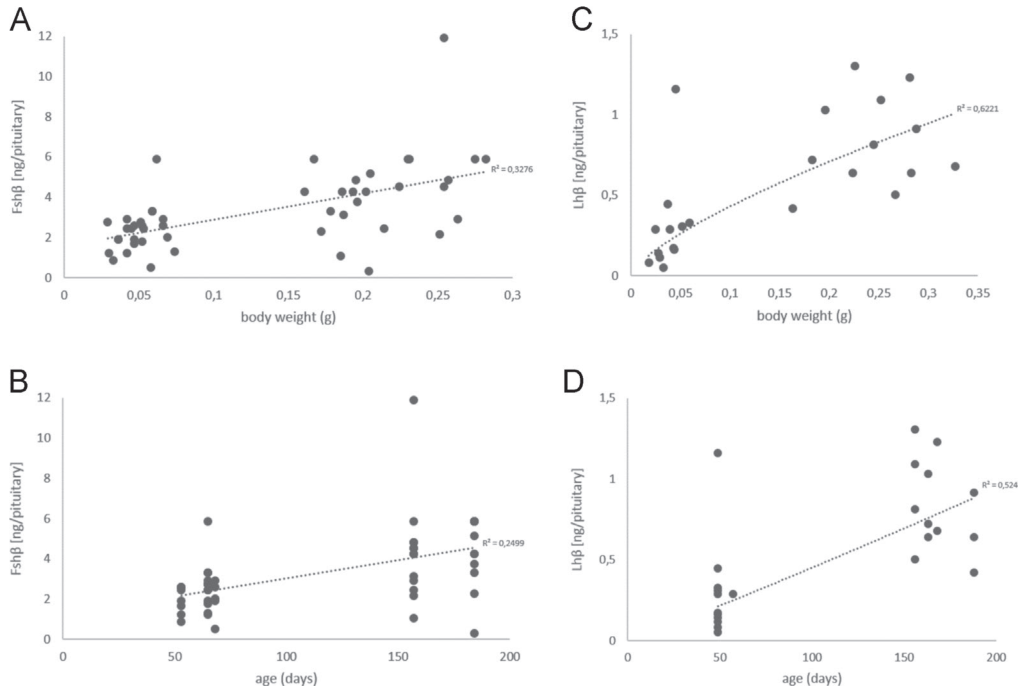

Fig. 4. Profile of Fsh and Lh protein levels in pituitaries from juvenile and adult male medaka as a functional study obtained by ELISA. Quantified amounts of Fshβ (Fig. 4A, B) and Lhβ (Fig. 4C, D) (in ng/pituitary). Data have been analyzed as a function of body weight (Fsh Fig. 4A, linear trendline; Lh Fig. 4C, power trendline), and age of the fish (Fsh Fig. 4B, linear trendline; Lh Fig. 4D, power trendline). Body weight (R^2 = 0,3276; Fig. 4A) explains the variance in the dependent variable (gonadotropin) better compared to age of the fish (R^2 = 0,2499; Fig. 4B) for Fshβ using a linear trendline. As for Fshβ, body weight (R^2 = 0,6221; Fig. 4C) explains the variance in the dependent variable better compared to age (R^2 = 0,524; Fig. 4D) for Lhβ using a power trendline. Comparing the R^2 of body weight and age between the Fshβ and Lhβ profiles, it is important to note that the R^2s are higher for Lhβ than for Fshβ, indicating that body weight explains the variance in the dependent variable for Lhβ better compared to Fshβ.

2.3. Quantification of Fsh and Lh in male medaka pituitaries using ELISA

To quantify the content of Fsh and Lh protein levels in male medaka pituitaries, the ELISA methodology described in Burow et al. [1] was performed. For the profile of Fsh, pituitaries from 24 juvenile males with standard length (SL) between 12 mm and 16.5 mm, and of 24 adult males between 21 mm and 25.5 mm were used. Pituitaries from 12 juvenile males with SL between 12 mm and 16 mm, and of 12 adult males between 22.5 mm and 26.5 mm were dissected for the profile of Lh. For both Fsh and Lh 1 pituitary in 40 μl 0.1% BSA in PBST per biological replicate was used. Within the two groups juveniles and adults, body weight and age were measured, and protein amounts were analyzed related to body weight and age of the fish.

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Transparency document. Supporting information

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II

Pharmacological characterization of gonadotropin receptors (Fshr and Lhr) in medaka, *Oryzias latipes*

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Abstract

Reproduction in vertebrates in controlled by the brain-pituitary-gonad axis, where the gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play a vital part by activating their cognate receptors in the gonads. In this work we have investigated the follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhr) in Japanese medaka (*Oryzias latipes*), including their tissue distribution, gonad expression levels during male sexual maturation, and receptor activation comparing recombinant homologous ligands (mdFshβα, mdLhβα, tiFshβα, tiLhβα) and receptors (mdFshr, mdLhr, tiFshr, tiLhr) from medaka and Nile tilapia (*Oreochromis niloticus*) to evaluate receptor kinetics and promiscuity. Medaka receptors, *fshr* and *lhr*, were highly expressed in ovary and testis. High levels of expression was found for *lhr* also in brain, while *fshr* was expressed at low levels. In addition, trace amounts for *fshr* and *lhr* were revealed in gills, gallbladder, intestine, spleen, pituitary, eyes (low levels of *lhr*), and for *lhr* in heart. *fshr* was not expressed in liver or heart, and *lhr* expression was not expressed in liver. Both *fshr* and *lhr* mRNA levels increased significantly during testis development. The characterization of intra-species ligand selectivity and cross-reactivity by receptor activation experiments revealed that each gonadotropin activated its own cognate receptor. Cross-reactivity was detected to some extent since mdFshβα was also able to activate the mdLhr, and mdLhβα the mdFshr. Furthermore, medaka pituitary extract (MPE) stimulated CRE-LUC activity in COS-7 cells expressing *mdlhr*, but could not stimulate cells expressing *mdfshr*. The study of inter-species ligand specificity of gonadotropins from medaka and Nile tilapia revealed that both recombinant tiFshβα and Nile tilapia pituitary extract (TPE) could activate the mdLhr, suggesting cross-species reactivity for mdLhr. Cross-species reactivity was also detected for mdFshr due to activation with TPE, and for tiFshr and tiLhr due to stimulation with MPE. Analysis of the amino acid sequence alignment and three-dimensional models of the ligands and receptors highlights conserved beta sheet domains of both Fsh and Lh between medaka and Nile tilapia, common alpha subunit for both Fsh and Lh with varying homology between the two species, and a higher structural homology and similarity of transmembrane regions of Lhr between both species, in contrast to Fshr, possibly related to an additional LRR inserted in the LRR region of medaka Fshr. Taken together, this is the first pharmacological characterization of medaka Fshr and Lhr using homologous ligands, and suggests partial ligand promiscuity and cross-species reactivity between gonadotropins and their receptors in medaka and Nile tilapia.

1. Introduction

In teleosts as in other vertebrates, reproduction is controlled by the brain-pituitary-gonad (BPG) axis (Norris, 1997; Schulz et al., 2000). The pituitary-derived gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play essential regulatory roles within the axis. Fsh and Lh are heterodimeric glycoproteins, comprising a common α-subunit and a β-subunit that confers biological specificity (Pierce and Parsons, 1981). In general, Fsh is important for early stages of gametogenesis, spermatogonial proliferation, and vitellogenesis in females, whereas Lh is mostly involved in processes leading to final gametogenesis, oocyte maturation and ovulation in females and spermiogenesis and spermiation in males (Yaron et al., 2003; reviewed by Levavi-Sivan et al., 2010). Fsh and Lh act via activation of their cognate receptors expressed on gonadal cells. The specific membrane-bound gonadotropin receptors were first reported in fish in the early 1970s from binding studies using mammalian gonadotropins (human chorionic gonadotropin, hCG) and partly purified hypophysial glycoprotein hormones (Breton et al., 1973). A two-receptor model for teleost gonadotropins was suggested by the mid-1990s (Miwa et al., 1994). This was supported by the molecular cloning of two distinct receptor cDNA in amago salmon (*Oncorhynchus rhodurus*) (Oba et al., 1999a,b), and thereafter in several other species: Atlantic salmon (*Salmo salar*) (Maugars and Schmitz, 2006), African catfish (*Clarias gariepinus*) (Bogerd et al., 2001; Kumar et al., 2001c; Kumar and Trant, 2001; Vischer and Bogerd, 2003), zebrafish (*Danio rerio*) (Kwok et al., 2005; So et al., 2005), European seabass (*Dicentrarchus labrax*) (Rocha et al., 2007a), Japanese eel (*Anguilla japonica*) (Jeng et al., 2007), and rainbow trout (*Oncorhynchus mykiss*) (Sambroni et al., 2007). Recently, a study based on genome analysis demonstrated the presence of a single follicle-stimulating hormone receptor (*fshr*) and duplicated luteinizing hormone receptor (*lhr*) genes in several teleost species resulting from a local duplication event early in the actinopterygian lineage (Maugars and Dufour, 2015). In the genome of Nile tilapia *(Oreochromis niloticus*), a single *fshr* and duplicate *lhr* genes, *lhr1* and *lhr2*, were found. In Japanese medaka (*Oryzias latipes*), another atherinomorph species, only single genes for *fshr* and *lhr* were conserved, the latter from the type1 (*lhr1*) whereas the second type (*lhr2*) was lost recently and after the emergence of the atherinomorph lineage (Maugars and Dufour, 2015).

Fshr and Lhr both are G-protein-coupled receptors (GPCRs), belonging to the family of rhodopsin-like receptors (family A) (Gether, 2000). They comprise, together with the thyroid-stimulating hormone receptors (Tshr), the subfamily of glycoprotein hormone receptors, which are

characterized by an N-terminal large extracellular domain (ECD), a seven transmembrane domain and a short intracellular C-terminal domain (Ji et al., 1998; Vassart et al., 2004). The extracellular domain comprises more than half of the length of the protein and is responsible for recognition of the hormone. It contains at the N-terminal multiple leucine-rich repeats (LRRs) that are involved in the high binding specificity, and ends by a hinge region connecting to the transmembrane domain. Characteristic for the perciform Fshr is an extra LRR that potentially effects their ligand binding mode (Maugars and Schmitz, 2006; Rocha et al., 2007b). The transmembrane domain is the most conserved part of these receptors, and is involved in receptor activation and signal transduction (Ulloa-Aguirre et al., 2007; Vassart et al., 2004). The intracellular C-terminal domain comprises residues related to G protein coupling and binding of different intracellular signaling molecules (Ulloa-Aguirre et al., 2007). The receptor activation results from specific hormone binding that promotes the rearrangement of the transmembrane domain from an inactive to an active conformational state, which is coupled to the activation mainly of the Gs/cAMP/PKA pathway (Ji et al., 1998; Vassart et al., 2004).

In mammalian species, FSHR and LHR bind their respective ligands specifically and with little cross-activation (0.01-0.1%), leading to functional specificity in the presence of physiologically relevant hormone concentrations (reviewed in Levavi-Sivan et al., 2010). Thereby, the activity of Fsh is guided to cells that express Fshr, i.e. Sertoli cells (testis) and granulosa cells (ovary). The activity of Lh is directed to steroidogenic cells, which express the Lhr, i.e. Leydig cells in testis as well as theca cells, preovulatory granulosa cells and postovulatory corpus luteum cells in the ovary (Levavi-Sivan et al., 2010). In some fish species like African catfish (García-López et al., 2009) and Senegalese sole (*Solea senegalensis*) (Chauvigné et al., 2012), the Leydig cells express both Fshr and Lhr. In contrast to mammals, where FSHR and LHR are highly specific to their cognate hormone, various studies in teleosts indicate that gonadotropin receptors are less specific. This was shown first for coho salmon (*Oncorhynchus kisutch*) by gonadotropin receptor ligand-binding studies using highly purified radiolabeled hormones. These studies demonstrated that Fshr, present on both theca and granulosa cells, did not distinguish between Fsh and Lh binding, whereas Lhr, localized on granulosa cells, bound only Lh (Miwa et al., 1994; Yan et al., 1992). Functionality studies revealed that chum salmon (*Oncorhynchus keta*) Fsh activates preferentially amago salmon Fshr but also revealed some degree of interactions with amago salmon Lhr, whereas chum salmon Lh activates only amago salmon Lhr (Oba et al., 1999a,b,

2001). Many functional studies on teleost gonadotropin receptors using transfected mammalian cell lines reported on the pharmacological characterization of cloned piscine gonadotropin receptors (African catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003; Vischer et al., 2004), channel catfish (*Ictalurus punctatus*) (Kumar et al., 2001a,b), zebrafish (Kwok et al., 2005; So et al., 2005), rainbow trout (Sambroni et al., 2007), European seabass (Rocha et al., 2007a; Molés et al., 2011), Japanese eel (Kazeto et al., 2008), Atlantic salmon (Andersson et al., 2009), and Nile tilapia (Aizen et al., 2012b)). From these studies, a more general pattern emerged. Teleost Lhrs tend to be specific for Lh, similar to their mammalian counterparts. Fshrs, however, possess a broader but still limited functional selectivity for both gonadotropins that may depend on the fish species or taxa (Aizen et al., 2012b; Cahoreau et al., 2015). Nevertheless, more work is necessary to assess the physiological significance of the gonadotropin receptor cross reactivity in fish, and to explain the evolutionary processes behind that gave rise to Lhr, which is selective for Lh, and Fshr that can be activated both by Lh and Fsh for some species (Levavi-Sivan et al., 2010).

Previous studies on medaka gonadotropin receptors (Ogiwara et al., 2013) and receptors of other teleost species (Levavi-Sivan et al., 2010; Nagahama and Yamashita, 2008; Yaron et al., 2003; Kwok et al., 2005) suggest that Fshr is absent in the large preovulatory follicles that are destined to ovulate, supporting the hypothesis that Fsh/Fshr determines early phases of gametogenesis, such as vitellogenesis. In contrast, Lh/Lhr is suggested to stimulate the final maturation stages, such as ovulation, and to be involved in the binding of the gonadotropins, which induce ovulation in medaka (Ogiwara et al., 2013). However, little is known about the expression of Fshr and Lhr during puberty in male medaka. Previous functional studies on the medaka recombinant Lhr and Fshr using mammalian heterologous gonadotropins suggest that they appear to be specific for their cognate gonadotropins, compared to those from other teleost species (Ogiwara et al., 2013). Further studies are required to establish the specificities of medaka gonadotropin receptors. Homology modeling provides an opportunity for revealing the details of interaction between GPCRs and their ligands (Brockhoff et al., 2010; Levit et al., 2012, 2014; Born et al., 2013), and structural information is crucial to elucidate the function of GPCRs (Katritch et al., 2013).

The current study aimed to gain more insight into the structure and differential specificity of medaka Fshr (mdFshr) and Lhr (mdLhr) by studying the selectivity of the activation response towards their cognate homologous hormones. A transactivation assay, followed by amino acid

sequence comparison and structural modeling of gonadotropins and receptors from medaka and the related Nile tilapia were performed, in order to model the intra- and interspecies ligand-receptor activation. Testicular expression profiles of the two gonadotropin receptor genes during puberty were analyzed to get a better understanding of the processes involved in the activation of the BPG axis in male medaka.

2. Materials and Methods

2.1 Animals

Japanese medaka (*Oryzias latipes*) of the dr-R strain were kept in recirculating systems with water temperature of 28 ± 1 °C and light-dark cycle of L14:D10. Embryos were incubated in embryo culture medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, all Sigma-Aldrich, St. Louis, U.S.A.) at 26 °C until hatching and transferred to system tanks. A combination of dry feed and live brine shrimp nauplii (*Artemia salina*) larvae was used to feed the fish three meals per day. Handling, husbandry and use of fish were in accordance with the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences.

2.2 qPCR gene expression analysis of *fshr* and *lhr* – tissue screening

A tissue screening of *fshr* and *lhr* gene expression was performed to investigate the distribution of gene expression in various tissues (brain, pituitary, testis, ovary, eyes, heart, intestine, liver, gills, gallbladder, spleen) according to the methodology described in detail in Burow et al. (2018) (see Table 1 for sequence details of the qPCR primers). Tissues from three adult 6 month-old males and females were dissected, using one fish per biological replicate. Total RNA was phenol-chloroform extracted from brain, testis, ovary, eyes, heart, intestine, liver, gills, gallbladder, spleen, and pituitary and resuspended in 14 µL of nuclease free water. cDNA was prepared from 25 ng – 500 ng of total RNA (brain 500 ng, pituitary 25 ng, testis 250 ng, ovary 500 ng, eyes 500 ng, heart 100 ng, intestine 500 ng, liver 50 ng, gills 250 ng, gallbladder 100 ng, spleen 500 ng) using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (Thermofisher scientific). qPCR was performed on a LightCycler 96 Real-Time PCR system (Roche, Mannheim, Germany) using LightCycler 480 SYBR Green I Master (Roche). cDNA

samples were run in duplicate, each composed of 5 µL mastermix, 5 µM of each of forward and reverse primer, and 3 µL of 10x diluted cDNA sample in a total volume of 10 µL. The cycling parameters of the qPCR were 10 min pre-incubation at 95 ºC, 40 cycles of amplification at 95 ºC for 10 s, 60 ºC for 5 s and 72 ºC for 4 or 5 s (4 s for *fshr, rpl7*, and *gapdh* and 5s for *lhr* and *18s*), followed by melting curve analysis 60 ºC to 95 ºC to assess the specificity of the qPCR products. Three non-template controls and three positive controls (calibrator) were included in every qPCR plate. The stability of the three candidate reference genes *18s*, *rpl7* and *gapdh* was evaluated using RefFinder, resulting in the use of *18s* and *rpl7* as the most stable combination of reference genes. Relative gene expression levels were calculated according to Weltzien et al. (2005) and Hodne et al. (2012).

2.3 qPCR gene expression analysis of *fshr* and *lhr* – testis developmental profile

To achieve a developmental profile of *fshr* and *lhr* gene expression in male medaka from juvenile to adult stage, testes from 30 males were selected and dissected according to standard body length and age: group 1 (14 - 16 mm, age 46-48 days post fertilization (dpf), group 2 (16 - 18 mm, age 79-84 dpf), group 3 (18 - 20 mm, age 100-111 dpf), group 4 (20 - 22 mm, age 129-134 dpf), group 5 (22 - 24 mm, age 159-167 dpf), and group 6 (24 - 26 mm, age 202-208 dpf). Total RNA extraction, cDNA synthesis (from 1000 ng RNA), and qPCR was performed as described above, with the exception that the combination of *18s*, *rpl7* and *gapdh* was found to be the most stable combination of reference genes and therefore used for normalization of expression data.

2.4 Vector construction of medaka and Nile tilapia follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone receptor (*lhr*)

Synthesis of full length coding cDNA sequences of medaka *fshr* and *lhr* was outsourced to GenScript (New Jersey, U.S.A.). The open reading frames of both receptors, *fshr* (*mdfshr*) (Accession Number NM_001201514) and *lhr* (*mdlhr*) (Accession Number NM_001201515), were synthesized with human codon optimization. The respective DNA fragments were cloned into pcDNA3.1(+) expression vector under the control of the CMV promoter using NheI (5' end) and HindIII (3' end) restriction sites. The entire coding regions of Nile tilapia *fshr* (*tifshr*) (Accession number AB041762.1) or *lhr1* (*tilhr*) (Accession number AB041763.1) were cloned also into pcDNA3.1(+), see Aizen et al. (2012b).

2.5 Production of medaka and Nile tilapia recombinant gonadotropins and pituitary extracts

Recombinant gonadotropins were produced in yeast methylotrophic *Pichia pastoris* cells as single chain polypeptides according to Burow et al. (2018) (medaka), and Kasuto and Levavi-Sivan (2005) and Aizen et al. (2007) (Nile tilapia). Preparation of medaka pituitary extract (MPE) or Nile tilapia pituitary extract (TPE) was according to Aizen et al. (2012a), using sexually mature fish. Pituitaries were homogenized on ice in 0.01 M PBS pH 7.0 containing 0.02 M phenylmethylsulfonyl fluoride and 0.05 M EDTA using a Polytron homogenizer. The homogenate was centrifuged at 4 ºC, 4100 *g* for 30 min. The pellet was stirred for 30 min to maximize extraction, and centrifuged again under the same conditions. The resulting combined supernatants were used as the pituitary extract.

2.6 Receptor-transactivation assay

To study the specificity of activation of the medaka and Nile tilapia gonadotropin receptors via the protein kinase A (PKA) signaling pathway, a sensitive luciferase (LUC) reporter gene assay was applied that makes use of the LUC transcriptionally regulated by a cyclic AMP (cAMP) response element (CRE; Invitrogen). Mammalian COS-7 cells were transfected with both CRE-LUC, and either mdFshr, mdLhr, tiFshr, or tiLhr and receptor activation was tested using recombinant medaka Fshβα (mdFshβα), medaka Lhβα (mdLhβα), Nile tilapia Fshβα (tiFshβα), Nile tilapia Lhβα (tiLhβα), MPE or TPE. Transient transfection, cell procedures and stimulation protocols were generally according to Levavi-Sivan et al. (2005). Briefly, COS-7 cells were grown in DMEM supplemented with 10 % FBS, 1 % glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Biological Industries, Israel) under 5 % CO_2 until confluent. COS-7 cells (American Type Culture Collection, Rockville, U.S.A.) was co-transfected either with pc-mdLhr (mdLhr cloned into pcDNA3.1 vector), pc-mdFshr, pc-tiLhr, or pc-tiFshr (3 µg/plate), together with a reporter plasmid (CRE-LUC; 3 µg/ plate), using TransIT-LT1 Transfection Reagent (Mirus, Milton Keynes, U.S.A.). The cells were serum starved for 18 h, stimulated with vehicle of two concentrations of either mdLhβα, mdFshβα, tiLhβα, tiFshβα (140, 550 ng/mL), or diluted pituitary extracts (1:100 for TPE and 1:10 for MPE, respectively) for 6 h, and then harvested and analyzed with Lumac Biocounter M2500 (Celsis, The Netherlands). Harvested COS-7 cell were lysed in 35 µL lysis buffer (Biological industries, Kibbutz Beit-Haemek, Israel) and assayed for luciferase

activity by assaying 10 µL with buffer containing luciferin and ATP for luciferase activity. Transfection experiments were performed in triplicate with three independently isolated sets.

2.7 Sequence comparison of gonadotropin β-subunits and receptors

To better understand the medaka gonadotropin receptor function we compared the amino acid sequences of medaka gonadotropin β-subunits and gonadotropin receptors with their homologous sequences of human and four other teleosts, including two other Acanthopterygii; the Nile tilapia and the European seabass. Gene sequences were retrieved from Genbank and deduced amino acid sequences were aligned using CLC Main workbench (QIAGEN) and a previous alignment as template for the gonadotropin receptors (Maugars and Dufour, 2015). Signal peptide and N-glycosylation sites were predicted using CLC Main workbench. Tyrosine sulfation sites were predicted using Sulfosite browser with 80 % prediction sensitivity (Chang et al., 2009).

2.8 Structural models of gonadotropin β-subunits and receptors

Sequence alignment was performed using *EMBOSS*-NEEDLE Pairwise sequence alignment tool (Rice et al., 2000). 3-D structures for medaka and tilapia gonadotropin β-subunits, α-subunits, and gonadotropin receptors were developed using I-Tasser online tool (a bioinformatics method for predicting 3D structural models of protein molecules (Roy et al., 2010; Zhang, 2008)) with the available sequences. Protein models were further rendered and prepared using Maestro tool in Schrödinger software. Structural alignment and superposition was done using Schrödinger Maestro (Schrödinger Release 2018-3) and verified using PyMOL (PyMOL, Molecular Graphics System, version 2.0 Schrödinger). Structural superposition was done using alpha carbon chain of the molecules. The root mean square deviation (RMSD) of atomic positions was calculated as a parameter of the divergence of two aligned structures from each other, hence to assess their structural similarity.

2.9 Statistical analysis

Data are presented as mean ± SEM. qPCR data were tested for normal distribution by the Shapiro-Wilk normality test. For normally distributed data, potential significance of differences in expression levels between body lengths groups was calculated by one-way analysis of variance (ANOVA) followed by Tukey´s multiple comparison test (post-hoc test) using the Graph-Pad

Prism software (version 7; GraphPad, San Diego, U.S.A.). For sample groups that did not follow a normal distribution, the data were first log-transformed. Data from the receptor transactivation assay was tested in the same manner, but using JMP software (version 10; North Carolina, U.S.A.). All data of the correlation analysis between gene expression levels were first tested for normality by means of D'Agostino-Pearson normality test. Since the datasets were normally distributed, Pearson correlation coefficients were calculated for all the analysis. Significance level was set to 0.05.

3. Results

3.1 Gene expression of *fshr* and *lhr* by qPCR – tissue screening

Both *fshr* and *lhr* were found to be highly expressed in ovary and testis (Fig. 1A and 1B, respectively). *fshr* was also found to be expressed at low levels in brain, and at trace amounts in eyes, gills, gallbladder, intestine, spleen and pituitary. *fshr* was not detected in heart and liver (Fig. 1A). High non-gonadal expression of *lhr* was revealed in brain. Low levels of *lhr* were observed in eyes, and trace amounts in gills, gallbladder, heart, intestine, spleen and pituitary (Fig. 1B). No *lhr* expression was detected in liver.

3.2 Gene expression of *fshr* and *lhr* by qPCR – testis developmental profile

The *fshr* expression data for the developmental profile targeting six different standard body length groups were not normally distributed, and thus needed to be log-transformed. Significant differences in *fshr* expression were detected between body length 14 - 16 mm (group 1) and 18 - 20 mm (group 3), between 16 - 18 mm (group 2) and 18 - 20 mm (group 3), and between 16 - 18 mm (group 2) and 24 - 26 mm (group 6) ($p < 0.05$) (Fig. 2A). Comparing them statistically, the difference between group 2 and 3 was the highest. The *lhr* expression data were normally distributed for all size groups. A significant difference of *lhr* expression was detected between body length 14 - 16 mm (group 1) and 18 - 20 mm (group 3), and between 16 - 18 mm (group 2) and 18 - 20 mm (group 3) ($p < 0.05$) (Fig. 2B). For both *fshr* and *lhr* the expression was the lowest in group 2, and highest in group 3. Correlation analysis showed a strong positive correlation between *fshr* and *lhr* expression levels during gonadal development (Pearson correlation coefficient $r = 0.9751$, $p < 0.0009$). Both *fshr* and *lhr* could be measured in all fish of body length

group 14 – 16 mm, 16 – 18 mm, and 18 – 19 mm that has been shown earlier to include male medaka of different testicular developmental stages including immature stage (Burow et al., 2018). Note that developmental stages cannot be used instead of group numbers since a histological analysis (Burow et al., 2018) revealed that the correlation between standard body length and testis developmental stage is low. Fish between 14 - 19 mm, clustered in different standard body length groups, contained different testicular developmental stages from immature (stage I) to late maturing (stage V), and fish between 19 - 26 mm contained only mature testes developmental stage V (stage I (spermatogonia type A (SPA), immature), stage II (SPA; spermatogonia type B (SPB), early maturing), stage III (SPA; SPB; spermatocytes (SC), maturing), stage IV (SPA; SPB; SC; spermatids (ST), late maturing), stage V (SPA; SPB; SC; ST; spermatozoa (SZ), mature).

3.3 Receptor-transactivation assay

The aim of this assay was to investigate the activation of the medaka receptors, mdFshr and mdLhr, and to compare their selectivity of activation with Nile tilapia receptors, Fshr and Lhr1 (tiFshr and tiLhr), to characterize the intra- and inter-species ligand specificity of gonadotropins from medaka and Nile tilapia. This study tested effects of recombinant gonadotropins from medaka (mdLhβα, mdFshβα; 140 and 550 ng/mL) and Nile tilapia (tiLhβα, tiFshβα; 140 and 550 ng/mL) or diluted pituitary extracts from medaka (MPE, 1:10) and Nile tilapia (TPE, 1:100) on their own cognate receptors (Fshr and Lhr) and on the receptors of the mutual species. The functionality of the receptors was studied by transient transfection assays in COS-7 cells expressing one of the receptors at the time. Since gonadotropins induce intracellular cAMP production upon binding to their receptors, the intracellular cAMP production was quantified using a cAMP-responsive luciferase reporter gene (CRE-LUC).

Each of the medaka recombinant gonadotropins, mdFshβα and mdLhβα, were able to activate its own cognate receptor. Recombinant mdFshβα significantly stimulated CRE-LUC activity in COS-7 cells expressing the mdFshr (Fig. 3B) and mdLhr (Fig. 3A). Recombinant mdLhβα significantly activated the mdLhr (lowest dose 140 ng/mL) (Fig. 3A), and the mdFshr (highest dose 550 ng/mL) (Fig. 3B). Recombinant tiFshβα was able to activate the mdLhr (550 ng/mL) (Fig. 3C), while tiLhβα had no effect on COS-7 cells expressing the mdLhr (Fig. 3C). Neither tiFshβα nor tiLhβα could activate the mdFshr (Fig. 3D). MPE managed to significantly activate the mdLhr (Fig. 3E), while MPE did not stimulate CRE-LUC activity in COS-7 cells

expressing the mdFshr (Fig. 3F). However, TPE managed to significantly activate both the mdLhr (Fig. 3G) and mdFshr (Fig. 3H). Regarding the activation of Nile tilapia receptors, both recombinant gonadotropins tiFshβα (Fig. 3J) and tiLhβα (Fig. 3I) were able to activate its own cognate receptor. The highest dose of recombinant tiFshβα significantly stimulated CRE-LUC activity in COS-7 cells expressing the tiFshr (550 ng/mL) (Fig. 3J). Similarly, the highest dose of recombinant tiLhβα managed to activate significantly the tiLhr (550 ng/mL) (Fig. 3I). Neither recombinant mdFshβα nor mdLhβα had any effect on the activation of tiFshr (Fig. 3J) or tiLhr (Fig. 3I). MPE and TPE stimulated CRE-LUC activity in COS-7 cells expressing the tiFshr (Fig. 3L) and tiLhr (Fig. 3K).

3.4 Sequence comparison of gonadotropin β-subunits and receptors

The deduced amino acid sequences of medaka Fshβ and Lhβ were compared with homologous subunits of human and four other teleosts, including two other Acanthopterygii, the Nile tilapia and the European seabass. The alignment of β-subunits, structured on alignment of cysteine residues, revealed that medaka and Nile tilapia Lhβ conserved the twelve cysteines common to the glycoprotein hormones, while Fshβ of medaka, contrary to most other Acanthopterygii, conserved the position of only 11 of the conserved cysteine residues (Fig. 4). In medaka Fshβ, the third cysteine is shifted three residues downstream, while it has been lost in both Nile tilapia and European seabass Fshβ. The latter two instead have an additional cysteine upstream of cysteine C1. Medaka and the other acanthopterygian Fshβ showed shorter length compared to Atlantic salmon Fshβ, European eel (*Anguilla anguilla*) Fshβ, and human FSHβ, including several amino acid deletions between C1 and C2, C5 and C7, and C7 and C8. For both medaka Fshβ and Lhβ, a single N-glycosylation site was revealed, positioned between C1 and C2 for medaka Fshβ, and between C10 and the C11 for medaka Lhβ (Fig. 4).

Alignment of the extracellular domain of the gonadotropin receptors determined that both medaka Fshr and Lhr conserved the typical structure of gonadotropin receptors with a long ECD including a conserved LRR region connected to the transmembrane domain by a hinge region highly variable in amino acid sequence composition and length. The ECD in medaka Lhr consisted of 11 successive conserved LRRs as in other vertebrate Lhr, while an additional LRR to the 11 LRRs was found positioned between the LRR2 and LRR3 in medaka Fshr. This extra LRR was observed also in Nile tilapia and European seabass Fshr. Three and four N-glycosylation sites were

predicted for medaka Fshr and Lhr, respectively. Among these N-glycosylation sites, both medaka Fshr and Lhr showed the site on LRR7 common to all glycoprotein hormone receptors. Medaka Fshr shared the 3 N-glycosylation sites with the Nile tilapia Fshr. The two first N-glycosylation sites of the medaka Lhr were conserved in Nile tilapia Lhr1. In contrast, no tyrosine sulfation site could be predicted in either medaka or Nile tilapia Fshr or Lhr (Fig. 5).

3.5 Structural models of gonadotropin β-subunits and receptors

The generated structures for medaka Fshβ (Fig. 6A) and Nile tilapia Fshβ (Fig. 6B), as well as medaka Lhβ (Fig. 6E) and Nile tilapia Lhβ (Fig. 6F), and their receptors Fshr (Fig. 6I-J) and Lhr (Fig. 6M-N), which all belong to the GPCR family, were screened and selected on the basis of high confidence score and stability. They were further prepared by processing the structures for structural alignment and superposition. A high structural similarity was observed between the predicted models for the β-subunits of both Fsh and Lh. The β sheet domains were seen to be highly conserved between medaka Fshβ (Fig. 6A) and Nile tilapia Fshβ (Fig. 6B), as well as between medaka Lhβ (Fig. 6E) and Nile tilapia Lhβ (Fig. 6F). Superposition of both gonadotropin β-subunits revealed structural homology between the Fshβ of both medaka and Nile tilapia (Fig. 6C), and between Lhβ of medaka and Nile tilapia (Fig. 6G). Structure alignment between medaka and Nile tilapia Fshβ (Fig. 6D) and between Lhβ of medaka and Nile tilapia (Fig. 6H) detected high similarity in β sheet domains. The α-subunit region, which is common for both hormones within the species, displayed a varying homology between medaka (Fig. 6Q) and Nile tilapia (Fig. 6R). The structural alignment revealed a similar but non-optimally aligned structure (Fig. 6S).

Superposition of Lhr displayed a higher structural homology between both species (Fig. 6O), in contrast to Fshr (Fig. 6K). Similarly structure alignment between Lhr of these species (Fig. 6P) displayed high similarity in transmembrane regions, compared to Fshr (Fig. 6L). A very good structural alignment was observed between Lhr of the two species (Fig. 6P). The structural alignment between the Fshr of medaka and Nile tilapia was observed to lack the similarity needed for a meaningful alignment (Fig. 6L). In addition, the receptors were revealed to be structurally similar comparing medaka Fshr (Fig. 6I) and Nile tilapia Fshr (Fig. 6J), as well as medaka Lhr (Fig. 6M) and Nile tilapia Lhr (Fig. 6N). Structure alignment and superposition have been verified using structural overlapping for medaka and Nile tilapia Fshβ (supplementary data Fig. S1a),

medaka and Nile tilapia Lhβ (supplementary data Fig. S1b), medaka and Nile tilapia Fshr (supplementary data Fig. S1c), and medaka and Nile tilapia Lhr (supplementary data Fig. S1d).

The sequence alignment revealed a sequence identity between medaka and Nile tilapia Fshβ (supplementary data Fig. S2) of 59 %, and a similarity of 71.8 %. The alignment score of the structural alignment was shown to be 0.121, and the RMSD value 1.74 Angstrom. Comparing medaka and Nile tilapia Lhβ (supplementary data Fig. S3), the sequence identity was observed to be 66.7 %, the sequence similarity 80.3 %, the alignment score of the structural alignment 0.474, and the RMSD value 3.44 Angstrom. The sequence alignment detected a sequence identity between medaka and Nile tilapia Fshr (supplementary data Fig. S4) of 61.9 %, and a similarity of 71.1 %. The alignment score of the structural alignment was reported to be 1.06, and the RMSD value 5.12 Angstrom. For Lhr (supplementary data Fig. S5), the sequence identity between medaka and Nile tilapia was observed to be 76.6 %, and the sequence similarity 85.7 %. The alignment score of the structural alignment was calculated to be 0.507, and the RMSD 3.56 Angstrom.

4. Discussion

In this study we have characterized the medaka gonadotropin receptors Fshr and Lhr, including i) a qPCR tissue screen to investigate the distribution of gene expression, ii) a testicular expression profile during sexual maturation, iii) a receptor-transactivation assay studying the activation of medaka Fshr and Lhr using recombinant medaka Fsh and Lh, and medaka pituitary extract, and iv) a characterization of the intra- and inter-species ligand specificities of gonadotropins from both medaka and Nile tilapia.

The tissue screening data revealed that both medaka *fshr* and *lhr* were highly expressed in ovary in female and testis in male 6 month-old medaka. This was expected and is in accordance with previous tissue distribution studies (e.g. Maugars and Schmidt, 2006 (Atlantic salmon); Kazeto et al., 2012 (Japanese eel); Maugars and Dufour, 2015 (European eel), underlining the importance of both glycoprotein hormones in the regulation of gonadal development. Non-gonadal *fshr* expression in the present study was detected at low levels in brain, and at trace amounts in eyes, gills, gallbladder, intestine, spleen and pituitary in medaka. Non-gonadal *lhr* expression was observed at high levels in brain, at low levels in eyes, but only at trace amount levels in gills, gallbladder, heart, intestine, spleen and pituitary. Even though the expression of *fshr* and *lhr* is found mainly in the gonads, *fshr* and *lhr* transcripts have been observed in non-gonadal tissues.

fshr transcripts were revealed in kidney of zebrafish (Kwok et al., 2005), and in brain of Atlantic salmon (Andersson et al., 2009), Atlantic halibut (*Hippoglossus hippoglossus*) (Kobayashi et al., 2008), and European eel (Maugars and Dufour, 2015). Gene expression of *fshr* was shown at low abundance in gills of Atlantic salmon (Maugars and Schmitz, 2006), European eel (Maugars and Dufour, 2015), and Senegalese sole (Chauvigné et al., 2010), in accordance with the results of the present work. Gene expression of *lhr* was shown in head kidney of African catfish (Kumar et al., 2001a; Vischer et al., 2003) and European seabass (Rocha et al., 2007a), and in brain of European seabass (*lhr2;* Rocha et al., 2007a), African catfish (*lhr2;* Vischer et al., 2003), Atlantic salmon (*lhr2;* Maugars and Schmitz, 2006), and Atlantic halibut (*lhr1;* Kobayashi et al., 2008). The observation of *lhr* transcripts in brain corresponds to the results of the present study. Furthermore, *lhr* transcripts were shown at low levels in gills and very low levels in heart and liver of Atlantic salmon (*lhr2;* Maugars and Schmitz, 2006), revealing overlapping results with the present study regarding gills and heart. Furthermore, *lhr* gene expression was shown in gills of channel catfish (*lhr1;* Kumar et al., 2001b), Atlantic halibut (*lhr1*; Nyuji et al., 2013), and European seabass (*lhr2*; Rocha et al., 2007b). In addition, *lhr* transcripts were revealed in eye of European eel (*lhr1* and *lhr2*; Maugars and Dufour, 2015) and European seabass (*lhr2;* Rocha et al., 2007b). The expression of *fshr* and *lhr* in gills suggests that gonadotropin receptors might participate in the complex multi-endocrine regulation of gill function in teleosts (Maugars and Dufour, 2015). The expression of *lhr1* and *lhr2* in eye of the European eel might reflect an ancestral function of this organ, which might have been conserved by both duplicated receptors (Maugars and Dufour, 2015). In human, *LHR* transcripts and LHR have been determined in retinal photoreceptors, where the receptor is suggested to be involved in local modulation of visual stimuli (Dukic-Stefanovic et al., 2012). Interestingly, *LHR* expression has been observed in the brain of amphibians (Yang et al., 2007), birds (You et al., 2000), and mammals including human (Lei et al., 1993). In mammals, several functions for brain LHR have been proposed, like GnRH regulation (Hu et al., 2006), sensory modulation, or fetal neurogenesis (AL-Hader et al., 1997). Due to the fact that *lhr* is highly expressed in medaka brain as in some other teleosts, investigations on the potential function of gonadotropin signaling in the vertebrate brain should be performed in future studies. Furthermore, non-gonadal expression of gonadotropin receptors in vertebrates caused suggestions of new functions to these hormones (Levavi-Sivan et al., 2010).

Both Fshr and Lhr were expressed in all testis stages from immature to fully mature. This is in agreement with Baron et al. (2005) showing that *fshr* of rainbow trout is already expressed before sex differentiation and the presence of both gonadotropin receptors in Atlantic salmon and European seabass in immature testis in juvenile males (Maugars and Schmitz, 2008; Rocha et al., 2009). Data on the expression of gonadotropin receptor genes in male fish are mainly focused on seasonal spawners, and still limited for fish species that are daily spawners, such as medaka. The developmental gene expression profile in the present study detected a significant increase of *fshr* mRNA levels from body length group 1 (14 - 16 mm) to 3 (18 - 20 mm), from group 2 (16 - 18 mm) to 3, and from group 2 to group 6 (24 - 26 mm). A significant increase of *lhr* transcripts was revealed from body length group 1 to 3, and from group 2 to 3. The results show that *fshr* and *lhr* mRNA levels increased during the transition leading to fully mature testis. In this context, we reported in our previous study that testis histology sections of medaka between 14 - 19 mm, classified in different standard body length groups, contained different testicular developmental stages from immature (stage I) to late maturing (stage V), and males between 19 - 26 mm comprised only sexually mature testes at the full spermiation stage (stage V) (Burow et al., 2018). The results of the present study are in concordance with various studies, since a peak of *fshr* and *lhr* expression during spermatogenesis was determined in various seasonal species. In salmonids, *fshr* is upregulated during early spermatogenesis because of the spermatogonial proliferation that is associated with Sertoli cell proliferation (Baron et al., 2005; Maugars and Schmitz, 2008). In male yellowtail and rainbow trout, *fshr* levels were shown to increase during early spermatogenesis, whereas at spermiation, mRNA levels decrease in yellowtail and fluctuate for rainbow trout (Rahman et al., 2003; Kusakabe et al., 2006). For rainbow trout, Atlantic salmon, and yellowtail males, *lhr* mRNA levels were shown to steadily increase as testicular maturation advances (Rahman et al., 2003; Kusakabe et al., 2006; Maugars et al., 2008). Interestingly, a correlation analysis detected a strong positive correlation between *fshr* and *lhr,* revealing a positive correlation of *fshr* and *lhr* mRNA expression levels during testis development in medaka. The peak at body length 18 - 20 mm for both *fshr* and *lhr* transcript level shown in this study could be explained by the peak of testicular maturation reaching maturity stage.

The reason for the division of fish in distinct standard body length groups for the developmental *fshr* and *lhr* gene expression profile of medaka in the present study was based on the evidence that initiation of puberty is strongly correlated with body length in female zebrafish

(Chen and Ge, 2013), which is well documented in mammals (Kennedy and Mitra, 1963). In order to target the period of puberty in this study, sampling was performed according to body length and age criteria, hence, fish was sampled between the age of 46 to 208 dpf according to the body length average. This classification was used in our previous study investigating a developmental profile for medaka *fshb* and *lhb* gene expression and protein levels (Burow et al., 2018). A histological analysis of testes was performed to assess the maturational stages of fish divided in six different standard body length groups. Spermatogenesis stages were defined according to the most advanced developed germ cell observed in the testis among the five main germ cell stages (SPA, SPB, SC, ST and SZ). According to the histology, fish between 14 - 19 mm contained fish with testis in different developmental stages from immature (stage I, SPA), to fully permeating males (stage V, SPA; SPB; SC; ST; SZ). Fish between 19 - 26 mm, with body weight above 130 mg, and age over 100 dpf contained only mature testes (stage V). A lower limit regarding body length, weight or age at which all fish were completely immature could not be determined, although the limit must be below 14 mm body length, 47 mg body weight, and 46 dpf. Important to note is that future studies should include even shorter specimens (< 14 mm) since fully mature specimens were found down to a body length of 15.5 mm.

Previously in Burow et al. (2018) both medaka *lhb* mRNA and Lh protein were shown to increase during development. Applying this on the present work, it indicates that both *lhr* mRNA as well as *lhb* mRNA and Lhβ protein increase during medaka testis development. Burow et al. (2018) found a significant increase of *lhb* expression from group 1 (14 - 16 mm) to group 6 (24 - 26 mm). Furthermore, it was reported a positive relation between *lhb* expression levels and body length demonstrating a significant increase of *lhb* expression with increased body length. However, *fshb* mRNA levels were not correlated with body length due to no significant difference for the *fshb* expression between any of the body length groups. Using homologous ELISA assays for medaka Fsh and Lh developed in our laboratory revealed that for both medaka Fsh and Lh, a highly significant increase of protein amount comparing juvenile male medaka (body length range from 12 - 16.5 mm) to adult male medaka (body length range from 21 - 26.5 mm) could be shown. For measuring protein levels (Lh, Fsh) body weight seemed to be the parameter with a better fit, whereas for measuring gene expression (*lhb*) body length was shown to have a positive correlation with expression levels. In the present study, it remains unclear whether the increase of *fshr* and *lhr* expression during development could be related to body length and/or body weight of the fish.

Not much is known about the regulation of gonadotropin receptors in male fish. Studies on mammals have demonstrated that *FSHR* and *LHR* mRNA levels are differentially regulated by their cognate hormones dependent on the stage of gonadal development (Ascoli et al., 2002; Walker and Cheng, 2005). *LHR* mRNA levels in Leydig cell progenitors in rats were significantly increased after presence of LH and testosterone (Shan et al., 1995). Oppositely, exposure of cultured immature porcine Leydig cells to recombinant LH resulted in downregulation of *LHR* mRNA (Lejeune et al., 1998). FSH upregulated *FSHR* transcript levels in Sertoli cells in 1 to 10 day old rats, whereas in 20 day old rats *FSHR* mRNA was downregulated and no effect was detected in adults (Maguire et al., 1997; Dahia and Rao, 2006). It is not known whether gonadotropin receptor genes in fish are controlled similarly, and whether other factors are involved (Maugars and Schmitz, 2008).

We further studied the receptor-transactivation analyzing the intra-species ligand promiscuity of medaka Fshr and Lhr. In both medaka and Nile tilapia, both gonadotropins could activate their own cognate receptor as expected. Interestingly, mdFshβα was also able to activate mdLhr, and mdLhβα the mdFshr. Generally, in contrast to mammals where FSHR and LHR are highly specific to their receptors, studies in teleosts suggest that the bioactivity of fish gonadotropins seems to be less specific due to promiscuous hormone-receptor interactions; in some teleosts, Fshr and Lhr can be cross-activated by Lh and Fsh, respectively, and thus teleost receptor specificity is unclear (Levavi-Sivan et al., 2010). This was first demonstrated for coho salmon where Fshr did not discriminate between Fsh and Lh binding, whereas the Lhr bound only Lh (Miwa et al., 1994; Yan et al., 1992). From later studies demonstrating the pharmacological characterization of cloned receptors of African catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003; Vischer et al., 2004), channel catfish (Kumar et al., 2001a,b), zebrafish (Kwok et al., 2005; So et al., 2005), rainbow trout (Sambroni et al., 2007), Japanese eel (Kazeto et al., 2008), and Atlantic salmon (Andersson et al., 2009) it can be suggested that the larger but still limited functional selectivity of the teleost Fshr for both gonadotropins could depend on the fish species or taxon, while the Lhrs seem to be specific for their corresponding ligand, like their mammalian counterparts (Aizen et al., 2012b). This seems not the case in medaka since we revealed that both mdLhβα and mdFshβα were able to activate the mdLhr, and both mdFshβα and mdLhβα could activate the mdFshr. This suggests that mdFshr and mdLhr are less specific to Fsh and Lh, respectively, compared to other species mentioned above. The reason why MPE did not activate

the mdFshr in this study could be due to the pituitary extract containing too low amounts of Fsh to activate the receptor. Inter-species ligand promiscuity in this study revealed interesting findings since tiFshβα and TPE could activate the mdLhr. Cross-species reactivity for the mdFshr was detected by activation with TPE only. MPE could activate the tiFshr and the tiLhr indicating cross-reactivity also for these receptors. Cross-species reactivity was also shown by Aizen et al. (2012b) detecting that tiLhr was activated by human chorionic gonadotropin (hCG), and Japanese eel Lhr could be activated by human FSH, hCG and Manchurian trout (*Brachymystax lenok*) Fsh. Furthermore, human FSHR was activated by porcine FSH and bovine FSH.

We could not find a complete rationalization for the interspecies cross-activation of medaka Fshr and Lhr by TPE, and activation of Nile tilapia Fshr and Lhr by MPE, which was shown in clear contrast to the specificity of recombinant gonadotropins (only 550 ng/mL tiFshβα was able to activate the mdLhr). Since recombinant gonadotropins and natural gonadotropins from pituitary extracts have similar sequences, it is expected that they would have shown the same cross-activation activity. Furthermore, recombinant gonadotropins should have a conformation that reflects the natural one in pituitary gonadotropins, since both recombinant gonadotropins were shown to be functional on their own cognate receptors. The interspecies cross-activation showed by the pituitary extracts and the specificity of the recombinant gonadotropins should therefore depend on other factors. Perhaps a difference in N-linked oligosaccharide composition between pituitary gonadotropins and recombinant gonadotropins could be one candidate. *P. pastoris* can glycosylate recombinant proteins in the same glycosylation sites as mammalian expression system, and has been previously used to generate biologically bioactive glycoproteins in mammals (e.g. Blanchard et al., 2008) and teleosts (e.g. Aizen et al., 2017), but the composition of the N-linked glycan could be different. Comparison of the gonadotropin β-subunit showed a difference in N-glycosylation site position between medaka Fshβ and Nile tilapia Fshβ that could explain the difference of activity between medaka and Nile tilapia Fshβα. A recent study analyzing the role of the individual N-glycosylation sites in recombinant Japanese eel Lh produced in mammalian CHO cells by N-glycosylation site mutagenesis revealed that glycosylation of the α and β-subunits play a pivotal role in signal transduction (Byambaragchaa et al., 2018) of the Japanese eel Lhr.

With regard to hypotheses of the biological meaning for the overlapping activity of Fsh and Lh in medaka, a recent study using Transcription Activator-Like Effector Nuclease (TALEN) reported interesting findings by disrupting gonadotropin receptor expression in zebrafish (Zhang

et al., 2015a). Neither Fshr nor Lhr deficient zebrafish mimicked deficiencies of their ligands. The study suggests that this is possibly because zebrafish Fshr can be activated by both Fsh and Lh proteins. It was revealed that Fshr was indispensable to folliculogenesis, and the disruption of the *fshr* gene caused a complete failure of follicle activation, which was followed by masculinization of females into males (Murozumi et al., 2014). Compared to this, it was indicated that Lhr is not crucial for zebrafish reproduction in both sexes (Zhang et al., 2015a). Furthermore, Fsh-deficient zebrafish (*fshb*$^{-/-}$) were revealed to be fertile in both sexes, but development of both ovary and testis was significantly delayed; puberty onset was observed to be significantly delayed in females, not in males. Compared to this, Lh-deficient zebrafish (*lhb*$^{-/-}$) were detected to show normal gonadal growth, but females failed to spawn and were therefore infertile. Neither the *fshb* nor *lhb* mutation alone seemed to influence gonadal differentiation. Nevertheless, the double mutation of the two genes resulted in all males, even though the development of the testis was shown to be significantly delayed. Furthermore, they observed that Fsh may play a role in maintaining female status, probably through regulation of ovarian aromatase (Zhang et al., 2015b; reviewed by Trudeau, 2018). In addition, Takahashi et al. (2016) reported that *fshb* and *lhb* deficiency had no effect on male fertility in medaka using TALENs. This could result from an overlapping action of Fsh and Lh in medaka.

Furthermore, we suggest that there are also regions in the receptors participating in recognition and/or activation, which could explain the activation profile. Amino acid sequences comparison of the extracellular domain, which is involved in the hormone recognition, between medaka and Nile tilapia Fshr and Lhr revealed some divergence. An additional N-glycosylation site was predicted positioned between the cysteine residue 2 and 3 of the hinge region in Nile tilapia Fshr. In contrast, an additional N-glycosylation site was predicted between the cysteine residue 3 and 4 of the hinge region in medaka Lhr. The hinge region of the glycoprotein hormone receptors has been shown to be involved in hormone binding and induction of signaling pathway (Jiang et al., 2012; Kleinau et al., 2017). In addition, implication of the hinge region in receptor activation has been reported for Tsh (Mueller et al., 2010). Furthermore, a previous study indicated that residues outside of the established hormone-receptor interface region may be involved in gonadotropin interactions with their receptors (Aizen et al., 2012b). To unravel possible causes for the divergence observed in the transactivation assay, an amino acid sequence alignment, as well as a protein structural super position and structural alignment between medaka Fshβ and Lhβ

ligands and Fsh and Lh receptors and other teleosts including Nile tilapia were performed. Another possible explanation for the activation by pituitary extract is the presence of other hormones than gonadotropins; possibly Tsh can activate the gonadotropin receptors.

Structural modeling is a powerful tool for analyzing and predicting ligand-receptor binding and activation (Niv et al., 2006; Brockhoff et al., 2010; Yarnitzky et al., 2010). Mechanisms of biological activity of a molecule is highly dependent on its structure. Structural superposition and alignments are key tools to understand the homology between two or more proteins and establish structural similarity and equivalence between them. These tools help to define the similarity of biological activity and affinity towards the similar binding regions on their receptors. In the present study, we used structural modeling and sequence analysis to reveal the molecular basis of cross-type hormone specificity and cross-species activity. Similar amino acid residues, which determine selectivity of ligands, can be present in both gonadotropins and their receptors, thus explaining the observed promiscuity (Levavi-Sivan et al., 2010). Related to the findings presented in this study, this can be a reason for cross-reactivity. Various studies have determined important regions of gonadotropins and their receptors, which are crucial for binding selectivity. These are for instance the "seatbelt region" of hCG (Campbell et al., 1991) and specific LRRs and residues in human LHCGR and human FSHR (Bogerd et al., 2005; Bogerd, 2007). Studies investigating chimeric analogs of the human gonadotropin β subunits demonstrated that particularly the region between the 10th and 12th conserved cysteine residues, named the seatbelt region, is essentially involved in determining receptor specificity. Especially the net charge difference on the region between the 10th and 11th cysteine residue (named determinant loop) between LH (and hCG) on the one hand, and FSH (and TSH) on the other hand were suggested to have separated LHR- from FSHR-/TSHR-activating properties (Campbell et al., 1997; Han et al., 1996). Additional sequence divergence within the C-terminal seatbelt region and outside the seatbelt segment were revealed as further separation of specific FSH, TSH and LH activities (Grossmann et al., 1997). Studying these regions in medaka and Nile tilapia gonadotropins and their receptors, resulted in residues that might partially explain the detected activity profile. The seatbelt region for both Fshβ and Lhβ in both species was well aligned and superpositioned. The net charge for the determinant loop was determined to be 1 for medaka Fshβ, -1 for Nile tilapia Fshβ, -1 for medaka Lhβ, and -2 for Nile tilapia Lhβ. Since only medaka Fshβ shows a positive charge, this might be a reason for its slightly promiscuous nature that our study advocates. There might be factors present in the pituitary

extracts that influence the net charge on this region or of the entire protein. The revealed high similarity in trans-membrane regions between Lhr of medaka and Nile tilapia compared to the Fshr, could partially be explained by the replacement of the conserved cysteine residue by tyrosine at position 617 in the transmembrane domain of medaka Fshr that leads to impairment of the transmembrane domain conformation.

Taken together, we present the first pharmacological and structural characterization of the interactions between gonadotropins and gonadotropin receptors between medaka and Nile tilapia. Our results demonstrate that each gonadotropin activated its own cognate receptor but also complex relations and cross-species reactivity between the ligands and receptors. A tissue screening of medaka *fshr* and *lhr* expression revealed that both receptor genes are highly expressed in ovary and testis, and at low levels in various non-gonadal tissues. A developmental gene expression profile in testes of *fshr* and *lhr* suggests that both transcript levels increase during pubertal development.

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7. Tables and table captions

Table 1. qPCR primers used in the present study.

Target	Reference	Primer sequence	Accession number	Amplicon size (nt)	Efficiency
lhr	This study	Forward: 5′-CAGTCTGAGGGGGATTGAAA-3′ Reverse: 5′-AGGAGACGGATGTGAAATCG-3′	NM_001201515	110	2.04
fshr	This study	Forward: 5′-ATCAGTGGCGATGCCTTCGTGG-3′ Reverse: 5′-CGGGCAAAGAGCTGATGGCTGT-3′	NM_001201514	79	2.00
18s	Burow et al., 2018	Forward: 5′-CCTGCGGCTTAATTTGACTC-3′ Reverse: 5′-AACTAAGAACGGCCATGCAC-3′	AB105163.1	118	2.02
rpl7	Burow et al., 2018	Forward: 5′-TGCTTTGGTGGAGAAAGCTC-3′ Reverse: 5′-TGGCAGGCTTGAAGTTCTTT-3′	NM_001104870	98	2.03
gapdh	This study	Forward: 5′-CCTCCATCTTTGATGCTGGT-3′ Reverse: 5′- ACGGTTGCTGTAGCCAAACT-3′	XM_004077972.3	75	2.01

8. Figure Captions

Fig. 1. Tissue distribution of *fshr* and *lhr* transcripts in medaka. Messenger RNA levels for *fshr* mRNA levels (A) and *lhr* mRNA levels (B) were assayed by qPCR in various tissues of medaka: brain, eyes, gills, gallbladder, heart, intestine, liver, spleen, testis, ovary, and pituitary. Three replicates per tissue were used (n=3). Detected (d.) and not detected (nd.) gene expression in tissue of interest are indicated. Means are given ± SEM.

Fig. 2. Developmental profile of *fshr* and *lhr* testes gene expression in male medaka. Transcript levels for *fshr* mRNA levels (A) and *lhr* mRNA levels (B) were studied in medaka classified in different standard body lengths groups (group 1 14 - 16 mm, group 2 16 - 18 mm, group 3 18 - 20 mm, group 4 20 - 22 mm, group 5 22 - 24 mm, group 6 24 - 26 mm). Five replicates per body length group were used (n = 5). Means marked by asterisks differ significantly (*p ≤ 0.05, ** p ≤ 0.01, significant difference between groups).

Fig. 3. Receptor-transactivation assay of medaka and Nile tilapia gonadotropin receptors for characterization of intra- and interspecies ligand specificity. COS-7 cells were transiently co-transfected with medaka gonadotropin receptors (mdFshr, mdLhr) (A-H) or Nile tilapia gonadotropin receptors (tiFshr, tiLhr) (I-L), each together with the reporter plasmid CRE-LUC. Cells were stimulated with 140 ng/mL or 550 ng/mL mdLhβα or mdFshβα (A, B, I, J), 140 ng/mL or 550 ng/mL tiLhβα or tiFshβα (C, D, I, J), medaka pituitary extract (MPE; 1:10) (E, F, K, L), or Nile tilapia pituitary extract (TPE; 1:100) (G, H, K, L). Since gonadotropins induce intracellular cAMP production upon binding to their receptors, the intracellular cAMP production was assessed using the cAMP-responsive luciferase reporter gene CRE-LUC. Each assay was repeated more than three times, and each treatment was conducted in triplicates. Data (mean ± SEM) are expressed as the increase in luciferase activity over basal activity. Means marked with different letters differ significantly (ANOVA followed by Tukey´s multiple comparison test, p < 0.05).

Fig. 4. Amino acid sequence alignment of medaka Fshβ and Lhβ with other teleost representatives as well as with the human Fshβ and Lhβ sequences. Residues of the signal peptide sequence are indicated in italic. Sequences were retrieved from GenBank and aligned using CLC bio main workbench. Conserved cysteine residues are numerated and indicated in red and other conserved residues are indicated in blue. Putative N-glycosylation sites are highlighted in green. The name of the sequences of Fsh and Lh subunit β of medaka are highlighted in yellow and Nile tilapia in blue. The accession numbers of the sequences are: human *FSHB* (NG_008144), European eel *fshb* (AY169722), Atlantic salmon *fshb* (LOC100136362), Nile tilapia *fshb* (LOC100534500), medaka *fshb* (NM_001309017.1), European seabass *fshb* (AF543314), human *LHB* (LOC3972), European eel *lhb* (X61039), Atlantic salmon *lhb* (LOC10038038), Nile tilapia *lhb* (LOC100534501), medaka *lhb* (AB541982.1), European seabass *lhb* (AF543315).

Fig. 5. Amino acid sequence alignment of extracellular domain of the medaka Fshr and Lhr with other teleost representatives as well as with the human Fshr and Lhr sequences. Sequences were retrieved from GenBank and aligned using CLC bio main workbench. Residues of the signal peptide sequence are indicated in italic. Leucine rich motifs are highlighted in orange. Arrows indicate the beginning of the different domains of the extracellular domain. Conserved cysteine residues are indicated in red and other conserved residues in blue. Putative N-glycosylation sites are highlighted in green and potential tyrosine sulfation sites are in grey box. The name of the sequences of Fshr and Lhr of medaka are highlighted in yellow and Nile tilapia in blue. The accession numbers of the sequences are: human FSHR (P23945), European eel *fshr* (LN831181), Atlantic salmon *fshr* (NM_001123610.1), Nile tilapia *fshr* (LOC100534395), medaka *fshr* (NM_001201514), European seabass *fshr* (FQ310507), human *LHCGR* (NP_000224), European eel *lhr1* (LN831182), Atlantic salmon *lhr1* (LOC106589743), Nile tilapia *lhr1* (LOC100534397), medaka *lhr* (NM_001201515), European seabass *lhr1* (HG916828), European eel *lhr2* (LN831183), Atlantic salmon *lhr2* (LOC100136406), Nile tilapia *lhr2* (LOC100705740), European seabass *lhr2* (AY642114.1/HG916828).

Fig. 6. Medaka and Nile tilapia gonadotropin β-subunit, α-subunit and gonadotropin receptor models. Structural three-dimensional model of medaka Fshβ (A), Nile tilapia Fshβ (B), medaka Lhβ (6E), Nile tilapia Lhβ (6F), medaka Fshr (I), Nile tilapia Fshr (J), medaka Lhr (M), Nile tilapia Lhr (N), medaka alpha subunit (Q), and Nile tilapia alpha subunit (R). Superposition of medaka Fshβ (red) with Nile tilapia Fshβ (green) (C), medaka Lhβ (red) with Nile tilapia Lhβ (green) (G), medaka Fshr (blue) with Nile tilapia Fshr (red) (K), and medaka Lhr (blue) with Nile tilapia Lhr (red) (O). Structure Alignment of medaka Fshβ (red) with Nile tilapia Fshβ (green) (D), medaka Lhβ (red) with Nile tilapia Lhβ (green) (H), medaka Fshr (blue) with Nile tilapia Fshr (red) (L), medaka Lhr (blue) with Nile tilapia Lhr (red) (P), and medaka alpha subunit (red) with Nile tilapia alpha subunit (green) (S). The gonadotropin subunits and gonadotropin receptors are shown in ribbon representation. 3-D structures were developed using the I-Tasser online tool. Protein models were further prepared using Maestro tool in Schrödinger software. Structural alignment and superposition was performed using Schrödinger Maestro (Schrödinger Release, 2018-3) and verified by Pymol (PyMOL, Molecular Graphics System, version 2.0 Schrödinger).

9. Figures

Fig. 1A

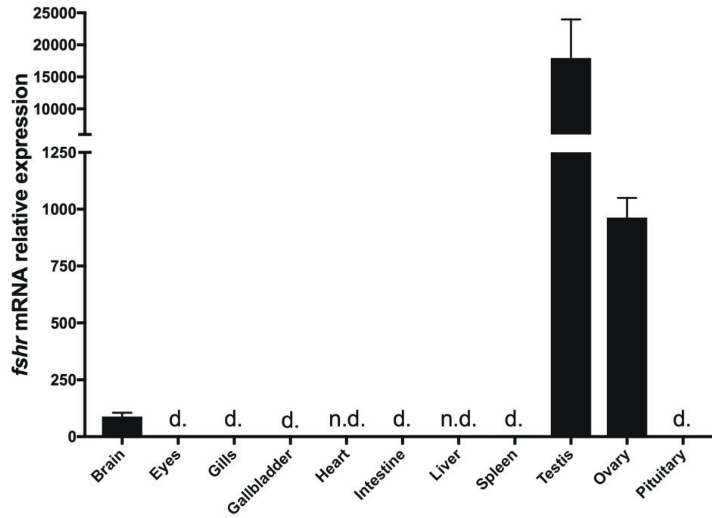


Fig. 1B

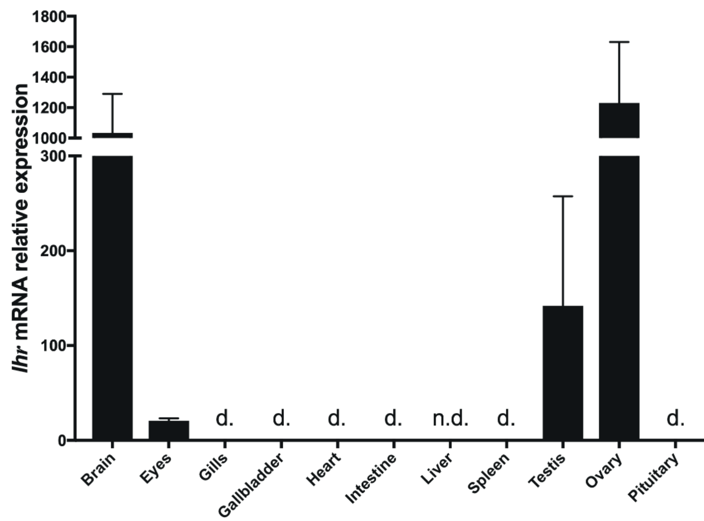


Fig. 2A

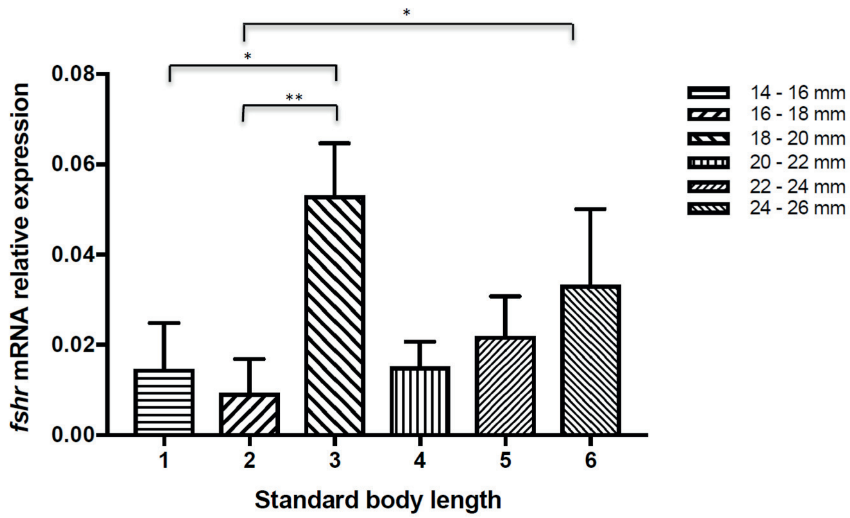


Fig. 2B

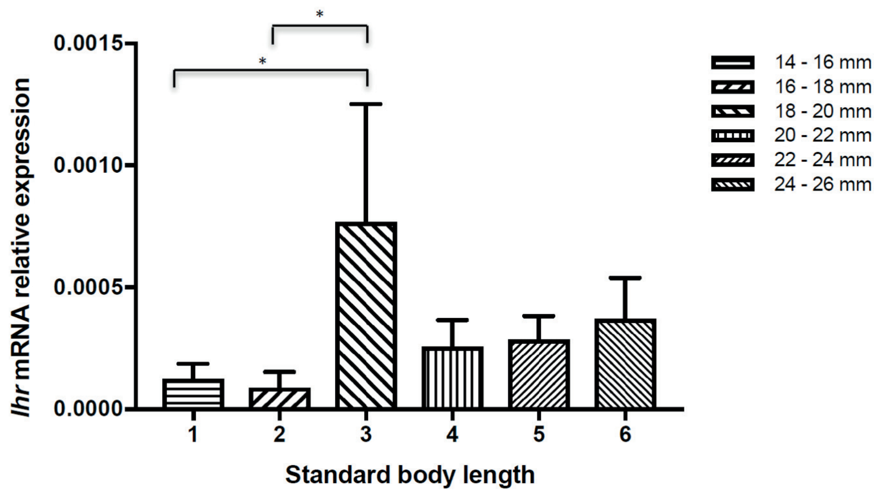
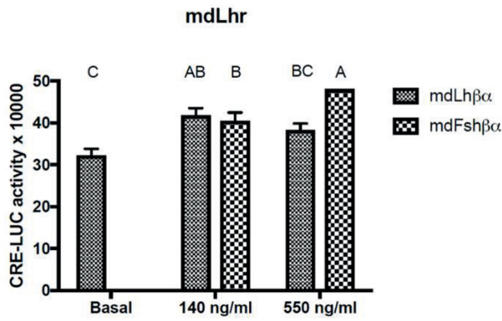


Fig. 3

3A



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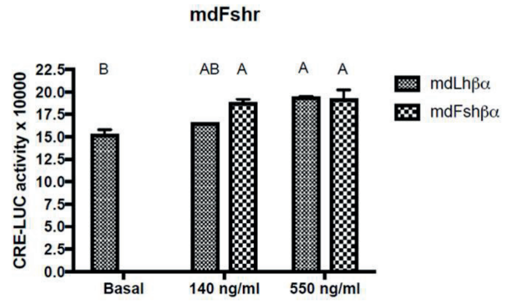

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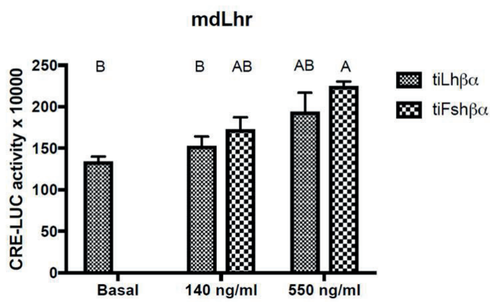
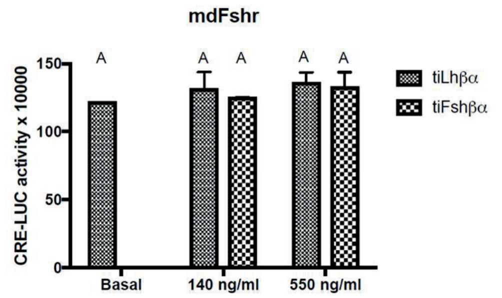


3D



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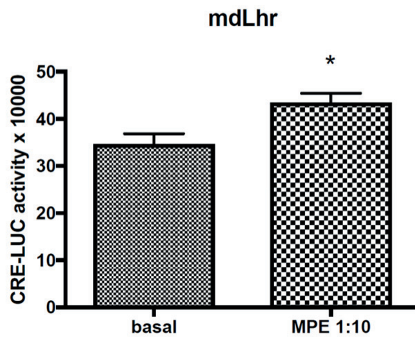

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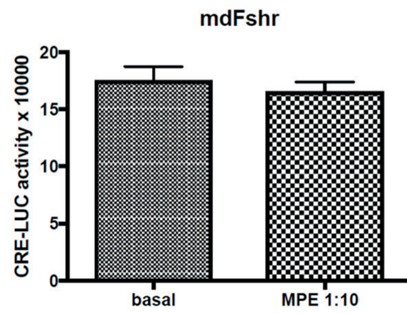

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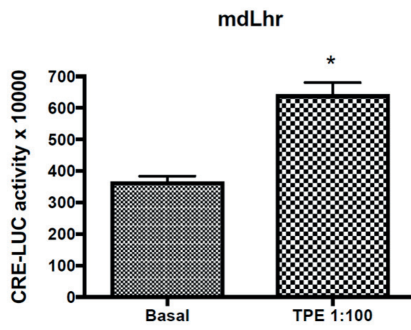

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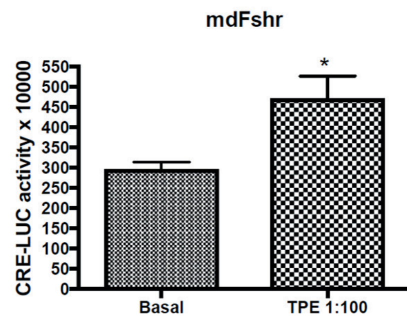

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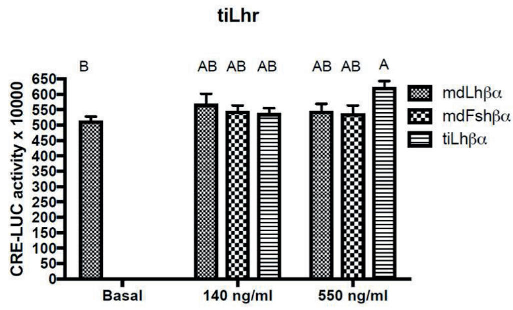

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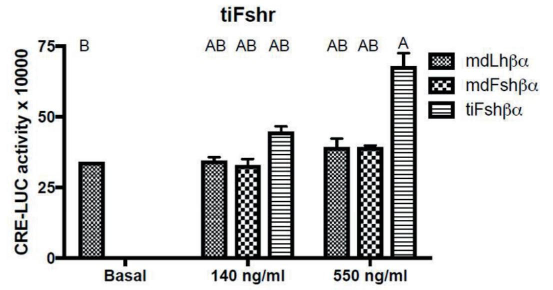

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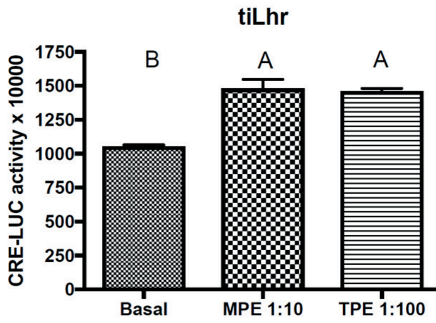

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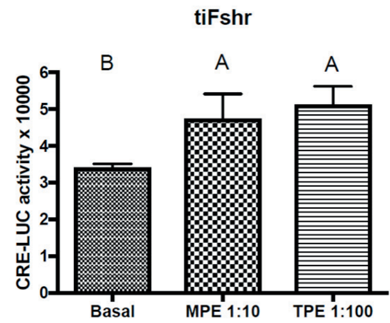

Fig. 4

```
                                                      1             2  3         4   5
Human FSHβ      -----------------MKTLQFFFLFCCWKAICCNSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDP   60
Eel Fshβ        -------------MHLAVTALCLTLAPILARA--STSCGLANISISVENEECGGCVTFNTTACAGLCFTQDSVYKSS   62
Salmon Fshβ     ------MYCTRLRTLQLVVMATLWVTPVRAGTDCRYGCRLNNMTITVEREDCHG--SITITTCAGLCETTDLNYQST   69
Tilapia Fshβ    ---------------MQLVVMAAVLALAGAEQDCSSGCRPKNISL--PVDTCGF---VDTTICEGQCFQKDPNFIHT   57
Medaka Fshβ     ---------------MQLVVMAAALVLAEVGQVSSFSCHPKNVSI--PVESCGISGCVHTTICEGRCYHEDPNYISY   60
Seabass Fshβ    ---------------MQLVVMVAVLALARAGQGCSFGCHPTNISI--QVESCGLTEVIYTTICEGQCYHEDLVYLSH   60
Human LHβ       ---------MEMLQGLLLLLLLSMGGAWASREPLRPWCHPINAILAVEKEGCPVCITVNTTICAGYCPTMMRVLQAV   68
Eel Lhβ         --------MSVYPECTWLLFVCLCHLLVSAGGSLLLPCEPINETISVEKDGCPKCLVFQTSICSGHCITKDPSYKSP   69
Salmon Lhβ      --------MLGLHVGT-LISLFLCILLEPVEGSLMQPCQPINQTVSLEKEGCPTCLVIQTPICSGHCVTKEPVFKSP   68
Tilapia Lhβ     MMAQISRMLLALMLSLFVGASTFI--LSPAAAFQLPPCQLINQTVSLEKEGCPSCHPVETTICSGHCITKDPVIKIP   75
Medaka Lhβ      MISRVSRVMFLLMLSFILGTSTFLWSLAPAAAFQLPYCQPVKQKLSLQKEGCSGCHTVETTVCSGHCLTKDPLMKI-   76
Seabass Lhβ     MAVQASRVMFPLVLSLFLGATSSIWPLATAEAFQLPPCQLINQTVSLEKEGCPKCHPVETTICSGHCITKDPVIKIP   77

                        6              7               8 9 10      11        12
Human FSHβ      ARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE-----     129
Eel Fshβ        LKPYPQQACNFRDVVYETVHLPGCPSGMDLHFTYPVALSCECSKCNTDSTDCGPLNTEVSGCLTH---------     127
Salmon Fshβ     WLPRSQGACNFKDWSYEKVYLEGCPSGVDP-FFIPVAKSCDCIKCKTDNTDCDRISMATPSCLGSPLEM-----     137
Tilapia Fshβ    DDWPKQKTCN-GEWSYEVKYTEQCPRG----FIYPVARKCECTACNA-NTDCGTLSGYIPSC------------     113
Medaka Fshβ     EDHPKEKICS-GDWSYEVKFIEGCPVG----FKYPVAKSCECTTCNTRTTYCGRLSADMPSC------------     117
Seabass Fshβ    YERPEQRICN-GDWSYEVKHIKGCPVG----VTYPVARNCECTTCNTENTDCGRFPGDIPSCLSF---------     120
Human LHβ       LPPLPQVVCTYRDVRFESIRLPGCPRGVDPVVSFPVALSCRCGPCRRSTSDCGGPKDHPLTCDHPQLSGLLFL-     141
Eel Lhβ         LSTVYQRVCTYRDVRYETVRLPDCRPGVDPHVTFPVALSCDCNLCTMDTSDCAIQSLRPDFCMSQRAS-L-PA-     140
Salmon Lhβ      FSTVYQHVCTYRDVRYETIRLPDCPPWVDPHVTYPVALSCDCSLCNMDTSDCTIESLQPDFCITHRALMDGNMW     142
Tilapia Lhβ     FSNVYQHVCTYRDLYYKTFELPDCPPGVDPIVTYPVALSCHCGRCAMDTSDCTFESMQPDFCMNDIPF---YY-     145
Medaka Lhβ      RSIQYQNVCTYRDFYYKTFELPDCLPGVDPSVTYPVALSCHCGACIMNASDCTFESLPPDFCVKHDSF---YY-     146
Seabass Lhβ     FSNVYQHVCTYRNSHYKTFELPDCPPGVDPTVTYPVAQSCHCGRCAMDTSDCTFESLQPNFCMNDIPF---YY-     147
```

Fig. 5

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                                                      ➤ LRR1            ➤
Human FSHR      ----------------MALLLVSLLAFLSLGSGCHHRICHC----------SNRVFLCQES--KVTEIPSDLPRNAIE  50
Eel Fshr        --------------------MTPLWVLFLGLVSGTSCV--AMHVC--LANGTTRSFFCVGS--RVNQMPAVIPKNTSY  52
Salmon Fshr     --------------------MMKMKKIMKMLLCVLGCVCVSQAEVAMVNSGTTFTYLCMG-NT-ITHMPTHIPKNTTN  56
Tilapia Fshr    ------------MMLVMTLMMLLIVTIKMAAASAHGSEMDIRPG-FHPSLAKQTSCLSYQVMFGVTAFPSNIS-NAQC  64
Medaka Fshr     -------------MVVMIQMMLVLFRLQMAGASLPETE---------------LDDVCFQVELGFSTFLRSISSNSTV  50
Seabass Fshr    --------------MMVMILIMLMILVIKTATASVPGPEMDVKPGVETSLAKRTLSFCYQLKFGVTEIPSSISSNTTC  64
Human LHCGR     --MKQRFSALQLLKLLLLLQPPLPRALR--EALCPE-PCNC---------VPDGALRCPGP-----------TAGLTR  53
Eel Lhr1        -----MSNLLLWTMWRLVMLSTVLQVRSGWTFSCP-VICQC---------TE-QSFRCTRETQLNSS---ARSSLINN  59
Salmon Lhr1     ------MAPARQTVWVLLALSGVLNLRSCWTFTCP-SICKCSLKSDTCYKDTGEKLSCTRETQLKSQ---AAPSFIRN  68
Tilapia Lhr1    --------MALREVWLLFALSGVLNARSCCAYTCP-AICRC---------TA-DSFQCSKETQLASR---TGPTSVLR  56
Medaka Lhr1     MSCGPPARMAPRVVWLLVALSGVLNARSCQAYPCH-PICRC---------TP-DTFQCNRGTQLA------AGTAEHR  61
Seabass Lhr1    --------MAPRAVWLLVALSGVLNVRSCWAYTCP-AICRC---------TA-DTFQCSRDTQLASR---AAAASVPR  56
Eel Lhr2        -----MDLRLSVLFLLFPSVMCMWGFMTISDFQCPG-VCMC---------SP-KTIRCNRVTELSLPV--AQRDSYRR  60
Salmon Lhr2     ----MMSISLLFLFYPSVLLFFGFGCRYASSFVCPG-ICRC---------SS-NTIRCNNITEKSVPM----SERGPR  59
Tilapia Lhr2    -------MWTSPSVSLLLFVSFFHGCR---NFVCPR-ICRC---------FS-NAIRCNNITQGSAPVM---DHRDRR  54
Seabass Lhr2    -------MWTSLLALLFLSVLGFYGCKCAPGFGCPR-ICRC---------FS-NTIRCNNVTQGSALMM---DHRDKR  57

                LRR2                 ➤extra percomorph Fshr LRR ➤ LRR3                  ➤ LRR4
Human FSHR      LRFVLTKLRVIQKGAFSGFG-------------------------DLEKIEISQNDVLEVIEADVFSNLPKLHEIRIE 103
Eel Fshr        IEFKLTQLRLIPRAAFSELH-------------------------ELSRIMVSENGALEGIAAHAFSNLSNLVEITIT 105
Salmon Fshr     LEFKQTHIRVFPREAFTNLL-------------------------QLTAIVLTENGMLESIGAFAFANLPRLTEITIT 109
Tilapia Fshr    LEVKQTQIREIQQGTLSSLQHLMELTISENDLLESIGAFAFSGLPHLTKILISKNAALRNIGAFVFSNLPELSEIIIT 142
Medaka Fshr     VNIKQTQITVIDQSIFTGLWHLEKLTILDNDKLLSICPSAFANLPRLFDVSIXQNMALKKIGAFAFSNLPALTEIEIT 128
Seabass Fshr    LEVKQTEIVVIPQGALNSLQHLRKLTIWENDKLESINEFAFASLSQLTDIFISGNVALKNIGAFAFSDLPELTEITIT 142
Human LHCGR     LSLAYLPVKVIPSQAFRGLN-------------------------EVIKIEISQIDSLERIEANAFDNLLNLSEILIQ 106
Eel Lhr1        IRLTHLPLQEVPSNTFRDLH-------------------------NVDRIEISQSDSIRIFRARAFHSLHSLQEVLIH 112
Salmon Lhr1     LRLTHLPLTEVPSNAFRGLI-------------------------NVSRIEISQSDCTRYIRNHAFLSLHSLTEIQIQ 121
Tilapia Lhr1    LRLTHLPLKRVPSHAFKELI-------------------------NITIIEISQSDCITHIQTHAFLSLYSLAQISVQ 109
Medaka Lhr1     LRLTHLPLKQVPTHAFKELM-------------------------NITIIEISQSDRITAIQRHAFLSLHSLQQILVL 114
Seabass Lhr1    LWLTHLPLKEVPTHAFKELI-------------------------NITIIEISQSDCITQIQRHAFLSLHSLAQISVQ 109
Eel Lhr2        LTLTHLSLKRIAGRAFEELG-------------------------GVLSIEITQSASLEIIEALAFNNLTNLSEILIQ 113
Salmon Lhr2     LVLKHLTMSTIASHTFDGLR-------------------------RVQHIEIGQSVALETIETLAFNNLLDLNEIFIK 112
Tilapia Lhr2    LFLYHLSLQTISSHSFEGLK-------------------------GVQRIEITQSVTLKTIETLAFNNLLSLFEISIQ 107
Seabass Lhr2    LFLYHLSLNTISSHSFDGLK-------------------------GVQRIEIAQSETLETIEALAFNNLLNLSEISVQ 110

                                   ➤ LRR5                 ➤ LRR6                          ➤
Human FSHR      KANNLLYINPEAFQNLPNLQYLLISNTGIKHLPDVHKIHSLQK----VLLDIQDNINIHTIERNSFVGLSFESV-ILW 176
Eel Fshr        KSKNLVFIEKGAFWNLSRLKYLTISNTGLKSLPDFSKINSAAK---DFLFDLQDNVNMKVIHPNAFLGLSSETIRELR 180
Salmon Fshr     KSKHLVVIHHQAFMGLPKLSHLTICNTGLRVLPNFSRIHSTAL---TFLLDLQDNVHIVIIPSNAFLGLTTNTIDELR 184
Tilapia Fshr    KSKHLSFIHPDAFRNMARLRFLTISNTGLRIFPDFSKIHSTAC----FLLDLQDNSHIKRVPANAFRGLCTQTFAEIR 216
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Medaka Fshr     KSRHLTSIHPDAFRSLVGLRRLIITNTGLRIFPDLSKIHSAVH---RFMFDLQDNIHIQAIPANAFRGLCTQTVEEIR 203
Seabass Fshr    KSKHLTHINPDAFKDIVKLKYLTIANTGLRLFPDFTKIHSTGL----LLFDLHDNSHIERVPANAFKGLCTQTIPEIR 216
Human LHCGR     NTKNLRYIEPGAFINLPRLKYLSICNTGIRKFPDVTKVFSSES---NFILEICDNLHITTIPGNAFQGMNNESVT-LK 180
Eel Lhr1        NMKNLEFIKKGAFSDLPKLKYLSICNTGLREFPDLSTISSLEP---LFFLEVGDNIKIDTIPPNAFLGMTEEET-YMN 186
Salmon Lhr1     NIRNLISIEKGAFTDLPRLRYLSICNTGIIHFPDFSTISSLVILE-YFFLELGDNIQLHSIPANAFQGITQESV-YMK 197
Tilapia Lhr1    NINSLRFIEKGAFADLPKLEYLSISNTGIAHFPDFTTISSLSP---NIILEMADNMEIDIIPANSFQGITEEYVD-MN 183
Medaka Lhr1     NINSLRGIERGAFTDLPRLEFLTISNTGMMHFPDFTSVSSLTP---SILLEMLDNMRIDVIPANSFRGMTKGHAN-MN 188
Seabass Lhr1    NINSLRVIEKGAFTDLPKLEYLSISNTGVIHFPDFTTISSLAP---NIILEIADNMRIDIIPANSFQGITEEYVD-MN 183
Eel Lhr2        NTRRLAHISRKAFNNLPKLQYLSISNTGISLFPDLSAISSLQS---NFILDICDNLNLQSIPSNAFIGMTKEHTD-MN 187
Salmon Lhr2     NTRSLVHIARRTFNNLPKLRYLSISNTGITVFPDMTSIHSLEPWNQNFVLDICDNLYLLSIPVNAFVGMTTEYTA-MN 189
Tilapia Lhr2    NTRSLMHIDRGAFNNLPKLRYLSISNTGIAVFPDVTSVSSLES---EFVLDICDNIFLLEIPTNAFTGMSKEYVT-MN 181
Seabass Lhr2    NTRSLMHISRRTFNNLPKLHYLSISNTGITLFPDITYINSLES---EFILDICDNLYLLEIPPNAFIGLTKEYVT-MN 184

                  LRR7           ➤ LRR8                   ➤ LRR9              ➤ LRR10
Human_FSHR      LNKNGIQEIHNCAFNGTQLDELNLSDNNNLEELPNDVFHGASGPVILDISRTRIHSLPSYGLENLKKLRARSTYNLKK 254
Eel Fshr        LTKNGITEVLNHAFNGTKLDRLLLMGNQQLRQIHSQAFSGAEGPVVLDVSRTSISVLPENILWRLKRLTAESVYTLKK 258
Salmon Fshr     LTKNGISEVESHAFNGTKIHKLFLMGNLQLSHMHNNSFKGAEGPGFLDISRTALSSLPESVLGEVEHLSAVSVFSLRA 262
Tilapia Fshr    LTRNGIKEVASDAFNGTKMHRLFLGGNRQLTHISPNAFVGSSELVVLDVSETALTSLPDSILDGLKRLIAESAFNLKE 294
Medaka Fshr     LTRNGIREVASDAFNGTKMHRLSLKGNKQLTHISGDAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKI 281
Seabass Fshr    LTRNGIKEVASDAFNGTKMHRLFLRGNKQLTHINPNAFVGSSELVVLDISQTALSSLPDYILGGLQKLIAESAPNLKE 284
Human LHCGR     LYGNGFEEVQSHAFNGTTLTSLELKENVHLEKMHNGAFRGATGPKTLDISSTKLQALPSYGLESIQRLIATSSYSLKK 258
Eel Lhr1        LVGNGFKEVQRHAFNGTKMSKLVLKGNRNLRKIHDEAFEGATGPSLLDVSSTALHSLPALGLQQVTVLTARSAQGLKT 264
Salmon Lhr1     IIKNGFKEIKSHAFNGTKLHKLILKDNRYLRNIHEDAFEGASGPTVLDVSSTALDTLPRRGLEHLKVLIARSTPYLKT 275
Tilapia Lhr1    LVRNGFKEIKSHAFNGTKLNTLVLRDNWYLRNIQEDAFEGATGPTLLDVSSTALRSLPPNGLRHVKFLKASHAYALKS 261
Medaka Lhr1     LVRNGFKEIQSHAFNGTKLNNLILRDNRFLSYIAEDAFEEATGPSYLDVSSTALSALPAKGLTRVQTLKATATFALKS 266
Seabass Lhr1    LVRNGFKEIKSHAFNGTKLNTLILRDNRYLSEIQENAFEGATGPSFLDVSSTALSSLPPKGLRQVRFLKASSAFALKT 261
Eel Lhr2        LYNNGFKEVQDHAFNGTRINKLVLKNNKKLKMIHSDAFKGAFGPSTLDVSATAVEALPERGLEAVLVLVARGAYALKS 265
Salmon Lhr2     LFNNGIREIQDYAFNGTKINKLVLKNNRNLRVIHREAFKGAVGPRILDVSSTAIETLPSHGLNSVVELVARTAYGLKR 267
Tilapia Lhr2    LYNNGIRKIHEHAFNRTKIDKLVLKNNRNLRVIHKDAFTGATGPGVLDVSATALTKLPSQGLESVLVLFALSAYTLKS 259
Seabass Lhr2    LYNNGIREIHDYAFNGTKIDKLVLKNNRNLRVIHRYAFEGATGPGVLDVSATALTKLPPQGLESVLVLFAQSAYALKS 262

                         ➤ LRR11    ➤ Hinge region
Human FSHR      LPTLEKLVALMEASLTYPSHCCAFANWRRQISELHPI------------CNKSILRQ---------EVDYM-TQARGQ 310
Eel Fshr        LPNLDLFTQLIEANLTYRSHCCAFANSKKNMSVVHEL------------CDKPNIKQE-------------------- 304
Salmon Fshr     LPPLSLFTKLRQANLTYPSHCCAFHKHQRNRTFRMNSA-----------CFKPGAQDN-------------------- 309
Tilapia Fshr    LPPIQLFTKLHQAKLTYPSHCCAFLNMHRNRSRWHSL------------CDNPEAKNN-------------------- 340
Medaka Fshr     LPPPQQFAKLRLAKLTYPSHCCAFKNKPRSRSKWSPL------------CSHPMAKYI-------------------- 327
Seabass Fshr    LPPLELFTKLHQANLTYSSHCCAFHNIHRNRSKWNSL------------CSHPDAQGN-------------------- 340
Human LHCGR     LPSRETFVNLLEATLTYPSHCCAFRNLPT--KEQNFSHSISENFSKQ--CESTVRKVNNKTLYSSMLAESE------- 325
Eel Lhr1        LPPLENLVNLQEAHLTYPSHCCAFHTWRRKHRENAFLG-SFGNLSRL--CNSGPPQGIGYPSASDMSYPEYSLDD--- 336
Salmon Lhr1     LPPLESLLELEVAEVTYPSHCCAFHTWRRKDKEKVVHA-LSPNLRRL--CDDDDEI--MDHSADNVTDLYD------- 341
Tilapia Lhr1    LPLLESLAELLEAELTYPSHCCAFHTWRRKQRESAL-----KNLTKF--CDLMNTE--IDPTADDTSLIND------- 323
Medaka Lhr1     LPPLQSLAELLEAELTYPSHCCAFDKWRRKQRENAL-----KNSTKL--CNLGETE--IEATDDGMNLVND------- 328
Seabass Lhr1    LPPLVSLAELLEAELTYPSHCCAFHTWRRKQRESAL-----KNFTKL--CDLSETE--TDPAGDGVNLIDD------- 323
```

```
Eel Lhr2          LPPLTGLQSLQEAQLTYPSHCCSL-------RE-ADVFAEWSNRS--AFCNDVSLLERMLALSVDLPLAP-------- 325
Salmon Lhr2       LPPFRGLGNLQKAHLTYNSHCCALLTWDT-HRD-SPINAAQHNGSRPTYCDDSQSE-KF-PAGMVDSSDTSLLVEI-H 340
Tilapia Lhr2      LPPLQGLWSLREAHLTYNSHCCALLSWNT-HRD-LSINPVWNNSS--TSCIERDPAGRVQPVI-GGSTDTSLLMGVQY 332
Seabass Lhr2      LPPLQGLWSLREAHLTYNSHCCALLSWNT-HRD-FSINPAYNNDS--TYCDESDQLARVQRVI-GGSADTTLVMDMPF 335
```

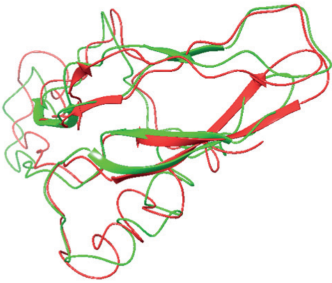
```
Human FSHR        RSSLAEDNESSYSRGFDMTYTEFDYDLCNEVVDVTCSPKPDAFNPCEDIMGYNILRV 367
Eel Fshr          -------------------EPQWHLEHC--TIEVSCHPKPDAFNPCEDIMGFTYLRV 340
Salmon Fshr       --------------------LHFFMDFCLNWTSVACSPAPDAFNPCEDIMGSAPLRV 346
Tilapia Fshr      --------------------LHFFREYCSNSTNITCSPAPDDFNPCEDIMSATPLRI 377
Medaka Fshr       --------------------TDFYRDHCSNSTSITCSPTPDNLNPCEDIMSPVPLRV 364
Seabass Fshr      --------------------LHFYRDYCSNSTSIICTPTQDDFNPCEDIMSAVPLRV 377
Human LHCGR       ---------------LSGWDYEYGF--CLPKT-PRCAPEPDAFNPCEDIMGYDFLRV 364
Eel Lhr1          ---------------FDFQYLNLEL--CLSDAPIKCTPEPDAFNPCEDLLGYAYLRT 376
Salmon Lhr1       ---------------INLQYPDLQLSLC-PGP-FQCTPESDAFNPCEDLLGYSFLRS 381
Tilapia Lhr1      ---------------INFQYPDLEFD-CFSNPFVKCSPKPDAFNPCEDLLGFSFLRC 364
Medaka Lhr1       ---------------IKFEYPDLEFD-CGSNPFVICTPTPDDFNPCEDLLRYAFLRC 369
Seabass Lhr1      ---------------INFEYPDLGFD-CLNNPFVKCTPKPDAFNPCEDLLGFPFLRC 364
Eel Lhr2          --ESVDPSPEV-YSSIDLHYPEFDF--CHSRRAPRCAPEADAFNPCEDIVGFGFLRV 377
Salmon Lhr2       GTN-KDV-EDESYGGVDFQYPELGLY-CQTRPTLQCTPEADAFNPCEDIAGFSFLRV 394
Tilapia Lhr2      FSEGDLLAEDEPYGDVNFHYPELDL--CQTTRTLVCTPEADAFNPCEDIAGFSFLRV 387
Seabass Lhr2      FSD-VDLSEDEGFGDVNFHYPELDF--CQTRPTLVCTPEADAFNPCEDIAGFSFLRV 389
```

Fig. 6

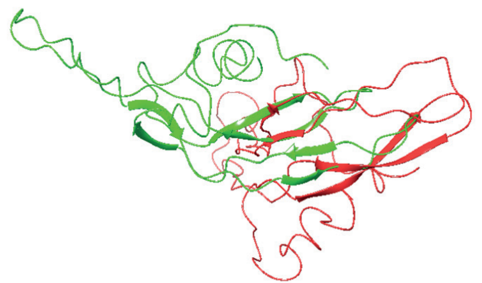
6A

6B

6C



6D



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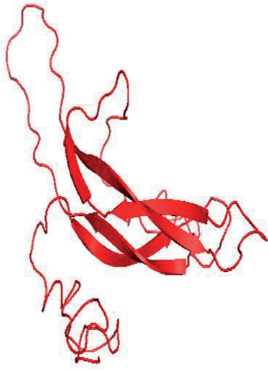

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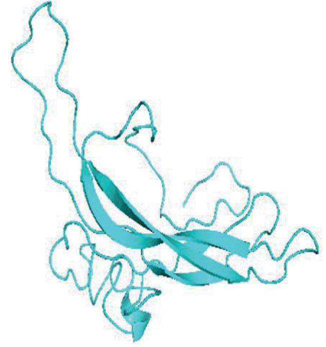

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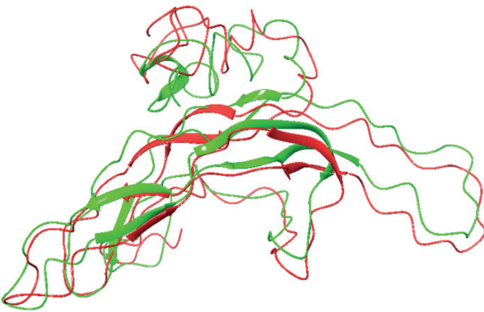

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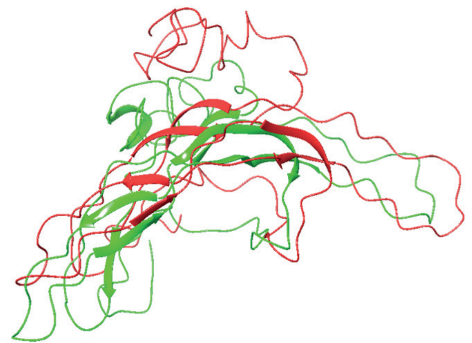
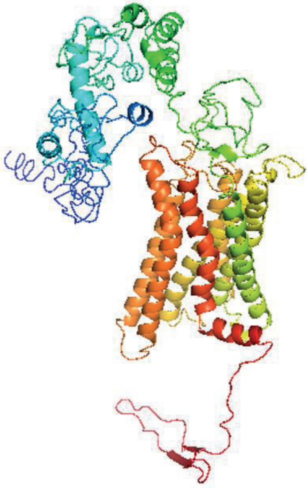


6I



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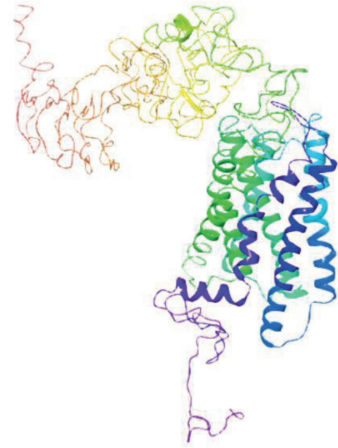

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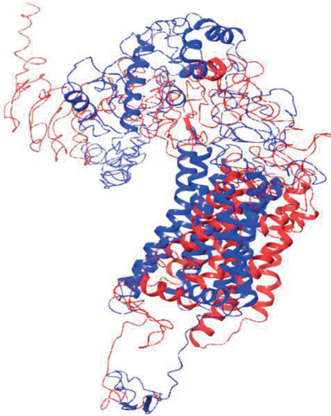

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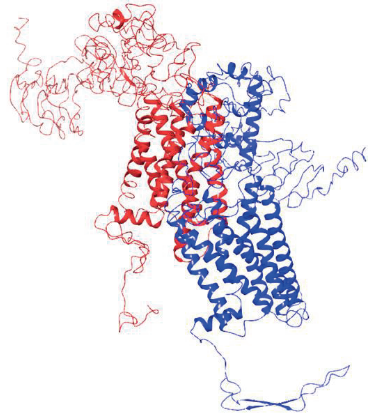

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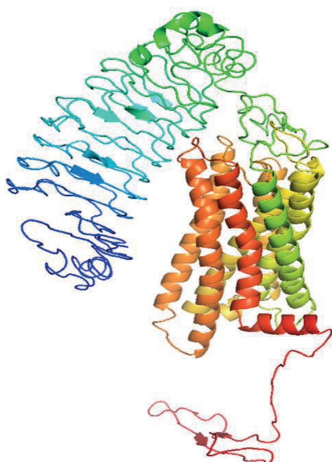

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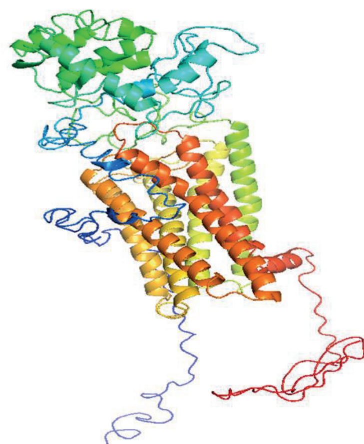

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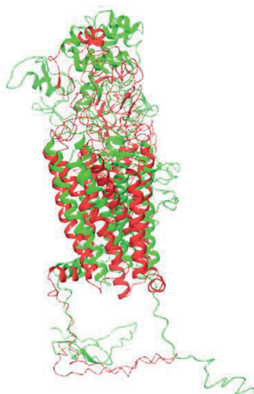

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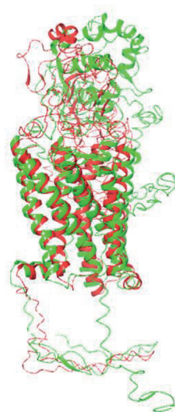
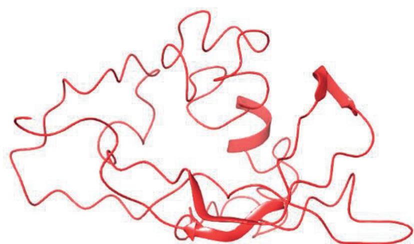
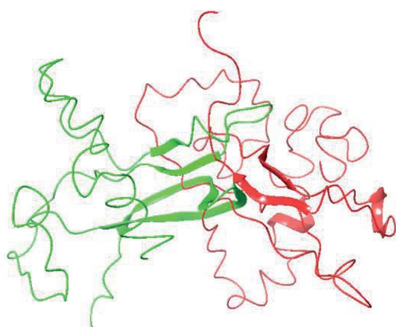


6Q



6R

6S

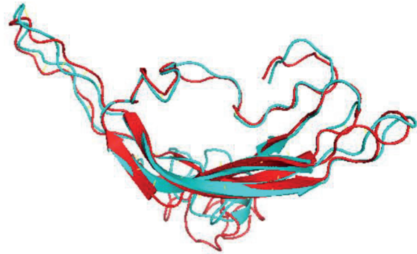


10. Supplementary data

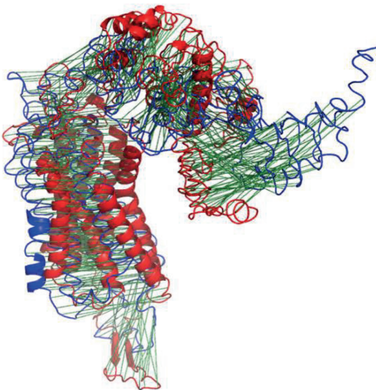
Fig. S1 Medaka and Nile tilapia gonadotropin β-subunit and gonadotropin receptor models. Structural overlapping of medaka Fshβ (blue) with Nile tilapia Fshβ (red) (a), medaka Lhβ (red) with Nile tilapia Lhβ (cyan) (b), medaka Fshr (blue) with Nile tilapia Fshr (red) (c), and medaka Lhr (blue) with Nile tilapia Lhr (red) (d). Green lines display the corresponding counterparts.

S1a

S1b



S1c



S1d

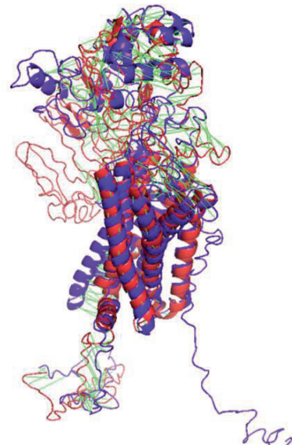


Fig. S2 Pairwise sequence alignment for medaka and Nile tilapia Fshβ using EMBOSS-NEEDLE pairwise sequence alignment tool

```
Medaka_Fshβ        1 MQLVVMAAALVLAEVGQVSSFSCHPKNVSIPVESCGISGCVHTTICEGRC     50
                     ||||||||.|.||...|..|..|.|||:|:||::||.   |.||||||:|
Tilapia_Fshβ       1 MQLVVMAAVLALAGAEQDCSSGCRPKNISLPVDTCGF---VDTTICEGQC     47

Medaka_Fshβ       51 YHEDPNYISYEDHPKEKICSGDWSYEVKFIEGCPVGFKYPVAKSCECTTC    100
                     :.:|||:|..:|.||:|.|:|:||||||:.|.||.||.||||:.||||.|
Tilapia_Fshβ      48 FQKDPNFIHTDDWPKQKTCNGEWSYEVKYTEQCPRGFIYPVARKCECTAC     97

Medaka_Fshβ      101 NTRTTYCGRLSADMPSC    117
                     |..|. ||.||..:|||
Tilapia_Fshβ      98 NANTD-CGTLSGYIPSC    113
```

Fig. S3 Pairwise sequence alignment for medaka and Nile tilapia Lhβ using EMBOSS-NEEDLE pairwise sequence alignment tool

```
Medaka_Lhβ         1 MISRVSRVMFLLMLSFILGTSTFLWSLAPAAAFQLPYCQPVKQKLSLQKE     50
                     |::::||::..||||..:|.|||:  |:||||||||.||.:.|.:||:||
Tilapia_Lhβ        1 MMAQISRMLLALMLSLFVGASTFI--LSPAAAFQLPPCQLINQTVSLEKE     48

Medaka_Lhβ        51 GCSGCHTVETTVCSGHCLTKDPLMKIR-SIQYQNVCTYRDFYYKTFELPD     99
                     ||..||.||||:|||||:||||::||. |..||:||||||.|||||||||
Tilapia_Lhβ       49 GCPSCHPVETTICSGHCITKDPVIKIPFSNVYQHVCTYRDLYYKTFELPD     98

Medaka_Lhβ       100 CLPGVDPSVTYPVALSCHCGACIMNASDCTFESLPPDFCVKHDSFYY    146
                     |.|||||.||||||||||||.|.|:.|||||||:.||||:....|||
Tilapia_Lhβ       99 CPPGVDPIVTYPVALSCHCGRCAMDTSDCTFESMQPDFCMNDIPFYY    145
```

Fig. S4 Pairwise sequence alignment for medaka and Nile tilapia Fshr using EMBOSS-NEEDLE pairwise sequence alignment tool

```
Tilapia Fshr       1 MMLVMTLMMLLIVTIKMAAASAHGSEMDIRPGFHPSLAKQTSCLSYQVMF     50
                      |:||..|||::..::||.||...:|:|             .:.:||..
Medaka Fshr        1 -MVVMIQMMLVLFRLQMAGASLPETELD-------------DVCFQVEL     35

Tilapia Fshr      51 GVTAFPSNI-SNAQCLEVKQTQIREIQQGTLSSLQHLMELTISENDLLES     99
                     |.:.|..:| ||:..:.:|||||..|.|...:.|.||.:|||.:||.|.|
Medaka Fshr       36 GFSTFLRSISSNSTVVNIKQTQITVIDQSIFTGLWHLEKLTILDNDKLLS     85

Tilapia Fshr     100 IGAFAFSGLPHLTKILISKNAALRNIGAFVFSNLPELSEIIITKSKHLSF    149
                     |...||:.||.|..:.||:|.||:.||||.|||||.|:||.||||:||:.
Medaka Fshr       86 ICPSAFANLPRLFDVSISQNMALKKIGAFAFSNLPALTEIEITKSRHLTS    135

Tilapia Fshr     150 IHPDAFRNMARLRFLTISNTGLRIFPDFSKIHSTA-CFLLDLQDNSHIKR    198
                     ||||||::..||.|.|.:|||||||||.|||||..  .|:.|||||.||:.
Medaka Fshr      136 IHPDAFRSLVGLRRLIITNTGLRIFPDLSKIHSAVHRFMFDLQDNIHIQA    185

Tilapia Fshr     199 VPANAFRGLCTQTFAEIRLTRNGIKEVASDAFNGTKMHRLFLGGNRQLTH    248
                     :||||||||||||..|||||||||:|||||||||||||||.|.||:||||
Medaka Fshr      186 IPANAFRGLCTQTVEEIRLTRNGIREVASDAFNGTKMHRLSLKGNKQLTH    235

Tilapia Fshr     249 ISPNAFVGSSELVVLDVSETALTSLPDSILDGLKRLIAESAFNLKELPPI    298
                     ||.:||||||||||||:||||::||||:|:.|||:|:||||::||.|||.
Medaka Fshr      236 ISGDAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILPPP    285

Tilapia Fshr     299 QLFTKLHQAKLTYPSHCCAFLNMHRNRSRWHSLCDNPEAKNNLHFFREYC    348
                     |.|.||..|||||||||||||.|..|:|||:|..||.:|.||....|:|::|
Medaka Fshr      286 QQFAKLRLAKLTYPSHCCAFKNKPRSRSKWSPLCSHPMAKYITDFYRDHC    335

Tilapia Fshr     349 SNSTNITCSPAPDDFNPCEDIMSATPLRILIWIISVLALLGNAVVLLVLL    398
                     ||||:|||||.||:.||||||||..|||:|||||||||||||.|||:|||
Medaka Fshr      336 SNSTSITCSPTPDNLNPCEDIMSPVPLRVLIWIISVLALLGNTVVLVVLL    385

Tilapia Fshr     399 GSRYKLTVPRFLMCHLAFADLCMGIYLVVIATVDMLTRGRYYNYAIDWQM    448
                     |||.||||||||||||||||||||||||::|||:||||.|.|||||||||.
Medaka Fshr      386 GSRSKLTVPRFLMCHLAFADLCMGIYLIIIATIDMLTHGHYYNYAIDWQT    435

Tilapia Fshr     449 GLGCNAAGFFTVFASELSVFTLTAITVERWHTITHALRLDRKLRLRHACI    498
                     ||||:|||||||||||||||||||||||||||||||||||||||:||||:
Medaka Fshr      436 GLGCSAAGFFTVFASELSVFTLTAITVERWHTITHALRLDRKLRMRHACV    485

Tilapia Fshr     499 IMTIGWIFSLLAALLPTVGISSYGKVSICLPMDVESLVSQFYVVCLLLLN    548
                     ||..|||||.|||||||||:|||.|||:||||||||||||.|:|.|||||
Medaka Fshr      486 IMAAGWIFSSLAALLPTVGVSSYSKVSVCLPMDVESLVSQVYLVSLLLLN    535

Tilapia Fshr     549 ILAFFCVCGCYLSIYLTFRKPSSAAAHADTRVAQRMAVLIFTDFICMAPI    598
                     ||||||||||||||||:||||||.||||||||||||:||||||:.|||:
Medaka Fshr      536 ILAFFCVCGCYLSIYLTYRKPSSAPAHADTRVAQRMAILIFTDFLGMAPV    585

Tilapia Fshr     599 SFFAISAALKLPLITVSDSKLLLVLFYPINSCSNPFLYAFFTRNFRRDFF    648
                     |||||||||||||
Medaka Fshr      586 SFFAISAALKLPL-------------------------------------    598

Tilapia Fshr     649 LLAARFGLFKTRAQIYRTESSSCQQPTWTSPKNSRVILYSLVNTLSLDGK    698

Medaka Fshr      599 --------------------------------------------------    598

Tilapia Fshr     699 QEC    701
```

```
Medaka Fshr      599 ---    598
```

Fig. S5 Pairwise sequence alignment for medaka and Nile tilapia Lhr using EMBOSS-NEEDLE pairwise sequence alignment tool

```
Tilapia Lhr        1 --------MALREVWLLFALSGVLNARSCCAYTCPAICRCTADSFQCSKE     42
                             ||.|.||||.|||||||||||.||.|..|||||.|:|||::.
Medaka Lhr         1 MSCGPPARMAPRVVWLLVALSGVLNARSCQAYPCHPICRCTPDTFQCNRG     50

Tilapia Lhr       43 TQLASRTGPTSVLRLRLTHLPLKRVPSHAFKELINITIIEISQSDCITHI     92
                     ||||:.|...   ||||||||||:||:||||||:|||||||||||.||.|
Medaka Lhr        51 TQLAAGTAEH---RLRLTHLPLKQVPTHAFKELMNITIIEISQSDRITAI     97

Tilapia Lhr       93 QTHAFLSLYSLAQISVQNINSLRFIEKGAFADLPKLEYLSISNTGIAHFP    142
                     |.||||||:||.||.|.||||||.||:|||.|||:||:|:|||||:.|||
Medaka Lhr        98 QRHAFLSLHSLQQILVLNINSLRGIERGAFTDLPRLEFLTISNTGMMHFP    147

Tilapia Lhr      143 DFTTISSLSPNIILEMADNMEIDIIPANSFQGITEEYVDMNLVRNGFKEI    192
                     |||::|||:|:|:|||.|||.||:||||||:|:|:.:.:|||||||||||
Medaka Lhr       148 DFTSVSSLTPSILLEMLDNMRIDVIPANSFRGMTKGHANMNLVRNGFKEI    197

Tilapia Lhr      193 KSHAFNGTKLNTLVLRDNWYLRNIQEDAFEGATGPTLLDVSSTALRSLPP    242
                     :||||||||||.|:||||.:|..|.|||||.||||:.||||||||.:||.
Medaka Lhr       198 QSHAFNGTKLNNLILRDNRFLSYIAEDAFEEATGPSYLDVSSTALSALPA    247

Tilapia Lhr      243 NGLRHVKFLKASHAYALKSLPLLESLAELLEAELTYPSHCCAFHTWRRKQ    292
                     .||..|:.|||:..:||||||.|:|||||||||||||||||||..|||||
Medaka Lhr       248 KGLTRVQTLKATATFALKSLPPLQSLAELLEAELTYPSHCCAFDKWRRKQ    297

Tilapia Lhr      293 RESALKNLTKFCDLMNTEIDPTADDTSLINDINFQYPDLEFDCFSNPFVK    342
                     ||:||||.||.|:|..|||:.|.|..:|:|||.|:||||||||.|||||.
Medaka Lhr       298 RENALKNSTKLCNLGETEIEATDDGMNLVNDIKFEYPDLEFDCGSNPFVI    347

Tilapia Lhr      343 CSPKPDAFNPCEDLLGFSFLRCLTWIIMVFAVAGNLAVLVILLIGHHKLT    392
                     |:|.||.||||||||.::|||||||||.:||||||||||||||:.|||||
Medaka Lhr       348 CTPTPDDFNPCEDLLRYAFLRCLTWIITIFAVAGNLAVLVILLVSHHKLT    397

Tilapia Lhr      393 VSRFLMCNLAFADLCMGLYLILIAFMDYHSHHEYYNHATDWQTGPGCGIA    442
                     :|||||||||||||||||||:||||||:||.|||||||||||||||||.|
Medaka Lhr       398 ISRFLMCNLAFADLCMGLYLMLIAFMDFHSRHEYYNHATDWQTGPGCGTA    447

Tilapia Lhr      443 GFLTVFSSELSVYTLTVISLERWHTITNAMHVNKRLRMHHVTAMMVGGWA    492
                     ||||||:||||||||||||:|||||||||||||||||||||||||..||.
Medaka Lhr       448 GFLTVFASELSVYTLTVISIERWHTITNAMHVNKRLRMHHVTAMMAAGWV    497

Tilapia Lhr      493 FSLLVALLPLVGVSSYSKVSICLPMDIDTLGAQVYVVAVLILNVVAFLVV    542
                     ||||||||||||||||||||||||||||||.:|||||..|||||.|||||
Medaka Lhr       498 FSLLVALLPLVGVSSYSKVSICLPMDIDTLSSQVYVVTFLILNVAAFLVV    547

Tilapia Lhr      543 CYCYICIYLSVHNPEHSTRRGDTKIAKRMAVLIFTDFLCMAPISFFAISA    592
                     |:|||.||:||.|||||||.||||||||||||||||||||||||||||||
Medaka Lhr       548 CFCYIGIYVSVRNPEHSTRNGDTKIAKRMAVLIFTDFLCMAPISFFAISA    597

Tilapia Lhr      593 ALRMPLITVSHSKILLILFYPINSLCNPFLYTIFTRAFRKDVCLLLSRCG    642
                     |||||||||||||||||||||||||||||||||.|||||||||.|.|||.
Medaka Lhr       598 ALRMPLITVSHSKILLILFYPINSLCNPFLYTISTRAFRKDVCRLASRCS    647

Tilapia Lhr      643 CCNSHADFYRSQTLGSHLTCTQKMSKREPHSLGFYAYHIKMKGCFLNKGT    692
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                         ||.::|:..||:  .|...|.::::.::||||.|||||||||||||::|.
Medaka Lhr      648 CCRANANSCRSK--ASQQACARRVTSQKPHSLNFYAYHIKMKGCFLSEGA    695

Tilapia Lhr     693 T    693
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Medaka Lhr      696 T    696
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III

Spatial and temporal expression of *fshb* during early development in medaka (*Oryzias latipes*) using a novel transgenic line tg(*fshb*:DsRed2)

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Keywords: Follicle-stimulating hormone; *fshb* promoter; Gonadotropins; *Oryzias latipes*; Red fluorescent protein; Transgenic fish

Abstract

The gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), produced in gonadotrope cells of the pituitary, play key roles in the endocrine control of vertebrate puberty and reproduction as part of the hypothalamo–pituitary–gonad axis. Contrary to in mammals, separate cell types produce the two gonadotropins in teleost fish, making them excellent models for investigating the development and regulation of the two hormones separately. In an attempt to fill the knowledge gap that is especially deep for Fsh, we have generated a transgenic line tg(*fshb*:DsRed2) of medaka expressing red fluorescent protein (Rfp, DsRed2) under the control of the endogenous medaka *fshb* promoter. The line was confirmed using fluorescence *in situ* hybridization (FISH) revealing co-localization of *dsred2* and *fshb* transcripts in adult pituitary cells. The ontogeny of *fshb*-producing gonadotropes using the transgenic line was followed by fluorescent imaging to determine spatial and temporal expression patterns. Cells expressing these genes were localized in the median part of the pituitary (*proximal pars distalis*), for the most part distributed from the ventral to the dorsal part. Fluorescent imaging analysis showed initial DsRed2 expression in larvae at the age of 8 days post fertilization (dpf). The number of Fsh cells increased gradually with development from embryonic to juvenile stages and further to adults, and there were no differences between sexes. Interestingly, some cells were found in the ventral pituitary surface in juvenile fish, while cells were mostly distributed inside the pituitary in mature fish. Compared to literature, *fshb* mRNA-positive cells have been first detected at 4 dpf in zebrafish (*Danio rerio*), and Fshβ cells at 6 dpf in medaka and at 15 days post hatching (dph) in salmonid (*Oncorhynchus mykiss*). Summarized, our results suggest a functional role for this early *fshb* expression in medaka, which is not unraveled yet. Taken together, we generated a powerful model to characterize the developmental regulation of Fsh-producing gonadotropes by establishing a novel transgenic line.

1. Introduction

Sexual maturation and reproduction in teleosts, as in other vertebrates, is controlled by the brain-pituitary-gonad (BPG) axis (Norris, 1997; Schulz et al., 2000). The pituitary hormones provide the connection between the brain (hypothalamus) and peripheral glands or organs, and are essential for important physiological processes, such as reproduction, but also growth and development, and regulation of homeostasis (Weltzien et al., 2014). The pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), are key players in the endocrine control of vertebrate puberty and reproduction. Fsh and Lh are part of a larger family of cysteine knot-forming polypeptide glycoproteins which form non-covalently linked heterodimers between a common α-subunit that is strongly but non-covalently linked to a hormone-specific β-subunit that defines the biological activity and specificity of the hormone. In their structure, both subunits consist of a cysteine knot, two *N*-linked glycosylation sites, stabilized by intramolecular disulphide bonds. All subunits Fshβ, Lhβ, and the common α-subunit are encoded each by a distinct gene (Yaron et al., 2003). In teleosts, Fsh and Lh are produced and released from different pituitary cell types (Nozaki et al., 1990; Naito et al., 1991; Naito et al., 1993; Kanda et al., 2011; Golan et al., 2016). In mammals, however, there is only one pituitary cell type that releases both gonadotropins (Nakane, 1970). The synthesis and secretion of Fsh and Lh is mainly controlled by gonadotropin-releasing hormone (Gnrh), in addition to a number of stimulating and inhibiting factors from the hypothalamus and preoptic area such as kisspeptins, neuropeptide y, dopamine, and gonadotropin-inhibitory hormone, and also by gonadal feedback loops (Levavi-Sivan et al., 2010; Clarke, 2011). Upon release, Fsh and Lh bind to their cognate receptors in the gonads stimulating steroidogenesis and gametogenesis (Weltzien et al., 2004). Although there are species differences and much is yet to be resolved regarding regulatory mechanisms, the two gonadotropic hormones have different functions in gonadal maturation. Fsh is involved in early gametogenesis and vitellogenesis through the stimulation of estradiol synthesis, whereas Lh is crucial for processes leading to final oocyte maturation and ovulation in females and spermiation in males (Yaron et al., 2003 and reviewed by Levavi-Sivan et al., 2010).

The pituitary gland or hypophysis is a vertebrate invention and key endocrine organ that produces and secrets eight to ten hormones from specific cell types (Weltzien et al., 2014). As in mammals, the pituitary in teleosts is composed of the neurohypophysis (NH) or posterior pituitary, comprising the pars nervosa (PN), and of the adenohypophysis (AH) or anterior pituitary,

containing the *pars distalis* (PD) and *pars intermedia* (PI) (Wingstrand, 1966). The PD is further divided into the *rostral pars distalis* (RPD) and *proximal pars distalis* (PPD). The neurohypophysis derives from the ventral diencephalon, constituting the neural compartment of the pituitary. The adenohypophysis on the other hand, has endothelial origin and represents the non-neural part of the gland (Levavi-Sivan et al., 2010). Compared to mammals, where the NH constitutes the posterior and AH the anterior lobe of the gland, the NH of teleosts is located dorsal of the AH (Chapman et al., 2005). In tetrapods, hypothalamic neurohormones are released to the median eminence portal system. However, in teleosts, the anterior pituitary endocrine cells are directly innervated by hypothalamic axons (Pogoda and Hammerschmidt, 2007). This enables a more precise control of individual endocrine cells, since hypophyseal axon terminals innervate the AH and abut the endocrine cells. Thus, this mode of regulation replaces the neurovascular mode of delivery that is common in most vertebrates with a more direct neuroglandular strategy (Holmes and Ball, 1974). In addition, the gonadotropes in mammals are distributed throughout the ventral portion of the PD, whereas in teleosts, both gonadotropes expressing *fshb* and *lhb* are located mainly in the ventral portion of the PPD, with some cells present in the PI of the pituitary gland (Weltzien et al., 2003).

Specific pituitary cell types are defined by differential expression of transcription factors during early embryonic development (Pogoda and Hammerschmidt, 2009). In zebrafish (*Danio rerio*), cell specification of many fish adenohypophysis cell types has been determined by gene knockdown studies and forward mutagenesis screens (Herzog et al., 2004; Nica et al., 2004, 2006; Pogoda et al., 2006). However, knowledge is limited regarding the early development of pituitary gonadotropes producing Fsh and Lh (Hildahl et al., 2012; Weltzien et al., 2014).

Both *fshb* and *lhb* gonadotropin subunit expression can be revealed from embryonic developmental stages in mammals (Asa et al., 1988; Brooks et al., 1992; Japon et al., 1994; Pope et al., 2006; Szarek et al., 2006) and fish (Nica et al., 2006; Chen and Ge, 2012; Hildahl et al., 2012). However, details regarding the function of this early expression are barely known (Weltzien et al., 2014). The ontogeny of pituitary cell types has been investigated in zebrafish (Nica et al., 2006; Chen and Ge, 2012), tilapia (*Oreochromis niloticus*, Sakai et al., 2005), rainbow trout (*Onchorhynchus mykiss*, Saga et al., 1993), American shad (*Alosa sapidissima*, Laiz-Carrion et al., 2003), and Ayu (*Plecoglossus altivelis*, Saga et al., 1999) by *in situ* hybridization or immunohistochemistry. These studies revealed that gonadotropins appear after the other pituitary

cell types, first appearing at hatching or post-hatch, similar to in mammals (Weltzien et al., 2014). Using the PCR method, *lhb* expression was revealed earlier by 72 hours post fertilization (hpf) (protruding mouth) in zebrafish larvae (Nica et al., 2006) and in medaka by 18 hpf (mid-gastrula, Hildahl et al., 2012). Fshβ cells were first detected at 6 days post fertilization (dpf) in medaka (Horie et al., 2014) and at 15 days post hatching (dph) in rainbow trout (*Oncorhynchus mykiss*) by means of immunohistochemistry, and at 4 dpf in female zebrafish by means of *in situ* hybridization (Chen and Ge, 2012). In short, the *fshb* and *lhb* ontogeny during fish larval development remains to be fully investigated.

The development of the *lhb*:Gfp transgenic line of medaka (established by Hildahl et al., 2012) enabled to investigate the medaka expression of *lhb* during embryonic development. With this transgenic line containing the Lh beta subunit gene (*lhb*) promotor driving Gfp expression, it was revealed that cells which are expressing *lhb* are initially localized outside the primordial pituitary in the developing gut tube as early as 32 hpf. At hatching, *lhb*-Gfp could be detected within the gut epithelium and in the anterior digestive tract. *lhb*-Gfp expression was shown to be consolidated in the developing pituitary by 2 weeks post fertilization. These results, indicating that *lhb* is expressed in the developing gut from an early stage long time before the first expression within the pituitary, suggest a function during gut development, possibly related to early steroidogenesis or osmoregulation (Hildahl et al., 2012). Concerning the expression of medaka Fsh, there are indications of very early expression also of *fshb* in medaka, but the functional role is not clear (Hildahl et al., 2012).

The Japanese medaka (*Oryzias latipes*) is a powerful model system for developmental and functional genomic studies due to its rapid development in transparent eggs, providing an excellent model for microinjection studies (Kinoshita et al., 2009). In addition, medaka is one of the few teleosts possessing the male heterogametic (XX-XY) sex-determining system (*dmy* gene in male), allowing genetic sex determination already from fertilization (Aida, 1921). Furthermore, there are distinct gonadotropes producing Lh and Fsh, as in all teleost fish (Kanda et al., 2011), thus providing a good model organism for characterizing gonadotropin regulation. Gonadotrope regulation is poorly understood, and characterization of these cells during embryonic development is still missing (Hildahl et al., 2012). Therefore, we chose to utilize medaka to characterize spatial and temporal expression patterns of *fshb* during early development. To address our aim, we produced a novel transgenic line of medaka, tg(*fshb*:DsRed2), with the endogenous *fshb* gene

promoter driving expression of Red fluorescent protein (DsRed2). The transgenic line will complement our tg(*lhb*:Gfp) line (Hildahl et al., 2012; Fontaine et al., in press.). This line provides a sensitive method to track expression patterns of *fshb* during development of medaka.

2. Materials and Methods

2.1 Animals

Japanese Medaka (*Oryzias latipes*) of the dr-R strain were used for all experiments. Wildtype and transgenic fish were kept in re-circulating systems with water temperature of 28 ± 1°C and light-dark cycle of L14:D10. Embryos were collected from female fish just after the lights were turned on and spawning began. They were rolled on paper to remove connecting fibers from chorion and placed in embryo culture (E3) medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ (all Sigma-Aldrich, St. Louis, U.S.A.)). For microinjections, embryos were kept on ice to cease development. Following injections, embryos were incubated at 26°C until hatching, and thereafter transferred to system tanks. A combination of dry feed and live brine shrimp nauplii larvae (*Artemia salina*) was used to feed the fish. Handling, husbandry and use of fish were in accordance with the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences.

2.2 Generation of stable transgenic line tg(*fshb*:DsRed2)

The medaka transgenic line, tg(*fshb*:DsRed2), was generated using the commercial vector DsRed2-N1 (Clontech, California, U.S.A.), which was digested partially by *NcoI* (New England Biolabs, Massachusetts, U.S.A.) and *NotI* (New England Biolabs) restriction enzymes. A DNA fragment (686 bp) coding for red fluorescent protein 2 (DsRed2) was purified by DNA gel extraction. This DNA fragment was cloned into *I-SceI*-MCS-leader-Gfp-trailer plasmid (gift from Felix Loosli, Karlsruhe Institute of Technology (KIT), Germany), replacing green fluorescent protein (Gfp) by *NcoI* and *NotI* sites, generating *I-SceI*-MCS-leader-Rfp2-trailer (transgenic construct, see Fig.1).

The medaka *fshb* promoter (3833 bp, for *fshb* promoter sequence see supplementary data) was retrieved from *Oryzias latipes* - Ensembl genome browser 90 (Genomic location 3:8375432-8379264). PCR primers with overhang to *KpnI* (forward:

GGCAACCTCCCTGACTGGCCTAAA) and *XhoI* (reverse: CAGTTAGCCGCCCATGCCGCTGCAG) were designed to amplify the *fshb* promoter. The PCR product was generated using Taq DNA polymerase (Invitrogen, California, U.S.A.) and a touchdown annealing PCR program. The PCR parameters were 1 cycle at 94°C for 2min, followed by 10 cycles at 94°C for 15s, 60°C to 50°C for 15s for 10 cycles, and 72°C for 3 min. Annealing started at 60°C and decreased by 1°C for each cycle. The amplification continued with 94°C for 15s, followed by 30 cycles at 50°C for 15s, 72°C for 3 min, and ended with 72°C for 5 min. The PCR product was cloned into pGEM-T Easy vector (Promega, Wisconsin, U.S.A.), digested with *KpnI* (New England biolabs) and *XhoI* (New England biolabs), purified by gel extraction and cloned into *I-SceI*-MCS-leader-Rfp2-trailer.

The optimal concentration of injected *I-SceI*-DNA was determined experimentally. With a manual microinjector (Picospritzer III, Parker Automation, Ohio, U.S.A.), about 1 to 2 nl with concentration of 10 µg/µl was injected into the cytoplasm of the one-cell stage medaka embryos together with the injection solution (0.5 x commercial meganuclease buffer (Roche Diagnostics, Germany or New England Buffer, U.S.A.); 1 units/µl meganuclease *I-SceI*; 0.1 % phenol red). About 25 embryos were microinjected in 10 batches with an approximate mortality rate of 50 % (survival was examined 24 h after injection). These F_0 fish were grown to adulthood and crossed with each other. The resulting F_1 embryos were checked for fluorescence on 5-6 days post fertilization (dpf) to identify founders (individuals with the *I-SceI*-DNA inserted into the germ cells). Following identification, the F_1 offspring of a F_0 founder fish were crossed with each other. The DsRed2-positive F_2 generation was further crossed with wildtype fish. F_2 transgenic fish that produced 90-100% DsRed2-positive progeny were defined to be homozygous for tg(*fshb*:DsRed2). A male and female individual among the identified homozygote F_2 fish were crossed with each other to produce a stable homozygous line. This crossing procedure was repeated with a second F_0 founder fish as a control to assure specificity of the observed Rfp expression pattern.

2.3 Fluorescence *in situ* hybridization (FISH) - Confirmation of the transgenic line tg(*fshb*:DsRed2)

Fluorescence *in situ* hybridization (FISH) was performed on free-floating parasagittal brain-pituitary sections as described previously (Fontaine et al., 2013). Briefly, after being sacrificed

with ice water, brain and pituitary from unsexed 6 months-old adult fish were dissected and fixated overnight with 4% PFA at 4°C. Tissues were then gradually dehydrated with a series of increasing concentrations of ethanol, and stored in 100% methanol until used. Tissues were rehydrated, embedded in 3% agarose (H_2O) and parasagittally sectioned (60 µm sections) using a vibratome (VT1000S Leica, Wetzlar, Germany). They were then treated with proteinase K (1 µg/ml; P6556, Sigma-Aldrich) for 30 min at 37°C. *fshb* riboprobe from a previous publication (Burow et al., 2018) was used. *dsred2* ribroprobe was cloned into PCR-II Topo (Invitrogen) using AGTTCATGCGCTTCAAGGTG and GTGTAGTCCTCGTTGTGGGA primers. Antisense riboprobes were conjugated with digoxigenin (DIG; 11277073910; Roche, Basel, Switzerland) for *dsred2* and with fluorescein (FITC; 11685619910 Roche) for *fshb* using SP6 or T7 RNA polymerase (Promega). Tissues were hybridized with both antisense riboprobes for 18 hours at 55°C and then incubated with sheep anti-DIG conjugated with peroxidase (POD; 1:250; 11207733910; Roche) and anti-FITC (POD; 1:250; 11426346910; Roche). *dsred2*-DIG probe signal was revealed using TAMRA-conjugated tyramide and *fshb*-FITC probes with FITC-conjugated tyramide constructed in our lab.

2.4 Fluorescent imaging - Ontogeny of the transgenic line tg(*fshb*:DsRed2)

In order to determine when Rfp could first be detected, 8 to 12 fish per stage (8 dpf, 10 dpf and 12 dpf larvae, 1 month and 2 months juveniles, and 4 month adults) were quickly fixed with 4% PFA for 4 hours at room temperature. After a few washes with PBST, brain and pituitaries were dissected, embedded in 3% agarose (H_2O) and parasagittally sectioned with a Leica VT1000S vibratome. Tissue sections were incubated for 20 min with DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride; 32670 Sigma) and mounted between glass slide and coverslip with Vectashield (H-1000 Vector Laboratories, United Kingdom) mounting medium.

2.5 Imaging

All confocal images were acquired using a Zeiss LSM710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) with 25X (N.A. 0.8) or 40X (N.A. 1.2) objectives. Channels were acquired sequentially to avoid signal crossover between the different filters. Images were processed using the ZEN software (version 2009, Zeiss). Z-projections from confocal stacks of images were

obtained using Image J software (http://rsbweb.nih.gov/ij/). Composites were assembled using Adobe Photoshop and Indesign CS6 (Adobe Systems, San Francisco, California). 3D reconstruction was built using 3D viewer plugin (Fiji software).

Cell counting and statistics. Counting was performed as described in Fontaine et al. (in press.) on sexed tg(*fshb:*DsRed2) juveniles (2 months) and mature adult fish (6 months), without knowing from which fish the files belonged. Pituitaries from 9 adult fish from each sex and 9 juvenile fish from each sex were mounted between slide and coverslip with spacers and Vectashield mounting medium. Briefly, images were acquired as described above and selected z-sections about one cell-size diameter apart were analyzed using Cell Profiler software (version 2.1.0). Potential significance between Rfp and DAPI cell numbers was calculated by one-way ANOVA followed by Tukey test using the Graph-Pad Prism software (version 7; GraphPad, San Diego, U.S.A.). Data are presented as mean ± SD.

3. Results

3.1 Generation of stable transgenic line tg(*fshb*:DsRed2)

In order to more accurately investigate *fshb* development, we established a transgenic medaka line tg(*fshb*:DsRed2) that express DsRed2 under the control of the endogenous medaka *fshb* promoter. Two transgenic founder fish were identified by crossing the 60 surviving F_0 generation fish that had been injected with the transgenic construct (Fig. 1). The two transgenic founder fish had identical spatial and temporal expression patterns in their F_1 offspring. Thus, the pattern of DsRed2 expression detected was unlikely to have been a consequence of a transgene position effect. The stable transgenic line tg(*fshb*:DsRed2) was made using the identified homozygote progeny of one founder fish in the F_3 generation.

3.2 Fluorescence *in situ* hybridization (FISH) - Confirmation of the transgenic line tg(*fshb*:DsRed2)

In order to confirm the transgenic line, FISH was performed on free-floating parasagittal brain-pituitary sections. Expression of *dsred2* in cells where *fshb* was transcribed was confirmed by revealing the co-localization of *dsred2* and *fshb* transcripts in specific adult pituitary cells by FISH.

It was observed that both *fshb-dsred2* (Fig. 2A) and *fshb* (Fig. 2B) transcripts were co-expressed in the same area of the pituitary since the endogenous fluorescence was detected in a similar area of the pituitary. The merge of both *fshb-dsred2* and *fshb* confirms this suggestion since an expression overlap of *fshb-dsred2* and *fshb* could be revealed (Fig. 2C). The DAPI staining together with staining of *fshb-dsred2* and *fshb* showed the nuclei of all *fshb-dsred2* expressing cells and the nuclei of all other cell types in the entire pituitary (Fig. 2D). Cells expressing *fshb* were localized in the median part of the pituitary (*proximal pars distalis*), mainly distributed from the ventral to the dorsal part (Fig. 2B). The expression of both markers *fshb* and *dsred2* was shown to be located mostly in the cytoplasm.

3.3 Fluorescent imaging - Ontogeny of the transgenic line tg(*fshb*:DsRed2)

To reveal temporal and spatial expression patterns of DsRed2, and most importantly to determine the first ontogenic appearance of *fshb* positive cells, a fluorescent imaging analysis was performed. Larvae (8 dpf, 10 dpf, 12 dpf), juvenile fish (1 month, 2 months), and mature adult fish (4 months) were used and the endogenous DsRed observed from larval stages to adulthood with a confocal microscope. In total, 8 to 12 fish per stage (8 dpf, 10 dpf, 12 dpf larvae; 1 month, 2 months juveniles; 4 months adults) were investigated.

Interestingly, while no DsRed2 was detected in 7 dpf embryos, DsRed2 was first expressed in larvae at the age of 8 dpf; at this stage, only one cell was found in 2 over 8 animals. The cell was located in the median part of the pituitary (*proximal pars distalis*) (Fig. 3 K, L).

Between the age of 10 dpf (Fig. 3 I, J), 12 dpf (Fig. 3 G, H), 1 month (Fig. 3 E, F), 2 months (Fig. 3 C, D), and 3 months old fish (Fig. 3 A, B), the intensity of DsRed2 in each cell was shown to increase. In mature adult fish, DsRed2 was detected in the median part of the pituitary, mainly distributed from the ventral to the dorsal part. Important to notice is that some cells appeared in the pituitary ventral surface in juvenile fish, while cells were mostly distributed inside the pituitary in mature adult fish. The DsRed2 cells seemed to be spread out at the different stages, and not clustered and congregated with other DsRed2 cells. Expression of DsRed2 was not observed outside the pituitary in any of the investigated stages.

Furthermore, the number of cells in the pituitary in 2 months-old and 6 months-old fish was determined. A comparison between the total number of cells per pituitary, the number of Fsh

cells (DsRed2) per pituitary, and the percentage of Fsh cells per pituitary for juveniles (2 months) and mature fish (6 months) for males and females was performed. The total number of cells per pituitary increased from 6400 (2 months; average) to 13000 (6 months; average), the number of Fsh cells (DsRed2) per pituitary raised from 330 (2 months) to 1300 (6 months), and there was an increase in the percentage of Fsh cells per pituitary from 5 % (2 months) to 10 % (6 months) (Fig. 4). Summarized, the number of Fsh cells increased significantly between juveniles and adults, with no differences between sex.

Due to technical reasons, we were not able to determine the exact location of the cells at each stage because tissue sections for both FISH and fluorescent imaging were utilized, missing a three dimensional structure of the pituitary. Whether there are indications for cell migration to or within the pituitary, or if cells seem to originate at one place before migrating to their final location cannot be answered in the present study. This is because cells and time lapses were not tracked, and therefore a distinction between moving cells and arising cells cannot be made (further methodological considerations in the Discussion below).

4. Discussion

In the current study, we (a) generated a transgenic line of medaka expressing DsRed2 under the control of the endogenous medaka *fshb* promoter, (b) confirmed the transgenic line using FISH, and (c) investigated the ontogeny of the transgenic line by fluorescent imaging to determine the spatiotemporal expression pattern from embryonic to adult stages. Together with our previously generated tg(*lhb*:Gfp) line, this transgenic line provides a valuable tool for studies on gonadotropin regulation in not only teleost fish, but vertebrates in general.

Methodological considerations

Potential sources of error can be introduced at various steps in the generation of a transgenic line. The method of choice to generate transgenic fish is the injection of plasmid DNA into the cytoplasm of one cell-stage embryos, providing a fast and easy technique due to the transparency and large size of most fish eggs, although rather inefficient (Thermes et al., 2002). The reason why *I-SceI* meganuclease was co-injected into the one-cell stage medaka embryos together with the injection solution in the present study is due to a strong enhancement of the promoter dependent expression already in F_0 and an increased transgenesis frequency due to integration at the one-cell

stage (Thermes et al., 2002). Generally, the injected DNA remains as long extrachromosomal concatamers transiently transcribed during early embryogenesis; an uneven distribution of the episomal DNA leads to a mosaic expression of the transgene in F_0. The DNA gets integrated into the genome at later cleavage stages, so that the DNA is stably inherited only by subsets of blastomeres (Etkin and Pearman, 1987; Westerfield et al., 1992). Therefore, transgenic founder fish transmit the transgene to only a low percentage of their offspring, resulting in low germline transmission rates (Collas and Aleström, 1998; Culp et al., 1991; Lin et al., 1994; Stuart et al., 1988, 1990; Tanaka and Kinoshita, 2001). Following the procedure of Thermes et al. (2002), a stable transgenic medaka line in the present study was generated by co-injection of the I-SceI meganuclease with a reporter construct flanked at both ends by the two corresponding recognition sites.

It was necessary to reveal that the DsRed2 expression specifically reflects the regulation of endogenous *fshb* gene and was not ectopic. In this regard, the use of controls was crucial, most importantly to check for co-localization of *dsred2* with *fshb*. During the microinjections, the transgene *I-SceI*-DNA was delivered into one-cell stage of medaka embryos to guarantee a homogeneous incorporation of the transgene across the germ cell line. On the other hand, the possibility of ectopic expression for either *fshb* or *dsred2* was reduced due to the incorporation of both genes under the control of the native *fshb* promoter, which warranted a tissue specific expression, as determined by co-localization of *dsred2* and *fshb* in adult pituitaries (Fig 2). Further possible sources of error regarding the transgenic line could be related to the possibility that the DsRed2 could be also expressed in other cells than those expressing *fshb*, and possibly not all *fshb* expressing cells express DsRed2. In this regard, another substantial control was performed by using two separate transgenic F_0 founder fish. Their Rfp expression was shown to be identical, which strongly suggests the specificity of the transgene.

Furthermore, to determine the exact location of the cells at each stage was difficult since tissue sections were used. In this regard, the distinction between the different structural parts in juvenile fish is not clear. It was impossible to determine whether there are indications for cell migration to or within the pituitary, or if cells seem to originate at one place before migrating to their final location. This is because cells and time lapses were not tracked in the present study, and therefore a distinction between moving cells and arising cells cannot be made. Investigations on changing cell shape or cell size have not been studied yet.

Colocalization of *fshb* transcripts and *dsred2* revealed by FISH in pituitaries from sexually mature 6 months-old fish confirms that *dsred2* expression specifically reflects the regulation of endogenous *fshb* gene and is not ectopic. For instance, Yoshizaki et al. (2000) generated a transgenic line in rainbow trout expressing green fluorescent protein (Gfp) under the control of the rainbow trout *vasa*-like gene *(RtVLG)* promoter. They revealed ectopic Gfp expression especially in the earlier stages, similarly to an ectopic expression of a transgene that was reported in a transient expression system in zebrafish with the *Gata1* gene promoter (Long et al., 1997). Possible explanation for the ectopic expression of the GFP gene is that *vasa*-GFP was not methylated properly during early embryogenesis, resulting in the loss of tissue specificity of the *RtVLG* promoter. In addition, the position effect (Bonnerot et al., 1990) could be a possible factor causing such ectopic gene expression. The position effect can be explained by the fact that the genetic program is determined by various developmental decisions taken during embryogenesis as a result of a combination of positional information, and also cell lineages and cell interactions. Therefore, gene expression can be correlated to the basic parameters of development, topography (related to position), cell type (related to function), and lineages (related to origin) (Bonnerot et al., 1990).

Scientific considerations

The presented ontogeny data obtained by fluorescent imaging indicate that *fshb* is expressed exclusively in the pituitary at all stages investigated, with first expression appearing at 8 dpf. Expression was not observed outside the pituitary at any stage investigated. Another study in medaka (d-rR [Shizuoka]) revealed by immunohistochemistry that Fshβ cells were first detected at 6 dpf, just after sex determination (using antiserum against mummichog Fshβ native, Fshβ protein purified from mummichog pituitary). This observation suggests that Fsh in medaka may play a role in the early stages of gonadal differentiation, indicating that Fsh cells become differentiated before hatching (Horie et al., 2014). Nevertheless, a different speed of development because of differing maintenance conditions, such as rearing temperatures or embryo culture medium, cannot be excluded between Horie et al. (2014) and the present study. In rainbow trout (*Oncorhynchus mykiss*), Fshβ cells were first detected at 15 days post hatching (dph) by means of immunohistochemistry, prior to morphological gonadal sex differentiation (Saga et al., 1993). In female zebrafish (*Danio rerio*), *fshb* mRNA expression was first observed at 4 dpf by means of *in situ* hybridization, prior to gonadal sex differentiation (Chen and Ge, 2012). This early expression

of *fshb* in development suggests that Fsh may be involved in the early stages of gonadal development as mentioned above. Regarding future studies, one interesting issue would be its potential involvement in primordial germ cell development, for instance in proliferation, migration, and survival. It cannot be excluded that Fsh may play a role in nonreproductive functions during early embryonic development (Chen and Ge, 2012). In mammals, Fsh has been demonstrated to directly increase osteoclastogenesis and bone resorption (Sun et al., 2006).

A developmental tracing in transgenic medaka detected that Gnrh neurons extend ventrally into the pituitary between 10 and 20 dpf (Okubo et al., 2006). Therefore, the present results suggest that *fshb* is expressed relatively early in the pituitary development before the gross morphology of brain and pituitary establishes and hypothalamic neurons innervate the pituitary. Therefore, we suggest that Fsh gonadotropes do not require hypothalamic input for final gonadotrope activation, which is in contrast to the gonadotrope maturation in rat (Aubert et al., 1985) and sheep (Brooks et al., 1992; Szarek et al., 2008). Partially conflicting to this is the observation that gonadotropin expression starts later than other anterior pituitary hormones in several teleost and mammalian species (Asa et al., 1988; Saga et al., 1993, 1999; Japon et al., 1994; Laiz-Carrion et al., 2003). Interestingly, the hypophysiotropic Gnrh3 neurons in zebrafish reach the pituitary earlier than the corresponding Gnrh1 neurons in medaka. Furthermore, the ontogeny of pituitary cell types has been studied in zebrafish (Nica et al., 2006; Chen and Ge, 2012), tilapia (Sakai et al., 2005), rainbow trout (Saga et al., 1993), American shad (Laiz-Carrion et al., 2003), and Ayu (Saga et al., 1999) by immunohistochemistry. These studies determined that gonadotropic hormones, similar to the situation in mammals, appear after the other pituitary cell types, first arising around the time of hatching or post-hatch.

The present study revealed that *fshb* expressing cells in adult medaka were localized in the median part of the pituitary (*PPD*), mainly distributed from the ventral to the dorsal part. This is in accordance with another study that detected *fshb* expressing cells to be located primarily to the *PPD* in adult medaka (strain d-rR [Shizuoka]) (Horie et al., 2014). Interestingly, some cells were found in the ventral surface in juvenile fish, while cells were mostly distributed inside the pituitary in mature fish. It was shown that in teleosts, both gonadotropes expressing *fshb* and *lhb* are located mainly in the PPD, as demonstrated in the present study, with some cells present in the PI of the pituitary gland (Weltzien et al., 2003). In Senegalese sole and Atlantic halibut (*Hippoglossus hippoglossus* L.) (Cerdà et al. 2008, Weltzien et al., 2003), *fshb* and *lhb* expression was revealed

in distinct gonadotropes of the PPD. In Chinese sturgeon (*Acipenser sinensis*), *fshb* cells were revealed in the middle area of the PPD and *lhb* in the middle and in the periphery of PPD and in PI (Cao et al., 2009). In European hake (*Merluccius merluccius*), the *fshb* and *lhb* expressing cells were shown to be homogenously distributed through the whole PPD and in PI (Candelma et al., 2017). In contrast to cells expressing *fshb* that were demonstrated to be arranged as small cell clusters or single cells, and *lhb* expressing cells as large clusters in zebrafish (So et al., 2005), both gonadotrope cell types were found to be arranged in large clusters in European hake (Candelma et al., 2017). This is in contrast to the present study showing DsRed2 cells not to be clustered with other DsRed2 cells. An overlapped expression of both cell types is rarely found in mammals (Nakane, 1970), as well as in teleosts where the colocalization of the two mRNAs in the same gonadotrope cell was only occasionally indicated (García Hernández et al. 2002; Weltzien et al., 2014; Candelma et al., 2017). This is in agreement with the present study since an overlapped expression of both cell types was not observed.

The present work revealed that the number of Fsh cells was observed to increase during development between juveniles and adults, and there were no differences between sexes. Regarding sex comparison, there are partly conflicting results from another study revealing that the *fshb* expression was significantly higher in XX female than in XY male medaka (d-rR [Shizuoka]) embryos at 0 dph (Horie et al., 2014). Sex differences in gonadotropin expression have been firmly demonstrated in a few other teleost species. In the adult cinnamon clownfish *Amphiprion melanopus*, *fshb*, *gpa*, and *lhb* transcript levels in the pituitary were shown to be significantly higher in females than in males, and the mRNA increased during sex determination with higher levels for females than for males (An et al., 2010). However, the expression profiles of the adult honeycomb grouper *Epinephelus merra* during sex change revealed that *fshb* expression levels in the pituitary are significantly higher in males than in females, suggesting that *fshb* is involved in the female-to-male sex change (Kobayashi et al., 2010). The participation of *lhb* in female to male sex change has also been demonstrated in some teleost fish (Yeung et al., 1993; Koulish and Kramer, 1989; Lee et al., 2000). Various reports on medaka have indicated that the sex-determining gene *dmy* regulates gonadal sex differentiation by *gonadal soma derived factor* (*gsdf*) expression (Matsuda et al., 2002; Shibata et al., 2010; Myosho et al., 2012). It was suggested that in medaka, sexual dimorphism in *fshb* expression appears after the onset of the sex-

determining gene cascade, like *dmy* and *gsdf*, indicating that this cascade regulates expression of the *fshb* subunit temporary soon after sex determination (Horie et al., 2014).

Interestingly, various studies in medaka have revealed developmental functions for multiple hormones in the HPG axis which are not involved in reproduction or development of reproductive organs, e.g. Gnrh (Okubo et al., 2006; Wu et al., 2006), Lh (Hildahl et al., 2012), and Kiss (Hodne et al., 2013). Gnrh and Kiss during early development are suggested to be implicated in axonal guidance and brain development, respectively (Weltzien et al., 2014). In zebrafish, *fshb* morpholino knockdown fish revealed abnormal morphology, like notochord distortion, tail swelling, no head, and growth retardation, suggesting that *fshb* is important for morphological development (Huang et al., 2008). Developmental aspects of reproductive endocrinology have been less in focus compared to its control of puberty, particularly for fish (Weltzien et al., 2014). The few studies that exist demonstrate that multiple hormone and receptor genes are expressed early during embryonic and larval development in mammals.

Specific pituitary cell types are determined by differential expression of several transcription factors during early embryonic development (Pogoda and Hammerschmidt, 2009). Cell specification of many cell types in the adenohypophysis has been determined in zebrafish by forward mutagenesis screens and gene knockdown experiments (Herzog et al., 2004; Nica et al., 2004, 2006; Pogoda et al., 2006). Knowledge regarding the early development of pituitary gonadotropes that produce Fsh and Lh is limited (Hildahl et al., 2012). The determination of pituitary cell types is guided by the *Bmp2-Fgf8* (mammals) or *hh-fgf3* (fish) ventral-dorsal expression gradient (Weltzien et al., 2014). According to the definition of Rathke´s pouch formation in mouse during embryonic day (E) 10-11, the pituitary cells begin to differentiate dependent on the location along the *Bmp2-Fgf8* ventral-dorsal gradient. Several factors are determining the differentiation of the gonadotrope lineage, however the coordinated program for gonadotrope maturation is largely unknown (Weltzien et al., 2014). *Gata2*, a zinc-finger transcription factor that is also expressed by ventrally located cells, stimulates in combination with *Gata4 Fshb* expression *in vitro* (Ingraham et al., 1994). Other transcription factors, like the homeobox protein *Pitx1* and transcription factor *Otx1* participate in the activation of *Fshb* expression (Tremblay et al., 1998; Acampora et al., 1998). *Msx1* also seems important in gonadotrope differentiation (Mackenzie et al., 1994), and in gonadotrope development by repressing *Gpa* expression (Xie et al., 2013). Homeobox protein ANF (*Hesx1*) is the first pituitary-

specific transcription factor that appears under the stimulation by *Otx2* (not expressed in the pituitary itself) and *Lhx3*. These are transcription factors, which are crucial for proper pituitary cell differentiation (Bach et al., 1995; West et al., 2004).

In order to determine whether there are indications for cell migration to or within the pituitary, or if cells seem to originate at one place before migrating to their final location, future investigations should include tracking of cells and time lapses which will enable a distinction between moving cells and arising cells. Furthermore, future studies should implement investigations on changing cell shapes or cell sizes, and a three-dimensional structure model of the pituitary that would allow determining the exact location of the cells at each stage. In addition, further investigations should include studies on identifying cell type(s) that expresses *fshb*, and studying further the potential functions of Fsh during early development.

In summary, we have established a powerful model to characterize the developmental regulation of Fsh gonadotropes by generating a transgenic line. The specificity of the transgenic line was confirmed by co-localization of *dsred2* and *fshb* transcripts in adult fish, and by comparing Rfp expression patterns in offspring from two separate F_0 founder fish. Cells expressing these genes were exclusively localized in the pituitary, with most cells located in the median part of the pituitary, mainly distributed from the ventral to the dorsal part, and a few cells in the posterior part. A fluorescent imaging analysis detected that DsRed2 was initially expressed in larvae at the age of 8 dpf. The number of Fsh cells was shown to increase during development with no differences between sexes. Interestingly, some cells were detected in the ventral surface in juvenile fish, while cells were mostly distributed inside the pituitary in mature fish.

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7. Figure captions

Fig. 1. Schematic overview of the *fshb*:DsRed2 transgenic construct. The medaka *fshb* promoter was amplified, cloned into pGEM-T Easy vector, digested with KpnI and XhoI, purified by gel extraction and cloned into IsceI-MCS-leader-Rfp2-trailer.

Fig. 2. Confirmation of the medaka *fshb*:DsRed2 transgenic line by double fluorescence *in situ* hybridization (FISH) of *fshb* and *dsred2* mRNA in adult medaka pituitaries. Labeling of *dsred2 (fshb-dsred2)* mRNA (A, magenta), *fshb* (B, yellow), merge of *dsRed2 (fshb-dsred2)* (magenta) and *fshb* (yellow) (C), and *dsred2 (fshb-dsred2)* (magenta) and *fshb* (yellow) and DAPI counter staining (grey) (D). Endogenous *fshb* expression was colocalized with *dsred2* expression (C). Scale bar represents 20 µm.

Fig. 3. Fluorescent imaging of the endogenous DsRed2 in transgenic medaka *fshb*:DsRed2 by confocal microscopy in order to reveal the ontogeny of endogenous DsRed2. DsRed2 detection after 4 months (A, B), 2 months (C, D), 1 month (E, F), 12 dpf (G, H), 10 dpf (I, J), and first expression at 8 dpf (K, L). Scale bar represents 20 µm.

Fig. 4. Cell counting measurement comparing the total number of cells per pituitary (A), the number of Fsh cells (DsRed2) per pituitary (B), and the percentage of Fsh cells per pituitary (C) for juveniles (2 months) and mature fish (6 months) males and females. Means marked by different letters differ significantly ($p < 0.05$).

8. Figures

Fig. 1

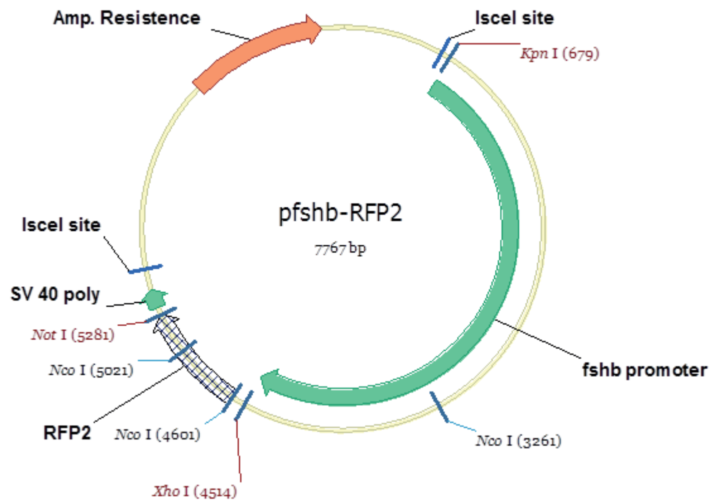


Fig. 2

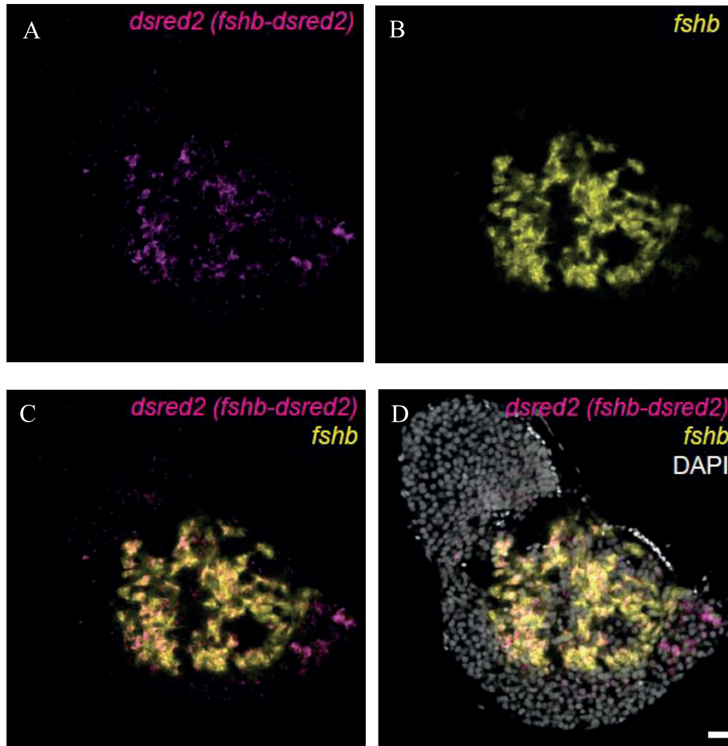


Fig. 3

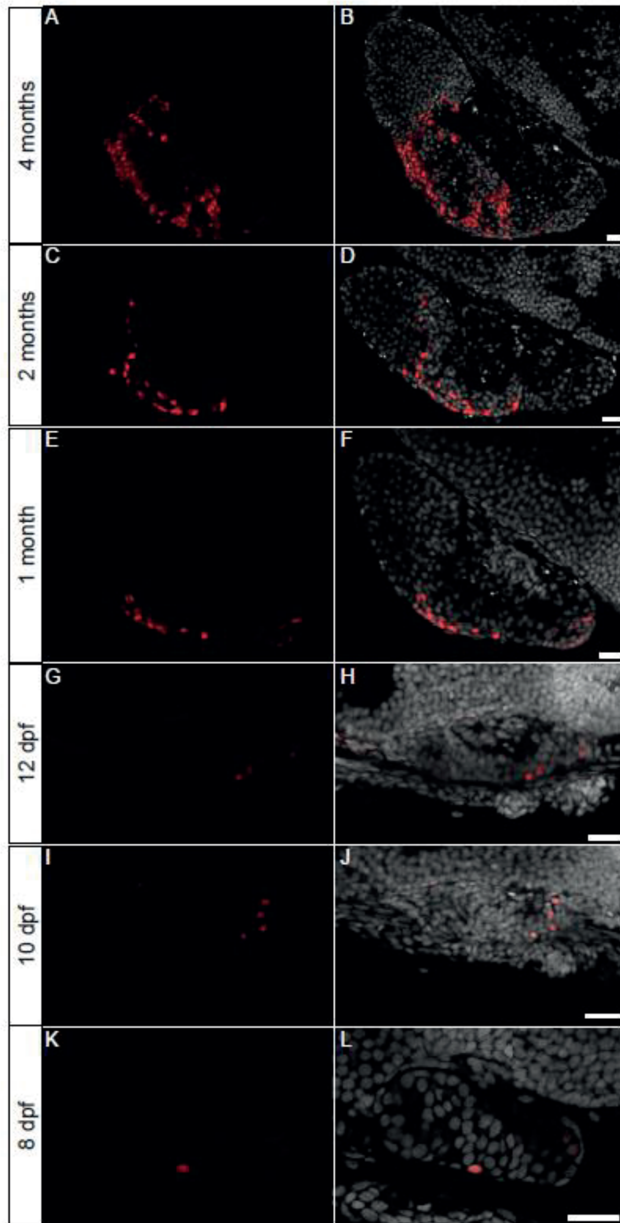


Fig. 4 A

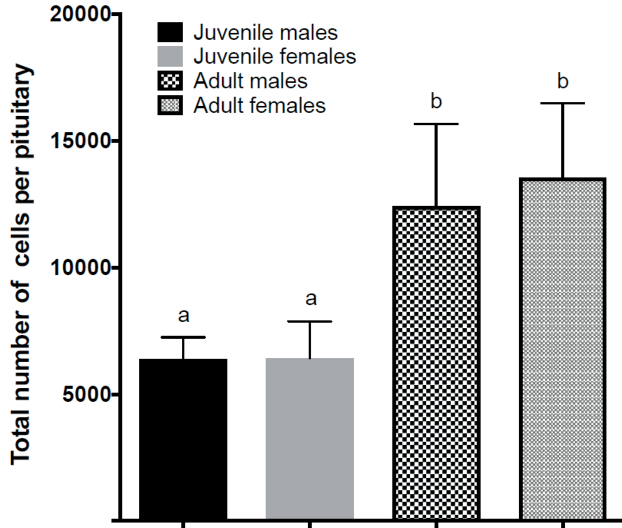


Fig. 4 B

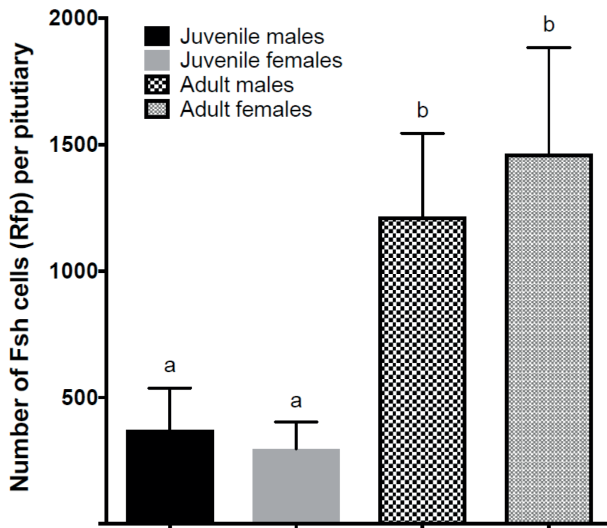
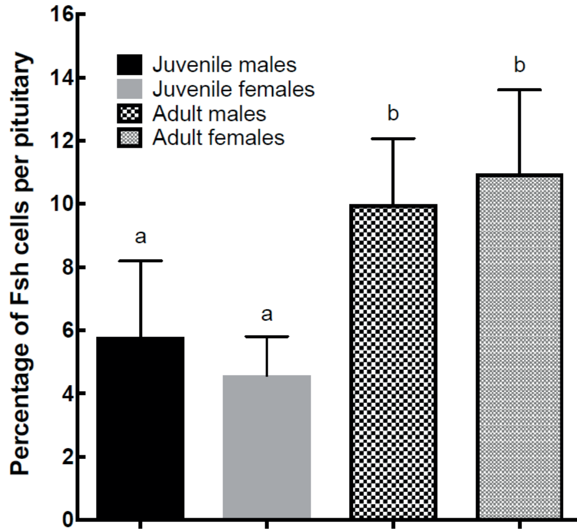


Fig. 4 C



9. Supplementary data

Medaka *fshb* promoter sequence (PCR amplicon: 3833bp)

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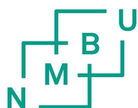

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