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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

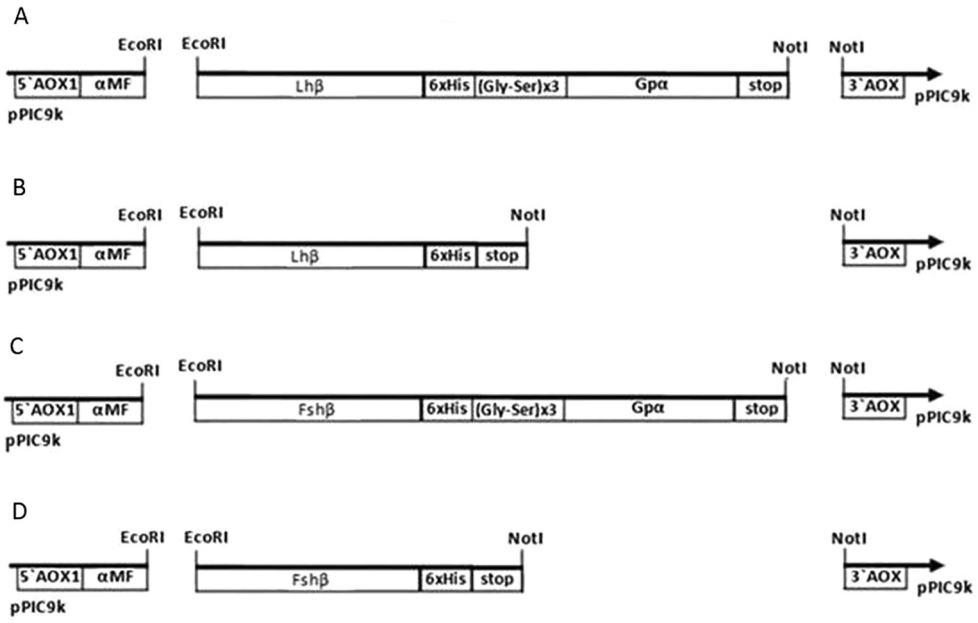

Fig. 1. Schematic overview of the synthesis of medaka *mdlhba* (A), *mdlhb* (B), *mdfshba* (C), and *mdfshb* (D). Fusion genes encoding "tethered" polypeptides in which one of the β-chains forms the *N*-terminal domain and the α-chain forms the C-terminal domain were produced for *mdlhba* and *mdfshba*. 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*

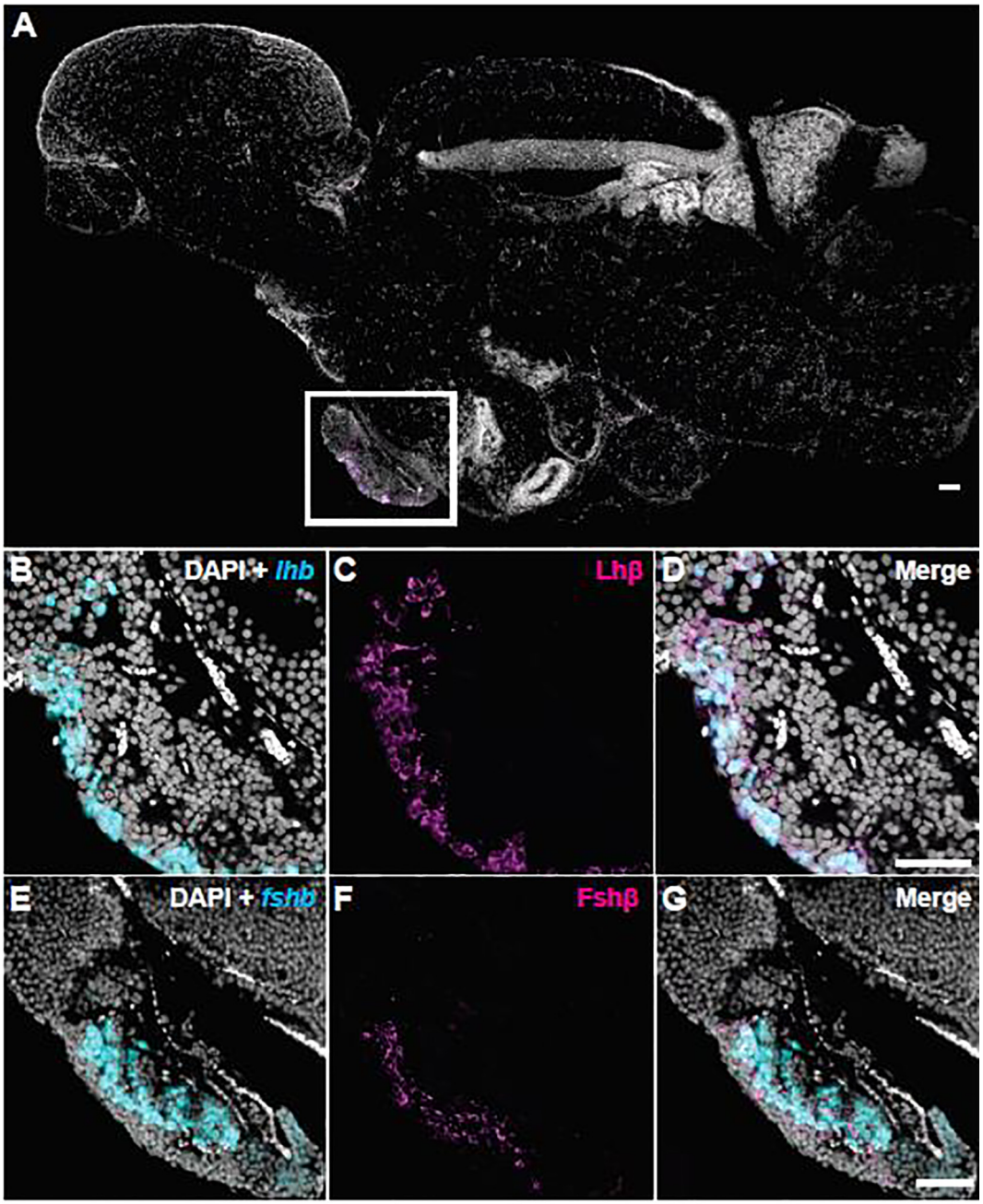

Fig. 2. Parasagittal section of brain with pituitary from 6 month-old medaka (A). Higher magnification of the pituitary representing the area from the square in (A) are presented in (B–G). Labeling of *lhb* (B) and *fshb* mRNA (E) by FISH (cyan), and Lhβ (C) and Fshβ protein (F) by IF (magenta), with 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

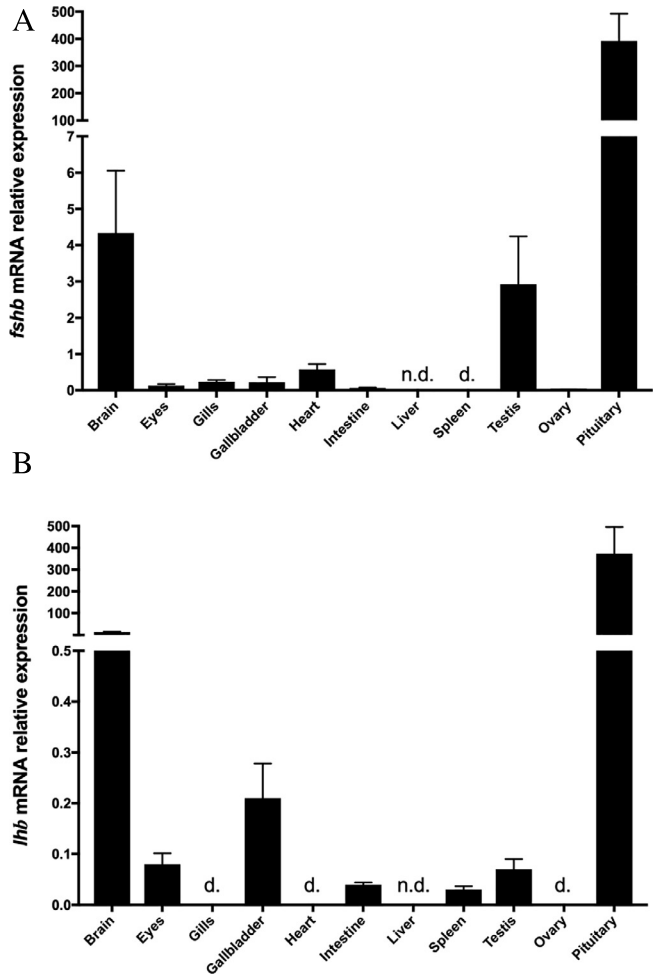

Fig. 3. Gene expression of *fshb and lhb* in various medaka tissues. (A) *fshb* relative expression, (B) *lhb* relative expression. Detectable but not quantifiable (d.), not detectable (n.d.). n = 3 per tissue.

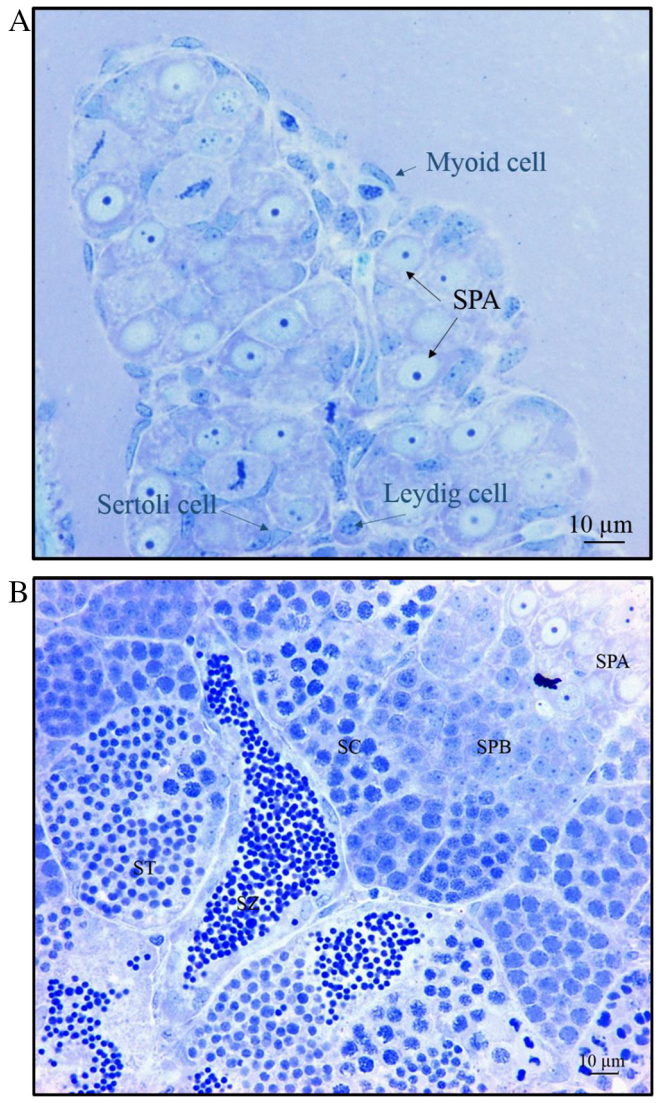

Fig. 4. Testis histology section from (A) sexually immature medaka and (B) sexually mature medaka. Section from sexually immature fish with SPA as the only germ cell present, testis section from sexually mature medaka with all germ cell stages present. Sagittal section, 3 µm thickness, stained with Toluidine 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

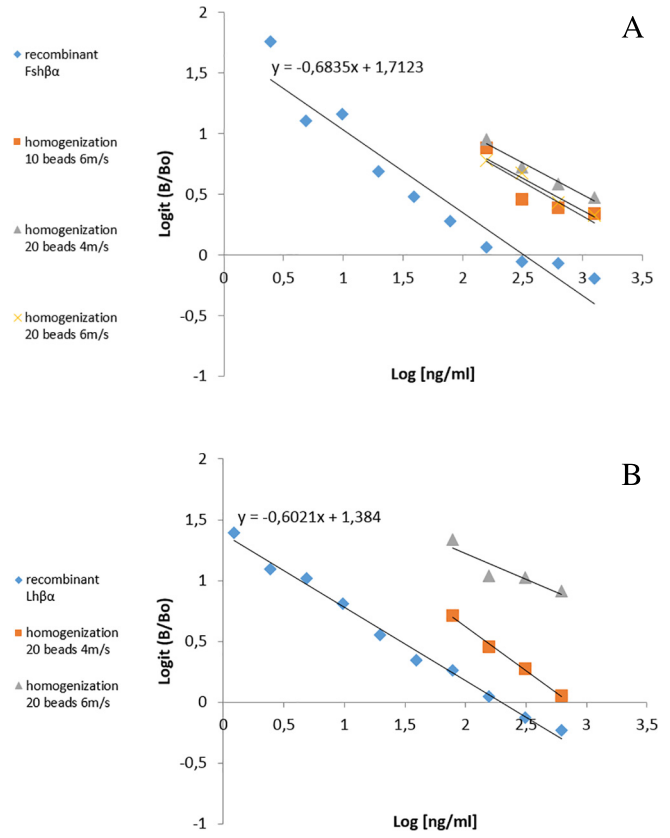

Fig. 5. Parallelism in ELISA for Fsh (A) and Lh (B) between the standard curve using recombinant mdFshβα or mdLhβα (labelled in blue) and displacement curves obtained by serial dilutions of pituitary extract from male medaka (labelled in orange, grey, yellow) using different homogenization conditions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

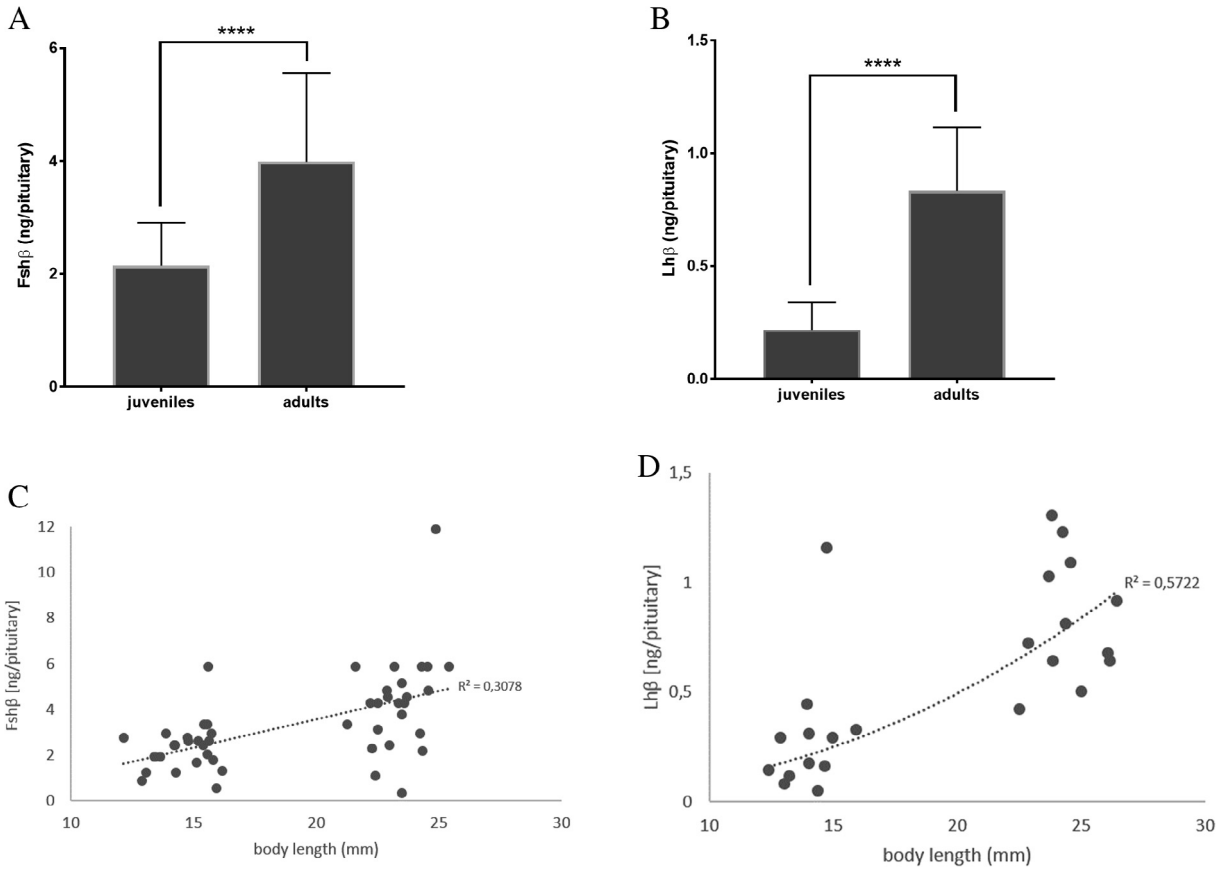

Fig. 6. Pituitary levels of Fsh (A) and Lh (B) in male medaka juveniles and adults. For juveniles, 24 fish with SL of 12–16.5 mm were sampled for quantification of Fsh, and 12 fish with SL of 12–16 mm for Lh. For adults, 24 fish with SL of 21–25.5 mm were sampled for quantification of Fsh, and 12 fish 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 $p \leq 0.0001$.

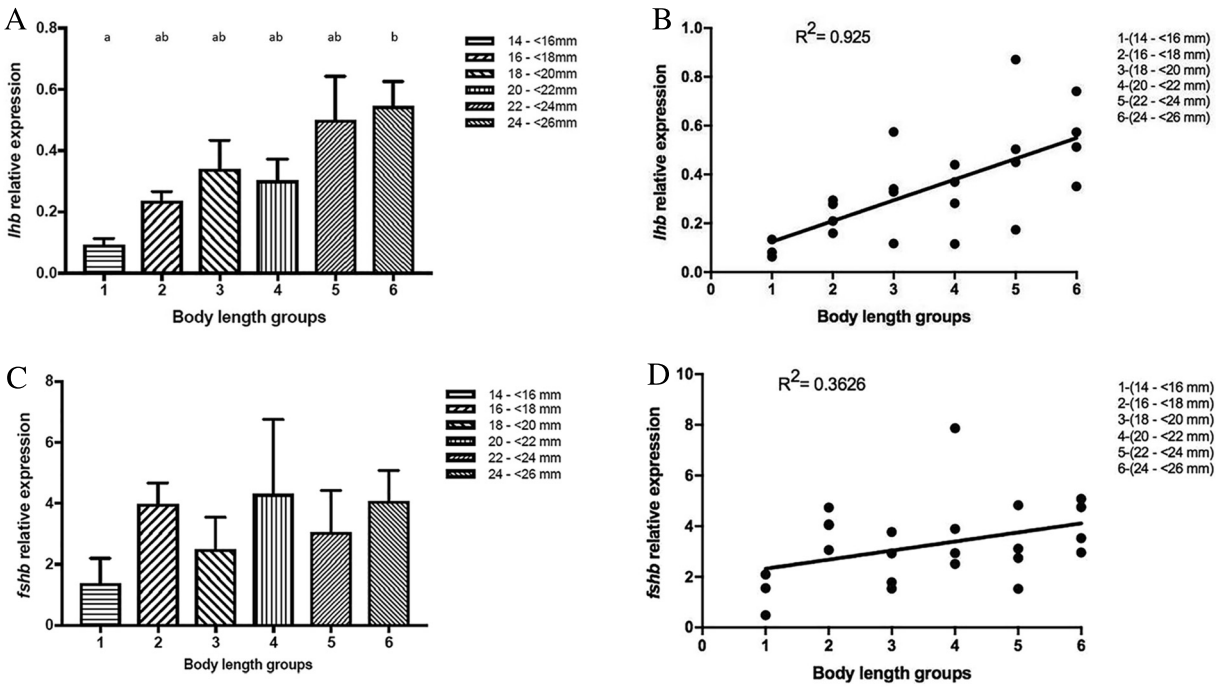

Fig. 7. Developmental profile of *fshb* and *lhb* gene expression levels from male medaka pituitaries as a function of body length, SL (14– < 16 mm; 16– < 18 mm; 18– < 20 mm; 20– < 22 mm; 22– < 24 mm; 24– < 26 mm), and presented as mean values ± SEM (A, n = 5) and (C, n = 5) or as linear regression curves 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
ctgcagtggccactgccttaccaaggacccccttgatgaagatacgatcaattcagtaccagaatgtgtgtacgtaccgggacttttactacaa
gacatttgagcttcctgactgcctgcctggcgtggatccgtcagtcacataccctgtggctctgagttgtcactgtggagcctgcatcatgaac
gcgtctgactgcacctttgagagcctgccaccagacttctgcgtgaaacatgattctttttattattag

fshb:

gatgcatcttagagaagagcagaggaagcaacactttcagccgagctcgcagtgacgctggagatctacaggcgtcggtactgcaccag
gcagaggatgcagctggttgtcatggcagctgcgttggtgctggcggaagtggggcaggtctccagctttcctgtcatcccaaaaacgtca
gcatccctgtggagagctgtggcatcagcgggtgcgtccacaccaccatatgcgaaggacggtgctaccatgaggatcccaactacatca
gctatgaagaccaccctaaagaaaagatctgcagtggggactggtcctacgaagttaaattcattgagggatgtccagtgggtttcaaatatc
ctgtggccaaaagctgcgagtgcactacatgcaacacaagaaccacatactgcggccgactttctgcagacatgccgagctgttaaagtga
ccttttgtttgcctcatgtggttaaattaccctaatgtcttcagcactgaaataaagaaactgtgccccccct

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
gactgcacctttgagagcctgccaccagacttctgcgtgaaacatgattctttttattatcatcatcatcatcattaa

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
gtggccactgccttaccaaggacccttgatgaagatacgatcaattcagtaccagaatgtgtgtacgtaccgggacttttactacaagacatt
tgagcttcctgactgcctgcctggcgtggatccgtcagtcacataccctgtggctctgagttgtcactgtggagcctgcatcatgaacgcgtct
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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