Journal of Chromatography B, 879 (2011) 591–598

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of 2-hydrazinopyridine derivatized steroid hormones in fathead minnow (*Pimephales promelas*) blood plasma using LC-ESI+/MS/MS

D. Hala, M.D. Overturf, L.H. Petersen, D.B. Huggett*

Department of Biology, Institute of Applied Sciences, University of North Texas, 1704 West Mulberry, Denton, TX 76203, United States

ARTICLE INFO

Article history:
Received 10 October 2010
Accepted 20 January 2011
Available online 27 January 2011

Keywords:
Androgen
Progestogens
Corticosteroid
Fathead minnow
Plasma
LC-ESI+/MS/MS

ABSTRACT

Fathead minnows (*Pimephales promelas*) comprise a species-of-choice for the hazard assessments of various environmental contaminants, including compounds capable of disrupting endocrine function. Towards this end, the use of liquid chromatography coupled with mass spectrometry (LC–MS) and/or tandem mass spectrometry (MS/MS) is gaining common use for the quantification of steroid hormones as biomarkers of endocrine stress in small-fish toxicological studies. In this work, 2-hydrazinopyridine (2-HP) was used to derivatize and quantify the physiologically relevant steroid hormones of: 17α-hydroxypregnenolone, progesterone, 11-ketotestosterone, 11-deoxycortisol and 17α,20β-dihydroxypregnenone, in the blood plasma of male and female fathead minnows. Liquid chromatographic separation was achieved using a Waters™ Sunfire C_{18} column (2.1 mm × 50 mm with a 3.5 μm particle size) and Milli-Q water:methanol (both with 0.1% formic acid) mobile phase over a gradient of 15 min. All mass analyses were conducted using electrospray ionization in the positive mode with tandem mass spectrometry (ESI+/MS/MS). This is the first such application of 2-HP derivatization for the quantifications of the structurally and functionally diverse C19 androgen of 11-ketotestosterone; C21 progestogens of 17α-hydroxypregnenolone, progesterone and17α,20β-dihydroxypregnenone; and C21 corticosteroid of 11-deoxycortisol, in fathead minnow blood plasma. The limits of detection (LOD) were set to the lowest calibration standard that gave a signal-to-background response of ≥3, and were: 0.16 ng/ml for progesterone, 0.63 ng/ml for 17α-hydroxypregnenolone, 11-deoxycortisol and 17α,20β-dihydroxypregnenone, and 1.25 ng/ml for 11-ketotestosterone. This study demonstrates the application of 2-HP derivatization for the analysis of a variety of steroid hormones representative of endocrine function in a species of fish commonly used in toxicological studies.

1. Introduction

Fathead minnows (*Pimephales promelas*) comprise a freshwater cyprinid fish species extensively used in regulatory testing and toxicological research. Their small size, relatively rapid life cycle (sexual maturity within 4–5 months), well characterized endocrinology and ease of culture have assisted in their wide acceptance as a model organism [1–6]. The relatively high fecundity (20.5 eggs/female/day) and spawning frequencies (every 3.9 days) of sexually mature and reproductively active male and female fathead minnows has also lead to their use as a species-of-choice for reproductive-toxicological studies assessing the hazards of various environmental contaminants [7–11]. In such studies, effects on survival, fecundity and plasma steroid hormone titers are commonly quantified as biomarkers of altered endocrine function and/or reproductive dysfunction [12–16].

Methods commonly used for the quantifications of steroid hormones in fish blood plasma include anti-body based techniques utilizing enzyme-labeled and/or radioactive-labeled steroids, such as enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs) [17–21]. Despite their sensitivity, these methods are cited as being prone to antibody cross-reactivity with hormones sharing conserved structural groups, with further limitations including the relatively small numbers of hormones that can be quantified per sample and the hazard associated with the use of radioactive materials [22–26]. In contrast to these methods, liquid chromatography coupled with mass spectrometry (LC–MS) and/or tandem mass spectrometry (LC–MS/MS) has increased in use. Their use has mainly been driven by the ease of automation, high specificity and simultaneous quantification of multiple compounds per sample along with the minimal use of radiolabelled substances [27–29]. Within the environmental sciences, LC–MS/MS has most commonly been used for the identification and quantitation of anthropogenic contaminants (including various steroid hormone analogues) in solid and liquid environmental matrices [30–37]. A more recent extension of such analyses has included the

* Corresponding author. Tel.: +1 940 891 6956; fax: +1 940 565 4297.
E-mail address: dbhuggett@unt.edu (D.B. Huggett).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.01.020

quantification of steroid hormones in blood plasma of small fish species, such as goldfish (*Carassius auratus*) and fathead minnow (*Pimephales promelas*) [38,39]. Despite these recent applications, only a limited number of steroid hormones have been quantified thus far.

In this study, we report the simultaneous quantification of five endogenous steroid hormones in the blood plasma of mature (≥5 months old) male and female fathead minnows (*Pimephales promelas*). The steroid hormones selected were: 17α-hydroxypregnenolone, progesterone, 11-ketotestosterone, 11-deoxycortisol and 17α,20β-dihydroxypregnenone. These steroid hormones included an androgen (11-ketotestosterone), progestogens (17α-hydroxypregnenolone, progesterone and 17α,20β-dihydroxypregnenone) and a corticosteroid (11-deoxycortisol). These steroid hormones constitute key regulators of endocrine functions and are proponents for reproductive viability [18,19]. This broad coverage of hormones was enabled by the derivatization of ketone-groups (mono and bi-oxosteroids) with 2-hydrazinopyridine. Derivatization enabled improved ionization efficiency and analysis by liquid chromatography-positive electrospray ionization–tandem mass spectrometry (LC-ESI+/MS/MS). This reported study is the first-application of 2-hydrazinopyridine derivatization for the analysis of sex steroid hormones in fathead minnow blood plasma.

2. Experimental

2.1. Chemicals and reagents

HPLC grade (≥99.8% purity) methanol (CAS# 67-56-1), ethyl acetate (CAS# 141-78-6) and chloroform (CAS# 67-66-3) were purchased from Fisher Scientific (USA). Anhydrous alcohol (VW0470-3; 90% ethanol) was purchased from VWR (USA). Formic acid (CAS# 64-18-6), 2-hydrazinopyridine (CAS# 4930-98-7) and trifluoroacetic acid (CAS# 76-05-1) were purchased from Sigma (USA).

2.2. Standards

Progesterone (CAS# 57-83-0; 4-pregnene-3,20-dione), 11-deoxycortisol (CAS# 152-58-9; 17α,21-dihydroxy-4-pregnen-3,20-dione) and 17α-hydroxypregnenolone (CAS# 387-79-1; 3β,17α-dihydroxy-5-pregnen-20-one) were purchased from Sigma–Aldrich (USA). Deuterated internal standards of d_3-17α-hydroxypregnenolone (CAS# 105078-92-0) and d_9-progesterone (Cat.# P755902) were purchased from Toronto Research Chemicals (Canada). 11-Ketotestosterone (CAS# 564-35-2; 4-androsten-17β-ol-3,11-dione) and 17α,20β-dihydroxypregnenone (CAS# 1662-06-2; 4-pregnen-17,20β-diol-3-one) was purchased from Steraloids (USA).

2.3. Standard stock and calibration curve preparations

Stock solutions (10,000 μg/ml) of d_9-progesterone, progesterone, 11-deoxycortisol and 11-ketotestosterone; 5000 μg/ml and 1000 μg/ml stocks of 17α,20β-dihydroxypregnenone and d_3-17α-hydroxypregnenolone were prepared using ethanol. 17α-Hydroxypregnenolone stock (10,000 μg/ml) was prepared in 1:1 (v/v) chloroform:methanol due to low solubility in either ethanol or methanol. All stocks were prepared in 2 ml screw-thread amber glass vials (Fisher Scientific) and stored at 4 °C. Working calibration ranges of standards were prepared by diluting stocks in methanol with deuterated internal standards of d_3-17α-hydroxypregnenolone and d_9-progesterone (either at 5 or 10 ng/ml). Calibration ranges used during method validation and sample quantification were 0.16, 0.31, 0.63, 1.25, 2.50, 5, 10, 20 and 40 ng/ml. Method validation standards were calibrated from 0.16 to 20 ng/ml (with 5 ng/ml internal standards), whereas standards for plasma analysis ranged from 0.31 to 40 ng/ml (with 10 ng/ml internal standards).

2.4. Fish culture and blood sampling: matrix spike for method validation

Male rainbow trout (*Oncorhynchus mykiss*) blood plasma was used as a representative matrix for spike recovery studies. This choice was dictated by the availability of large volumes of plasma (≥50 μl) from these fish. Rainbow trout were sourced from Greers Ferry National Fish Hatchery (Heber Springs, Arkansas) and were maintained in re-circulating streams at 12 °C. Fish were fed thrice weekly with commercial dense culture feed and maintained under day:night duration of 16 h light:8 h dark cycle. Blood was sampled using a 23 gauge needle and a heparinized 1 ml syringe by caudal vein puncture after euthanizing with 300 mg/l ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma, CAS# 886-86-2). Blood samples were centrifuged at 12,000 × *g* for 5 min with plasma supernatant transferred to a fresh microfuge tube and stored at −80 °C.

2.5. Fish culture and blood sampling: fathead minnow plasma analysis

Adult male and female (≥5 months) fathead minnows (*Pimephales promelas*) were purchased from Osage Catfisheries (Osage Beach, Missouri) and were maintained inflow-through tanks at 25 ± 1 °C with a 16 h light:8 h dark cycle. Male and female fish were housed in separate tanks. Fish were fed twice per day with dried flake food (Ocean Star International Inc.) and once with brine shrimp, *Artemia franciscana* (San Francisco Bay Brand). Nine females and eight males were euthanized in 200 mg/l ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma, CAS# 886-86-2) buffered with 0.5 M sodium bicarbonate (Sigma, CAS# 144-55-8). Blood was sampled through caudal-cut using heparinized blood collecting tubes (Kimble-Chase, #42E603) and transferred to 1.5 ml microfuge tubes. Tubes were stored on ice until centrifugation at 12,000 × *g* for 5 min with plasma supernatant transferred to fresh microfuge tubes for storage at −80 °C.

2.6. Steroid extraction and derivatization

Plasma samples were thawed on ice, and aliquots from male rainbow trout (50 μl) and fathead minnows (20–25 μl) were spiked with internal standards (d_3-17α-hydroxypregnenolone and d_9-progesterone). Prior to liquid:liquid extraction with 5 ml ethyl acetate, plasma samples were suspended in a 1 ml volume using ultra-pure (Milli-Q) water. This addition of Milli-Q water enabled effective phase separation between aqueous and solvent layer. Samples were liquid:liquid extracted twice with 5 ml ethyl acetate. Pooled solvent layers were dried down under a gentle stream of nitrogen with the residue re-suspended in ethyl acetate and transferred to 2 ml amber glass vials. These amber glass vials were further dried under nitrogen and residue was reconstituted with a mix solution (100 μl) containing 100 ng/ml 2-hydrazinopyridine and 250 ng/ml trifluoroacetic acid (in ethanol). This suspension was derivatized by sonication at 42 kHz in a water bath at room temperature for 15 min. Derivatized samples were nitrogen dried-down and reconstituted in methanol (with 0.1% formic acid) and transferred to small-volume spring inserts (Grace, #98018) for analysis using LC-ESI+/MS/MS.

Table 1
Masses of precursor and product ions obtained subsequent to collision induced dissociation (CID) with multiple reaction monitoring (MRM) mass transitions of precursor > product ions used for analysis. Chromatographic retention times used for steroid hormone quantifications are also listed {11-KT = 11-ketotestosterone; 11-DC = 11-deoxycortisol; 17,20-DP = 17α,20β-dihydroxypregnenone}.

Steroid (HP-derivatized)	ESI+-MS precursor ion (m/z)	CID-MS/MS product ions (m/z)	MRM transitions	Chromatographic retention times (min)
11-DC	529	364; 405	529 > 364	6.09
11-KT	394	95; 106	394 > 95	6.20
d_9-Progesterone	506	354; 395	506 > 354	6.31
Progesterone	497	348; 389	497 > 348	6.32
d_3-17αOH-Pregnenolone	427	253; 409	427 > 253	6.73
17αOH-Pregnenolone	424	253; 406	424 > 253	6.74
17,20-DP	424	95; 120	424 > 95	7.47

2.7. Liquid chromatography-positive electrospray ionization–tandem mass spectrometry (LC-ESI+/MS/MS)

The liquid chromatography (LC) system comprised a Waters™ 2695 separations module coupled to a Waters™ 2998 UV/vis detector. The mobile phase was comprised of Milli-Q water (A) and methanol (B) (both containing 0.1%, v/v formic acid). The initial mobile phase gradient during chromatographic separation was 70% (A), transitioned to: 30% (A) over 3 min, 5% (A) over the next 6 min, 30% (A) over 3 min and finally back to initial conditions of 70% (A) for the final 3 min. The total run-time was 15 min at a flow rate of 0.2 ml/min. A 5 μl sample volume was injected onto a Waters™ (#186002533) Sunfire C_{18} column (dimensions of 2.1 mm × 50 mm with 3.5 μm particle size). Electrospray ionization (positive mode) and tandem mass spectrometry (MS/MS) was conducted using a quadrupole–hexapole–quadrupole instrument, the Micromass Quattro Ultima mass detector (Manchester, U.K.). Low-energy collision tandem mass spectrometric analysis using the multiple reaction monitoring mode (MRM), consisted of measuring the transition of precursor ion fragmentation (via collision gas) to product ion(s). Electrospray ionization was conducted in the positive ionization mode (ESI+) with a capillary voltage of 3 kV and cone voltage of 60 V. The source and desolvation temperatures were set to 150 °C and 350 °C, respectively. Nitrogen gas generated from a nitrogen generator (Peak Scientific, USA) was used to supply cone (144 l/h) and desolvation (670 l/h) gas with the supply of argon as collision gas from a stand-alone cylinder (Praxair, TX, USA). The hexapole ion tunnel and aperture voltages were set to 1 V. Quadrupole (Q1 and Q2) low/high mass resolution was set to 10 arbitrary units with ion energies at 0.5 V. The entrance and exit potentials for the collision cells were set to 10 V with collision energy of 30 eV and photomultiplier voltage of 650 V. The multiple reaction monitoring (MRM) spectra selected for steroid identification and quantitation included the transition of m/z 497 > 348 for progesterone, m/z 506 > 354 for d_9-progesterone, m/z 424 > 253 for 17α-hydroxypregnenolone, m/z 427 > 253 for d_3-17α-hydroxypregnenolone, m/z 529 > 364 for 11-deoxycortisol, m/z 394 > 95 for 11-ketotestosterone and m/z 424 > 95 for 17α,20β-dihydroxypregnenone (Table 1). Mass detection (span of ±1 amu) and quantification was conducted using MassLynx software (version 4.1). All chromatograms were smoothed twice with relative response ratios calculated from the slope equations of linear calibrated standard curves (origin excluded) with a $1/x$ weighting (no axis transformations).

2.8. Method validation: establishing limits of detection

Limits of detections (LODs) for steroid hormones were set to the lowest nominal concentration of standard that gave a signal-to-background ratio of ≥3.This was calculated from analyzing a mix standard curve (ranging from 0.16 to 20 ng/ml) in two batches. The first batch was run (n = 4 per standard) to enable the calculation of intra-assay variability (data not shown), subsequently stored at 4 °C for 48 h and re-analyzed (n = 4 per standard) for inter-assay quantification (data not shown). Two-way Analysis of Variance (ANOVA) was conducted to test for significant differences between the two time-points. The absence of statistically significant differences allowed the calculation of mean total percentage accuracy and precision by pooling values from the two time-points (n = 8). Percentage precision and accuracy were calculated using the EPA method validation guideline as: % precision = (sample standard deviation/sample mean) × 100; and % accuracy = (sample mean/sample nominal concentration) × 100 [40]. Furthermore, the signal to noise intensities (counts per second) associated with the lowest standard concentrations giving a signal-to-background ratio of >3 were also statistically tested to establish whether the limits were significantly distinguishable from background.

2.9. Method validation: percentage recovery

Male rainbow trout (*Oncorhynchus mykiss*) plasma (n = 4) was used as a representative matrix for spiking experiments. Plasma samples were spiked at 10 ng/ml of final concentration for steroid hormones and samples were processed as described in Section 2.6. *Steroid extraction and derivatization*. Percentage accuracy (or recovery) was calculated subsequent to blank correction as (sample mean/sample nominal concentration) × 100 (data not shown).

2.10. Statistical testing

All statistical analyses were conducted using GraphPad Prism (version 5.01). Parametric two-way analysis of variance or paired t-tests were conducted subject to majority normal distribution of datasets (Shapiro–Wilk normality test, $p \geq 0.05$) with statistically significant differences declared at the 95% level ($p \leq 0.05$). Non-parametric pair-wise comparisons were analyzed using the Mann–Whitney test (significance at $p \leq 0.05$).

3. Results and discussion

3.1. Steroid hormone mass transitions

A limitation towards the analyses of steroid hormones in small-fish plasma samples are the low volume of sample procured, with average volumes ranging from 10 to 20 μl [39]. This agrees with our study, where the blood sampling of adult female (n = 9) and male (n = 8) fathead minnows yielded an average (± standard error) plasma volume of 20.6 ± 2.4 μl and 29.4 ± 5.5 μl, respectively. These low yields prioritize the use of methods that are capable of maximizing the quantification of multiple steroid hormones in a single sample. Towards this end, sample derivatization was used to enable increased ion-

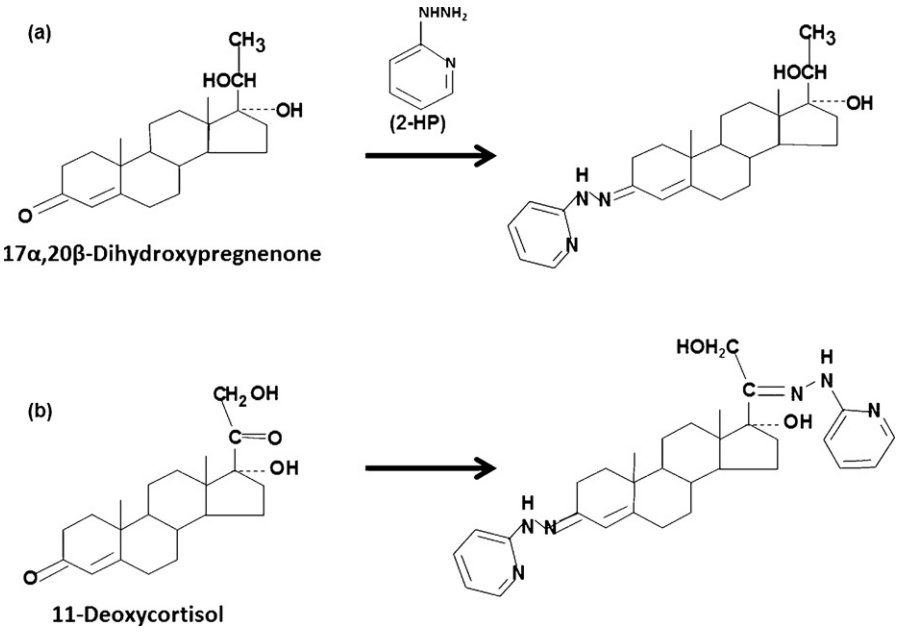

Fig. 1. Chemical structures demonstrating 2-hydrazinopyridine (2-HP) derivatization of: (a) 17α,20β-dihydroxypregnenone (mono-oxosteroid on carbon 3) and (b) 11-deoxycortisol (bi-oxosteroid on carbons 3 and 20).

ization efficiency (such as during electrospray ionization) and sensitivity for the detection of multiple analytes [41]. The use 2-hydrazinopyridine (2-HP) has been successfully applied towards the derivatization of ketone groups in mono- and/or bi-oxosteroids, which constitute prevalent structural features in a majority of steroid hormones (Fig. 1) [42–44]. The electrospray ionization (ESI+/MS) and collision-induced (CID-MS/MS) mass spectra of the protonated ($[M+H]^+$) 2-HP derivatized steroid hormones of progesterone, d_9-progesterone, 17α-hydroxypregnenolone, d_3-17α-hydroxypregnenolone, 11-deoxycortisol, 11-ketotestosterone and 17α,20β-dihydroxypregnenone are listed in Table 1. Introduction of a pyridyl group subsequent to derivatization commonly results in the formation of a product ion (after collision) with m/z 95 which constitutes the dissociated hydrazine N–N bond yielding $[\text{pyridine} + NH_2]^+$ group [42]. The remainder product ions were selected on the criteria of highest abundance subsequent to collision (and described in Higashi et al. [43]). A survey of published literature suggested this study to be the first account of 11-ketotestosterone and 17α,20β-dihydroxypregnenone derivatization with 2-HP. This is an important consideration as both these steroid hormones are key regulators of sexual maturation in male and female fish and constitute good biomarkers of reproductive competence [45–48]. Consequent to MS/MS tune parameter and MRM precursor ion to product ion selections, liquid chromatographic retention times were determined using a C_{18} column and a ultra-pure water and methanol mobile phase (both containing 0.1% formic acid) for a run duration of 15 minutes (Table 1 and Fig. 2). All steroid hormones were shown to elute between 5 and 8 min (Fig. 2).

3.2. Method validation: limits of detection (LOD) and percentage recovery analysis

Mixed calibration standards were prepared over a concentration range spanning 0.16–20 ng/ml withd_9-progesterone and d_3-17α-hydroxypregnenolone (5 ng/ml) as internal standards. d_9-Progesterone was used as internal standard for progesterone, 11-ketotestosterone, and 11-deoxycortisol; whereas d_3-17α-hydroxypregnenolone was used as internal standard for 17α-hydroxypregnenolone and 17α,20β-dihydroxypregnenone. Each standard was run four consecutive times to obtain intra-assay variation, after which standards were stored at 4 °C for 48 h and re-analyzed to obtain inter-assay variations (data not shown). Statistical analysis (two-way ANOVA) of pooled datasets ($n = 8$) showed no significant differences between the two time points ($p \geq 0.05$). Subsequently, mean total percentage precision, percentage accuracy and signal-to-background ratios were calculated for the pooled datasets ($n = 8$) (Table 2). Using a signal-to-background ratio of ≥3 as selection criteria, the following LODs were established: 0.16 ng/ml for progesterone; 0.63 ng/ml for 17α-hydroxypregnenolone, 11-deoxycortisol and 17α,20β-dihydroxypregnenone; 1.25 ng/ml for 11-ketotestosterone. The efficacy for selecting a signal-to-background ratio ≥ 3 was statistically tested to confirm for significant differences between the LOD standard intensity as compared to background (Mann–Whitney test, $p \leq 0.05$) (Fig. 3). Statistically significant differences for the LOD standards versus background intensities gave confidence in the quantifications of values at or above the selected LOD levels. A statistical method described by Armbruster et al. [49] for setting limits of detection (LOD) and limits of quantitation (LOQ) states; the LOD as the mean of blankplus two-or-three standard deviations; and the LOQ as mean of blank plus ten standard deviations [49]. In our study the signal intensity (counts per second) associated with the selection of a signal-to-background ratio of >3 was inclusive (and above) the criteria for LOD and LOQ described by Armbruster et al. [49](data not shown). Furthermore, in order to bolster the statistical robustness of the analysis (95% confidence level) comparing LOD standard intensity to background (shown in Fig. 3), the datasets were re-tested at the 99% confidence level with significant differences maintained (data not shown). Finally, rainbow trout plasma matrix spike analysis ($n = 4$) gave percentage recoveries (or % accuracy) ranging from 81 to 110% for steroid hormones (data not shown). Subsequent to this analysis, ethyl acetate was used for liquid:liquid extractions of steroid hormones from fathead minnow blood plasma.

3.3. Quantitation of steroids in plasma of male and female fathead minnows

After establishing effective LODs, the steroid hormones of 17α-hydroxypregnenolone, progesterone, 11-ketotestosterone,

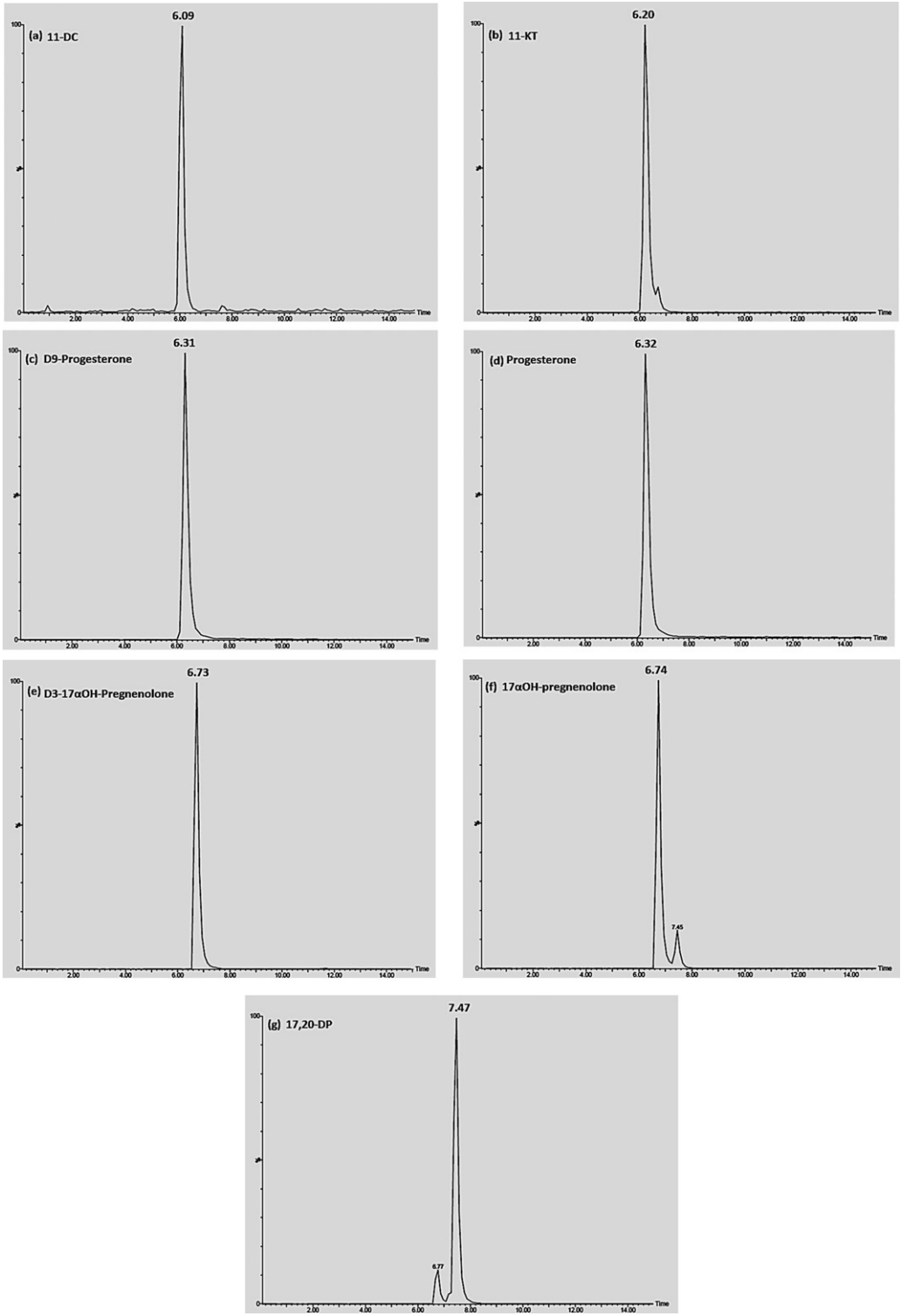

Fig. 2. Chromatograms showing retention times (in minutes) over a 15 min chromatographic run-time for (a) 11-deoxycortisol (11-DC), (b) 11-ketotestosterone (11-KT), (c) d_9-progesterone, (d) progesterone, (e) d_3-17αOH-pregnenolone, (f) 17αOH-pregnenolone and (g) 17α,20β-dihydroxypregnenone (17,20-DP). Steroid hormones were injected onto C_{18} column as a mix sample at 1 μg/ml final concentration.

11-deoxycortisol and 17α,20β-dihydroxypregnenone were quantified in the blood plasma of male and female fathead minnows (Table 3). To our knowledge there are currently no published accounts attempting to quantify 17α-hydroxypregnenolone, progesterone, 11-deoxycortisol and 17α,20β-dihydroxypregnenone in the blood plasma of fathead minnows. Statistical analysis indicated significant differences in plasma steroid titers for progesterone and 11-ketotestosterone between male and female fathead minnows. The average 11-KT concentration (19.11 ng/ml) measured in male fish in this study were in range with those reported in other studies using liquid chromatography and mass spectrometry. For example, in cyprinid small-fish species such as goldfish and fathead minnow, plasma 11-ketotestosterone in male fish have been reported from 19.38 to 32.2 ng/ml [38,39]. Elevated concen-

Table 2
Mean total percentage precision, accuracy and signal-to-background values for (a) 17αOH-pregnenolone, (b) progesterone, (c) 11-ketotestosterone (11-KT), (d) 11-deoxycortisol (11-DC) and (e) 17α,2β-dihydroxypregnenone (17,20-DP).

Steroids	Nominal (ng/ml)	Mean total performance				
		Average (n = 8)	St.Dev	% precision	% accuracy	Signal:background ratio
(a) 17αOH-pregnenolone						
	0.16	0.13	0.14	108	83	2
	0.31	0.30	0.17	57	96	2
	0.63	0.69	0.45	65	111	3
	1.25	1.42	0.30	21	113	4
	2.50	2.62	0.36	14	105	7
	5.00	5.22	0.57	11	104	14
	10.00	10.67	0.81	8	107	27
	20.00	18.92	1.94	10	95	47
(b) Progesterone						
	0.16	0.11	0.07	69	69	4
	0.31	0.29	0.04	14	94	5
	0.63	0.68	0.07	11	109	9
	1.25	1.36	0.12	9	109	15
	2.50	2.81	0.12	4	113	30
	5.00	5.42	0.26	5	108	59
	10.00	10.56	0.20	2	106	111
	20.00	18.62	0.68	4	93	191
(c) 11-KT						
	0.16	0.17	0.08	49	108	1
	0.31	0.40	0.16	40	129	2
	0.63	0.56	0.17	31	89	2
	1.25	1.33	0.35	26	106	3
	2.50	2.75	0.43	15	110	4
	5.00	5.36	0.85	16	107	8
	10.00	11.05	2.13	19	111	15
	20.00	18.80	2.30	12	94	23
(d) 11-DC						
	0.16	0.12	0.08	64	75	2
	0.31	0.24	0.13	55	78	2
	0.63	0.83	0.33	40	132	3
	1.25	1.04	0.20	20	83	4
	2.50	2.68	0.51	19	107	8
	5.00	6.20	0.63	10	124	18
	10.00	10.76	1.52	14	108	29
	20.00	18.06	1.32	7	90	46
(e) 17,20-DP						
	0.16	0.06	0.03	52	37	2
	0.31	0.27	0.15	56	87	2
	0.63	0.81	0.31	39	129	3
	1.25	1.58	0.38	24	126	5
	2.50	3.13	0.79	25	125	9
	5.00	6.14	0.61	10	123	18
	10.00	10.35	1.65	16	104	31
	20.00	18.03	3.66	20	90	54

trations of this hormone in male fish tend to correlate with late stages of sexual maturation implicating a key role of this androgen in stimulating spermatogonial proliferation prior to spawning [50]. In seasonal spawning fish, such as male gilthead seabream (*Sparus aurata*) and winter flounder (*Pseudopleuronectes americanus*), elevated concentrations of 11-KT from 11.36 to 170 ng/ml have been reported during sexual maturation, highlighting the importance of this steroid during later stages of spermatogenesis and sperm maturation [50,51]. Significantly elevated progesterone titers in female fathead minnows (0.73 ng/ml) relative to male (0.25 ng/ml) in our study was interesting though it is difficult to speculate on a sex-specific functional role for such elevation. Increasing concentrations of up to 17 ng/ml progesterone have been reported in sexually maturing male carp (*Cyprinus carpio*) with no changes in plasma concentrations (1 ng/ml) reported in female winter flounder (*P. americanus*) undergoing oocyte maturation [50,52]. Progesterone may play a role in stimulating oocyte maturation, as demonstrated in a study by Atteke et al. [53] where progesterone was shown to stimulate gonadotropin levels (luteinizing hormone or LH) in immature female rainbow trout (*Oncorhynchus mykiss*). This highlights regulatory role on the brain–pituitary axis mediating the production of a gonadotropin which is highly participant

Table 3
Steroid hormone concentrations (ng/ml) of (a) 17α-hydroxypregnenolone (17αOH-pregnen), (b) progesterone, (c) 11-ketotestosterone (11-KT), (d) 11-deoxycortisol (11-DC) and (e) 17α,20β-dihydroxypregnenone (17,20-DP) quantified in blood plasma of male and female fathead minnows (*Pimephales promelas*). Concentrations shown as average ± standard errors with asterisks (*) indicating significantly elevated concentrations (p < 0.05).

	Plasma steroid hormone concentrations (ng/ml)				
	(a) 17αOH-pregnen	(b) Progesterone	(c) 11-KT	(d) 11-DC	(e) 17,20-DP
Male fish (n = 8)	1.27 ± 0.44	0.25 ± 0.04	19.11 ± 5.47*	1.61 ± 0.17	1.47 ± 0.21
Female fish (n = 9)	0.92 ± 0.12	0.73 ± 0.34*	5.08 ± 3.26	1.67 ± 0.63	1.31 ± 0.17

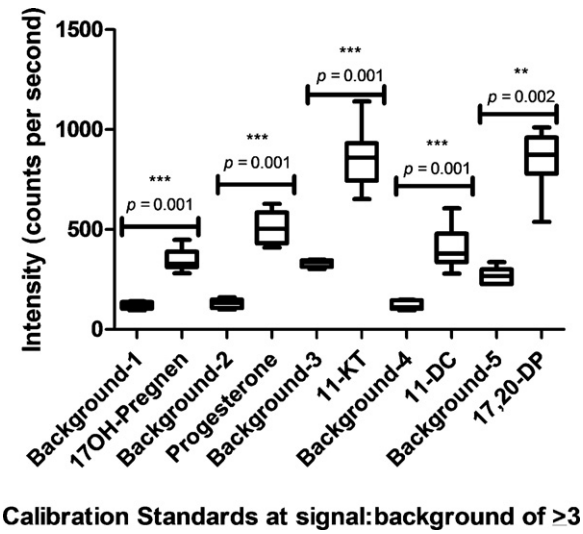

Fig. 3. Comparison of lower limits of quantitations (LLOQs) for steroid hormone standards relative to their respective backgrounds (1–5) at intensities (counts per second) giving a signal-to-background ratio of ≥3. Asterisks indicate statistically significant differences ($p \leq 0.05$) {17OH-Pregnen = 17α-OH-pregnenolone; 11-KT = 11-ketotestosterone; 11-DC = 11-deoxycortisol; 17,20-DP = 17α,20β-dihydroxypregnenone}.

during the final stages of sexual maturation. This may explain the significantly elevated progesterone concentrations seen in female fish in our study, however a meticulous assessment of this would only be provided by profiling plasma progesterone concentrations in spawning fathead minnows. Aside from its possible stimulatory role on gonadotropin production, progesterone also constitutes a key steroidogenic intermediate metabolite whose catalysis is responsible for the productions of various steroid hormones, such as corticosteroids androgens and estrogens. The catalysis of these steroid hormones from progesteronetends to constitute a favored reaction pathway (also known as the 4-ene pathway) due to the thermodynamic stability of 4-ene steroids [48]. The 4-ene pathway derives its name from the catalysis of steroidogenic intermediates containing a carbon-carbon double bond (-ene) from the fourth to the fifth carbon on the A-ring of steroid molecules. The lack of significant differences in 17α-hydroxypregnenolone concentrations between male and female fathead minnows in our study could be due to a possible preference of steroid hormone catalysis via the 4-ene pathway. The production of 17α-hydroxypregnenolone (from steroidogenic precursor of pregnenolone) characterizes the catalysis of 5-ene steroids with carbon-carbon double bonds from the fifth to sixth carbon of the B-ring of steroid molecules. The data presented in this study does not refute or confirm a preference in adult male and female fathead minnows for steroid hormone catalysis via the 4-ene pathway versus the 5-ene. Our observations are consistent with those from Budzinski et al. [54] reporting no significant differences in plasma titers of 17α-hydroxypregnenolone between mature male (0.6–1.3 ng/g) and female (0.5–0.9 ng/g) flounder (*Platichthys flesus*). It is possible that 17α-hydroxypregnenolone plays a functional role during early stages of reproductive cycling. Ponthier et al. [55] have shown elevated concentrations of 17α-hydroxypregnenolone in early vitellogenic ovaries of female channel catfish (*Ictalurus punctatus*) with rapid decrease in production during late vitellogenic stages (i.e. prior to spawning). Further more, developmental stage dependent preferences for either the 4-ene or 5-ene pathways for steroid hormone catalysishave also been demonstrated in the African catfish (*Clarias gariepinus*), where pubertal life-stages utilize the 5-ene pathway and adult life-stages the 4-ene [56]. The investigation of 4-ene versus 5-ene pathway preference for steroid hormone catalysis could also explain the low concentrations of 17α-hydroxypregnenolone quantified, and the resolution of which provides an avenue for further research.

Corticosteroids (such as 11-deoxycortisol) play a key role in stress response and energy metabolism with potential participation in oocyte maturation [57]. The participation of 11-deoxycortisol in mediating oocyte maturation is expected to be highly species-specific. For example, the administration of high concentrations of 11-deoxycortisol (up to 1 μg/ml) failed to induce oocyte maturation in Japanese catfish (*Silurus asotus*), whereas it was able to induce oocyte maturation in mummichog (*Fundulus heteroclitus*) at the same concentration [58,59]. Furthermore, blood plasma concentrations of 11-deoxycortisol have also been shown to vary between adult male (0.06 ng/ml) and female (1.9 ng/ml) winter flounder (*Pseudopleuronectes americanus*) [50]. Overall, elevated concentrations of 11-deoxycortisol are reported in reproductively cycling fish with concentrations spanning 8–31 ng/ml quantified in female rainbow trout (*Salmo gairdneri*) and 9–18 ng/g (plasma) in female perch (*Perca fluviatilis*) during the spawning and post-ovulatory period (i.e. after spawning) [60–62]. The lack of significant differences between male and female fathead minnows quantified in our study may be attributed to the fact that fish were maintained as non-spawners in separate tanks. The progestogen, 17α,20β-dihydroxypregnenone has been demonstrated to be a key regulator of oocyte and sperm maturation in teleost fish with elevations in plasma concentrations commonly measured in seasonally spawning fish [46,47]. Maximal concentrations of 17α,20β-dihydroxypregnenone in female post-vitellogenic fish (just prior to spawning) such as Indian major carp (*Labeo rohita*) and lake whitefish (*Coregonus clupeaformis*) have been reported to range from 5 to 14 ng/ml, however an elevated concentration as high as 317 ng/ml has also been reported for female rainbow trout (*Salmo gairdneri*) [17,63,64]. At present it is not known whether 17α,20β-dihydroxypregnenone titers would show such dramatic variations over the spawning cycle of fathead minnows. In our study, the absence of significant differences between male and female fathead minnows could be attributed to the fact that fish were maintained in separate tanks and were non-spawning, however it could be expected that in actively spawning fish, cycling of this key maturation inducing hormone would be seen. For example, Jensen et al. [3] have shown significant elevations in plasma concentrations of 17β-estradiol to be concomitant with increasing numbers of vitellogenic oocytes with days post spawn in spawning female fathead minnows. A similar trend for 17α,20β-dihydroxypregnenone could also be expected in spawning fathead minnows prior to the onset of oocyte maturation, though the demonstration of this would require the quantification of this progestogen with days post spawn. This constitutes an avenue of further work, as characterizing changes in the plasma titers of thesesteroid hormones would provide invaluable insights into their functional significances during the reproductive cycle. Furthermore, as shown from our study, the uses if derivatizing agents such as 2-hydrazinopyridine (2-HP) will greatly enable the pursuit of such an endeavor.

4. Conclusion

This study demonstrated the first application of 2-hydrazinopyridine derivatization for the simultaneous quantifications of an androgen, progestogens and corticosteroid in fathead minnow (*Pimephales promelas*) blood plasma. This method could prove invaluable for studies assessing endocrine function in fish as it enables the analysis of steroid hormones with diverse functional roles. The application of this method for reproductive-

toxicological and basic endocrinology studies is apparent and provides an avenue of further research and development.

References

[1] US-EPA, Guideline# EPA/600/3-87/001 (1987).
[2] OECD, Guideline# 203 (1992).
[3] K. Jensen, J. Korte, M. Kahl, M. Pasha, G. Ankley, Comp. Biochem. Physiol. Part C 128 (2001) 127.
[4] G. Ankley, R. Johnson, ILAR 45 (2004) 469.
[5] R. Van Aerle, T. Runnalls, C. Tyler, J. Fish Biol. 64 (2004) 355.
[6] G. Ankley, D. Villeneuve, Aquat. Toxicol. 78 (2006) 91.
[7] W. Gale, G. Buynak, J. Am. Fish Soc. 111 (1982) 35.
[8] J. Harries, T. Runnalls, E. Hill, C. Harris, S. Maddix, J. Sumpter, C. Tyler, Environ. Sci. Technol. 34 (2000) 3003.
[9] G. Ankley, K. Jensen, M. Kahl, J. Korte, E. Makynen, Environ. Toxicol. Chem. 20 (2001) 1276.
[10] US-EPA, 2007, http://www.epa.gov/endo/pubs/att-f_fish_assay_protocol.pdf.
[11] K. Watanabe, K. Jensen, E. Orlando, G. Ankley, Comp. Biochem. Physiol. Part C 146 (2007) 348.
[12] K. Nichols, S. Miles-Richardson, E. Snyder, J. Giesy, Environ. Toxicol. Chem. 18 (1999) 2001.
[13] J. Giesy, S. Pierens, E. Snyder, S. Miles-Richardson, V. Kramer, S. Snyder, K. Nichols, D. Villeneuve, Environ. Toxicol. Chem. 19 (2000) 1368.
[14] G. Ankley, M. Kahl, K. Jensen, M. Hornung, J. Korte, E. Makynen, R. Leino, Toxicol. Sci. 67 (2002) 121.
[15] G. Ankley, K. Jensen, E. Makynen, M. Kahl, J. Korte, M. Hornung, T. Henry, J. Denny, R. Leino, V. Wilson, M. Cardon, P. Hartig, E. Gray, Environ. Toxicol. Chem. 22 (2003) 1350.
[16] T. Runnalls, D. Hala, J. Sumpter, Aquat. Toxicol. 84 (2007) 111.
[17] A. Scott, J. Sumpter, P. Hardiman, Gen. Comp. Endocr. 49 (1983) 128.
[18] D. Kime, Rev. Fish Biol. Fisher. 3 (1993) 160.
[19] D. Kime, Sci. Total Environ. 225 (1999) 3.
[20] M. McMaster, J. Jardine, G. Ankley, W. Benson, M. Greeley, T. Gross, L. Guillette, D. MacLatchy, E. Orlando, G. Van Der Kraak, K. Munkittrick, Environ. Toxicol. Chem. 20 (2001) 2081.
[21] S. Jobling, N. Beresford, M. Nolan, T. Rodgers-Gray, G. Brighty, J. Sumpter, C. Tyler, Biol. Reprod. 66 (2002) 272.
[22] J. Nisbet, P. Jomain, Clin. Chem. 33 (1987) 1672.
[23] L. Guillette, A. Rooney, D. Crain, E. Orlando, in: D. Henshel, M. Black, M. Harrass (Eds.), Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment, 8th ed., 1999, p. 254.
[24] J. Taieb, C. Benattar, A. Birr, A. Lindenbaum, Clin. Chem. 48 (2002) 583.
[25] T. Sink, R. Lochmann, K. Fecteau, Fish Physiol. Biochem. 34 (2008) 95.
[26] J. Ström, A. Theodorsson, E. Theodorsson, Scand. J. Clin. Lab. Invest. 68 (2008) 806.
[27] M. Kushnir, A. Rockwood, J. Bergquist, Mass Spectrom. Rev. 29 (2010) 480.
[28] S. Soldin, O. Soldin, Clin. Chem. 55 (2009) 1061.
[29] J. Pitt, Clin. Biochem. Rev. 30 (2009) 19.
[30] T. Croley, R. Hughes, B. Koenig, C. Metcalfe, R. March, Rapid Commun. Mass Spectrom. 14 (2000) 1087.
[31] M. Alda, D. Barceló, J. Chromatogr. A 938 (2001) 145.
[32] P. Ferguson, C. Iden, A. McElroy, B. Brownawell, Anal. Chem. 73 (2001) 3890.
[33] A. Gentili, D. Perret, S. Marchese, R. Mastropasqua, R. Curini, A. Di Corcia, Chromatographia 56 (2002) 25.
[34] D. Kolpin, E. Furlong, M. Meyer, E. Thurman, S. Zaugg, L. Barber, H. Buxton, Environ. Sci. Technol. 36 (2002) 1202.
[35] M. Petrovic, S. Tavazzi, D. Barceló, J. Chromatogr. A 971 (2002) 37.
[36] M. Alda, S. Díaz-Cruz, M. Petrovic, D. Barceló, J. Chromatogr. A 1000 (2003) 503.
[37] R. Williams, A. Johnson, J. Smith, R. Kanda, Environ. Sci. Technol. 37 (2003) 1744.
[38] M. Blasco, P. Carriquiriborde, D. Marino, A. Ronco, G. Somoza, J. Chromatogr. B 877 (2009) 1509.
[39] F. Zhang, M. Bartels, D. Geter, M. Carr, L. McClymount, T. Marino, G. Klecka, Rapid Commun. Mass Spectrom. 23 (2009) 3637.
[40] US-EPA, 2003, http://www.epa.gov/wastes/hazard/testmethods/pdfs/methdev.pdf.
[41] T. Santa, O. Al-Dirbashi, T. Fukushima, Drug Discov. Ther. 1 (2007) 108.
[42] T. Higashi, T. Nishio, N. Hayashi, K. Shimada, Chem. Pharm. Bull. 55 (2007) 662.
[43] T. Higashi, T. Nishio, S. Uchida, K. Shimada, M. Fukushi, M. Maeda, J. Pharm. Biomed. 48 (2008) 177.
[44] Y. Shibayama, T. Higashi, K. Shimada, K. Kashimada, T. Onishi, M. Ono, K. Miyai, S. Mizutani, J. Chromatogr. B 867 (2008) 49.
[45] R. Schulz, T. Miura, Fish Physiol. Biochem. 26 (2002) 43.
[46] Y. Nagahama, M. Yamashita, Dev. Growth Differ. 50 (2008) S195.
[47] A. Scott, J. Sumpter, N. Stacey, J. Fish Biol. 76 (2010) 183.
[48] D. Kime, I.I. Chester-Jones, in: I. Ingleton, J. Phillips (Eds.), Fundamentals of Comparative Vertebrate Endocrinology, 1987, p. 3.
[49] D. Armbruster, M. Tillman, L. Hubbs, Clin. Chem. 40 (1994) 1233.
[50] C. Campbell, J. Walsh, D. Idler, Gen. Comp. Endocr. 29 (1976) 14.
[51] E. Chaves-Pozo, F. Arjona, A. García-López, A. García-Alcázar, J. Meseguer, A. García-Ayala, Gen. Comp. Endocr. 156 (2008) 531.
[52] G. Degani, R. Boker, K. Jackson, Comp. Biochem. Phys. C 120 (1998) 433.
[53] C. Atteke, A. Vetillard, A. Fostier, D.-H. Garnier, P. Jego, T. Bailhache, Comp. Biochem. Phys. A 134 (2003) 693.
[54] H. Budzinski, M. Devier, P. Labadie, A. Togola, Anal. Bioanal. Chem. 386 (2006) 1429.
[55] J. Ponthier, C. Shackleton, J. Trant, Gen. Comp. Endocr. 111 (1998) 141.
[56] J. Cavaco, J. Lambert, R. Schulz, H. Goos, Fish Physiol. Biochem. 16 (1997) 129.
[57] S. Milla, N. Wang, S. Mandiki, P. Kestemont, Comp. Biochem. Phys. A 153 (2009) 242.
[58] M. Greeley, D. Calder, M. Taylor, H. Hols, R. Wallace, Gen. Comp. Endocr. 62 (1986) 281.
[59] T. Miwa, G. Yoshizaki, H. Naka, M. Nakatani, K. Sakai, M. Kobayashi, T. Takeuchi, Aquaculture 198 (2001) 179.
[60] E. Noaksson, B. Gustavsson, M. Linderoth, Y. Zebühr, D. Broman, L. Balk, Toxicol. Appl. Pharm. 195 (2004) 247.
[61] E. Noaksson, M. Linderoth, B. Gustavsson, Y. Zebühr, L. Balk, Sci. Total Environ. 340 (2005) 97.
[62] C. Bry, Gen. Comp. Endocr. 57 (1985) 47.
[63] U. Sen, D. Mukherjee, S. Bhattacharyya, D. Mukherjee, Gen. Comp. Endocr. 128 (2002) 123.
[64] J. Rinchard, K. Dabrowski, J. Ottobre, Comp. Biochem. Phys. C 129 (2001) 65.