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RECEIVED 17 July 2023

ACCEPTED 23 August 2023

PUBLISHED 11 September 2023

## CITATION

Soffer O, Rubin O, Levy Y and Aizen J  
(2023) Development of an enzyme-linked  
immunosorbent assay for determining  
FSH plasma concentrations in green  
sea turtle (*Chelonia mydas*), using  
recombinant gonadotropins.  
*Front. Mar. Sci.* 10:1260205.  
doi: 10.3389/fmars.2023.1260205

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# Development of an enzyme-linked immunosorbent assay for determining FSH plasma concentrations in green sea turtle (*Chelonia mydas*), using recombinant gonadotropins

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Follicle-stimulating hormone (FSH) is involved in the regulation of essential reproductive processes such as gametogenesis and follicular growth. There are presently no immunoassays for measuring FSH in turtles. Recently we produced green sea turtle (*Chelonia mydas*) recombinant (r) FSH as a single-chain polypeptide using the methylotrophic yeast *Pichia pastoris* expression system, and polyclonal antibodies for the recombinant FSH. In this work we developed a competitive enzyme-linked immunosorbent assay (ELISA) for the measurement of FSH concentrations in plasma samples from green sea turtles. We used the rFSH $\beta\alpha$  for standard, rFSH $\beta$  for coating and a cmFSH $\beta$  antibody. The sensitivity of the assay was 0.13 ng/ml and the intra-assay and inter-assay coefficients of variation were 5.54% and 13.52% respectively. Parallelism was observed between the linearized FSH standard curves and the corresponding serial dilutions of green sea turtle plasma samples. We also observed parallelism between the linearized standard and serial dilutions of plasma samples from the loggerhead sea turtle (*Caretta caretta*), hawksbill sea turtle (*Eretmochelys imbricate*), and African softshell turtle (*Trionyx triunguis*). The ELISA was used to study the FSH plasma concentrations during the reproductive cycles and was compared to hormonal steroid concentrations (Testosterone, Estradiol and Progesterone). This revealed a positive correlation between FSH and estradiol concentrations in females; estradiol concentrations were increased immediately after FSH elevation. In addition, nested females presented an increase in FSH concentrations prior to progesterone elevation in January to April, slightly before egg laying. This ELISA will increase our understanding of gonadotropin functions, and their effects on reproduction in the green sea turtle.

## KEYWORDS

FSH, ELISA, sea turtle, nesting, progesterone, reproduction

## Introduction

Reproductive cycles in vertebrates are regulated by the hypothalamus. The secretion of gonadotropin releasing hormone (GnRH) stimulates the pituitary gland to release the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Licht, 1980; Kang et al., 2020). Gonadotropins (GTH) are composed of a common  $\alpha$ -subunit, and a unique  $\beta$ -subunit with a biological role specific to each hormone (Aizen et al., 2007a; Aizen et al., 2007b). Both gonadotropins regulate gonadal functions and thus are essential for reproduction. In mammals, FSH and LH have a distinct effect on endocrine regulation traits. In females, LH stimulates estrogen and progesterone production in the ovary. FSH is responsible for the development of the ovarian follicle, and stimulates secretion of estrogen from the follicle (Kluge et al., 2012).

Green turtles (*Chelonia Mydas*) have been subject to a long, and worldwide, history of human exploitation, resulting in endangerment of the global green turtle's population (Seminoff et al., 2015). Since the green turtles exhibit high natal homing behavior and due to their long and complex life cycle, human intervention is needed for to increase the green turtle population of nesting females. The Israel National Sea Turtle Rescue Center established a captive breeding stock in 2002 to provide this needed help. To rehabilitate the Mediterranean green turtle population, a deeper understanding and prediction of reproductive stages are essential. Although the reproductive ecology and endocrinology of green turtles have been the subject of numerous studies, little is known regarding their reproductive biology.

There is little information on the cycling and function of FSH in green sea turtles, and the physiological significance in this species is not yet fully understood. Several studies in female reptiles have shown a stimulation of follicular development following injection of mammalian FSH (Kawazu et al., 2014; Tripathy and Rai, 2017; Kang et al., 2020). Previous studies by Licht, 1980 and Wibbels et al., 1992 regarding FSH function in green sea turtle supported these results. An increase in FSH concentration at the time of ovulation in green sea turtles from April to August has also been observed (Licht et al., 1979; Wibbels et al., 1992).

In males, FSH was shown to stimulate testicular growth, and increased production of an androgen-binding protein by Sertoli cells that are necessary for sustaining the maturing sperm cell. Furthermore, FSH also stimulates spermatogonial mitosis (Licht et al., 1979; Ng et al., 1986; Bousfield et al., 2007). In males, an increase in FSH concentration was concurrent with an increase in testosterone, reaching maximal concentration after summer testicular recrudescence (Licht et al., 1985; Mendonca and Licht, 1986).

Gonadotropins regulate second messenger pathways leading to the production and secretion of sex steroids (Aizen et al., 2007a; Aizen et al., 2007b). There are three main sex hormones that affect behavior and reproduction in vertebrates: progesterone, estradiol, and testosterone. In females, progesterone is a potential ovulation inhibitor; testosterone affects mating receptivity towards males, and estradiol, is the primary stimulus for vitellogenin (Licht et al., 1979). In males, testosterone is significantly correlated with testicular

growth and spermiation, and estradiol is essential for spermatogenesis (Mahmoud and Licht, 1997).

The development of tools such ELISA or Radioimmunoassay (RIA) for the measurement of gonadotropins until now has required purified protein in large amounts and specific antibodies (Aizen et al., 2007b). Obtaining high quality and quantity of native gonadotropins requires purifying GTHs from pituitary glands, which would involve the killing of a significant number of animals. This is very problematic, especially for the green sea turtle that is an endangered species (Seminoff et al., 2015).

Recombinant FSH production has a distinct advantage since production is not dependent on pituitary availability (Kasuto and Levavi-Sivan, 2005). The yeast host system is one of the most powerful tools for expressing recombinant proteins. Methylophilic yeast species such as *Pichia pastoris* have been developed for this purpose and are very popular due to their low cost (Karbalaei et al., 2020). This yeast line can produce high yields of recombinant proteins with glycosylation (Balamurugan et al., 2007). The advantages of this expression system include appropriate folding in the endoplasmic reticulum, and secretion of recombinant proteins to the medium, making the harvesting easier, and avoiding yeast cell protein contamination in the purification process (Li et al., 2007; Yang et al., 2013). Furthermore, they have high growth rate, coupled with ease of high cell-density fermentation, high levels of productivity in an almost protein-free medium, ease of genetic manipulation of well characterized yeast expression vectors as in *Escherichia coli*. (Li et al., 2007). The time needed to assess recombinant gene expression with the *P. pastoris* is approximately 3-4 weeks. This time frame includes transformation of the yeast, screening the transformants for integration, and an expression time-course analysis (Aizen et al., 2007a; Aizen, 2011). *P. pastoris* culture can reach 120 g/L of dry cell weight density (Aizen, 2011).

There are many immunoassay platforms available to quantify protein concentrations in biological fluids. ELISAs are preferred in many cases due to their sensitivity, specificity, accuracy, and ability to tolerate harsh buffers or pre-treatments (Molés et al., 2012). Both indirect and sandwich ELISAs allow detection of antigen or antibody at very low concentrations hence their specificity. The main advantage of competitive ELISA is that the method detects differences in antigen-antibody complex with high sensitivity, even when the specific detecting antibody is present in relatively small amounts. Competitive ELISA is less sensitive to sample dilution and sample matrix effects than the sandwich ELISA, and there is less variability between duplicate samples and between assays (Barlow et al., 1986). Taken together one should consider using a sandwich ELISA. The use of two specific antibodies, one against the beta subunit and the other against the alpha subunit allows us to specifically detect each glycoprotein hormone, depending on the anti-beta used, in its natural quaternary structure. (i.e. associated with alpha and excluding free beta detection). With regards to the alpha subunit in glycoproteins, it holds some challenges, as it is not very immunogenic and highly conserved among glycoproteins among different species and the same species (identical alpha for FSH, LH and TSH).

RIA methods were widely used for the measurement of gonadotrophin concentrations based on the principle of

competitive binding. In this method, an unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity (Webster et al., 1990). RIA is often based on a polyclonal antibody and may show cross-reactivity to substances other than the test analyte. ELISA, on the other hand, is based on two antibodies, which ensures high specificity for the analyte. In addition, the ELISA method can utilize antibody quantities that are higher than those allowed by RIA's radioactive tracer (Wewer Albrechtsen et al., 2014), and ELISA exhibits lower coefficient of variation (CV) than RIA (Warnken et al., 2016).

ELISA also offers excellent performance with small sample volumes, and the procedure takes about half the amount of time as the RIA assay. In addition, RIA uses radiolabeled reagents that require a license for storage and special waste disposal. The ELISA method avoids these constraints of using radioactive chemicals (Webster et al., 1990; Wewer Albrechtsen et al., 2014).

In recent years the use of analytical methods to determine concentrations of gonadotropins such as LC-MS/MS and LC-MRM/MS were implemented due to the limited availability of high-quality antibodies, and ability to distinguish between different FSH glycovariants and showed comparable sensitivity to ELISA assays, with advantage of being faster and more selective (Melchiorre et al., 2021). The majority of assays LC-MS is currently at least complementary if not superior to immunoassay and that the benefits LC-MS offers will see its increased use over immunoassay, especially as the technology advances but we are not there yet (Cross and Hornshaw, 2016).

The reproductive biology of sea turtles has been studied both in the wild and in captivity; most of the research is based on nesting females, due to the ease of sampling during the nesting season. Only a few studies on hormonal cycles of adult males have been investigated in the wild (Wibbels et al., 1990; Tim et al., 2004; Blanvillain et al., 2008) and others have been studied in captivity (Licht et al., 1979; Licht et al., 1985; Rostal et al., 1998). To study GTH biological function we need assays for measuring GTH concentrations, and in order to achieve that we need to produce properly folded and biologically active recombinant GTHs.

In this study, we used the yeast *Pichia pastoris* expression system, since this system provided sufficient quantities of recombinant protein (Daly and Hearn, 2005) for the development and validation of an ELISA. We report on the production of a recombinant green sea turtle FSH (rcmFSH) and the development of a competitive ELISA for measuring green sea turtle FSH using the recombinant protein. For the first time, we were able to directly measure FSH concentrations in plasma samples, by comparing plasma FSH concentrations with steroids concentrations. We observed that FSH concentrations increased during the prior and during the nesting season from January to June (nesting season is April to August) along with progesterone concentrations. We were able to identify a pattern whereby FSH concentrations rise followed by rising estradiol concentrations. This helps us predict the reproductive stages of the turtles in the breeding facility, which can enhance successful breeding in captivity. Our main goal is to monitor annual concentrations of sex steroids and gonadotropins to characterize green turtle hormonal profiles. A better understanding of the green sea turtle reproductive

biology may boost future efforts on population rehabilitation and conservation.

## Materials and methods

### Research population and site

The Sea Turtle Rescue Center under was established by the Nature & Parks Authority of Israel is located in Alexander River National Park (Coordinates: 32.4016667, 34.8666667)

The reproduction facility features two 450 cubic meters tanks and an artificial nesting beach. Water temperature is kept at 20–22°C, and periodically altered. The turtle's diet is composed of romaine lettuce (2% BW) and frozen fish (Herring, Flounder and Salmon, 0.2% BW). All turtles are tagged with metal plates on either their front or back limb. The research population consists of 12 females (weight: 83.58 ± 22.74 Kg; CCL: 82.81 ± 7.52 cm; CCW: 75.31 ± 7.44 cm) and 11 males (weight: 66.16 ± 17.89 Kg; CCL: 76.09 ± 8.16 cm; CCW: 69.5 ± 7.2 cm), from the captive breeding stock in Mikhmoret (Table S1). 6 of the 12 females nested in 2 nesting seasons: 474, 495, 729 and 760 in 2019; 173 and 1055 in 2020.

### Blood sample collection

Blood sampling was performed approximately every two months in the Israel National Sea Turtle Rescue Center. Five ml of blood were taken from the dorsal vein of the turtle's neck using a sterile 21-gauge 8.9 cm needle. Blood was moved into a biobased blood collection tube that contains an anticoagulant agent heparin sodium. The blood samples were stored at 4°C during the collection (no more than one hour). Once collection ended, hematocrit count of each turtle was first examined to obtain information on their health. We centrifuged two capillaries for each sample at 17800 RCF for 12 min and calculated the average of the two capillaries. For blood fractionation, we centrifuged the 5 ml tubes at 3200 RCF for 15 min. Each tube contained approximately 3 ml of plasma. We divided each individual plasma sample into three different 1.5 ml Eppendorf tubes for hormone measurement, blood biochemistry and backup. The plasma was then stored at -80°C.

### Steroid extraction and measurement

For steroid extraction we used a 500 µl plasma sample and added 5 ml diethyl ether to glass tubes and mixed 3 times for 15 seconds with vortex. We transferred the glass tubes to -80°C for 45 minutes, the upper phase in the glass tube was transferred to a new glass tube and the diethyl ether was evaporated by heating the tubes in a 55°C water bath. Extraction products were dissolved in 500 µl Steroid Assay Buffer (SAB). The concentrations of sex steroids (estradiol, progesterone and testosterone) were measured by commercial Cayman ELISA kits<sup>®</sup>. (501890, 582701 and 582601 respectively). Concentration detection levels were 6.6–4,000, 7.8–

1,000 and 3.9-500 pg/ml for estradiol, progesterone and testosterone respectively, with sensitivity range of 6-15 pg/ml. In order to assess the concentration range of each steroid in the research population, preliminary tests were conducted. For each steroid assay at least three dilutions were tested, with samples from both sexes and different sampling dates to assess the annual concentration variation (data not shown).

## Hormone production and purification

Recombinant FSH was produced using the methylotrophic yeast *P. pastoris*, as a biologically active, single-chain polypeptide according to Aizen et al. (2007a) protocol, green sea turtle (*Chelonia mydas*) FSHB (GenBank Accession No. XM\_007067910.1) and green sea turtle (*Chelonia mydas*) CGA (GenBank Accession No. XM\_007052575.1) subunits coding gene sequences were translated to amino acid sequences using the ExPASy Proteomics Server (<http://ca.expasy.org/tools/dna.html>). The presence of signal peptide cleavage site was predicted by SignalP 4.1 Server (Nielsen, 2017). The protein coding gene sequences were used to design two expression vectors: FSH  $\beta$ -subunit peptide chain for future antibody production, and FSH $\beta\alpha$  peptide chain for standard curve. FSH $\beta\alpha$  vector was constructed with a flexible linker allowing a smooth rotation of the two subunits to reach the natural structure of the entire gonadotropin. To allow flexibility to the construct tethered polypeptide, we used 2xGS-6His-2xGS between the  $\beta$ -subunits and  $\alpha$ -subunits. Both vectors were transfected into chemically competent *E. coli*, JM109 strain via heat shock based on manufacturer's protocol (Promega). Selection and expression were generally performed according to (Aizen et al., 2007a). Recombinant proteins were purified using nickel-nitrilotriacetic acid-agarose (Ni-NTA; Qiagen). As a negative control, *P. pastoris* was transformed with an expression vector that did not contain the cmFSH cDNA, and fractions were prepared in the same manner. The eluted proteins were concentrated by lyophilization, and protein concentration was measured using Bradford reagent. The eluted proteins were stored at  $-80^{\circ}\text{C}$  until use. Recombinant proteins were deglycosylated using PNGaseF (Peptide N-glycosidase F) and analyzed by Western blot with anti-HIS staining.

## Antisera

The antiserum against green sea turtle FSH  $\beta$ -subunit was produced by a commercial company ADAR Biotech Ltd. (Rehovot, Israel). The antiserum was raised in two rabbits with approximately 40 ml of sera harvested from each rabbit. Sera antibodies titers against the antigen were determined by direct ELISA by ADAR Biotech Ltd. resulting in high titer of antibodies against the FSH  $\beta$ -subunit. Final bleeding was performed, and the antiserum was then aliquoted, and stored at  $-80^{\circ}\text{C}$ .

## Characterization of FSH by western blot analysis

FSH protein samples were electrophoresed on 16% SDS-polyacrylamide unstained gels with 6-8% stacking gel. We added Laemmli SDS reducing buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol concentrated at 4X to the protein samples for SDS-PAGE under reducing conditions. Samples were heated in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany) for 10 min at  $100^{\circ}\text{C}$ . The electrophoresis was carried in TGS (Tris-Glycine-SDS) running buffer (Bio-Rad, Hercules, CA) at 110V for one hour. The SDS PAGE gels were then washed once with DD  $\text{H}_2\text{O}$ . Gels were blotted to nitrocellulose membranes (BioRad) using Bio-Rad Trans-Blot<sup>®</sup> Turbo TM Blotting System. Membranes were blocked with Anti-His HRP blocking (Qiagen, Limburg, Netherlands) or 5% low-fat milk powder in PBS. Bound antibodies were detected with either penta-His antibody (Qiagen, Limburg, Netherlands) or with turtle FSH $\beta$  antibody. When using anti-His antibody, the membranes were treated with penta-His-HRP antibody (1:2000 dilution) overnight at  $4^{\circ}\text{C}$ . When using anti-FSH $\beta$ , the membranes were incubated in PBS plus 1% low-fat milk powder with the antibodies (diluted 1:5000) overnight at  $4^{\circ}\text{C}$  and then with goat-antirabbit horseradish peroxidase conjugate (GAR-HRP) (diluted 1:5000) for 1h at room temperature (RT). After washing, all membranes were treated with enhanced chemiluminescence reagent, EZ-ECL kit (Biological Industries, Beit Haemek, Israel) to reveal immunoreactive bands.

## Development of ELISA for FSH

Competitive ELISA for green sea turtle FSH was developed using FSH $\beta$ -subunit primary antibody, recombinant FSH $\beta$ -subunit for coating ELISA microplates, and recombinant single chain  $\beta\alpha$ FSH for the standard curve. The protocol was based on (Aizen et al., 2007b).

### Coating

We coated ELISA 96-well microplates (MaxiSorp<sup>™</sup> Nunc-Immuno, Thermo Scientific<sup>™</sup>, Denmark) with 100  $\mu\text{l}$ /well of recombinant green sea turtle FSH $\beta$  diluted 1.5 ng/10ml (0.015 ng/well) in a solution of carbonate buffer (50 mM sodium carbonate ( $\text{NaHCO}_3$ ), pH 9.6). The plates were incubated in complete darkness overnight at RT without shaking. For non-specific binding, two wells (A12 and B12) were not coated with the FSH $\beta$  solution.

### Washing and blocking

The next day, coated microplates were washed (3 times X 5 min) with 200  $\mu\text{l}$ /well of PBST buffer (10 mM  $\text{Na}_2\text{PO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$  (pH 7.4), 140 mM NaCl, 3 mM KCl, and 0.05% Tween 20). The microplates were blocked with 200  $\mu\text{l}$ /well of 1% bovine serum albumin (Sigma, Rehovot, Israel) in PBST buffer for 1 h in complete darkness at RT without shaking to reduce background.

## Pre-incubation of standards and samples

Standard was diluted in 0.1% BSA in PBST with 10% of normal goat serum (NGS, Biological Industries). Unknown plasma samples were diluted (1:15 for males, 1:15 for females and 1:30 for nested females) with 0.1% BSA in PBST, each sample was diluted in a volume of 300  $\mu$ l allowing duplicate analysis. The unknown samples and FSH $\beta$  $\alpha$  standards were pre-incubated in 96-well microplates (Sarstedt AG & Co., Nümbrecht, Germany) with the primary antibody against FSH $\beta$  in final dilution of 1:5000 dissolved in 0.1% BSA in PBST in complete darkness overnight at RT without shaking.

## Samples and standards dispensed

After the pre-incubation of standard and unknown samples with the primary antibody, each sample was dispensed into the coated and blocked microplates (100  $\mu$ l/well) and incubated in complete darkness overnight at RT without shaking.

## Incubation with secondary antibodies

The next day the plates were washed (3 X 5 min) with PBST. 100  $\mu$ l/well of GAR-HRP diluted 1:5000 in PBST 0.1% BSA buffer were added and incubated for 2 h in complete darkness at RT without shaking to detect the formed antigen–antibody complexes.

## Color development

Before color development the plates were washed again (3 times X 5 min) with PBST. The visualization of enzyme complexes was executed by addition of 100  $\mu$ l/well of ABTS<sup>®</sup> 2-Component Microwell Peroxidase Substrate (KPL, Zotal, Israel). The reaction was carried out in complete darkness at RT for 20 min. Absorbance was read at 405 nm, using a Synergy HTX Multi-Mode Reader (BioTek, USA).

## Validation of the ELISA for green sea turtle FSH

The ELISA was validated for cmFSH determinations in plasma samples of *Chelonia mydas* and was tested for cross-reactivity with additional turtle species (loggerhead, hawksbill and african softshell turtle). To conduct the parallelism analysis, whole blood samples were collected from sexually mature turtles held in captivity at the NPA Center. Displacement curves for plasma samples were obtained by serial dilutions of the plasma in the ELISA buffer (PBST buffer containing 10% NGS) and compared with the ELISA standard curve. The ELISA was also validated for FSH in other turtle species. Plasma samples were diluted 1:2. All samples were used in the ELISA at a total volume of 100 $\mu$ l solution per well. Intra assay coefficient of variation (CV) was determined by assaying 24 replicates of green sea turtle samples on the same assay plate. Inter assay variation was determined by assaying the same sample 9 times on different assay plates.

## Statistical analysis

All data are presented as means  $\pm$  SEM (Standard Error of the Mean). To test parallelism between displacement curves, we used linear regression in Graph-Pad Prism 9.0 software (GraphPad, San Diego, CA) and compared slopes. The significance in differences of hormone level means between groups was determined by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. To test for parallelism between various regressions lines, we performed logit transformation, where  $\text{logit}(B/B_0) = \log[r/(1-r)]$ , and  $r = B/B_0$ , B represents the binding at each point, and  $B_0$  represents the maximum binding. We used the linear regression parameter in Graph-Pad Prism 9.0 software to compare the slopes.

## Results

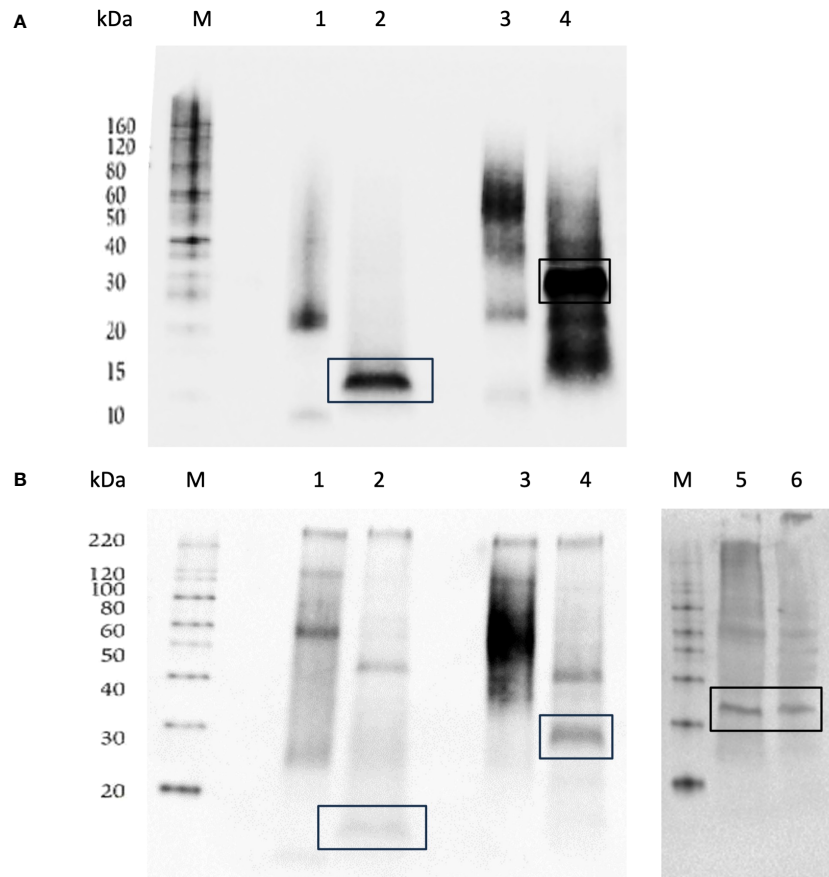
### Recombinant FSH $\beta$ $\alpha$ and FSH $\beta$ validation

Western-blot analysis was performed on green sea turtle pituitary extract and the supernatant derived from yeast transformed with an expression vector containing cmFSH $\beta$  $\alpha$ , cmFSH $\beta$ .

These proteins were immunoreacted with antibodies against Penta-His antibody (QIAGEN) (Figure 1A) or against cmFSH $\beta$  antibody (Figure 1B) for antigen specificity conformation. The anti-HIS staining FSH $\beta$  yielded bands of  $\sim$ 25kDa, and after deglycosylation FSH $\beta$  yielded a band of 15 kDa (Figure 1A, lane 2). FSH $\beta$  $\alpha$  protein analysis yielded bands of  $\sim$ 60- 75 kDa (Figure 1A, lane 3) and a band of 27-30 kDa after deglycosylation (Figure 1A, lane 4). The anti-cmFSH $\beta$  antibody staining yielded bands for cmFSH $\beta$  of  $\sim$ 60-120 kDa, and after deglycosylation a band of 15-25 kDa (Figure 1B, lane 2). The cmFSH $\beta$  $\alpha$  protein analysis yielded bands of  $\sim$ 60-80 kDa (Figure 2B, lane 3) and a band of 25-30 kDa after deglycosylation (Figure 1B, lane 4). Green sea turtle pituitary yielded bands around 30-35 kDa (Figure 1B, lane 5-6) validating the expected size of the dimer protein in the pituitary. Transformation with vector alone served as a negative control and yielded no bands (data not shown).

### Development and validation of competitive ELISA for green sea turtle FSH

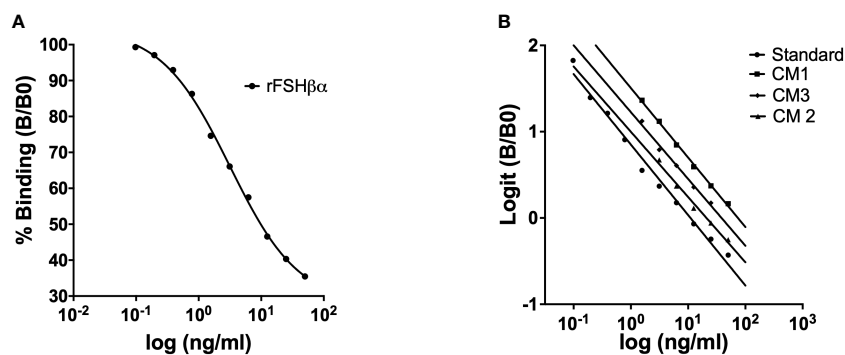
A competitive ELISA was developed for FSH determination in plasma samples of green sea turtle, using rcmFSH $\beta$  $\alpha$  as standard, rcmFSH $\beta$  for coating and primary antisera against rcmFSH $\beta$  for detection. Dilution tests were carried out to determine appropriate dilutions for antigen (coating step) and antibody (competition step) to achieve optimal results. Antibody dilutions (1:5000, 1:10000, 1:20000) and four FSH $\beta$  coating concentrations (0.5 ng/ml, 1 ng/ml, 1.5 ng/ml and 5 ng/ml) were first tested to determine the combination that would result in optical density closest to 1 OD. The optimal dilution was 1:5000 for first antibody, 1.5 ng/ml for coating (rcmFSH $\beta$ ), standard curve was generated using rcmFSH $\beta$  $\alpha$ .



**FIGURE 1** Western blot stained with anti-His antibody (A) or anti-FSHβ antibody (B) of Ni-NTA nickel agarose beads purified FSHβ and FSHβα protein expressed by *P. pastoris* (GS115) and green sea turtle pituitary gland extracts, western blots were carried out under reducing conditions. M: Protein size marker, 1: FSHβ protein, 2: deglycosylated FSHβ protein, 3: FSHβα protein, 4: deglycosylated FSHβα protein, (5-6): pituitary gland protein extractions from green sea turtle (*Chelonia mydas*). (B) Marker (M) and lane 5,6 were spliced and grouped from [Supplementary Image S3](#), all original westerns are in the [Supplementary Images S1–S3](#).

at starting concentrations of 50 ng/ml that were serially diluted (x2) down to 0.0488 ng/ml. Percentage binding showed the sigmoidal curve typical in competitive ELISAs ([Figure 2A](#)). After logit-transformation, the percentage binding data showed a linear

relationship with the logarithm of the standard concentration ( $r^2 = 0.97298$ , [Figure 2B](#)). Sensitivity of the assay was defined as the amount of FSH sufficient to reduce the optical density determined in the absence of FSH by two standard deviations. Sensitivity of



**FIGURE 2** Competitive binding curve for green sea turtle FSH standard. Each point represents the mean value of a duplicate (A). Parallelism between the standard curve of ELISA for green sea turtle FSH measurements and displacement curves obtained with serial dilutions of plasma samples from three of green sea turtle females (CM: *Chelonia mydas*) (B).

FSH measuring was 0.13 ng/ml, with the optical density decreasing as a linear function of FSH concentration ( $r^2 = 0.97298$ , Figure 2B), with percent binding B/Bo > 96%. The intra-assay CV was calculated by measuring replicates of the same sample within the assay. The intra-assay CV was estimated at 5.54% for FSH. The inter-assay CV was calculated by measuring replicates of the same sample in different assays. The inter-assay CV was estimated at 13.52%. The sensitivity of the ELISA we developed was 0.13 ng/ml for measuring FSH concentrations in plasma samples.

Assay validation was confirmed by parallelism of the standard curve and the curves of serial dilutions of green sea turtle plasma (Figure 3A). No significant difference was found between the slopes of the standard curve ( $-0.8168 \pm 0.03318$ ) and the curves of CM plasma serial dilutions ( $-0.7787 \pm 0.03997$ ). These data indicate that the recombinant FSH is immunologically like the endogenous gonadotropin found in the turtle plasma. To determine ELISA applicability in other species we diluted plasma from loggerhead sea turtle (*Caretta caretta* - CC, Figure 3A), hawksbill sea turtle (*Eretmochelys imbricate* - EI, Figure 3B) and African softshell turtle (*Trionyx triunguis* - TT, Figure 3B). Results showed no significant difference between the slopes of the standard curve and the slope of CC plasma samples ( $-0.7847 \pm 0.09212$ ), EI plasma concentrations ( $-0.9605 \pm 0.08680$ ) and TT plasma concentrations ( $-0.8301 \pm 0.1789$ ).

## Seasonal variations in the FSH plasma concentrations compared to testosterone, progesterone and estradiol

The analysis presented in this section focuses on three categories: nested females, non-nested females, and males. The data represents the seasonal variations of three sex steroids and FSH. The objective is to explore the relationship between the concentrations of the variables under examination and the reproductive activity of female green turtles. To achieve this, the turtles were classified based on their nesting behavior. In the Mediterranean region, the nesting season typically spans from May to September (Mazor et al., 2013). Considering that vitellogenesis, the process of yolk formation,

typically initiates 10-12 months before nesting, the nesting females in our study were defined as those that laid eggs between May and September in our reproduction facility. Females that did not engage in nesting activities during the analyzed year were categorized as non-nesting. In terms of the nested females, the graphs display data from both a year prior to and a year following the nesting season, highlighting the shifts in the analyzed parameters.

The four females that have nested in 2019 season presented a rise in FSH concentrations from November 2018 towards June 2019 (60-237 ng/ml), then a decrease towards November 2019 (86 ng/ml) and a rise towards April 2020 that maintained same concentrations until the end of 2020 (150-204 ng/ml) (Figure 4A). The testosterone concentrations in 2019 season peaked before the nesting season (April to August) from June 2018 to April 2019 (147-1856 pg/ml) and then decreasing to 40 pg/ml in July 2019 maintaining low concentrations until September 2020 (Figure 4A). The progesterone concentrations presented a rise from November 2018 peaking in June 2019 (27-4513 pg/ml) and then decreasing to 30pg/ml maintaining low concentrations below 100pg/ml until the end of 2020. The concentrations of Estradiol remained low from January 2018 to January 2020 (6-56 pg/ml) and started to rise from April 2020 to June 2020 (50- 143 ng/ml) (Figure 4A).

The two females that nested in the following 2020 season also presented a rise in FSH concentrations from January 2020 to April 2020 (156-293 ng/ml) following a decrease until August 2020 and a rise in FSH concentrations until September 2021 reaching 337ng/ml (Figure 4B). The testosterone concentrations in 2020 season peaked before the nesting season (April to August) from June 2019 to September 2019 (42-948 pg/ml) and then decreasing to 273 pg/ml in April 2020 maintaining low concentrations until August 2021(30-185 pg/ml) (Figure 4B). The progesterone concentrations presented a rise from January 2020 peaking in April 2020 (46-2930 pg/ml) and then decreasing to 65pg/ml maintaining low concentrations below 111pg/ml until the end of 2021. The concentrations of Estradiol peaked prior to the nesting season in September 2019 reaching 143pg/ml and from January 2020 to November 2021 ranged from at low concentrations of 73 to 4pg/ml (Figure 4B).

The non-nesting females from January 2018 to November 2021 presented FSH concentrations ranging from 50-383ng/ml. a pattern

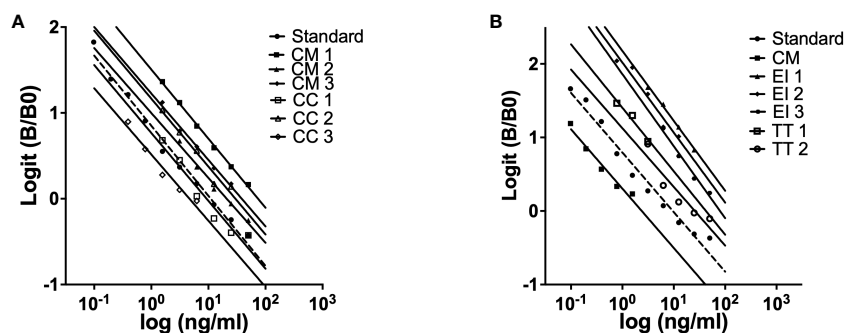


FIGURE 3

Parallelism between the standard curve of ELISA for green turtle FSH and displacement curves obtained with serial dilutions of plasma samples from three individuals loggerhead sea turtles (CC: *Carreta caretta*) and green sea turtle (CM: *Chelonia mydas*) (A) three individuals hawksbill sea turtle (EI: *Eretmochelys imbricate*), two African softshell turtle (TT: *Trionyx triunguis*) and green sea turtle (CM: *Chelonia mydas*) (B).

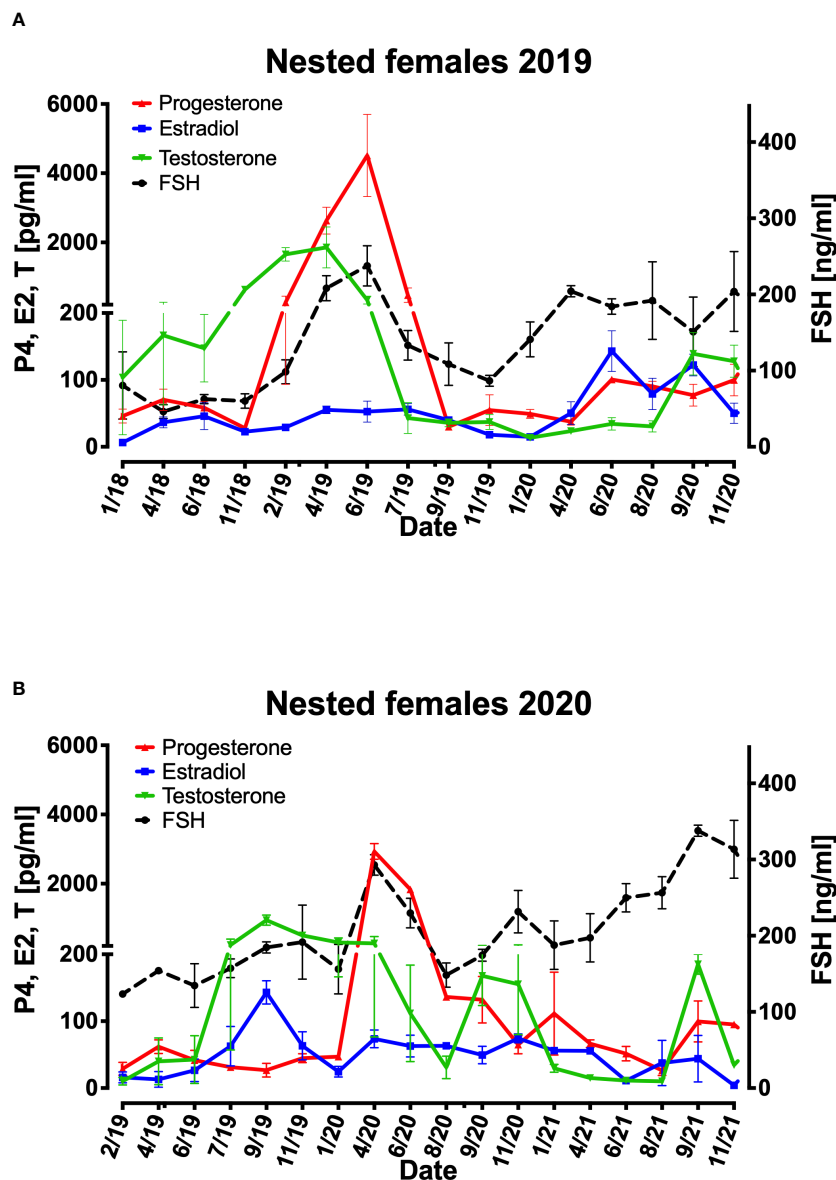


FIGURE 4

Mean variations of FSH, progesterone, estradiol, and testosterone plasma concentration from 4 females that nested in 2019 (A) and 2 females that nested in 2020 (B). X axis represent each sampling date in the period. Right Y axis represents FSH (ng/ml) in females, Left Y axis represents progesterone (P4) estradiol (E2) and testosterone (pg/ml) concentrations.

of rise in FSH concentrations was shown every year prior to the nesting season (April to August) from September or November reaching a peak in June, different from 2018 and 2019, 2020 season FSH concentrations were higher reaching 383ng/ml compared to 135ng/ml in prior seasons (Figure 5A). The testosterone concentrations were below between 24-156pg/ml and peaked every year excluding 2018 in September reaching the highest in September 2021 159pg/ml (Figure 5A). Progesterone presented no significant pattern over 2018 to 2021 and concentrations of 40-128pg/ml. The concentrations of Estradiol remained very low during 2018-2019 (8-15 pg/ml). In 2020 estradiol peaked during the nesting season reaching 74pg/ml and from November 2020 decreased to 17pg/ml in November 2021 (Figure 5A).

Throughout 2018 to 2021 males showed high concentrations of FSH in January (122-260 ng/ml), September (100-374 ng/ml), and November (150-200 ng/ml) compared to the rest of the year (50-150 ng/ml), and their estradiol concentrations corresponded to the rises in FSH concentrations. Regarding testosterone the concentrations were between 3-45 ng/ml, the progesterone concentrations were higher during the nesting season (April to August) (Figure 5B).

## Discussion

Herein we describe for the first time the development of an ELISA for FSH in green sea turtles, with recombinant gonadotropin



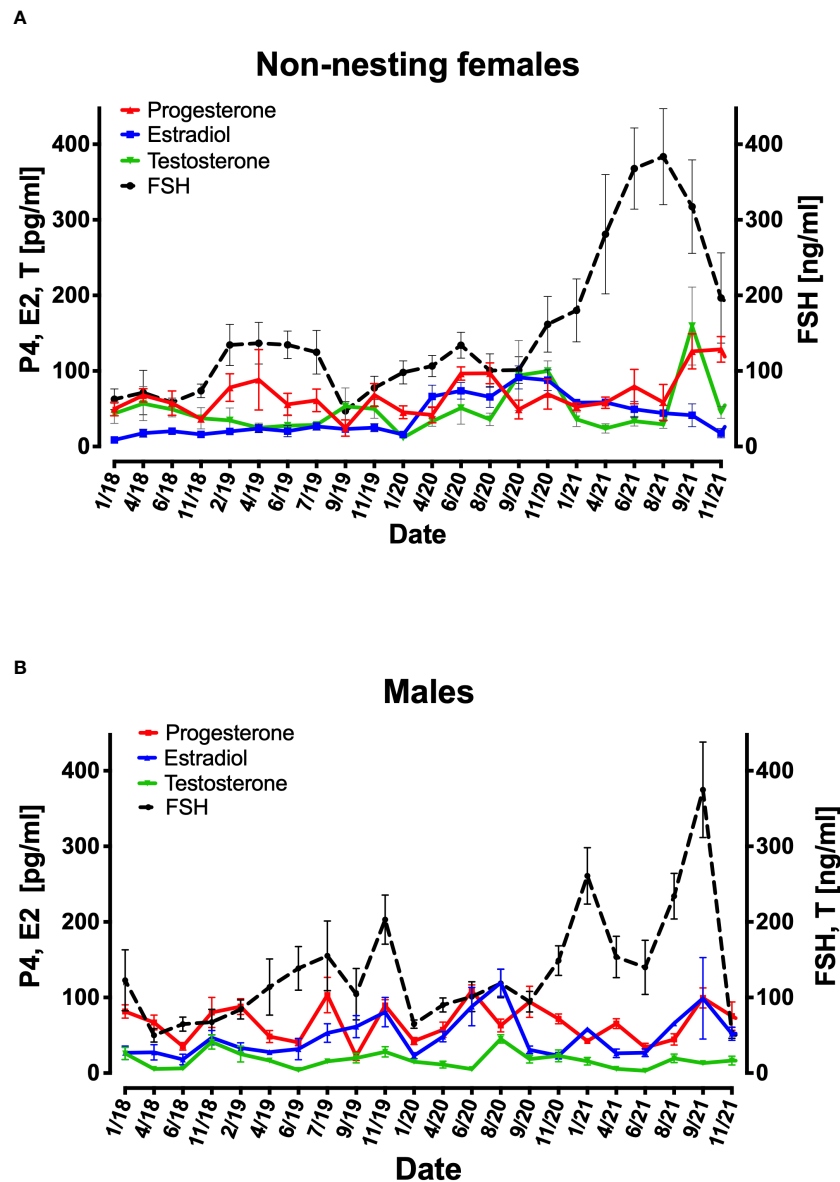


FIGURE 5

Mean variations of FSH, progesterone, estradiol, and testosterone plasma concentration from 6 females that have not nested from 2018 to 2021 (A) and 12 males (B). X axis represent each sampling date in the period. Right Y axis represents FSH (ng/ml) in females (A). Left Y axis represents progesterone (P4) estradiol (E2) and testosterone (pg/ml) concentration in females (A). In males Right Y axis represents FSH and testosterone (ng/ml) concentration and Left Y axis represents progesterone (P4) and estradiol (E2) (pg/ml) concentration (B).

we produced using the yeast *P. pastoris* expression system. The ELISA was also found to be applicable for other sea turtle species. We also observed plasma steroid concentration during the reproductive cycles that revealed a positive correlation between FSH and estradiol concentrations in females; estradiol concentrations were increased immediately after FSH elevation. In addition, nested females presented an increase in FSH concentrations prior to progesterone elevation in January to April, slightly before egg laying.

We must emphasize that the turtles in this research have been in the breeding facility since hatching. Our results describe patterns of FSH and steroids concentrations for turtles that were kept in captivity their entire life. With that we believe that our findings

can help to better understand what is occurring during the time periods before nesting and after nesting especially for turtles that will not nest every year.

Measuring hormone concentrations in turtles is challenging on many levels. Taking blood from females arriving on the beaches to lay eggs may interfere with their normal behavior during this critical period. Finding males on the beach is rare, and so measuring their hormone concentrations may not be feasible (Goldberg et al., 2013). When injured turtles arrive at the Sea Turtle Rescue Center, they are usually young, and distinguishing between males and females is not possible, Owens et al. (1985) showed that injecting mammalian FSH in immature turtles increased the testosterone concentration in the blood and was helpful to determine if the turtles are truly males, but

this method has not been tested since then (Owens and Morris, 1985). Measuring testosterone concentrations in young turtles does not correlate with their gender, especially in young age.

Therefore, studying the breeding stock at Mikhmoret was important for achieving a hormonal profile of the green sea turtles. In this research we developed and validated a competitive ELISA for measuring FSH concentrations in plasma samples from green sea turtle based on the approach of production recombinant FSH as a single chain molecule using the *P. pastoris* expression system. Earlier research using the *P. pastoris* expression system has shown that the main advantage of this system is the ability to produce a recombinant protein that is similar to the natural hormone and can elicit an *In-vitro* and *In-vivo* biological response (Kasuto and Levavi-Sivan, 2005). In addition, the protein can be efficiently purified from the culture medium (Aizen et al., 2007a; Aizen et al., 2007b). The use of a recombinant gonadotropin can increase its immunogenicity, as well as the specificity of the generated antibodies (Daly and Hearn, 2005).

Generally, immunoassays that are developed to determine GTHs concentrations have been based on native GTHs that were purified from pituitary glands (Molés et al., 2012). Purification of native FSH is a very demanding process, and to purify a sufficient amount of GTHs we would need a large number of pituitary glands, which would require killing a large number of turtles. Recombinant proteins can be produced continuously, independent of turtle pituitary gland availability. Additionally, recombinant FSH subunits can ensure there is no cross-reactivity with similar GTHs that share the same  $\alpha$ -subunit (Aizen, 2011). The open reading frame for FSH  $\alpha$ -subunit is 120 amino acids, and for FSH  $\beta$ -subunit is 138 amino acids. The subunits were cloned and validated for their predicted protein sequences from NCBI. The sequences were compared to different turtle species (The common box turtle; *T. carolina*, Painted turtle; *C. picta*, Chinese pond turtle; *C. reevesii*, and Chinese softshell turtle; *P. sinensis*) and were found to be practically identical. In different studies on the FSH molecular structure resemblance in FSH  $\alpha$ -subunit between vertebrates was found (Chien et al., 2005; Shen et al., 2006). To date, an ELISA for the measurement of FSH in sea turtles or in any other reptile has not been developed.

Different studies for development of ELISA to measure FSH were done in several fish and mammalian species: tilapia, rat, mice, flatfish, yellowtail kingfish, Japanese rice fish, Japanese eel and more (Pappa et al., 1999; Shimizu et al., 2012; Chauvigné et al., 2015; Burow et al., 2019; Kazeto et al., 2019; Nocillado et al., 2019; Pappas, 2019; Ongaro et al., 2021). FSH in green sea turtles was last measured in the early 90's (Wibbels et al., 1990) using radioimmunoassay (RIA), which is based on the principle of all immunoassays: a known amount of antigen is labeled with a radioactive element and mixed with a known amount of antibody directed against that antigen (Webster et al., 1990).

The RIA method is problematic because it uses radiolabeled reagents that require a special license for storage and waste disposal (Sancho et al., 2013). In addition, ELISA is more accurate and reproducible at lower concentrations than RIA (Webster et al., 1990).

LC-MS/MS and LC-MRM/MS techniques present the potential for surpassing the accuracy of RIA and ELISA, exhibiting heightened sensitivity towards extremely low concentrations. It's crucial to consider the requirements for regular biomolecule quantitation from a broad range of fields. While immunoassays have been instrumental, they might need to evolve to match the escalating throughput demands. As long as the goals involve reduced per-sample expenses and enhanced precision, the prospects of LC and LC-MS appear promising as a prospective solution.

The FSH concentrations measured in our ELISA were 17-650 ng/ml for females and 24-710 ng/ml for males. The results for FSH level ranges in females do not concur with studies by P. Licht et al., 1979 (0.3-10.6 ng/ml) and Wibbels et al., 1992 (0.1-2 ng/ml). In these studies green sea turtle males did not reveal measurable concentrations of FSH, but observed females FSH concentrations of 3-10 ng/ml in the snapping turtle (*Chelydra serpentina*) (Mahmoud and Licht, 1997), 2-16 ng/ml in the musk turtle (*Sternotherus odoratus*) (Mendonca and Licht, 1986) and 6-26 ng/ml in the painted turtle (*Chrysemys picta*) (Licht et al., 1985).

Our results are higher than those reported in these previous studies. The RIA method was used in all these studies. The antisera used in these studies were not specific enough for FSH and failed to maintain selective neutralization between the gonadotropins (Licht et al., 1977). Licht and Wibbels have conducted measurements on FSH levels in females during the nesting period (20 to 50 hours after each turtle had nested). Our measurements encompass both the months preceding nesting and the period following nesting completion (every two months). It appears that FSH, as previously observed in reptiles, plays a role in elevating the levels of estradiol and testosterone and vitellogenin (Smelker et al., 2014; Bruno et al., 2021). This can explain the high concentrations of FSH observed months prior to nesting. In contrast, non-nesting females exhibited consistently lower FSH concentrations compared to the nesting females.

The ELISA developed in this study showed sensitive characteristics compared to other GTH immunoassays. High precision represented by low intra- assay and inter-assay coefficients of variation. The CV values are similar to those from immunoassays for measuring FSH reported in other species, with a range of 5-8% and 7-15% for intra-assay and inter-assay (Aizen et al., 2007b; Molés et al., 2012; Caron et al., 2018; Nocillado et al., 2019). These findings indicate that recombinant FSH was produced as expected, and that the antiserum correctly recognized the target FSH in the plasma (Kazeto et al., 2019).

The sensitivity of the ELISA was 0.13 ng/ml for measuring FSH concentrations in plasma which was similar or more sensitive in comparison to the sensitivities reported for measuring FSH in fish ELISA's; 0.24 pg/ml for tilapia (Aizen et al., 2007b), 78 pg/ml for yellowtail kingfish (Nocillado et al., 2019) and 10 pg/ml for flatfish (Chauvigné et al., 2015). The differences in assay sensitivity may be affected by the purification level of the recombinant antigens and antisera (Nocillado et al., 2019).

To increase the specificity limitations of the ELISA presented herein, one should consider developing a sandwich ELISA that will permit us to detect each glycoprotein hormone with 2

antibodies, one for the beta subunit and the other for the alpha specific epitope even though the immunogenicity of the alpha subunit is not very high.

Validation of the assay was demonstrated by parallelism between serial dilutions of standard curve and serial dilutions of displacement curves of green sea turtle FSH in plasma. These results indicate that the structure of the rcmFSH we produced is immunologically identical to the native FSH of green sea turtle (Aizen et al., 2007b; Nocillado et al., 2019). We also tested parallelism between serial dilutions of standard curve and serial dilutions of displacement curves for potential measurements of gonadotropins in other turtle species. Parallelism in tested plasma samples of three other species was observed: loggerhead sea turtle, hawksbill sea turtle and African softshell turtle. As seen in several studies on fish species, when parallelism is observed between the standard curve and several dilutions of the same sample it indicates similar protein structure between the different species (Aizen et al., 2007a; Aizen et al., 2007b; Aizen, 2011). Therefore, the structure of green sea turtle FSH is similar to the FSH of the other three turtle species. One should keep in mind that the developed ELISA assay presented herein cannot distinguish between free beta and beta associated with alpha. The two subunits of the gonadotropins are co-translated and they non-covalently combine in the endoplasmic reticulum of gonadotrope (FSH, LH), thyrotroph (TSH), or trophoblast (CG) cells (Cahoreau et al., 2015). Probably, if free FSH $\beta$  is present in the blood it can be a result of FSH dissociation.

This discovery will allow us the use of this assay for measuring FSH in these species and possibly more turtle species. In mammals, FSH in females controls the menstrual cycle and stimulates growth of follicles in the ovaries, whereas males FSH is an important hormone regulating spermatogenesis (Licht et al., 1979; Sairam and Krishnamurthy, 2001; Kluge et al., 2012). It is estimated that the mechanism is similar in most vertebrates, but the specific functions of FSH in reptiles are not fully understood (Tripathy and Rai, 2017; Kang et al., 2020). It was suggested that FSH has a distinct function in the stimulation of estradiol during the inter-nesting period that stimulates follicular development in females, and spermatogenesis in males (Wibbels et al., 1992; Mills et al., 2008; Kawazu, 2015; Tripathy and Rai, 2017).

After the development and calibration of the ELISA, we used it to measure concentrations of FSH from turtles in the Mikhmoret breeding facility using plasma samples taken since 2018. Our results show that successful nesting females that laid eggs (2019 and 2020) had an elevation in FSH concentrations during and before nesting season (January- August) compared to non-nesting females that presented relatively low FSH concentrations. FSH concentrations in non-nesting females were highest from April 2021 to September 2021. The females did not lay eggs in that year even though mating attempts were observed. We assumed that the females that haven't nested yet didn't reach sexual maturity and therefore the pituitary continues to secrete FSH at high concentrations in order to enter the puberty phase as in the case of other vertebrates.

High FSH concentrations increase the secretion of progesterone due to the ruptured follicle that forms a corpus luteum and

produces high concentrations of progesterone (Wibbels et al., 1992; Al-Habsi et al., 2006). In female green sea turtles the high progesterone concentrations were reported slightly before nesting (Al-Habsi et al., 2006; Ikonomopoulou et al., 2014), associated with early stages of ovulation that follow the formation of new active corpora luteum (Licht et al., 1979; Licht, 1980; Al-Habsi et al., 2006). In the loggerhead turtle on the other hand high concentrations were also recorded in April prior to nesting similar to our results in the green sea turtle, but much higher concentrations were recorded when female was sampled 3 days after egg laying for 5 consecutive egg laying sessions with concentrations between 12-15ng/ml (Kakizoe et al., 2010). Additionally, increased progesterone concentrations stimulates albumin that is needed for the egg production (Owens and Morris, 1985). As shown in our results, in nested females, and relatively low in non-nested females.

Nested females showed elevated testosterone concentrations six months prior to mating (November) until the concentrations peaked three months prior to nesting in April, and gradually decreased to lower concentrations at the beginning of the nesting season (June). It has been suggested that a rise in testosterone concentrations can act as a breeding behavior initiator, and a possible trigger to male-attractive pheromones (Licht et al., 1979; Owens and Morris, 1985). Licht et al. (1979) conducted research with captive green turtles in the Cayman farm and reported that testosterone elevation was two months prior to mating until they peaked at the beginning of mating activity (mean >700 pg/ml) followed by testosterone level decrease during nesting activity. loggerhead turtle on the other hand showed testosterone concentrations elevation from February to May (Kakizoe et al., 2010) different from the green turtle.

Our results show a consistent rise in estradiol concentrations as FSH concentrations rises in both males and females. In females, we observed that estradiol concentration start to rise in November during follicular growth, declining towards July, concurring with observations from a study by Blanvillain et al. (2011) for green sea turtles and loggerhead turtles (Kakizoe et al., 2010). High concentration of estradiol were observed in April slightly before the females nested and concentration remain at the same concentrations until they decreased in September. The secretion of estradiol may serve both behavioral and physiological functions; it might increase the drive of migration to nesting beaches, regulate ovarian follicle maturation (Al-Habsi et al., 2006) and stimulate vitellogenesis (Kawazu et al., 2016).

In males, we observed a decrease in estradiol concentrations during August and September - consistent with the study by G. D. Blanvillain et al. (2011), in which the FSH concentrations dropped after the nesting season along with the drive to seek a mating partner. The gradual increase in testosterone concentrations in males during the several months before the onset of mating activity, August to November in our case, was expected as described earlier by Licht (Licht et al., 1979),

Overall FSH role in the green sea turtle was found to be correlated with the release of ovarian and testicular steroids. Further development of GTH ELISA for LH and TSH will

contribute to a better understanding of the endocrine controls of the reproductive system in green sea turtle and other related species as well, clarifying the functional differentiation between these GTHs and their effects on other sex hormones. Future work should be directed towards the development and production of recombinant LH and TSH of the green sea turtle, this will help to better test and validate the assay specificity compared to other turtles' glycoproteins hormones. This research has the potential to encourage the development of methods for increasing the fertility and reproduction of the turtles in the Mikhmoret breeding facility.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was approved by Israel Nature and Parks Authority. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

OS: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. OR: Investigation, Methodology, Validation, Visualization, Writing – original draft, Formal Analysis, Writing – review & editing. YL: Conceptualization, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. JA: Conceptualization, Data curation, Formal

Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

## Funding

This research was supported by the Israel Science Foundation (ISF) (Grant No. 451/21) and the internal research funding program of Faculty of Marine Sciences, Ruppin Academic Center, Israel.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1260205/full#supplementary-material>

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