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Development of specific enzyme-linked immunosorbent assay for determining LH and FSH levels in tilapia, using recombinant gonadotropins

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Abstract

We recently produced Oreochromis niloticus recombinant LH and FSH as single-chain polypeptides in the methylotrophic yeast Pichia pastoris. Glycoprotein subunit α was joined with tilapia (t) LHβ or tFSHβ mature protein-coding sequences to form a fusion gene that encodes a “tethered” polypeptide, in which the gonadotropin β-subunit forms the N-terminal part and the α-subunit forms the C-terminal part. Recombinant (r) gonadotropins were used to develop specific and homologous competitive ELISAs for measurements of FSH and LH in the plasma and pituitary of tilapia, using primary antibodies against rtLHβ or rtFSHβ, respectively, and rtLHβα or rtFSHβα for the standard curves. The wells were coated with either rtLHβ (2 ng/ml) or rtFSHβ (0.5 ng/well), and the final concentrations of the antisera were 1:5000 (for tLH) or 1:50,000 (for tFSH). The sensitivity of the assay was 15.84 pg/ml for tLH and 0.24 pg/ml for tFSH measurements in the plasma, whereas for the measurements in the pituitary, the sensitivity was 2.43 ng/ml and 1.52 ng/ml for tLH and tFSH, respectively. The standard curves for tFSH and tLH paralleled those of serially diluted pituitary extracts of other cichlids, as well as of serially diluted pituitary extract of seabream, European seabass and hybrid bass.

We examined plasma tFSH and tLH levels in the course of one reproductive cycle, between two successive spawnings, in three individual tilapia females. Plasma levels of both FSH and LH increased during the second day after the eggs had been removed, probably related to the vitellogenic phase. LH levels increased toward spawning, which occurred on the 11th day. FSH levels also increased on day of cycle, probably due to recruitment of a new generation of follicles for the successive spawning. The development of specific ELISAs using recombinant gonadotropins is expected to advance the study of the distinct functions of each of these important hormones. © 2007 Published by Elsevier Inc.

Keywords: ELISA; Tilapia; LH; FSH; Spawning; Reproductive cycle

1. Introduction

Despite the taxonomic distance between fish and mammals, their different modes of reproduction, and the peculiar morphology of the hypothalamo-hypophyseal connection, the two classes share many features in gonadotropin structure, function, and regulation (Yaron and Levavi-Sivan, 2006). The two gonadotropins, FSH and LH, stimulate growth and development of the gonads through gonadal biosynthesis of steroid hormones and growth factors (Yaron et al., 2003). Through binding to membrane receptors, FSH stimulates follicular development in the ovary and gametogenesis in the testes, while LH stimulates processes leading to final oocyte maturation and ovulation in females, and spermatiation in males. To date, cDNA sequences encoding gonadotropin subunits have been isolated and characterized from more than 19 fish species representing seven teleostean orders (Yaron et al., 2001), and the number is rising. The two gonadotropins have distinct temporal profiles of expression and release and are synthesized in two distinct cell types in the pituitary of the teleost fish studied to date (Nozaki et al., 1990), including tilapia (Yaron et al., 2001). As in
mammals, both gonadotropins are heterodimeric glycoproteins, sharing an identical α-subunit, and distinct β-subunits which confer their biological specificity. Single, separate genes encode each of the subunits of FSH and LH (Weltzien et al., 2004), and phylogenetic studies suggest that the α- and β-subunits diverged from a common ancestral gene more than 900 million years ago (Li and Ford, 1998). Carbohydrates account for approximately 30–35% of the hormone’s molecular mass (Fares, 2006). In most vertebrates, the cytochrome-rich subunits have one or two N-linked glycosylation sites that play an important role in the signal transduction of the hormone (Moyle et al., 1975).

We have recently produced Oreochromis niloticus recombinant LH and FSH as single-chain polypeptides in the methylotrophic yeast Pichia pastoris. This yeast expression system possesses the genetic manipulability and growth characteristics of prokaryotes and the subcellular machinery for post-translational modification of eukaryotes. Passage of the expressed protein through the secretory pathway allows post-translational modification events such as proteolytic maturation, glycosylation and disulfide-bond formation to occur (Hamilton et al., 2006). Tilapia glycoprotein subunit α and τLHβ or tFSHβ mature protein-coding sequences were joined to form a fusion gene that encodes a “tethered” polypeptide. A “linker” sequence of six amino acids was placed between the β- and α-chains to assist in the chimerization of the subunits, and a six-His tail was placed to enable purification of the recombinant protein (Aizen et al., 2007; Kasuto and Levavi-Sivan, 2005). The recombinant LH (τLHβα) and FSH (τFSHβα) could stimulate the release of 11-ketotestosterone (11-KT) from tilapia testes. Both recombinant gonadotropins could also stimulate the release of estradiol from vitellogenic ovaries of the same fish (Aizen et al., 2007; Kasuto and Levavi-Sivan, 2005).

Among the teleosts, homologous immunoassays for both FSH and LH have only been developed for salmonids (Suzuki et al., 1988a; Swanson et al., 1989), while ontogeny and quantification of FSH in non-salmonids currently relies on the expression levels of GnIH subunit genes in the pituitary (Yaron et al., 2003). The objective of the present study was to produce glycosylated, properly folded and biologically active recombinant tilapia gonadotropins using the yeast expression system, since this system provided sufficient quantities of rτLH and rτFSH for the development and validation of a homologous enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Fish and experimental design

Nile tilapia (Oreochromis niloticus) were kept and bred in our fish facility unit in 500-L tanks at 28 °C under a photoperiod of 14L:10D. They were fed every morning ad libitum with commercial pellets and flakes containing 50% protein, 6% fat, 5.6% ash, and 2.6% cellulose (Zemach Feed Mills, Zemach, Israel). Under these conditions, reproductive activity occurred mainly in the afternoon and each spawning cycle lasted between 11 and 13 days. The fish (300–400 g BW) were maintained in 120-L aquariums at the ratio of six females to each male. Fish were checked every morning for the presence of newly mouthbrooding females: these were immediately transferred to a bucket and their incubated eggs were removed before returning to the aquarium. Fish sampling for the reproductive cycle study was performed every 2 days, always in the morning (10:00 h). Each fish was sampled six times. Blood was collected from the caudal vasculature of anesthetized fish into heparinized syringes. After centrifugation, the plasma was stored at 20 °C until further analysis.

All experimental procedures were in compliance with the Animal Care and Use Guidelines of the Hebrew University and were approved by the local Administrative Panel on Laboratory Animal Care.

2.2. Hormone production and purification

Using the methylotrophic yeast P. pastoris, a recombinant tLH and tFSH were produced as a biologically active, single-chain polypeptide according to (Kasuto and Levavi-Sivan, 2005; Aizen et al., 2007). The τLHβ (GenBank Accession No. AY541609) or τFSHβ (GenBank Accession No. AF289174) and α (GenBank Accession No. AF303087) mature protein-coding sequences were joined to form a fusion gene that encodes a “tethered” polypeptide in which one of the β-chains forms the N-terminal part and the α-chain forms the C-terminal part. A “linker” sequence of six amino acids (three Gly-Ser pairs) was placed between the β- and α-chains to assist in the chimerization of the subunits, and a six-His tail was placed at the end of the β-subunit to enable purification of the recombinant protein (Fig. 1). Recombinant (r) τLHβα and τFSHβα were able to stimulate the release of 11-KT from the homologous testes, demonstrating their biological activity (Kasuto and Levavi-Sivan, 2005) and (Aizen et al., 2007).

2.3. Antisera

The antiserum against rτLHβα was raised in rabbit and was described by Melamed et al. (1999). An antiserum against rτFSHβα was also raised in rabbits by intradermal injection with rτFSHβα (50 µg) emulsified in an equal volume of complete Freund’s adjuvant. Injections were performed four times at 3-week intervals. The rabbits were bled 2 weeks after the final injection, and the serum was aliquoted and lyophilized.

2.4. Gel electrophoresis and Western-blot analysis

Native non-reduced samples of tilapia pituitary extract (TPE) were electrophoresed on 15% polyacrylamide running gels, with a 5% stacking gel. Gels were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), and blocked with 5% low-fat milk. Tilapia pituitary extract, tFSHβα, tFSHβ, τLHβα, and τLHβ were visualized with either the antiserum against rτFSHβα (Section 2.3) or that against τLHβα (Melamed et al., 1999). The membranes were incubated in PBS plus 1% non-fat milk with the antisera (1:10,000) for 1 h at RT, and then with goat anti-rabbit horseradish peroxidase conjugate (GAR-HRP; Jackson, ImmunoResearch Laboratories, Inc. West Grove, PA; 1:5000) for 1 h at RT. After washing, all membranes were treated with enhanced chemiluminescence reagent (ECL; Biological Industries, Beit Ha’emek, Israel) to reveal immunoreactive bands.

2.5. ELISAs for tFSH and tLH

Competitive ELISAs for determinations of tLH or tFSH (generally according to Mananos et al. (1997)) were developed using specific β-subunit primary antibodies, recombinant β-subunit for coating ELISA microplates, and recombinant yoked τLHβα or rτFSHβα for the standard curve.

2.5.1. Coating

Polystyrene ELISA microtiter plates (Nunc-Immuno™ Plates; Nunc, Denmark) were coated with 100 µl/well of 5 ng/ml (0.5 ng/well) tFSH or 20 ng/ml (2 ng/well) tLH. The solution was prepared in carbonate
buffer (50 mM sodium carbonate, pH 9.6) and incubated overnight at RT without shaking. Two wells for non-specific binding were not coated.

2.5.2. Washing and blocking

The next day, plates were washed (3 × 5 min) with 200 μl/well PBST buffer [10 mM Na2HPO4, 2 mM KH2PO4 (pH 7.4), 140 mM NaCl, 3 mM KCl, and 0.05% Tween 20]. To reduce background, plates were blocked for 1 h at RT without shaking with 200 μl/well of PBST buffer containing 1% bovine serum albumin (BSA, Sigma, Ness Ziona, Israel).

2.5.3. Preincubation of standards and samples

Due to the large difference in the amount of LH and FSH between plasma and pituitary samples, we developed two different standard curves for plasma and pituitary measurements.

For gonadotropin measurement in plasma samples: each hormone standard was diluted in 0.1% BSA in PBST with 10% normal goat serum (NGS, Biological Industries). Plasma samples (unknowns) were diluted 1:1000 with 0.1% BSA in PBST, in sufficient volume to allow duplicate analysis. For gonadotropin measurement in pituitary samples: each hormone standard was diluted in 0.1% BSA in PBST with 10% 199 medium (Biological Industries). Pituitary samples (unknowns) were diluted 1:1000 with 0.1% BSA in PBST, in sufficient volume to allow duplicate analysis.

Unknown samples and standards were first pre-incubated overnight at RT with the primary antibodies (final dilution 1:50,000 for tFSHβ and 1:250,000 for tLHβ) in PBST containing 1% BSA and 0.05% Tween 20. After pre-incubation, each sample was dispensed into the wells (100 μl/well) of the coated microtiter plates and incubated for 3 h at RT without shaking. Following incubation, the plates were washed (3 × 5 min) with PBST.

2.5.4. Incubation with secondary antibodies

The formed antigen-antibody complexes were detected by addition of 100 μl/well of GAR-HRP diluted 1:5000 in PBST-0.1% BSA buffer for 2 h at RT without shaking. The plates were washed again (3 × 5 min) with PBST.

2.5.5. Color development

The presence of enzyme complexes was visualized by addition of 100 μl/well of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Zotal, Israel) diluted 1:4. The reaction was carried out in complete darkness at RT and was stopped after 15 min with TMB stop solution (100 μl/well). Absorbance was read at 450 nm, using a Spectra II ELISA reader (SLT, Salzburg, Austria).

Fig. 1. Characterization of tilapia pituitary extract and Pichia-expressed recombinant tilapia gonadotropins by Western-blot analysis. Total proteins from tilapia pituitary or supernatants of transformed Pichia cultures were separated by 10% semi-native PAGE and immunoreacted with antibodies raised against recombinant rtFSHβ; lane 1, tilapia pituitary extract; lane 2, rtFSHβ; lane 3, rtFSHβ. Total proteins from tilapia pituitary or supernatants of transformed Pichia cultures were separated by 15% semi-native PAGE and immunoreacted with antibodies raised against recombinant tLHβ; lane 2, rtLHβ; lane 3, rtLHβ.

Preliminary experiments (data not shown) demonstrated that standard curves obtained in plates coated with rtLHβ or rtFSHβ subunits were similar to those obtained in plates coated with the intact molecule (rtLHβ or rtFSHβ, respectively); this allowed the use of rtLHβ or rtFSHβ for the coating step.

2.6. Validation of the ELISA for tilapia FSH and LH

The ELISA was validated for tLH and tFSH determinations in plasma and pituitary samples of tilapia and was tested for cross-reactivity with additional fish species. Displacement curves for plasma and pituitary samples were obtained by serial dilutions of the sample in the ELISA buffer (PBST buffer containing 10% NGS, for plasma; PBST buffer containing 1% medium for pituitaries) and compared with the ELISA standard curve.

For the parallelism analysis, whole pituitary glands were collected from sexually mature cultivated fish. Pituitaries were homogenized on ice in 0.01 M PBS pH 7.0 containing 0.02 M phenylmethylsulfonyl fluoride and 0.05 M EDTA using a Polytron homogenizer. The homogenate was stirred for 30 min at 4°C and centrifuged at 15,000g for 30 min. The pellet was re-extracted to maximize recovery. The resulting supernatant was used as the pituitary extract.

The ELISA was validated for FSH and LH measurements in pituitary samples of Tilapia nilotica, Tilapia zillii, and the Malawi cichlid Electric blue Hap (Haplochromis ahl). The ELISA was also validated for FSH and LH measurements in other fish: carp (Cyprinus carpio), Russian sturgeon (Acipenser gueldenstaedti), seabream (Sparus aurata), European sea bass (Dicentrarchus labrax), hybrid striped bass (Morone saxatilis × M. chrysops), rainbow trout (Oncorhynchus mykiss), zebrafish (Danio rerio) and eel (Anguilla anguila). Plasma was diluted 1:2, while pituitaries were diluted first 1:10, and then 1:2.5. All samples were used in the ELISA at a total volume of 100 μl of solution per well.

Intraassay coefficient of variation (CV) was determined by assaying eight replicates of one of the standard concentrations (1.25 ng/ml) on the same assay plate. Interassay variation was determined by assaying the same sample seven times on different plates.

2.7. Statistical analysis

Data are presented as means ± SEM. The significance of differences between group means of hormone levels was determined by one-way analysis of variance (ANOVA) followed by Newman–Keuls test using the
Graph-Pad Prism 4.02 software (GraphPad, San Diego, CA). To test for parallelism between various regressions lines, we used the analysis of covariance at: http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/ANOCOV.htm.

For data calculations in the ELISA, sigmoid curves were linearized using the logit transformation, where \[ \text{logit}(B/Bo) = \log [r/(1−r)] \] where \( r = B/Bo \), \( B \) represents the binding at each point, and \( Bo \) the maximum binding.

### Results

#### 3.1. Recombinant tFSHβα, rtFSHβ, rtLHβα, and rtLHβ

Western blot analysis was performed on tilapia pituitary extract and the supernatant derived from yeast transformed with an expression vector containing rtFSHβα, rtFSHβ, rtLHβα, or rtLHβ cDNAs. These proteins were immunoreacted with antibodies against rtLHβ (Melamed et al., 1999; Fig. 1b) or against rtFSHβ (Aizen et al., 2007; Fig. 1a). When the homogenate of female tilapia pituitaries was subjected to SDS–PAGE under reducing conditions, proteins of approximately 17, 30, and 55 kDa reacted specifically with anti-rtFSHβ (Fig. 1a, lane 1), while proteins of approximately 17 and 30 kDa reacted specifically with anti-rtLHβ (Fig. 1b, lane 1). Transformation with vector alone served as a negative control and yielded no bands (data not shown). Under reducing conditions, the immunoreactive rtFSHβα and rtFSHβ were revealed as bands of 26–30 kDa (Fig. 1a, lane 2) and 17–18 kDa (Fig. 1a, lane 3), respectively. A protein of approximately 12 kDa also specifically reacted with anti-rtFSHβ (Fig. 1b, lane 3).

The immunoreactive rtLHβα was revealed as a band at 25 kDa and a smear, probably due to a high glycosylation rate (Fig. 1b, lane 2), while rtLHβ was revealed as two bands, at 25 and 35 kDa, probably reflecting the nascent and glycosylated forms of the protein (Fig. 1b, lane 3), respectively.

Positive staining bands were not observed when the anti-rtFSHβ was replaced by serum that was preabsorbed with rtLHβ, or when anti-rtLHβ was replaced by serum that was preabsorbed with rtFSHβ (data not shown).

#### 3.2. Development and validation of ELISA for tFSH and tLH

Competitive ELISAs were developed for tFSH and tLH determination in pituitary and plasma samples of tilapia, using rtFSHβα and rtLHβα as standards, rtFSHβ or rtLHβ for coating and specific primary antisera against rtFSHβ or rtLHβ for detection.

Dilution tests were carried out to determine appropriate dilutions for antigen (coating step) and specific antibody (competition step) in order to achieve optimal results. The optimal concentration of tilapia antigen for coating the wells was determined by incubating various concentrations of rtLHβ (for the tLH ELISA) or rtFSHβ (for the tFSH ELISA) in coating buffer for 18 h at RT. Coating conditions that yielded a reading close to an optical density of 1.0 by using a 1:5000 dilution of antiserum tLH, or a 1:50,000 dilution of antiserum tFSH, were selected.

Gonadotropin levels in the plasma are in the nanogram range, and in the pituitary in the microgram range. Therefore, since we aimed to process all samples of an experiment in the same assay, we used two different standard curves for plasma and pituitary. The standard curve used for plasma samples ranged from 0.02 to 10 ng/ml for tFSH and from 0.04 to 20 ng/ml for tLH (Fig. 2a). The standard curve calibrated for tFSH and tLH determination in the pituitary ranged from 0.98 to 500 ng/ml for both gonadotropins (Fig. 2b). Under the above described routine conditions, all the standard curves typically showed a sigmoidal dose–response (Fig. 2).

Sensitivity of the assay was defined as the amount of tLH or tFSH sufficient to reduce the optical density determined in the absence of tLH or tFSH by 2 standard deviations. For measuring LH in the plasma, the sensitivity of the assay was 15.84 pg/ml, with the optical density decreasing as a linear function of LH concentration \((r^2 = 0.9997; \text{Fig. 2a})\). The sensitivity of the assay for tilapia FSH was 0.24 pg/ml, with the optical density decreasing as a linear function of FSH concentration \((r^2 = 0.9968; \text{Fig. 2a})\). For measuring tLH in the pituitary, the sensitivity of the assay was 2.43 ng/ml \((r^2 = 0.9962; \text{Fig. 2b})\), while the sensitivity of the assay for pituitary tFSH was 1.52 ng/ml \((r^2 = 0.9954; \text{Fig. 2b})\).

The intraassay CV, calculated by measuring replicates of the same sample within the assay, was estimated at 7.2% for tLH and 8.0% for tFSH. The interassay CV, calculated by measuring replicates of the same sample in different assays, was 14.8% and 12.5% for tLH and tFSH, respectively.

Validation of the assay for the determination of tFSH and tLH was performed by testing the parallelism between the standard curves and displacement curves obtained by serial dilutions of tilapia plasma. Serial dilutions of plasma from tilapia males or females were found to parallel both LH and FSH standard curves (Fig. 3). The slope of the displacement curve obtained with rtFSHβα (slope ± SEM, -0.205 ± 0.016) was not significantly different from that obtained with native plasma FSH (slope ± SEM, -0.209 ± 0.056). The slope of the displacement curve obtained with rtLHβα (slope ± SEM, -0.356 ± 0.031) was not significantly different from that obtained with native plasma LH (slope ± SEM, -0.418 ± 0.029). These data suggest that the recombinant gonadotropins are immunologically similar to the authentic glycoproteins present in the fish plasma.

Graded volumes of tilapia pituitary extract were used for measuring LH or FSH by the ELISA method. The dose–response curves obtained for each hormone (Fig. 4) were parallel to the respective hormone’s standard curve. Using this ELISA system, tilapia (O. niloticus) pituitaries were estimated to contain 7.16 µg LH and only 0.45 µg FSH/fresh pituitary tissue. It should be noted, however,
that these values correspond to females during the reproductive phase, and they are likely to show seasonal fluctuations. Not surprisingly, pituitary extracts from other cichlids were found to parallel that of tilapia nilotica for both LH and FSH (Fig. 4).

To test the possibility of using the tFSH and tLH ELISAs for gonadotropin measurements in other fish species, displacement curves obtained with serial dilutions of pituitary extracts from several fish species were compared with the tFSH and tLH standard curves (Fig. 5). A linear parallelism with the tFSH and tLH standard curves was observed for species in the same order (Perciformes) but in different families (labrax, seabream, and hybrid bass), but not with species from other orders (carp, zebrafish, eel, sturgeon, trout; Fig. 5). rLH did not show detectable cross-reactivity (<0.001%) in the LH ELISA, and rLH did
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not show detectable cross-reactivity (<0.01%) in the FSH ELISA.

3.3. Tilapia FSH and tLH levels during the reproductive cycle

We examined plasma tFSH and tLH levels in O. niloticus females in the course of one reproductive cycle, between two successive spawnings. Eggs were removed from the female’s oral cavity immediately after spawning, and individual blood samples were taken on alternate days until the second spawning occurred. Generally, all three females examined showed a similar pattern of gonadotropin secretion during the reproductive cycle (Fig. 6). Plasma levels of both FSH and LH increased on the second day after the eggs had been removed, declined toward the sixth to eighth day, and increased thereafter. In all three females, LH levels increased toward spawning, that occurred on the 11th day. FSH levels also increased on the day of spawning, but to a lesser extent (Fig. 6).

4. Discussion

In this study, we report the development of a sensitive and specific ELISA for monitoring tilapia FSH and LH levels using tethered recombinant gonadotropins as standards and their specific antibodies. Until now, all ELISAs and RIAs developed for the determination of FSH and LH levels in fish were based on native FSH and LH purified from fish pituitaries by various methods. Purifying FSH and LH is a complex procedure from both a quantitative and qualitative perspective. The amount of gonadotropins, and particularly FSH, in the pituitary of fish is very limited. Moreover, since LH, FSH and another pituitary glycoprotein, TSH, share the same z-subunit—it is very hard to achieve a high degree of separation. These limitations make it very difficult to purify FSH and LH in order to produce specific antibodies. Therefore, immunoassays for both FSH and LH were only developed for chum salmon (Suzuki et al., 1988a) and rainbow trout (Govoroun et al., 1998).

In other fish species, such quantitative tools for gonadotropins were restricted to LH, a fact that hampered studies on the differential role of LH and FSH in fish.

The recombinant gonadotropins generated in this study yielded several products of different sizes. This might be due primarily to different degrees of glycosylation, as reported in Ben-Menahem et al. (1999). Tilapia LHβ and tFSHβ each have only one putative N-glycosylation sequence (Asn-X-Ser/Thr), (Rosenfeld et al., 1997), while the α-subunit has two such sites (Gur et al., 2001). Moreover, carbohydrate modifications of the tilapia gonadotropins occur exclusively through N-linked, and not O-linked glycosylation (Kasuto and Levavi-Sivan, 2005), in tLH and (Aizen et al., 2007), in tFSH. This could explain the different degrees of glycosylation by Pichia of rtLHβ and rtLHβα. The rtLHβ appeared as a distinct band on a Western-blot, probably due to the smaller degree of glycosylation relative to rtLHβα, which appeared as a smear. Although not required for receptor binding, N-linked glycosylation is known to influence gonadotropin bioactivity both in vitro and in vivo, involving receptor coupling to adenylate cyclase (Arey et al., 1997).

The molecular-mass estimates of the recombinant tilapia gonadotropins, as well as of the pituitary-derived gonadotropins, were in the range of those reported for other teleost species, although mass estimates vary considerably, between 15 and 28 kDa for the subunits, and between 29 and 50 kDa for the intact hormones (chum salmon (Suzuki et al., 1988b); coho salmon (Swanson et al., 1991); common carp (Van der Kraak et al., 1992); seabream (Pagrus major) (Tanaka et al., 1993); bonito (Katsuwonus pelamis) (Koide et al., 1993); tuna (Thunnus obesus) (Okada et al., 1994); Mediterranean yellowtail (Seriola dumerili) (Garcia-Hernandez et al., 1997); hybrid striped bass (Mananos et al., 1997); killifish (Fundulus heteroclitus) (Calman et al., 2001); Japanese eel (Kamei et al., 2003); Atlantic halibut (Hippoglossus hippoglossus) (Weltzien et al., 2003); goldfish (Kobayashi et al., 2006), and Cichlasoma dimerus (Pandolfi et al., 1997).
inant band recognized by the anti-tFSHβ was the β-subunit (17 kDa). A major difference in stability between FSH and LH has been previously reported: whereas LH dissociates to its two subunits in the presence of 0.1% TFA, SDS or heating, FSH does not dissociate at all (Garcia-Hernandez et al., 1997; Wetzstein et al., 2004). In this work, we subjected the pituitary samples to SDS–PAGE under native conditions, with SDS in the sample buffer. These conditions may have caused dissociation of LH to its subunits, while not affecting FSH. Another option is that the quantitative relationship between the heterodimers and their subunits is a direct result of their native low content in the pituitary.

The recombinant tilapia gonadotropins analyzed by Western-blot were found to be active in bioassays based on steroid production by gonadal tissue. Both rtFSHβ2 and rtLHβ2 increased the release of 11-KT or estradiol from tilapia testes or ovaries, respectively ((Kasuto and Levavi-Sivan, 2005), for tLH and (Aizen et al., 2007), for tFSH). Moreover, exposure of female tilapia or dispersed pituitary cells to a GnRH superactive analog increased the release of pituitary products measured by our ELISA as LH and FSH (Aizen et al., 2007; Levavi-Sivan et al., 2006). This indicates that the production of both gonadotropins in the tilapia pituitary is under hypothalamic regulation of this peptide.

The characteristics of the ELISA developed in the present study for tilapia FSH and LH were comparable to those reported for other GtH immunoassays. The sensitivity of our ELISA was 0.24 pg/ml and 15.84 pg/ml for FSH and LH, respectively. In comparison, the range of sensitivities reported for fish LH RIAs is: 100 pg/ml for tilapia (Bogomolnaya et al., 1989), 156 pg/ml for hybrid striped bass (Mananos et al., 1997), 780 pg/ml for red seabream (Tanaka et al., 1993), and 580 pg/ml for silver carp (Kobayashi et al., 1985). The sensitivities of the two ELISAs described for fish GtHs are 125 pg/ml for goldfish GtH ELISA (Kah et al., 1989), 0.65 ng/ml for European seabass (Dicentrarchus labrax) LH (Mateos et al., 2006), 0.15 ng/ml for trout FSH, 0.87 ng/ml for trout LH (Goverou et al., 1998), 0.1 ng/ml for trout FSH (Santos et al., 2001), and less than 2 ng/ml for both gonadotropins of salmon (Suzuki et al., 1988a). Similar sensitivities were obtained by (Pappa et al., 1999) in an ELISA developed for rat LH (0.8 ng/ml) and FSH (1.25 ng/ml).

It should be noted that a potential problem exists in the use of ELISAs for monitoring GtH in plasma samples (Mananos et al., 1997). We solved this problem by developing two parallel standard curves, each with the appropriate solvent. The reproducibility of our ELISA was relatively high, as shown by the reasonably low intra- and interassay CVs. The intraassay CV was 7–8%, and the interassay CV ranged from 12.5% to 15%, depending on the binding. These variations are comparable to those previously reported for RIAs of GtH in other fish species, in the range of 5–8% and 7–15% for the intra- and interassay CVs, respectively, for tilapia (Bogomolnaya et al., 1989), silver

![Fig. 6. Plasma gonadotropin (FSH and LH) concentration as a function of days between two successive spawnings. Eggs were taken from the female mouth at day 0. a, b, and c represent three individual females.](image-url)
carp (Kobayashi et al., 1985), chum salmon (Suzuki et al., 1988a), and red seabream (Tanaka et al., 1993)). For similar ELISAs, the reported intra- and interassay CVs are, respectively, 4% and 6% for the rainbow trout GtH ELISA (Salbert et al., 1990), 7% and 15% for hybrid striped bass LH ELISA (Mananos et al., 1997), 10% and 11.5% for rat LH ELISA, and 5.5 and 9.5 for rat FSH ELISA (Pappa et al., 1999).

The antisera used in our ELISAs were developed against the recombinant β-subunits of the hormones and were shown to differentiate between the glycoproteins in the pituitary. Moreover, since both the standards and the antigens that the antisera were raised against were recombinant proteins, it is very unlikely that the antisera would cross-react with other pituitary hormones. Nevertheless, RIAs developed for fish LH, using antibodies against highly purified β-subunits present rather low cross-reactivity with FSH: 0.001% for trout (Prat et al., 1996), 4.4% for salmon (Swanson et al., 1989), 3.1% for red seabream (Tanaka et al., 1993), and 6% for carp (Van der Kraak et al., 1992). In the ELISAs developed in the current work the cross-reactivity between rtLH in the FSH ELISA was very low (<0.001%). Moreover, the same antibodies were recently shown to stain different specific cells in the pituitary of tilapia (Aizen et al., 2007). Taken together these results strengthen the specificity of the antibodies used in this study.

The dilution curves of the tilapia pituitary and plasma extracts were found to parallel those of the rtFSHβ and rtLHβ standards. We have previously shown that these recombinant gonadotropins can increase the secretion of estradiol and 11-KT from tilapia ovaries and testes, respectively (Aizen et al., 2007; Kasuto and Levavi-Sivan, 2005). Taken together, these results indicate that the form of the gonadotropins, in both the circulation and the pituitary, is immunologically similar to that of the tethered recombinant gonadotropins.

We also tested the validity of the tFSH and tLH ELISAs for potential measurements of gonadotropins in other fish species. Good parallelism was observed for pituitary samples from other perciform species, such as bass, seabream and seabass (where the percent homology of their β-subunits was 86–88 for LH and 67–70 for FSH), allowing the use of this assay for both FSH and LH measurements in these species. However, little or no parallelism was shown with serial dilutions of pituitaries from sturgeon, zebrafish, carp, eel, or trout.

Females of the genus Oreochromis are characterized by mouthbrooding behavior. Their ovarian recrudescence, which shows a transient arrest while brooding, is accelerated in females deprived of their eggs and expressing typical parental care (Smith and Haley, 1987) in O. mossambicus (Tacon et al., 2000, 1996) in O. niloticus. In O. niloticus, the mean duration of the cycle of non-mouthbrooding females is thus condensed relative to mouthbrooding females (Tacon et al., 1996).

Under the conditions prevailing in our holding facilities, one spawning cycle lasted 11 days. We analyzed the hormonal profiles of FSH and LH during one full reproductive cycle in three individual females. Generally, LH levels (measured by the present ELISA) were very similar to the values measured in tilapia by RIA in unstimulated females (Gissis et al., 1991; Levavi-Sivan et al., 2004a). Two clear peaks were recorded in the plasma of tilapia females between two successive spawns. The first was evident 2–3 days after spawning, at a stage that is probably the vitellogenic phase. According to the salmon model, it is not surprising to find an increase in FSH levels during the vitellogenic phase (reviewed by (Yaron and Levavi-Sivan, 2006)). However the relatively high levels of LH during the vitellogenic phase concomitant with the surge of FSH suggest that in tilapia, LH may play a role not only during final oocyte maturation, but also during vitellogenesis. Nevertheless, one should bear in mind that FSH has never been monitored in the circulation of fish with asynchronous ovaries that contain, at the same time, oocytes at various stages of development, which are probably under the control of each gonadotropin.

The second peak in gonadotropin levels was evident just before the next spawning. High levels of LH prior to or concomitant with final oocyte maturation have been reported in many fish species: salmon (Suzuki et al., 1988c), trout (Gomez et al., 1999), silver perch (Bidyanus bidyanus) (Levavi-Sivan et al., 2004b), and carp (Levavi-Zermonsky and Yaron, 1986) (reviewed by (Yaron and Levavi-Sivan, 2006)). Together with the second increase in LH levels, just before ovulation, an increase in FSH levels was evident. This increase probably reflects the hormone profile during the recruitment of a new generation of follicles for the next cycle. These results corroborate findings on gonadotropin gene expression: we recently found that tilapia females injected with low levels of DHP (the dominant steroid during final oocyte maturation) show an increase in LHβ gene expression, whereas high levels of the same steroid cause an increase in FSHβ gene expression (Levavi-Sivan et al., 2006).

A parallel fluctuation during the reproductive cycle of the gene expressions of both FSHβ and LHβ has also been found in other non-salmonid fish such as goldfish (Huggard-Nelson et al., 2002), seabream (Meiri et al., 2004), and blue gourami (Trichogaster trichopterus) (Jackson et al., 1999). However, alteration in the level of gonadotropin subunit gene expression is not necessarily a reliable yardstick for plasma level of the hormones (Swanson et al., 2003).
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