Original Article

A Rapid ELISA Method for 17, 20β-dihydroxy-4-pregenen-3-one (17, 20βP) Hormone Using Acetylcholinesterase Enzyme as Tracer

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Abstract

Background: During the past 15 years Enzyme Linked ImmunoSorbent Assay (ELISA) has been described as an alternative to radioimmunoassay for steroid detection. In addition to gonads, sperm itself is capable of producing reduced progesterone metabolites. In this study we introduced a method to extend the applicability of previous measures by describing a general preparation procedure for the enzyme label which is applicable to any steroid hormone.

Methods: A simple and rapid Enzyme Linked Immunosorbant Assay (ELISA) is described and validated for 17,20β-dihydroxy-4-pregnen-3-one (17,20βP). A general procedure for preparation of the acetylcholinesterase labelled steroid is described which is applicable to any steroid.

Results: Use of acetylcholinesterase tracer increased the sensitivity of assay so that reliable measurements of each steroid could be achieved with only 10 µl of plasma. ELISA was applied to measure of 17,20βP steroid production by sperm of trout which has sufficient amount of potent and active 20βHSD enzyme to convert 17α-hydroxy-4-pregnen-3-one (17αP) substrate to 17,20βP product. The results showed that a clear shift in 17,20βP production was found with increase in substrate concentration in all in vitro incubations.

Conclusion: ELISA method presented in this study has greater sensitivity and accuracy compared to previously described method that uses radiolabelled substances.

Key Words: Immunoassay, ELISA, Steroids, Hormone, Assay

Although radioimmunoassay has been used as a standard method of measurement for steroids as well as other hormones in plasma for over 20 years[1, 2], it has a number of serious disadvantages that restrict its applicability including disposal of radiolabeled tracers which is an increasing problem in many countries where it is restricted to sites and personnel approved by national regulatory authorities. Also it requires expensive and sophisticated equipment for measurement and radioactive counting of large numbers of samples which is time-consuming and requires large amounts of scintillation fluid, and finally the availability of radiolabeled steroids from commercial sources is very limited and severely restricts the range of steroids that can be measured, especially in non-mammalian vertebrates in which the reproductive hormones differ from those in mammals[1, 3-5].

During the last 15 years there have been a number of publications in which Enzyme Linked ImmunoSorbent Assay (ELISA) has been described as an alternative to radioimmunoassay for the mammalian and nonmammalian steroids detection [6-14]. In addition to interest in measurement of steroids in plasma as an aid to clinical diagnostics, there is an increasing interest in such measurements to monitor the effects of endocrine disrupting chemicals on reproductive function[15-21]. The need for a simple, rapid and reliable method for the measurement of hormones as the basis for regular testing of the effects of potential endocrine disrupters on humans and wildlife has already been highlighted[22].

Recent studies have shown that in addition to gonads, in some species, sperm itself is capable of producing 20α or 20β - reduced progestogen metabolites[23-31]. Although the role of the 20β-reductase

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in sperm, (converts progesterone to 17,20β-dihydroxy-5-pregnen-20-one (17,20βP) metabolite), is a matter of speculation, yet the enzyme activity has been used as a basis for regulatory testing of the effects of potential endocrine disrupters chemicals pollution[32, 33].

Cuisset et al[3] have described a simple and very sensitive ELISA method for the assay of 11-ketotestosterone using acetylcholinesterase as tracer, but the applicability was limited by use of electric eel as a source of acetylcholinesterase. In this study we extend the applicability of the methodology to measure the specific progestogen metabolite, (17,20βP), and describe a general preparation procedure for the enzyme label which can be applicable to any steroid hormone. The method was applied to the determination of 20βHSD enzyme activity in in vitro incubation of trout sperm because sperm in this species has pure potent active 20βHSD enzyme producing large amounts of 17,20βP hormone[33-36].

Materials and Methods

Chemicals and equipment

Acetylthiocholine, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), acetylcholinesterase (Sigma C2888, 1000-2000 units mg-1), trypsin and all steroids were obtained from Sigma chemical company (Poole, UK). The antiserum to 17,20βP (against 3-CMO-BSA in sheep) was kindly donated by Dr. D. E. Kime (Sheffield). Cross reactivities of these antiserum is described by Scott et al[37, 38].

Ninety-six well polystyrene high-binding microtiter plates (Costar Catalogue No. 3590) were used for ELISA. Plates were sealed for storage with Anachem Sealplate sealing film. Plates were processed using a Chelsea Instruments shaker, an Anthos Model AW-1 plate washer and read with an Anthos Model HTII Plate Reader. 96-well low binding microtiter plates (Costar Cat. No. 2501) were used for sample dilution and preparation of standard curves.

Preparation of steroid-carboxymethyloxime (CMO)

The preparation of the 3-CMO of 17,20αP was based on the method of Simpson and Wright[39].

Generation of G4-acetylcholinesterase

Acetylcholinesterase (AChE, 1 mg) in 500 µl 0.1M phosphate buffer pH 7.0 was treated overnight with 25 µl trypsin solution (25µg ml-1 in 0.1 M phosphate buffer, pH 7.0) at room temperature. The solution was dialysed against 100 ml 0.1M borate buffer pH 8.5 using an 80 mm length of dialysis tubing (Sigma D2272) for 24 hours using several changes of borate buffer. The contents of the dialysis tubes were removed and the tubes were washed with clean borate buffer to give a final volume of G4-AChE solution of 1 ml which could be stored at 4°c for at least two weeks. The activity of enzyme was checked with Ellmans reagent as described later.

Preparation of steroid-CMO-AChE conjugate

Four hundred nmol (174 µg) of the steroid-CMO in 38µl of freshly made N-hydroxysuccinimide solution (1 $mg.ml^{-1}$ in anhydrous dimethylformamide) was treated overnight with 32 µl freshly made N,N'-dicyclohexylcarbodiimide solution (2 $mg.ml^{-1}$ in anhydrous dimethylformamide) in the dark (Cuisset et al. 1994). It is essential that all traces of moisture are excluded from this step. Thirty microliters of this solution were reacted for 2 hours in the dark with 200 µl of the 1 $mg.ml^{-1}$ G4-AChE stock and then mixed with 1 ml of steroid assay buffer (0.1M K2HPO4.3H2O, 0.1M g KH2PO4, 0.15M NaCl, 1 mM EDTA, 0.1% bovine serum albumin (BSA), 0.15mM sodium azide, pH 7.4). The product was chromatographed on a 900 x 15 mm column of BioGel A 15-m (BioRad) with elution buffer (0.01M Tris, 0.01M MgCl2, 1M NaCl, 0.15mM NaN3 pH 7.4) at a flow rate of 300 $\mu l.min^{-1}$. The eluate was discarded for 5 hours, then eighty 1.5 ml fractions were collected into tubes containing 1 ml steroid assay buffer. Five microliters of every 5th fraction was treated with 200 µl Ellmans reagent (215 mg 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 200 mg acetylthiocholine in 20 ml 1M potassium phosphate buffered saline (PPBS) kept as 400µl frozen stock aliquots for up to six weeks and diluted 1:50 for use and absorption read at 405 nm after 2 hours. In the region of peak activity, 5 µl from all fractions was tested with Ellmans reagent and an activity curve was plotted. Fractions showing >30% of the maximum Ellman activity were combined and stored in stock aliquots at -20°C. The column was flushed overnight with elution buffer, stored at 4°C and flushed again overnight before reuse.

Milt preparation and incubation

Milt of six mature male trout was hand stripped by gentle abdominal pressure. Twenty microliters of

milt from each male fish (512 ± 73g, mean ± SD) was incubated with gentle shaking for 3 hours at 20°C in 2 ml trout incubation medium (40) containing 0, 0.1, 1 or 10 μg/ml of 17α-hydroxy-5-pregnen-20-one (17P).

Steroid extraction

Steroids were extracted from incubation media as described previously[33, 35].

Plate coating

ELISA plates were coated with anti-rabbit because a competition method was used here. Eight microliters of polyclonal anti-rabbit IgG (affinity purified, Sigma R2004; 1 $mg.ml^{-1}$) was dissolved in 15 ml 0.05M potassium phosphate buffer, pH 7.4, 150 μl added to each well of the plate and incubated overnight at room temperature in a humid container. The plate was blocked by addition of a further 100 μl of blocking buffer (0.1M PPBS pH 7.4 containing 3% bovine serum albumin (BSA), 1 mM EDTA and 25 mM sodium azide) and incubation overnight at room temperature. Plates were sealed and stored at 4°C in a humid container. Immediately before use, plates were washed 3 times with wash buffer (100 ml PPBS and 2.5 ml Tween-20 in 5 l water).

Checkerboard titration

A checkerboard titration was carried out for antiserum and also after each preparation of new AChE label. Serial dilutions of the steroid antiserum (from 1:1000 dilution; plate columns 1-10) were made against serial dilutions of AChE label (from 1:4; plate rows A-H) in a microtiter plate. Plates were processed as in the Standard Assay Procedure and plots made of AChE dilution against absorption for antiserum dilution to determine optimal dilutions of the two components[41]. For the tracers and antiserum described here, this was 1:20 for AChE tracer and 1:20,000 for antiserum to 17, 20αP.

Standard assay procedure

Serial dilutions of standard steroid (400 to 0.78 pg) in 100 μl assay buffer were made in the first 10 wells of rows A and B of the coated plate. The remaining 2 wells in these rows were reserved for B0 (0 pg) and non-specific bound (NSB). Twenty-five microliters of the extracted steroids from the samples (5 μl incubation media equivalent) were pipetted into the remaining 6 rows in duplicate. Twenty-five microliters of the diluted steroid label was added to all wells, and 25 μl antiserum to all wells except for the NSB at the dilutions determined by the checkerboard titration. All wells were made up to 150 μl in steroid assay buffer; the plates sealed and incubated 2 hours at room temperature in a humid chamber. Plates were then washed three times with wash buffer. 200 μl per well of Ellmans reagent were added, the plates incubated overnight in the dark at room temperature and read at 405 nm. Picograms per well were calculated for the samples from the standard curve using Stingray software (Dazdaq, Ringmer, UK).

Assay validation

Pool plasma (500μl) was treated with activated charcoal as described by Cuisset et al. (1994) and extracted twice with 5 ml dichloromethane. The combined extracts were evaporated and reconstituted in 3 ml assay buffer. One hundred microliter aliquots were pipetted in duplicate into wells on two rows of a microtiter plate. 17, 20αP was added to one pair of wells to give a concentration of 400 pg/well, serial dilutions (x2) performed and the samples assayed according to standard procedure.

The same procedure was used to assay 17,20αP production by roach sperm and SPSS 6 for Windows software (SPSS Inc., 444 N. Michigan Avenue, Chicago, Illinois 60611, USA) was used for the statistical analysis of 20αHSD activity in milt of control fish (0 μg/ml 17P) with other groups.

Results

Cross-reactivity of antiserum

The antiserum to 17,20βP cross-reacted with 17P (1.9%), progesterone (4.3%), 5α-androstane-3α,17β-diol (3.7%), 11β-hydroxy-testosterone (0.33%), 5α-androstane-3β,17β-diol (0.27%), 5β-androstane-3α,17β-diol (0.25%),11-ketotestosterone (0.85%), estradiol (0.54%), 4-androstenedione (0.47%), 4-androstenetrione (0.31%) and 17,20αP (4.1%) at the 50% displacement level.

Standard curves and assay sensitivity

Typical standard curves for 17,20βP steroids showed a workable range (detection limit) from 0.8 to 400 pg/well (figure 1). The sensitivity of the assay, taken as the concentration of steroid that induced 90% of B/B0, was 1.5 pg.

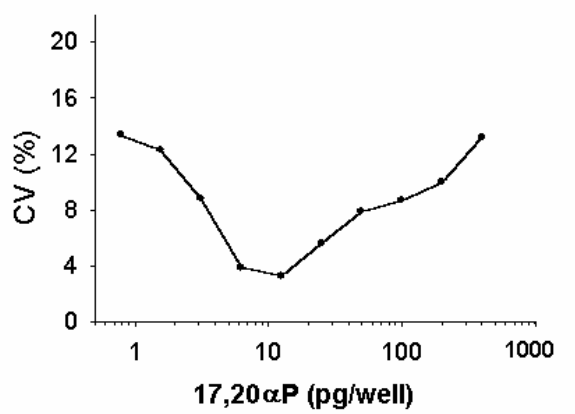
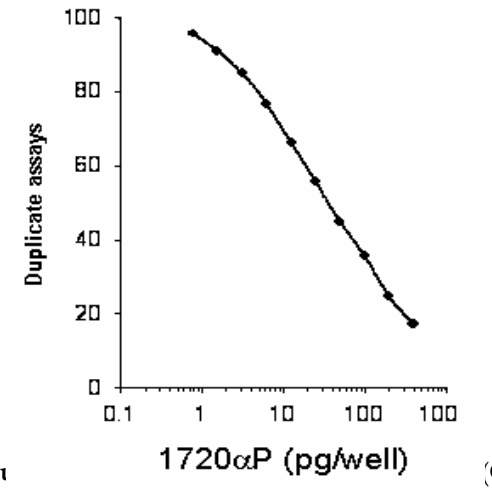


Figure 1. Typical standard curves for ELISA of 17, 20αP. Data points are means of duplicate assays.

Inter-assay variations measured on female carp plasma samples that gave approximately 50% displacement was 9.2% for 10 replicates.

Intra-assay variations were determined from standard curves in which 10 replicates of each concentration were used (figure 2). The coefficient



Figı (CV) for ELISA of 17,20αP determined from a standard curve with 10 replicates at each concentration. The coefficient of variation was less than 10% over the central part of the standard curve between 3 and 200 pg/well

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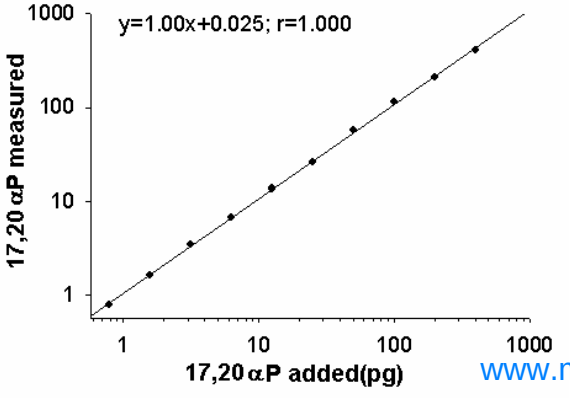

Assay accuracy

There was a strong positive correlation (r>0.999) between the amount of steroid added to plasma and the amount measured. (figure 3).

Figure 3. Accuracy test for 17, 20βP assay. A known amount of the steroid was added to an aliquot of a steroid free extract of pooled plasma and 2 x serial dilutions made with the extract. Assays were performed in duplicate. There was a strong positive correlation (r>0.999) between the amount of steroid added to plasma and the amount measured

Application to measuring 17,20βP production by sperm

17,20βP was measured in duplicate by ELISA using 10 µl of incubation media equivalent. The amounts of 17,20βP detected by this method were 1, 4.2, 13.2 and 18.66 µg/ml for 0.0, 0.1, 1.0 and 10 µg/ml 17P added to milt incubations respectively. A clear shift in 17,20βP production was found with increase in substrate concentration in all incubations.

Discussion

Assay validation

Here the procedure described by Cuisset et al[3] for validation of the 11-ketotestosterone ELISA using the same enzyme label was closely followed.

Sensitivity: Sensitivity of the assays, which has been taken as 90% of the binding with 0.0 pg was just over 1 pg for 17,20βP. Using identical conditions for label preparation and assay we also obtained a sensitivity of 0.39 pg for hormone comparable to that obtained by Cuisset et al3 As with radioimmunoassay, the sensitivity is a function of both the specific activity of the label and the quality of the antiserum, and will therefore vary with the material available to the user. Therefore in this study, we used the antiserum that were available to us but have little reason to expect that any antiserum which gives good results with radioimmunoassay will not give similar results to those that we describe for ELISA.

Previous reports on ELISA for steroid hormones measurements have generally used horseradish peroxidase as the enzyme label[42-49]. This has a major disadvantage in that the enzymatic reaction must be irreversibly stopped before photometric measurement can be made[3, 50]. A major advantage of the use

of acetylcholinesterase is that such an arrest is unnecessary and the sensitivity can be greatly increased by using even smaller concentrations of enzyme label and allowing the reaction to continue for several days until sufficient colour has developed. Plates can, in fact, be read daily until the absorption is considered sufficient[3]. The higher turnover rate of acetylcholinesterase compared to other enzyme labels also gives a potentially more sensitive assay[50]. In this paper a method that is sufficiently sensitive for the rapid measurement of steroid hormones in mammals and nonmammalian was described.

Accuracy and reproducibility: A major advantage of ELISA is the ability to minimize errors resulting from multiple pipetting by use of multichannel pipettes. Inter and intra-assay coefficient of variations (CV) reported in radioimmunoassay are frequently 15-20% even when measured at the steepest part of the curve. For the assays reported here we obtain intra-assay CVs around 5% in the steep part of the curve, rising to 15-20% at the flatter end regions (figure 2), while inter-assay variation (at 50% displacement) was around 8%, comparable to that reported by Cuisset et al[3] for 11-ketotestosterone. There was a highly significant correlation between the steroid added to and that measured in stripped plasma (figure 3). A further advantage of ELISA is that sample handling can also be made more rapid and reproducible by the use of low binding microtiter plates for sample storage and dispensing with multichannel pipettes.

Tracer preparation

A major problem with the assay of steroids in plasma is the availability of tracer[1]. 17, 20βP must be prepared chemically from other materials and must then be purified chromatographically. Such conversion of radioactive materials is beyond the expertise of many laboratories and has severely restricted measurement of these hormones in plasma. Availability of commercial enzymatically labelled steroids, including acetylcholinesterase labels (Cayman Chemical Company, Ann Arbor), is similarly restricted to those of mammalian steroids that are routinely assayed in clinical labs. Cuisset et al[3] prepared acetylcholinesterase from electric eel, but in this communication we describe a simple procedure for making enzyme label from commercially available acetylcholinesterase which requires no facility more complicated than a simple chromatographic column. Using such a procedure we have made acetylcholinesterase labelled 17, 20βP which has similar sensitivity to that was described previously[3].

A major advantage of ELISA is that, since the steroid is coupled to the enzyme by exactly the same reaction as that used to link it to the bovine serum albumin to stimulate the immunological reaction in the host animal, it is possible to make an enzyme label for any steroid for which it is possible to raise an antiserum. In addition to the steroid described in this paper, acetylcholinesterase label and obtained standard curves of similar sensitivity estrodial, testosterone, 11-ketotestosterone, 17,20α-dihydroxy -4-pregnen-3-one, 11-deoxycortisol, 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), cortisol and 17α-hydroxyprogesterone have been prepared in our lab which demonstrate the widespread applicability of the methodology. This greatly expands the number of steroids that are possible to be measured by ELISA, since only the labels for testosterone and estradiol are available commercially. This flexibility is particularly important in animals in which a large number of steroids are produced by gonadal tissue[1] which cannot be measured by radioimmunoassay due to lack of availability of radiolabel except by a prohibitively expensive custom preparation involving very high levels of radioactivity. ELISA may therefore play a major part in clarifying the role, for example, of progestogen, 20α-, 20β-, 5α- and 5β-reduced steroids. As with radioimmunoassay it is essential that ELISA be fully validated for each new species used. This is especially true for a new method since it is not known whether other chemicals extracted from plasma may interfere with binding or enzymatic processes. The present validation suggests that there are no such problems in this assay.

Application to the measurement of 17, 20βP

To demonstrate an application of the ELISA method we have measured the steroid 17,20βP in in vitro incubations of sperm and a clear shift in 20βHSD enzyme activity found when the concentration of substrate (17P) increases. The same shift has been also reported in gonadal[34, 51-56] and non-gonadal organs[33].

In this paper we presented the data from in vitro sperm incubation as an application, but additional information was also obtained using plasma hormonal assay for other steroids, therefore the higher sensitivity of ELISA using acetylcholinesterase as enzyme label compared with radioimmunoassay was confirmed.

It has been also shown that this method can be readily used for the measurement of other steroids simply by using a small volume of plasma (especially when steroid levels in plasma are low)[3]. The rapid determination of several steroids from the very small volumes of blood allows the examination of profiles of steroids at even higher sampling frequencies or over longer periods. This methodology will therefore be of great interest to workers examining the temporal endocrine mechanisms involved in photoperiodic, circadian and other chronobiological processes.

References

1. Kime DE, 'Classical' and 'non-clssical' reproductive steroids in fish. Reviews in Fish Biology and Fisheries, 1993. 3: p. 160-180.
2. Idler DR, Steroids in nonmammalian vertebrates. 1972, New York and London: Academic Press. 504.
3. Cuisset B, et al., Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as label: Application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. Comparative Biochemistry and Physiology C Pharmacology Toxicology and Endocrinology, 1994. 108(2): p. 229-241.
4. Weltzien FA, et al., Spermatogenesis and related plasma androgen levels in Atlantic halibut (Hippoglossus hippoglossus L.). Comp Biochem Physiol A Mol Integr Physiol, 2002. 132(3): p. 567-75.
5. Larsson DG, et al., Seasonal variations of vitelline envelope proteins, vitellogenin, and sex steroids in male and female eelpout (Zoarces viviparus). Gen Comp Endocrinol, 2002. 125(2): p. 184-96.
6. Yamada H, et al., Circadian changes in serum concentrations of steroids in Japanese char Salvelinus leucomaenis at the stage of final maturation. Zoolog Sci, 2002. 19(8): p. 891-8.
7. Pall MK, Mayer I, and Borg B, Androgen and Behavior in the Male Three-Spined Stickleback, Gasterosteus aculeatus. II. Castration and 11-Ketoandrostenedione Effects on Courtship and Parental Care during the Nesting Cycle. Horm Behav, 2002. 42(3): p. 337-44.
8. Dunlap KD, Hormonal and body size correlates of electrocommunication behavior during dyadic interactions in a weakly electric fish, Apteronotus leptorhynchus. Horm Behav, 2002. 41(2): p. 187-94.
9. Rohr DH, et al., 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, Anguilla australis. Comp Biochem Physiol A Mol Integr Physiol, 2001. 130(4): p. 701-14.
10. Melamed P, et al., Reproductive development of male and female tilapia hybrids (Oreochromis niloticus x O. aureus) and changes in mRNA levels of gonadotropin (GtH) Ibeta and IIbeta subunits. J Exp Zool, 2000. 286(1): p. 64-75.
11. Manire CA, Rasmussen LE, and Gross TS, Serum steroid hormones including 11-ketotestosterone, 11-ketoandrostenedione, and dihydroprogesterone in juvenile and adult bonnethead sharks, Sphyrna tiburo. J Exp Zool, 1999. 284(5): p. 595-603.
12. Mayer I, Rand-Weaver M, and Borg B, Effects of gonadectomy and steroids on plasma and pituitary levels of somatolactin in Atlantic salmon, Salmo salar. Gen Comp Endocrinol, 1998. 109(2): p. 223-31.
13. Canario AVM and Scott AP, Levels of 17 alpha,20 alpha-dihydroxy-4-pregnen-3-one, 3 beta,17 alpha,20-trihydroxy-5-BETA-pregnane, and other sex steroids, in blood plasma of male dab, Limanda limanda (marine flatfish) injected with human chorionic gonadotropin. General And Comparative Endocrinology, 1991. 83(2): p. 258-264.
14. Kime DE and Manning NJ, Seasonal patterns of free and conjugated androens in the brown trout Salmo trutta. General and Comparative Endocrinology, 1982. 48: p. 222-231.
15. Wade MG, et al., Effects of subchronic exposure to a complex mixture of persistent contaminants in male rats: systemic, immune, and reproductive effects. Toxicological Sciences : An Official Journal of the Society of Toxicology, 2002. 67(1): p. 131-43.
16. van den Heuvel MR and Ellis RJ, Timing of exposure to a pulp and paper effluent influences the manifestation of reproductive effects in rainbow trout. Environ Toxicol Chem, 2002. 21(11): p. 2338-47.
17. Wainwright SE, et al., Chlorinated hydrocarbons and biomarkers of exposure in wading birds and fish of the lower Rio Grande Valley, Texas. Arch Environ Contam Toxicol, 2001. 40(1): p. 101-11.
18. Sepulveda MS, et al., An evaluation of biomarkers of reproductive function and potential contaminant effects in Florida largemouth bass (Micropterus salmoidesfloridanus) sampled from the St. Johns River. Sci Total Environ, 2002. 289(1-3): p. 133-44.

19. Olsen KH, et al., Both ovarian fluid and female urine increase sex steroid hormone levels in mature Atlantic salmon (Salmo salar) male parr. J Chem Ecol, 2001. 27(11): p. 2337-49.
20. MacLatchy DL and Van Der Kraak GJ, The phytoestrogen beta-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol, 1995. 134(2): p. 305-12.
21. Laskey JW, et al., Age-related dose response of selected reproductive parameters to acute cadmium chloride exposure in the male Long-Evans rat. Journal Of Toxicology And Environmental Health, 1986. 19(3): p. 393-402.
22. Tattersfield L, et al., SETAC-Europe/OECD/EC Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing (EMWAT). Proceeding of SETAC-Europe/OECD/EC Expert Workshop, Veldhoven, The Netherlands, 10-13th April 1997. SETAC-Europe, Amsterdam., 1997. 1.
23. Kakai N, et al., Involvement of sperm in the production of 17-alpha, 20-beta-dihydroxy-4-pregnen-3-one in the testis of spermiating rainbow trout, Salmo gairdneri. Biomedical Research, 1989. 10(2): p. 131-138.
24. Sakai N, et al., Involvement of sperm in the production of 17a,20b-dihydroxy-4-pregnen-3-one in the testis of spermiating rainbow trout, Salmo gairdneri. Biomedical Research, 1989. 10: p. 131-138.
25. Barry TP, et al., Steroid profiles during spawning in male common carp. Gen Comp Endocrinol, 1990. 80(2): p. 223-31.
26. Koldras M, et al., Study of the composition of seminal fluid and of sperm motility along the genital tract, during a spawning season, in the rainbow trout (Oncorhynchus mykiss). Aquat. Living Resour. Ressour. Vivantes Aquat., 1996. 9(4): p. 337-345.
27. Koldras M, Bieniarz K, and Kime DE, Sperm production and steroidogenesis in testes of the common carp, Cyprinus carpio L., at different stages of maturation. Journal Of Fish Biology, 1990. 37(4): p. 635-646.
28. Asahina K, Lambert JGD, and Goos HJT, Bioconversion of 17-alpha-hydroxyprogesterone into 17-alpha,20-alpha-dihydroxy-4-pregnen-3-one and 17-alpha,20-beta-dihydroxy-4-pregnen-3-one by flounder (Platichthys flesus) spermatozoa. Zoological Science, 1994. 11(6): p. 859-863.
29. Asahina K, et al., Biosynthesis of 17-alpha,20-alpha-dihydroxy-4-pregnen-3-one from 17-alpha-hydroxyprogesterone by spermatozoa of the common carp, Cyprinus carpio. Journal Of Experimental Zoology, 1990. 255(2): p. 244-249.
30. Asahina K, Aida K, and Higashi T, Biosynthesis of 17-alpha,20-alpha-dihydroxy-4-pregnen-3-one from 17-alpha-hydroxyprogesterone by goldfish (Carassius auratus) spermatozoa. Zoological Science, 1993. 10(2): p. 381-383.
31. Tan AMC, et al., 17-alpha,20-alpha-dihydroxy-4-pregnen-3-one, not its 20-beta isomer, is produced from 17-alpha-hydroxyprogesterone by spermatozoa of secondary male groupers (Epinephelus tauvina) derived from females implanted with 17-alpha-methyltestosterone. Journal of Experimental Zoology, 1995. 271(6): p. 462-465.
32. Kime DE, et al., Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish; application to effects of heavy metals. Aquatic toxicology, 1996. In press.
33. Ebrahimi M, Scott AP, and Kime DE, Extragonadal production of 17,20-dihydroxy-4-pregnen-3-ones in vitro in cyprinid fish. General and Comparative Endocrinology, 1996. 104(3): p. 296-303.
34. Lee ST, et al., Synthesis of 17,20alpha/beta-dihydroxy-4-pregnen-3-one and 5beta-pregnanes in spermatozoa of primary and 17alpha-methyltestosterone-induced secondary male grouper (Epinephelus coioides). General and Comparative Endocrinology, 1998. 112(1): p. 1-9.
35. Ebrahimi M, Singh PB, and Kime DE, Biosynthesis of 17,20alpha-dihydroxy-4-pregnen-3-one, 17,20beta- dihydroxy-4-pregnen-3-one, and 11-ketotestosterone by testicular fragments and sperm of the roach, Rutilus rutilus. General and Comparative Endocrinology, 1995. 100(3): p. 375-384.
36. Kime DE, The effect of temperature on the testicular steroidogenic enzymes of the rainbow trout, Salmo gairdneri. General and Comparative Endocrinology, 1979. 39(3): p. 290-296.
37. Scott AP, Sumpter JP, and Hardiman PA, Hormone changes during ovulation in the rainbowe trout (Salmo gairdneri Richardson). General and Comparative Endocrinology, 1983. 49: p. 128-134.
38. Scott AP, et al., Seasonal variations in plasma concentrations of 11-ketotestosterone and testosterone in male rainbow trout, Salmo gairdneri Richardson. Journal of Fish Biology, 1980. 17: p. 495-506.
39. Simpson TH and Wright RS, A radioimmunoassay for 11-ketotestosterone: its application in the measurement of levels in blood serum of rainbow trout (Salmo gairdneri). Steroids, 1977. 29: p. 383 - 398.
40. Jalabert B, Szollosi D, and Fostier A, Étude comparée de l'action des hormones hypophysaires et stéröides sur la maturation in vitro des ovocytes de la truite et du carassin (Poissons Téléostéens). Ann. Biol. Anim. Biochim, 1973. 13: p. 59-72.
41. Diamandis EP and Christopoulos TK, Immunoassay. Academic Press, San Diego., 1996.
42. Nishi K, et al., Development and application of a monoclonal antibody-based sandwich ELISA for quantification of Japanese medaka (Oryzias latipes) vitellogenin. Comp Biochem Physiol C Toxicol Pharmacol, 2002. 132(2): p. 161-9.
43. Basu A and Shrivastav TG, One step enzyme linked immunosorbent assay for direct estimation of serum cortisol. J Immunoassay, 2000. 21(1): p. 39-50.
44. Bolton J, et al., Measurement of thyroid-stimulating hormone receptor autoantibodies by ELISA. Clin Chem, 1999. 45(12): p. 2285-7.
45. Madersbacher S, et al., Increased ELISA sensitivity using a modified method for conjugating horseradish peroxidase to monoclonal antibodies. J Immunol Methods, 1992. 152(1): p. 9-13.

46. Karawajew L, et al., Production and ELISA application of bispecific monoclonal antibodies against fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP). J Immunol Methods, 1988. 111(1): p. 95-9.
47. Zhiri A, Wellman-Bednawska M, and Siest G, ELISA of 6-beta-hydroxycortisol in human urine: diurnal variations and effects of antiepileptic therapy. Clin Chim Acta, 1986. 157(3): p. 267-76.
48. Preti MS, et al., ELISA for salivary and plasma estriol in pregnancy. Steroids, 1984. 43(5): p. 469-79.
49. Rajkowski KM, et al., The conjugation of testosterone with horseradish peroxidase and a sensitive enzyme assay for the conjugate. Steroids, 1977. 29(5): p. 701-13.
50. Pradelles P, Grassi J, and Maclouf J, Enzyme immunoassays of eicosanoids using acetylcholinesterase as label: an alternative to radioimmunoassay. Analyt. Chem., 1985. 57: p. 1170-1173.
51. Ebrahimi M, Singh PB, and Kime DE, Biosynthesis of 17,20 alpha-dihydroxy-4-pregnen-3-one, 17,20 beta-dihydroxy-4-pregnen-3-one, and 11-ketotestosterone by testicular fragments and sperm of the roach, Rutilus rutilus. Gen Comp Endocrinol, 1995. 100(3): p. 375-84.
52. Kime DE and Ebrahimi M, Synthesis of 17,20 alpha - and 17,20 beta -dihydroxy-4-pregnen-3-ones, 11-ketotestosterone and their conjugates by gills of teleost fish. Fish Physiology and Biochemistry, 1997. 17(1-6): p. 117-121.
53. Kime DE and Abdullah MA, The in vitro metabolism of 17-hydroxyprogesterone by ovaries of the goldfish, Carassius auratus, is affected by substrate concentration. General and Comparative Endocrinology, 1994. 95(1): p. 109-16.
54. Kime DE, et al., Substrate concentration affects the in vitro metabolism of 17-hydroxyprogesterone by ovaries of the carp, Cyprinus carpio. Fish Physiology and Biochemistry, 1994. 13(4): p. 317-324.
55. Manning NJ and Kime DE, Temperature regulation of ovarian steroid production in the common carp, Cyprinus carpio L., in vivo and in vitro. General and Comparative Endocrinology, 1984. 56(3): p. 376-88.
56. Abdullah MA and Kime DE, Increased substrate concentration causes a shift from production of 11-oxygenated androgens to 17,20-dihydroxyprogestogens during the in vitro metabolism of 17-hydroxyprogesterone by goldfish testes. Gen Comp Endocrinol, 1994. 96(1): p. 129-39.