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Development of specific enzyme-linked immunosorbent assay for yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone using recombinant gonadotropins

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ABSTRACT

We developed a specific competitive enzyme-linked immunosorbent assay (ELISA) for yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone (FSH). We previously produced a full-length single chain recombinant yellowtail kingfish FSH using the *Pichia pastoris* expression system. We used the same method to produce the β subunit of the hormone, against which polyclonal antibodies were raised in rabbits. We first confirmed immunoreactivity of the polyclonal antibodies with the recombinant full length FSH and FSHβ as well as plasma and pituitary FSH of sexually immature and mature yellowtail kingfish by Western blot analysis. We then developed a precise and reproducible ELISA for yellowtail kingfish FSH and validated the assay in plasma and pituitary extracts. The intra- and inter-assay coefficients of variation was < 2.2% and 10.2%, respectively. The sensitivity of the assay was 78 pg/ml. For further validation of the assay, we measured the plasma FSH in immature yellowtail kingfish treated with increasing doses (blank, 50, 100 and 150 μg/kg) of kisseptin2-10 peptide from a previous study. The dose response observed in treated females was not significant, however the increased plasma FSH levels coincided with the significantly higher estradiol levels we previously reported in the treated groups. We assessed the applicability of the assay in measuring circulating FSH in other species. We observed parallelism between the linearized FSH standard curve and displacement curves of serially diluted plasma from Atlantic bluefin tuna (*Thunnus thynnus*) and tilapia (*Oreochromis niloticus*). We also observed similar parallelism with full length recombinant giant grouper (*Epinephelus lanceolatus*) FSH. The ELISA we developed for yellowtail kingfish FSH will be useful in understanding the reproductive biology of the species as well as enhancing its aquaculture.

1. Introduction

The success in producing biologically active recombinant piscine pituitary gonadotropins is helping unveil the physiological roles of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in various species of fish (reviewed by Levavi-Sivan et al., 2008; Levavi-Sivan et al., 2010; Aizen et al., 2012b; Mazón et al., 2015). The significance of using recombinant gonadotropins to overcome reproductive dysfunctions associated with captive breeding of fish for aquaculture is also growing (for instance in common carp, *Cyprinus carpio;* (Aizen et al., 2017); European eel, *Anguilla anguilla* (Peñaranda et al., 2018); Japanese eel, *Anguilla japonica* (Kobayashi et al., 2010); yellowtail kingfish, *Seriola lalandi* (Sanchís-Benlloch et al., 2017); Senegalese sole, *Solea senegalensis* (Chauvigné et al., 2015; 2017; 2018).

As in other vertebrates, gonadotropins in fish are heterodimers of a common α subunit and a β subunit, each encoded by a distinct gene (reviewed by Levavi-Sivan et al., 2010). Fish gonadotropins are regulated mainly by the hypothalamic gonadotropin-releasing hormone (GnRH) (Zohar et al., 2010). In fish, FSH generally stimulates the early stages of gonadal development while LH controls the final maturational stages as well as spawning (Yaron et al., 2003; Levavi-Sivan et al., 2010). The advantage of using recombinant gonadotropins in stimulating gonadal development and spawning is that they act downstream of GnRH, which can be inhibited upstream by dopamine in some species

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(reviewed by Dufour et al., 2005; Levavi-Sivan et al., 2010).

Recombinant gonadotropins have been successfully produced in at least 15 species of fish using various expression systems (reviewed by Levavi-Sivan et al., 2010; Moles et al., 2012; Chauvigné et al., 2015; Nyuji et al., 2016; Sanchís-Benlloch et al., 2017; Yom-Din et al., 2016; Hollander-Cohen et al., 2018; Peñaranda et al., 2018; Palma et al., 2018). Previously, the process of preparing the expression construct was tedious, from molecular cloning of the cDNA sequences of the two gonadotropin subunits, to several steps of DNA purification, transformation, restriction digestion and ligation (for instance in tilapia *Oreochromis niloticus*; Kasuto and Levavi-Sivan, 2005). With the advent of next-generation sequencing and availability of commercial providers for DNA synthesis at a reasonable price, the long process of generating the expression construct has been greatly shortened, hence facilitating the production of recombinant gonadotropins. Consequently, species-specific antibodies are generated, and specific non-radioactive immunoassays for FSH and LH have been developed, without requiring large numbers of fish to be sacrificed for pituitary glands as the source of native gonadotropins (examples are Senegalese sole, Chauvigné et al., 2015; Russian sturgeon, *Acipenser gueldenstaedtii* (Yom-Din et al., 2016); carp (Hollander-Cohen et al., 2018); and European eel (Peñaranda et al., 2018).

We previously reported the production of a biologically active recombinant yellowtail kingfish follicle stimulating hormone (ryktFSH) using the yeast (*Pichia pastoris)* expression system (Sanchís-Benlloch et al., 2017). Yellowtail kingfish (YTK) is an aquaculture species of high market value (Whatmore et al., 2013; Symonds et al., 2014). As such, YTK genetics is being investigated extensively to identify economically important traits (Whatmore et al., 2013; Knibb et al., 2016; Nguyen et al., 2018). Achieving genetic gain of valuable traits may be delayed due to the late maturing nature of female YTK, which may take 3–5 years to reach sexual maturity depending on geographic location. We observed that rytkFSH can stimulate gonadal development in immature female YTK, however optimisation of the treatment is needed as complete sexual maturation was not achieved (Sanchís-Benlloch et al., 2017).

We report here the development of a competitive enzyme-linked immunosorbent assay (ELISA) for YTK FSH. This assay is applicable in studies that aim to manipulate sexual maturation and gonadal development of YTK as well as in advancing the overall understanding of the reproductive biology of this species. We validated the assay in sexually immature and mature male and female YTK. We also used the assay to measure the levels of circulating FSH in immature YTK that responded to increasing doses of kisspeptin-2 decapeptide from a previous study (Nocillado et al., 2012). We further validated its applicability as a heterologous assay to measure circulating levels of FSH in other closely related and commercially important species, such as Atlantic bluefin tuna (*Thunnus thynnus*), giant grouper (*Epinephelus lanceolatus*) and tilapia (*Oreochromis mossambicus*).

2. Materials and methods

2.1. Source of YTK plasma, gonads and pituitary samples

Where fish were used, care and handling followed the ethical regulations by New South Wales Department of Primary Industries (NSW DPI), Animal Research Authority, ACEC ref. 93/3, Marine Fish Breeding, and by the University of the Sunshine Coast animal ethics committee (AN/A/12/69).

Sixteen sexually immature YTK, which were bred at the hatchery of Port Stephens Fisheries Institute, NSW DPI, were sampled for blood, pituitary and gonads. Four sexually mature fish were similarly sampled from a cohort of broodstock held in captivity. Total body weight and gonad weight were obtained at sampling. Plasma from blood was obtained after centrifugation at 4000 rpm for 15 min and stored at −80 °C until analysis. Pituitary glands were dissected and immediately frozen on dry ice and stored at −80 °C. For gonad histology, tissue samples were fixed in 10% buffered formalin for 24 h and then stored in 70% ethanol. Gonadosomatic index (GSI) was determined by dividing the gonad weight by the total body weight multiplied by 100.

Fixed gonad tissues for histology were processed in an automatic tissue processor and embedded in paraffin. Sections (6–7 μm thickness) were stained with hematoxylin and eosin using standard procedures and viewed under Leica Microsystems microscope equipped with DFC550 camera system. Microscopic examination revealed that of the four sexually mature fish, two were males and two were females. Among the sexually immature fish, 12 were females, with two of them showing early stage of development, while four were males. Two of the immature males were developing and the other two were immature. Stage classification of the gonads was according to Poortenaar et al. (2001) and Gillanders et al. (1999).

2.2. Production of recombinant YTK FSHβ (rytkFSHβ)

The protocol for the production of rytkFSHβ generally followed those described by Kasuto and Levavi-Sivan (2005), Aizen et al. (2007) and Palma et al. (2018).

An expression construct was designed consisting of the mature region of YTK FSHβ (GenBank Accession number HQ449731). Six Histidine (His) residues were added between the last amino acid and the stop codon as a tag. The DNA sequence was optimized according to *P. pastoris* coding preference, synthesized (Genscript, Piscataway, NJ, USA) and then ligated in frame into the EcoRI and NotI sites of pPIC9K expression vector. The construct was verified by Sanger sequencing.

Purified Sal1-linearised construct (10 μg) was used to transform *P. pastoris* (SuperMan$_5$His$^-$ strain, Biogrammatics, Carlsbad, Ca, USA) by electroporation (BioRad GenePulser, Hercules, CA, USA) following Invitrogen's *P. pastoris* manual. As a negative control for downstream analysis, empty pPIC9K plasmid vector was also electroporated into *P. pastoris*, which were then grown in media supplemented with histidine.

Cells transformed with the expression construct were grown in agar regeneration dextrose medium without histidine. Colonies were then cultured in liquid regeneration dextrose medium without histidine and subsequently screened with Geneticin (G418, Sigma-Aldrich, St Louis, MO, USA) at 1 mg/ml and 2 mg/ml concentrations. From 285 colonies, four colonies were found Geneticin-resistant at 2 mg/ml. These were grown in 5 ml buffered minimal glycerol media overnight at 30 °C. To induce expression, cells were then cultured in 50 ml buffered minimal methanol medium for 3 days. Methanol was added each day at a final concentration of 1%. On the third day, supernatant was collected by centrifugation at 3000 rpm for 5 min. The supernatant pH was adjusted to 8.0 with 10 M potassium hydroxide and then centrifuged at 10000 rpm for 10 min. The recombinant protein was harvested from the supernatant using nickel nitriloacetic acid-agarose (Ni-NTA, QIAgen) according to the manufacturer's instructions. The eluted recombinant protein was lyophilised and resuspended in 0.1X PBS. The suspension was centrifuged at 5000 rpm for 15 min to isolate and discard the salts. The aqueous solution was used for Western blot analysis. Steps in processing the supernatant after methanol induction were carried out at 4 °C. The primary antibody for Western blot analysis was mouse anti-His (Penta-His, Qiagen), while the secondary antibody was goat anti-mouse IR Dye 800CW (Li-cor Bioscience, Lincoln, NE, USA). Western blot analysis protocol was performed as previously described (Sanchís-Benlloch et al., 2017).

Large scale production of rytkFSHβ was conducted in a bio-fermenter (BioFlo CelliGen 115, Eppendorf New Brunswick) following Invitrogen's *Pichia* Fermentation Process Guidelines Version B 053002, with modifications according to Palma et al. (2018). The basal salts fermentation media volume was 2 L. After adaptation to methanol (24 h after the methanol feed), the temperature was gradually adjusted down to 24 °C. The supernatant was harvested 72 h after continuous methanol induction. The recombinant protein was purified using Ni-NTA

Superflow beads (QIAgen) and then dialyzed in dialysis cassettes (3 kDa molecular weight cut off; ThermoFisher Scientific) in two changes of 0.1X PBS for 2 h each and a final change of 0.01X PBS for 1 h at 4 °C throughout dialysis. Western blot analysis was conducted as previously described (Sanchís-Benlloch et al., 2017) to confirm production of rytkFSHβ using His tag antibodies (QIAgen).

2.3. Production of polyclonal antibodies

The polyclonal antibodies against rytkFSHβ were raised in rabbits by a commercial provider (Abbiotec, CA, USA). Briefly, pre-immune serum was collected from two rabbits. Four immunizations were conducted at 21 days interval. The first immunization was performed with 200 μg rytkFSHβ in complete Freund's adjuvant while the subsequent three immunizations injected 100 μg rytkFSHβ in incomplete Freund's adjuvant. Rabbits were bled after the third immunization and titers were confirmed by direct ELISA, after which, the fourth immunization was performed. Rabbits were bled 3 weeks after the final injection. The IgG fraction of the antibodies were purified by Protein-A affinity chromatography.

2.4. Characterisation of rytkFSHβ polyclonal antibodies by Western blot analysis

Reactivity of the polyclonal antibodies was evaluated on native, non-denatured YTK pituitary extracts as well as on full length rytkFSH (either deglycosylated with PNGaseF or glycosylated recombinant) and in rytkFSHβ under denaturing and reducing conditions. Pituitary extracts were obtained following Aizen et al. (2007). Pituitaries were homogenised for 1 min in 500 μl, ice cold, extraction buffer (0.01 M PBS; 1 mM PMSF; 50 mM EDTA) using QIAgen's Tissue Ruptor blades. The homogenised tissue was incubated at 4 °C with gentle shaking for 30 min. The mixture was then centrifuged for 30 min at 15,000 × *g* at 4 °C. The supernatant was collected and stored at −80 °C. The pellet was extracted a second time with 250 μl of the extraction buffer.

Electrophoresis of the pituitary extract and full length rytkFSH was performed on 12% precast polyacrylamide gel (Biorad) while the rytkFSHβ was electrophoresed on 16.5% precast Tris-tricine gel (Biorad). Membrane wash and blocking buffers were as described by Sanchís-Benlloch et al. (2017) but washes were done for 5 min each time. Incubation with the rytkFSHβ antibodies (diluted 1:4000 in the blocking buffer) was done overnight at 4 °C with gentle shaking. Bands were detected via the secondary antibody (goat antirabbit IRDye 680LT, Li-cor Bioscience) diluted 1:5000 in a 50:50 solution of TBST:Odyssey blocking buffer. Membranes were viewed on the 700 nm channel of Odyssey CLx Infrared Imaging System (Li-cor Bioscience).

2.5. Development of competitive ELISA for YTK FSH

The competitive ELISA was developed following Mañanós et al. (1997) and Aizen et al. (2007) with modifications. Plate washes were done three times, first by discarding the content of the wells, and then adding 200 μl/well PBST buffer (10 mM Na_2PO_4; 2 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, and 0.05% Tween-20; pH 7.4), and incubating for 2 min. In developing the assay, the coating concentration and primary antibody dilution were first optimised to obtain a maximum binding absorbance value close to 1. For all assays, the rytkFSHβ antibodies were prepared a day prior to the pre-incubation step on day 1.

On day 1, plate coating and pre-incubation of standard and samples were performed. High protein-binding capacity 96 well flat bottom ELISA plates (Nunc MaxiSorp™; ThermoFisher Scientific) were coated with 100 μl of 10 ng/ml rytkFSHβ in 50 mM sodium carbonate coating buffer (pH9.6). Non-specific binding (NSB) wells were not coated. The plate was incubated overnight without shaking at room temperature.

Standard and samples were pre-incubated with an equal volume of primary antibody diluted 1:15,000 in PBST-0.1% BSA. The standard was the single chain rytkFSH serially diluted from 100 ng/ml down to 50 pg/ml in PBST-0.1% BSA. The single chain rytkFSH consisted of the mature β subunit at the amino terminal and the mature α subunit at the carboxy terminal linked with GSGSHHHHHHGSGS (Sanchís-Benlloch et al. (2017). As initial assays showed most plasma samples had low levels of FSH, plasma samples (750–1000 μl) were lyophilised and resuspended in a smaller volume (250 μl) of PBST-0.1% BSA. The FSH concentration was then calculated back and expressed on per ml of plasma. For Bo and NSB, equal volume of PBST-0.1% BSA and primary antibody were mixed. Incubation was done overnight at room temperature without shaking.

On day 2, the coated plate was washed and then wells were blocked with PBST-1% BSA. The plate was incubated for 1 h at room temperature. After blocking, the plate was washed again and the preincubated standard and plasma samples were dispensed in duplicate wells (100 μl/well) of the coated plate (NSB were in uncoated wells). The plate was incubated for 3 h at room temperature without shaking and then washed. After washing, 100 μl of the secondary antibody (peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:5000 in PBST-0.1% BSA) was added into the wells. The plate was incubated for 2 h at room temperature without shaking and then washed. Antibody complexes were detected by addition of 100 μl/well 3,3′,5,5′-tetramethylbenzidine (TMB; diluted 1:4 in double distilled water) peroxidase substrate (Sigma). The plate was incubated for 20 min at room temperature whilst protected from light. To stop the reaction, 100 μl 1 N phosphoric acid were added into the wells. Plate was immediately read with Enspire multimode plate reader (Perkin Elmer, MA, USA,) at 450 nm after the reaction was stopped.

Intra-assay coefficient of variation (CV) was determined in six replicates of standard of known concentration (3 ng/ml) assayed on one plate. Inter-assay CV was determined in duplicates of the same sample on three separate assays.

The assay was validated using YTK pituitary extracts and plasma samples, which were serially diluted up to 32-fold in PBST-0.1% BSA. Additional validation was conducted by determining the FSH levels from plasma of sexually immature YTK (557 ± 85 g mean body weight) treated with increasing doses (blank, 50, 100 and 150 μg/kg) of kisspeptin2-10 decapeptide from a previously conducted study (Nocillado et al., 2012). Briefly, fish were implanted with the peptide contained in slow release Evac implants and reared for 2 weeks, after which, fish were sacrificed, and plasma samples collected. Plasma were stored at −80 °C until analysis.

To assess the applicability of the assay to other species, cross-reactivity was tested in plasma from Atlantic bluefin tuna (*Thunnus thynnus*) and tilapia (*Oreochromis mossambicus*), as well as in recombinant giant grouper (*Epinephelus lanceolatus*) FSH (Palma et al., 2018).

2.6. Statistical analysis

Data are presented either as mean ± SE or mean ± SD. Where means were compared, analysis was conducted using SPSS v.24 (IBM SPSS, Ottawa, ON) following the univariate general linear model analysis of variance. Regression and slope analyses of ELISA data were conducted on Graph Pad Prism 8 (GraphPad Software, San Diego, Ca). Data were first logit transformed prior to analysis, that is logit(Bi/Bo) = log(r/(1 − r)), where r = Bi/Bo with Bi representing the OD of each sample or standard and Bo the maximum binding OD. The FSH concentration of samples were calculated according to the linear regression equation generated for the standard.

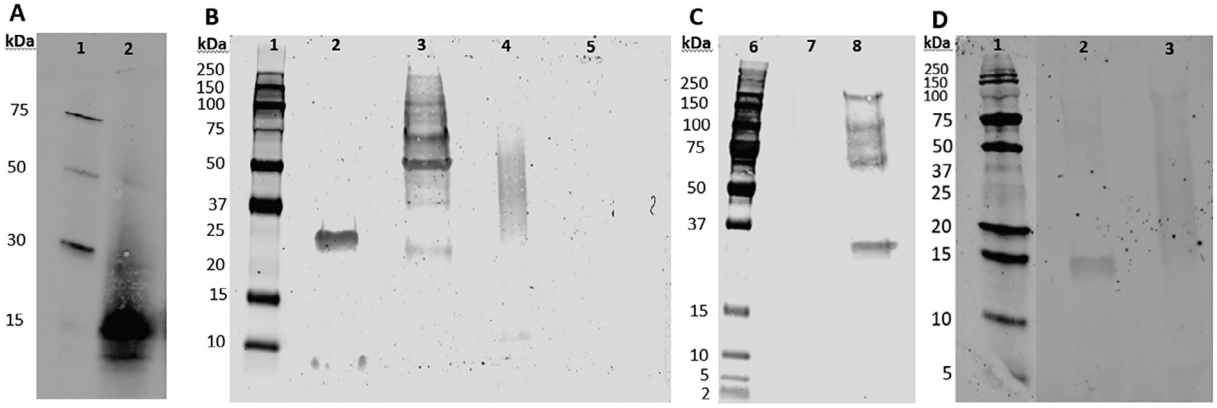


Fig. 1. Western blot analysis of (A) rytkFSHβ using antibodies against the His tag. The predicted 13 kDa was observed. (B, C, D) Characterisation of rytkFSHβ polyclonal antibodies by Western blot analysis. Lanes in B: 1 – protein size marker; 2 – denatured, PNGAseF-treated (4 h) single chain rytkFSH; 3 – non-denatured pituitary extract; 4 – denatured single chain rytkFSH not treated with PNGaseF; 5 – negative control. Lanes in C: 6 – protein size marker; 7 – pituitary extract pre-absorbed with rytkFSHβ polyclonal antibodies; 8 – PNGaseF-treated (2 h) single chain rytkFSH as positive control; Lanes in D: 1 – protein size marker; 2 – PNGAseF-treated rytkFSH β showing the expected 13 kDa band 3 – untreated rytkFSH β.

3. Results

3.1. Production of rytkFSHβ and characterization of polyclonal antibodies

A rytkFSHβ was produced using the yeast expression system (Fig. 1A). The predicted band size of 13 kDa was visualised using antibodies against the 6 His tag of the recombinant hormone, indicating successful production. Polyclonal antibodies produced in rabbits against rytkFSHβ were found immunoreactive to the single chain deglycosylated rytkFSH. Under reducing and denaturing conditions, an intense band of about 24 kDa (Fig. 1B Lane 2; Fig. 1C Lane 6) was observed. The glycosylated full length rytkFSH was revealed as a smear covering the size range of 25–75 kDa (Fig. 1B Lane 4). From native, non-denatured pituitary extract, intense bands of 50 and 75 kDa were observed, as well as a light band between 20 and 25 kDa (Fig. 1B Lane 3). These bands were not observed in pituitary extract previously incubated with the antibodies (Fig. 1C Lane 7). The positive control of this Western blot showed the expected size of deglycosylated rytkFSH (24 kDa band), as well as a smear from 70 to 150 kDa, which may represent residual non-deglycosylated rytkFSH due to the shorter PNGaseF incubation (Fig. 1C Lane 8). Reactivity was not observed in extract from yeast transformed with the expression vector only (Fig. 1B Lane 5). When the polyclonal antibodies were tested against the rytkFSHβ, the expected 13 kDa band was observed both in deglycosylated (PNGaseF-treated) (Fig. 1D Lane 2) and non-deglycosylated recombinants (Fig. 1D Lane 3).

3.2. Development and validation of competitive ELISA for YTK FSH

Three antibody dilutions (1:5000, 1:15000, 1:25000) and six rytkFSHβ coating concentrations (from 160 ng/ml diluted 2-fold down to 5 ng/ml) were first tested to determine the combination that would result in optical density closest to 1. The optimal antibody dilution was 1:15000 with coating concentration at 10 ng/ml. A standard curve was generated using the full length rytkFSH at concentrations from 100 ng/ml serially diluted down to 39 pg/ml. Percentage binding showed the sigmoidal curve typical in competitive ELISAs (Fig. 2A) (Aizen et al., 2007; Chauvigné et al., 2015). After logit-transformation, the percentage binding data showed a linear relationship with the logarithm of the standard concentration (r^2 = 0.9811; Fig. 2B). Intra-assay variation was 2.2% (n = 6) while inter-assay variation was 10.2% (n = 3). The sensitivity of the assay was 78 pg/ml with percentage binding Bi/Bo > 95%.

Validity of the assay was confirmed by parallelism of the standard curve and the displacement curves of serially diluted YTK plasma and

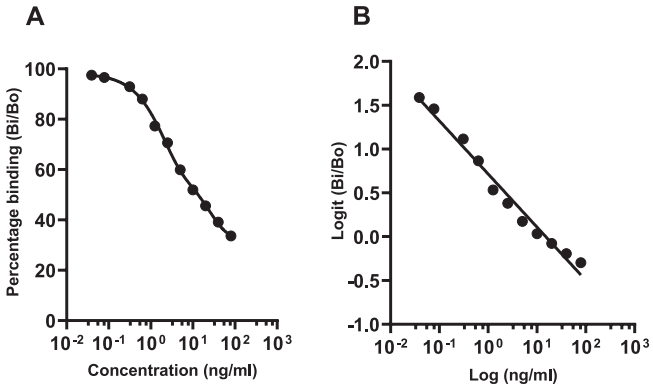


Fig. 2. Competitive binding curve for YTK FSH standard. (A) Percentage binding; (B) Linearised (logit-transformed) percentage binding. Each point represents mean of duplicates.

pituitary extracts (Fig. 3A). The slope of the standard curve (−0.71 ± 0.07) was not significantly different from the slope of the displacement curves of serially diluted YTK plasma (−0.79 ± 0.22) and serially diluted pituitary extract (−0.84 ± 0.19).

To determine the applicability of the ELISA in other species, we evaluated the displacement curves of serially diluted plasma from Atlantic bluefin tuna and tilapia, and the recombinant giant grouper FSH. Results showed parallelism between the rytkFSH standard and displacements curves from the heterologous samples (Fig. 3B). The slopes obtained were −0.60 ± 0.03 for the standard curve, −0.54 ± 0.15 for ABT, −0.46 ± 0.08 for tilapia and −0.57 ± 0.01 for rggFSH.

We determined the circulating levels of FSH in male and female YTK at different stages of gonadal maturation. Male YTK had between 3 ng/ml and 30 ng/ml plasma FSH, with highly variable levels observed between stages (Fig. 4). FSH concentration among the females ranged between 1 ng/ml and 14 ng/ml (Fig. 5). From a previously conducted study, we assessed the effect of increasing doses of kisspeptin2-10 peptide in pre-pubertal YTK. A dose response trend was observed in females; however, the differences were not significant (Fig. 6).

4. Discussion

The availability of recombinant fish gonadotropins has facilitated a deeper understanding of the modes of action of FSH and LH (Moles et al., 2011; Aizen et al., 2012b) and their roles in reproductive

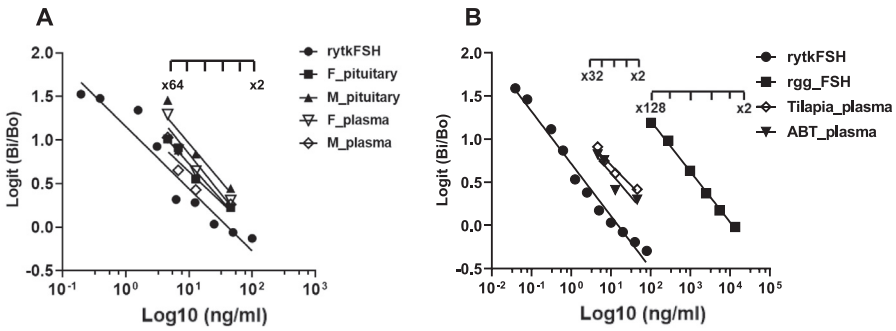


Fig. 3. Parallelism of standard curve (rytkFSH) with displacement curves obtained from serially diluted YTK plasma and pituitary extract diluted 2- to 64-fold (A) and other species (B). M-male; F-female; rgg_FSH – recombinant giant grouper FSH; ABT – Atlantic bluefin tuna. Tilapia and ABT plasma samples were diluted 2- to 32-fold; rgg_FSH was diluted 2- to 128-fold. Each point represents mean of duplicates.

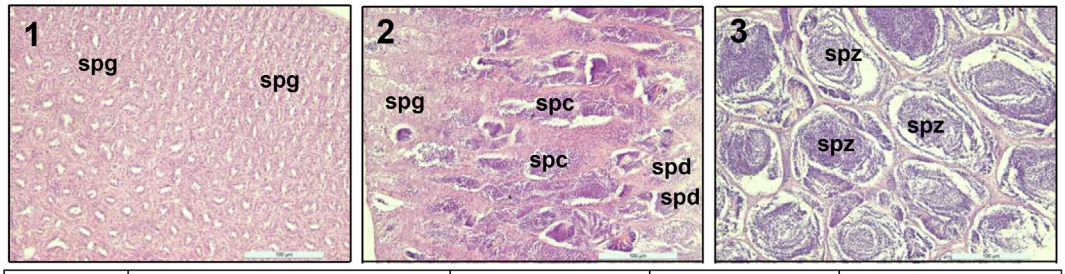
development (Levavi-Sivan et al., 2010; Aizen et al., 2012a; Chauvigné et al., 2018; Palma et al., 2018). The development of reliable, non-radioactive, enzyme-linked immunoassays from antibodies generated against recombinant gonadotropins has also enabled the determination of the physiological levels of FSH and LH in fish at different maturational stages (Aizen et al., 2007; Moles et al., 2012; Chauvigné et al., 2015; Yom-Din et al., 2016; Nyuji et al., 2016; Hollander-Cohen et al., 2018; Burow et al., 2019). This advanced knowledge is proving vital in designing hormonal therapies in species for aquaculture (Aizen et al., 2012a; Chauvigné et al., 2018).

We report here the development of a specific ELISA for FSH in yellowtail kingfish. We previously produced a recombinant, full length, single chain YTK FSH using the yeast expression system (Sanchís-Benlloch et al., 2017). In the present study, we also generated in yeast the recombinant β subunit, from which polyclonal antibodies were raised in rabbits. Recombinant gonadotropins from several fish species have been successfully produced using the methylotrophic yeast, *Pichia pastoris* (Kamei et al., 2003; Kasuto and Levavi-Sivan, 2005; Aizen et al., 2007; Chen et al., 2012; Chauvigné et al., 2015; Yom-Din et al., 2016; Sanchís-Benlloch et al., 2017; Hollander-Cohen et al., 2018). This has enabled the development of specific ELISAs, such as in tilapia (Aizen et al., 2007), European seabass (Moles et al., 2012), Senegalese sole (Chauvigné et al., 2015), Russian sturgeon (Yom-Din et al., 2016), carp (Hollander-Cohen et al., 2018), and medaka Burow et al. (2019). The first specific ELISAs for piscine gonadotropins were developed in salmonids (Suzuki et al., 1988; Swanson et al., 1991). These assays used native gonadotropins that required a long process of isolation and purification from large quantities of fish pituitaries, for instance up to 40 g of pituitaries in coho salmon (Swanson et al., 1991). Sacrificing large number of fishes in developing quantitative gonadotropin assays is no longer necessary with the availability of recombinant gonadotropins.

As in other glycoprotein hormones, FSH is heterodimeric, consisting of two subunits (α and β), which are encoded by separate genes in separate chromosomes (reviewed by Gharib et al., 1990). The α subunit is conserved for all glycoprotein hormones of a species. In contrast, the β subunit is unique for each glycoprotein hormone, conferring functional specificity. Therefore, when developing ELISAs, antibodies are raised against the β subunit (for instance in tilapia, Aizen et al. (2007); orange-spotted grouper, Chen et al. (2012); European seabass, Moles et al. (2012); Russian sturgeon, Yom-Din et al. (2016); carp, Hollander-Cohen et al. (2018)). In Senegalese sole, the antibodies were raised against a full length recombinant FSH, which was a chimera of the flatfish FSH β subunit and chicken FSH α subunit, however the antiserum had to be depleted of the anti-chicken FSH α to enrich for the antiserum against the FSH β subunit (Chauvigné et al., 2015). As in these studies, we found the antibodies raised against the β subunit to be effective for development of a species-specific ELISA.

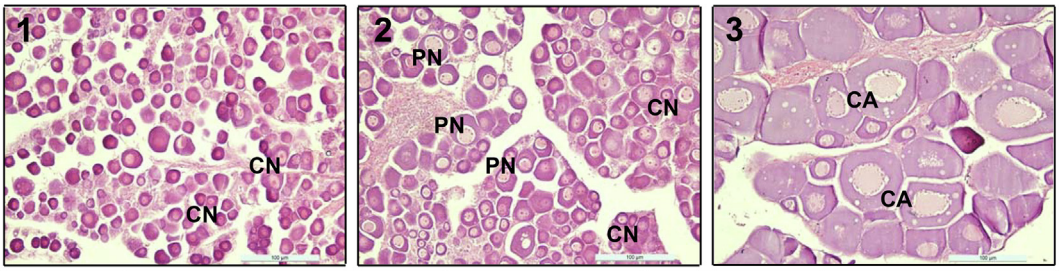
In the present study, results of the Western blot analysis confirmed the specific reactivity of anti-rytkFSHβ with rytkFSH. Intact non-deglycosylated rytkFSH presented as a smear (25–75 kDa) when reacted with rytkFSHβ polyclonal antibodies. A defined band (about 24 kDa) was detected after treatment with PNGAseF, an amidase which removes N-linked carbohydrate moieties in glycoproteins (Plummer and Tarentino, 1991). The results confirm glycosylation of the rytkFSH expressed in *Pichia pastoris* similar to those identified in other species of fish (Levavi-Sivan et al., 2008). The type and degree of carbohydrate chains can affect the stability and bioactivity of glycoprotein hormones (reviewed by Gharib et al., 1990). The bioactivity of rytkFSH was previously confirmed not only *in vitro* but also *in vivo* (Sanchís-Benlloch et al., 2017).

From native, non-denatured and non-reduced pituitary extract, the



Number	Stage (n=number of fish)	Mean body weight (g±SD)	Mean GSI ±SD	Mean plasma FSH (ng/ml; ±SD)
1	Immature (n=2)	870±164	0.035±0.007	12.3±7.2
2	Spermatogenic (n=2)	1,564±4	0.075±0.049	8.2±3.6
3	Mature; spermiating (n=2)	3,978±187	1.645±0.120	14.8±13.0

Fig. 4. Gonad histology of male YTK evaluated for circulating levels of FSH (scale bar is 100 μm). The table shows the corresponding descriptive fish data and plasma FSH concentration. (1) Immature testis contained spermatogonia (SPG) only. (2) Spermatogenic testis contained spermatogonia (SPG), spermatocytes (SPC) and spermatids (SPD). (3) Mature, spermiating testis mainly contained spermatozoa (SPZ). Testicular stages were according to Gillanders et al. (1999) and Poortenaar et al. (2001).



Number	Stage (n=number of fish)	Mean body weight (g±SD)	Mean GSI ±SD	Mean plasma FSH (ng/ml; ±SD)
1	Immature, CN (n=10)	906±300	0.36±0.49	8.1±4.1
2	Immature, PN (n=2)	1,315±247	0.62±0.56	4.8±0.2
3	Developing, CA (n=2)	7,469±954	0.80±0.06	3.1±1.8

Fig. 5. Gonad histology of female YTK evaluated for circulating levels of FSH (scale bar is 100 μm. The table shows the corresponding descriptive fish data and plasma FSH concentration. (1) Immature – most oocytes are at chromatin nucleolar (CN) stage. (2) Immature – oocytes at chromatin nucleolar (CN) and perinucleolar (PN) stages (3) Developing – most oocytes are at cortical alveoli (CA) stage. Oocyte stage definitions were according to Gillanders et al. (1999) and Poortenaar et al. (2001).

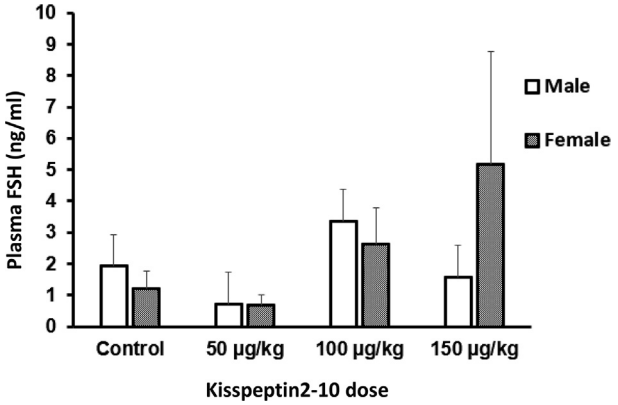


Fig. 6. Mean plasma FSH levels of sexually immature YTK treated with increasing doses of kisppeptin2-10 peptide. Bars represent mean ± SEM (n = 3–7 for males; n = 4–5 for females).

most intense immunoreactive bands were approximately 50 and 75 kDa in size, together with a smear from 37 to 150 kDa, and a band of 23 kDa, which we showed previously as the molecular mass size of single chain rytkFSH using His tag antibodies (Sanchís-Benlloch et al. (2017). In this study, we obtained a slightly bigger band (24 kDa) when rytkFSHβ antibodies were used to detect the full length rytkFSH. The slight variation might be attributed to minor differences in the degree of glycosylation between production batches of rytkFSH that may affect the binding of the polyclonal antibodies. Previous studies have indicated that variation in glycan content does occur in recombinant gonadotropins (reviewed by Bousfield and Harvey (2019)). In Mediterranean yellowtail (*Seriola dumerilii*), the estimated molecular weight of native non-reduced pituitary FSH was 47 kDa, while under reducing conditions, 22 kDa and 17 kDa bands were observed (García-Hernández et al. (1997). In the present study, the bigger immunoreactive bands obtained from the pituitary extract could represent glycosylated heterodimers, as also described in other species (Aizen et al., 2007; Chen et al., 2012; Chauvigné et al., 2015; Yom-Din et al., 2016). The bands were no longer visible when the pituitary extract was preabsorbed with rytkFSHβ antiserum, indicating specificity of the antibodies. Specificity of anti-rytkFSHβ was further confirmed on Western blot where the predicted 13 kDa molecular weight of rytkFSHβ was revealed.

From the generated rytkFSHβ antibodies, we developed a precise and reproducible ELISA for YTK FSH, with < 2.2% intra-assay CV and 10.2% inter-assay CV. These values are comparable with those obtained from other species (Aizen et al., 2007; Moles et al., 2012; Chauvigné et al., 2015). The assay sensitivity was 78 pg/ml, which is more sensitive than those in European seabass (500 pg/ml, Moles et al., 2012); greater amberjack, (200 pg/ml, Nyuji et al., 2016); and Russian sturgeon (1.56 ng/ml, Yom-Din et al., 2016). However, lower detection limits were reported in tilapia (0.24 pg/ml, Aizen et al., 2007), Senegalese sole (10 pg/ml, Chauvigné et al., 2015), carp (7.54 pg/ml, Hollander-Cohen et al., 2018); medaka (44.7 pg/ml, Burow et al., 2019). Differences in assay sensitivity may be attributed to the level of purity of the recombinant antigens and antisera, or variation in the available epitopes of the recombinant antigen.

The validity of the assay was demonstrated by parallelism of the serially diluted standard with displacement curves of serially diluted plasma and pituitary extracts. We previously demonstrated the bioactivity of rytkFSH both *in vitro* and *in vivo* (Sanchís-Benlloch et al., 2017). These results indicate that the form of FSH in the pituitary and plasma of YTK is immunologically identical to the rytkFSH.

We tested the cross reactivity of the rytkFSHβ antibodies against the plasma of Atlantic bluefin tuna and tilapia as well as the full length recombinant giant grouper FSH. Comparison of the mature YTK FSHβ peptide sequence showed identity of 71% with that of giant grouper, 65% to Atlantic bluefin tuna and 59% to tilapia (data not shown). Parallelisms were obtained between the displacement curves of serially diluted Atlantic bluefin tuna and tilapia plasma, and recombinant giant grouper FSH, suggesting applicability of the developed FSH ELISA for these species. These results are remarkable considering the divergent characteristic of piscine FSH (reviewed by Levavi-Sivan et al., 2010). However, YTK, Atlantic bluefin tuna and tilapia all belong to order Perciformes. When parallelism was evaluated between the recombinant Senegalese sole FSH that belongs to Pleuronectiformes and plasma and pituitary samples in species representing three different orders, namely Salmoniformes, Perciformes and Cypriniformes, little or no parallelism was observed (Chauvigné et al., 2015). Similar results were reported when the FSH ELISA developed for tilapia was tested for applicability in sturgeon, zebrafish, carp, eel and trout (Aizen et al., 2007). Our results and those of others indicate that the applicability of piscine FSH ELISAs may be limited among species within the same order.

In the southern hemisphere, the spawning season of YTK is spring to summer (Gillanders et al., 1999). Oocyte development in female YTK is classified as multiple group synchronous, indicating capacity for spawning several times during the season (Poortenaar et al., 2001). In the present study, plasma samples were collected in the summer months (February and March). Results showed that FSH is detectable in plasma at the different stages identified, both in male and female, suggesting a

role of FSH for spermatogenesis in males and oocyte development in females, but statistical analysis could not be performed due to the limited number of samples. Nevertheless, the levels of FSH as well as the observed trend are similar to what has been reported in other species. In female greater amberjack, plasma FSH levels remained approximately between 5 ng/ml and 25 ng/ml in female fish from recrudescent stage and until spawning (Nyuji et al., 2016). In European seabass, plasma FSH did not also vary significantly in pre-, late- and post-vitellogenic fish (Moles et al., 2012). The presence of circulating FSH is associated with its role in stimulating vitellogenesis via estradiol production in the ovary as well as in the production of androgens in the testis (Levavi-Sivan et al., 2010; Mazón et al., 2014).

In Australia, YTK aquaculture is well established and genetic studies have been conducted to achieve genetic gain of commercially important traits (Whatmore et al., 2013; Knibb et al., 2016; Nguyen et al., 2018). Late maturation of the females is an issue for aquaculture, therefore previous studies aimed to advance the onset of sexual maturation in this species (Nocillado et al., 2013; Sanchís-Benlloch et al., 2017). We previously observed significantly higher estradiol production in prepubertal female YTK treated with kisspeptin2-10 peptide (Nocillado et al., 2012). In that study, we concluded the optimal kisspeptin2-10 dose to be 100 μg/kg (compared with 50 μg/kg and 150 μg/kg) based on higher plasma estradiol levels and stimulation of ovarian development. In the present study, although not significantly different, the plasma FSH levels in treated female fish increased with increasing kisspeptin2-10 dose. Our results here further support our previous conclusion.

In summary, the FSH ELISA developed in the present study will be significant in determining the critical time, dose and duration at which hormonal manipulation must be administered if the onset of puberty in female YTK is to be advanced. Together with our rytkFSH (Sanchís-Benlloch et al., 2017), we have the tools to advance the understanding of the endocrine control of reproduction in YTK as well as fine-tune its captive breeding technology.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113208.

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