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Steroids accumulate in the rearing water of commercial recirculating aquaculture systems

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ABSTRACT

Little information is available on steroid concentrations in the rearing water of aquaculture systems and whether they accumulate in recirculating aquaculture systems (RAS). Therefore this study aimed at determining (1) the concentrations and variation of cortisol and sex steroids in RAS, (2) the contribution of fish rearing conditions to steroid concentrations in seven commercial RAS. Each RAS was sampled twice at three different points: (1) make-up water; (2) influent and (3) effluent of the rearing unit. The results showed significant higher steroid concentrations in the influent and effluent when compared with the make-up water. On average cortisol concentration was 15.7% higher in the effluent when compared with the influent. Mean steroid concentrations in the rearing unit effluent varied between: 3.8–217.0 ng/L for cortisol, 3–12.5 ng/L for testosterone, 0.9–7.1 ng/L for 11-ketoteststerone and 1.8–12.8 ng/L for 17,20β-dihydroxypregn-4-en-3-one. Stocking density, Total Ammonia-Nitrogen concentration and orthophosphate-P concentration (a measure of make-up water usage) showed a positive correlation with sex steroids in the water. The steroid concentrations from the present study were orders of magnitude lower than initial estimations indicating a water treatment efficiency of >99%. The results suggest that an intensification of fish production through decrease of make-up water use and increase of stocking density will lead to a build-up of steroids in the water. Although intensification is critical for the economical success of RAS, this ultimately could affect fish performance as steroids accumulates in the water of RAS at levels that can potentially be detected by some fish species.

1. Introduction

Recirculating aquaculture systems (RAS) are among the most environmentally sustainable systems to culture fish due to their reduced make-up water usage (new water supply) and release of nutrients to the environment (Martins et al., 2010). In response to the increasing demand for aquaculture products, production methods in RAS have been intensified. Intensification of fish production is achieved by increasing stocking densities, which increases the amount of metabolites released into the water (Fanouraki et al., 2008). However, intensification of fish production may not always result in optimal rearing conditions.

It is known that situations eliciting the production of fish steroids, will increase their release into the water and ultimately bioaccumulation in RAS (Scott et al., 2008). Studies with rainbow trout *Oncorhynchus mykiss* (Ellis et al., 2004) and Atlantic salmon *Salmo salar* (Ellis et al., 2007) showed that the stress hormone cortisol is released at higher quantities into the water after exposure to acute handling stress. European sea bass *Dicentrarchus labrax* kept at stocking densities of 50 kg/m^3 increased both blood plasma concentrations and cortisol release rates into water when compared to fish kept at 20 kg/m^3 (Fanouraki et al., 2008). Other steroid hormones potentially accumulating in RAS are sex steroids such as testosterone, 11-ketotestoterone and the maturation-inducing steroid 17,20β-dihydroxypregn-4-en-3-one (17,20β-*P*). Sex steroids can be transferred from one group of fish to another group of fish (Budworth and Senger, 1993) as reported

Abbreviations: RAS, recirculating aquaculture systems; 17,20β-*P*, 17,20β-dihydroxypregn-4-en-3-one; TA-N, Total Ammonia-Nitrogen; TFA, trifluoroacetic acid; RIA, radioimmunoassay; TLC, thin-layer chromatography; CV, coefficient of variation.

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Table 1
Overview of the rearing unit, water treatment unit and water quality parameters of the seven sampled RAS. Values are mean ($N = 2$, except RAS 1 where $N = 1$).

	RAS 1	RAS 2	RAS 3	RAS 4	RAS 5	RAS 6	RAS 7
Rearing unit[a]							
Species	*Solea solea*	*Anguilla anguilla*	*Psetta maxima*	*Stizostedion lucioperca*	*Clarias gariepinus*	*Oreochromis niloticus*	*Seriola lalandi*
Common name	*Dover sole*	*European eel*	*Turbot*	*Pike-perch*	*African Catfish*	*Nile tilapia*	*Yellowtail amberjack*
Fish tanks	Raceways	Circular	Raceways	Circular	Rectangular	Rectangular	Circular
Standing stock (kg)	20,000		65,000	7500	6000	4750	19,000
Stocking density (kg/m^3)	104	175	59	43	162	68	50
Feed load (kg/d)	60	300	331	48	100	48	180
Weight range (g)	16–555	5–1500	8–2500	370–1800	100–1500	100–800	400–1800
Sex ratio (M:F)	1:3	9:1	Unknown	1:1	1:1	1:1	Unknown
Volume (m^3)	193	200	1100	175	37	70	378
Water treatment unit[a]							
Mechanical filtration	Drum	Drum	Drum	Drum	Settling	Sieves/settling	Drum
Bio-reactor	Trickling	Trickling	Moving bed	Trickling	Trickling	Trickling	Trickling
Ozone	Present	–	Present	–	–	–	–
UV	–	–	–	–	Present	–	Present
Make-up water (L/kg feed/d)	1000	480	920	450	100	74	1000
Volume (m^3)	7	30	2500	25	13	10	122
Water quality							
Temperature (°C)[a]	19	24	17	25	26	24	21
pH[a]	5.9	5.5	7.7	6.2	6.6	7.5	7.7
Conductivity (μs/cm)[a]	40,600	2500	40,000	1900	4000	4200	36,300
TA-N (mg/L)[b]	5.7	63.5	0.3	1.4	48.8	5.9	1.0
Nitrite-N (mg/L)[b]	0.14	0.15	0.05	0.22	4.6	1.3	0.31
Nitrate-N (mg/L)[b]	64.5	92.3	27.0	91.1	53.5	72.3	73.6
Orthophosphate-P (mg/L)[b]	4.9	21.6	2.1	7.1	13.1	6.5	5.0

Fish sexual maturation: immature in RAS 1, 2, 3, 4, 5 and 7 and 90% of the standing stock mature in RAS 6, according to information provided by the facility managers.
[a] Value or information provided by the facility manager.
[b] Value measured.

for rainbow trout (Vermeirssen and Scott, 1996) and tench *Tinca tinca* (Scott et al., 2005).

Besides acting as endogenous signals, steroids are also used by fish as exogenous signals, e.g. pheromones that synchronize gamete maturation or spawning interactions (Stacey, 2003). For instance, three steroids (androstenedione, 17,20β-*P* and 17,20β-*P* sulphate) that are released by female goldfish *Carassius auratus* can elicit behavioural and physiological changes in males at very low concentrations (nM threshold) (Stacey and Sorensen, 2006). In addition, testosterone is reported to be a potent odorant in precocious male Atlantic Salmon parr (Moore and Scott, 1991).

Whether steroids in RAS occur at concentrations that can be sensed or taken up by fish remains to be investigated. Therefore the present study aimed at determining (1) the concentrations and variation of cortisol and sex steroids in RAS (2) the contribution of fish rearing conditions to the concentration of steroids in RAS.

2. Materials and methods

2.1. Sampling sites and sample collection

Water samples were collected from seven commercial recirculating aquaculture systems (RAS) in full operation located in The Netherlands. None of these RAS were in the start up phase. Details of the systems (rearing unit, water treatment unit and water quality) provided by the facilities managers are presented in Table 1. Five different commercial RAS were sampled twice with an interval of ±15 months, one sampled twice (RAS 7) with and interval of ±2 months and one RAS (1) was sampled once (the farm closed down during the second sampling period). Three different points were sampled in each RAS: (1) make-up water; (2) influent of rearing unit and; (3) effluent of rearing unit (Fig. 1).

Water samples for steroid analysis were collected in 1 L containers and immediately placed on ice water, transported to the laboratory and stored at −20 °C. Additional water samples (10 mL) were taken (Fig. 1), placed on ice water and transported to the laboratory for Total Ammonia-Nitrogen (TA-N), Nitrite-N, Nitrate-N and Orthophosphate-P analysis using a SAN autoanalyzer (Skalar, The Netherlands). Temperature, pH, and conductivity were measured at the sampling sites using portable meters or provided by the facilities managers. Nitrile gloves were used during all water sampling and processing activities to prevent contamination with steroids.

2.2. Steroid analysis

Steroid hormones were measured by radioimmunoassay of their free forms. For this it required extraction of steroids from the water and hydrolysis of conjugates to release the free forms as previously reported (Canario and Scott, 1989; Scott and Sorensen, 1994). Briefly, water samples for steroid analysis were first paper filtered (2 μm; VWR, France) followed by a membrane filter (0.45 μm; Millipore, Ireland). The sample volume (±500 mL) was determined gravimetrically and pumped (±12 mL/min) through an Oasis HLB Plus solid phase extraction cartridge (Oasis®, Waters, Milford, U.S.A.) previously activated with methanol (5 mL) and washed with distilled water (5 mL). Cartridges were eluted (3 mL 100% ethanol) and the eluate evaporated in a dry bath at 45 °C under a gentle flow of nitrogen. The dried residue was re-dissolved in 0.1 mL distilled water, 3× 3 mL of diethyl ether was added, the tubes vigorously shaken, and centrifuged at low speed to separate the organic and water phases. The water phase was frozen in liquid nitrogen. The diethyl ether was transferred to another tube and evaporated under nitrogen. To the residue was re-constituted in radioimmunoassay (RIA) buffer (sodium phosphate 0.05 M, pH 7.6, containing 1% gelatine) and stored (−20 °C) until assay.

The remaining aqueous fraction containing the conjugated steroids was evaporated at 40 °C and 1 mL of trifluoroacetic acid (TFA)/ethyl acetate (1/100, v/v) was added to the dried residue and incubated in a water bath at 40 °C overnight for the chemical hydrolysis of the sulphate steroids. The TFA/ethyl acetate was subsequently evaporated in a dry bath at 45 °C under a gentle nitrogen flow and the dried residue was re-dissolved in 0.5 mL sodium

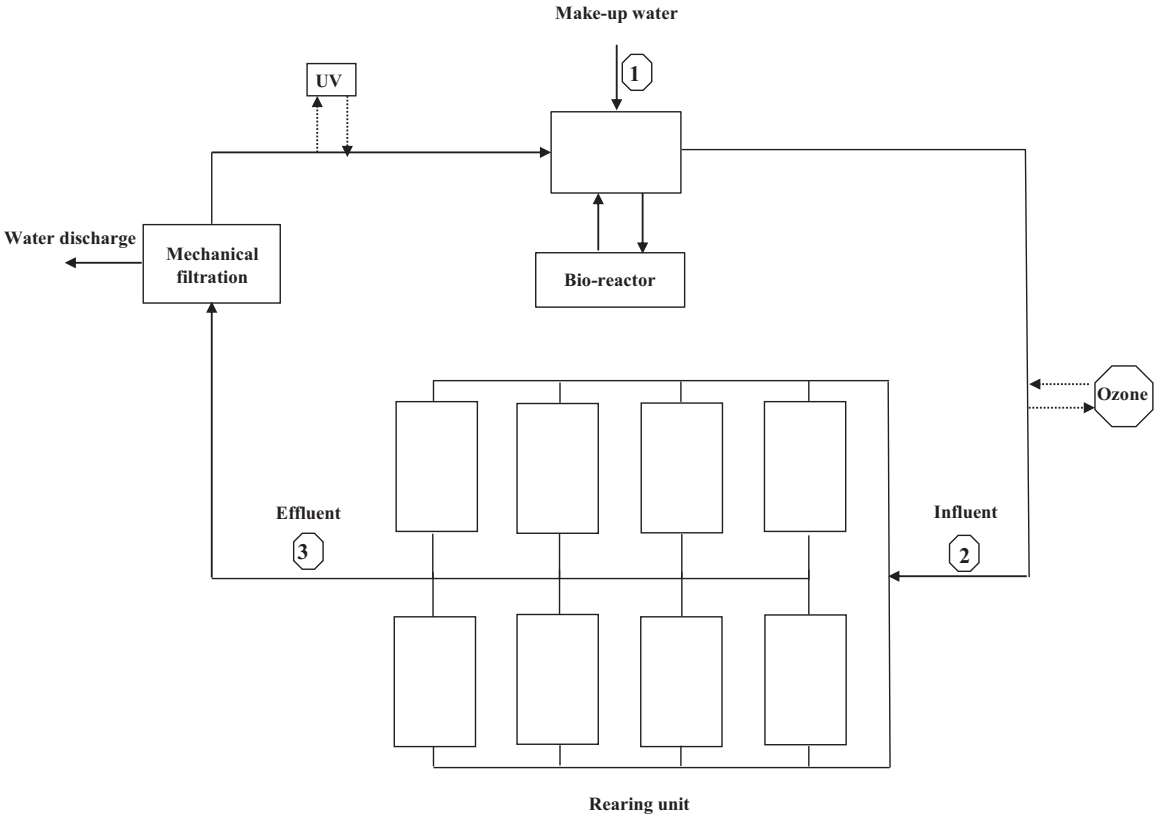

Fig. 1. Schematic design of a general recirculating aquaculture system. Numbers represent sampling points: (1) make-up water; (2) influent of rearing unit and; (3) effluent of rearing unit. Arrows indicate the direction of the water flow. Not all components may be present in a specific recirculating system or arranged as shown (see Table 1 for detailed information).

0.1 M acetate buffer pH 4.5. The sulphate steroid fraction (now free steroids) was extracted and radioimmunoassay buffer added as described above.

Finally, traces of diethyl ether were removed from the sodium acetate by a gentle stream of nitrogen and β-glucuronidase (10 μL) from Helix pornatia (Sigma–Aldrich, U.S.A.) was added and incubated at 37 °C overnight for the enzymatic hydrolysis of the steroid glucuronides. The steroid glucuronide hydrolysates were extracted 3× with diethyl ether as described above, radioimmunoassay buffer added and stored at −20 °C until assay.

The methodology for steroid RIA is described by Scott et al. (1982). For cortisol antiserum 20-CR50 (Fitzgerald Industries International, Concord, USA) and tritiated cortisol (GE Healthcare Europe GmbH, Carnaxide, Portugal) were used. Cross-reactions were 54% for 11-desoxycortisol, 10% for cortisone, 16% for 17,21-dihydroxy-5β-pregnan-3,11,20-trione, 5% for 11β,17,21-trihydroxy-5β-pregnan-3,20-dione, 0.05% for 11β-hydroxytestosterone and less than 0.001% for testosterone (Rotllant et al., 2005). The testosterone and 11-ketotestosterone antisera were kindly donated by Dr. David Kime (University of Sheffield, UK). The testosterone antiserum cross-reactions were 63% for androstenedione, 35% for 11-ketotestosterone, 55% for 11β-hydroxytestosterone, 40% for 5α-androstan-17β-ol-3-one, 31% for 5β-androstan-17β-ol-3-one, 12% for 5β-androstane-3α,17β-diol, 25% for 5α-androstane-3α,17β-diol. The 11-ketotestosterone antiserum cross-reactions are given elsewhere (Kime and Manning, 1982) and were 20.1% for 11β-hydroxytestosterone, 20.6% for testosterone, 76.9% for androstenetrione, 30.1% for 11β-hydroxyandrostenedione, 52% for dihydrotestosterone, 3.3% for cortisol and 1.3% for cortisone. The 17,20β-*P* antiserum was donated by Dr. A.P. Scott and characterized by Scott et al. (1982, 1997).

The mean recovery efficiency for steroids was 84% (unspiked RAS (without fish) water samples contained 0.7 ± 0.2 ng/L and contained 76.4 ± 4.8 ng/L after samples were spiked with 87 ng/L cortisol (hydrocortisone H4001, Sigma–Aldrich, The Netherlands). The observed recovery is in line with the reported recovery in literature 87% (Ellis et al., 2004) and 85% (García-López et al., 2006). RIA detection limit was 36 pg/L for cortisol, 60 pg/L for testosterone, 50 pg/L for 11-ketotestosterone and 80 pg/L 17,20β-*P*.

Cross-reactivity of water samples with antisera was verified by thin-layer chromatography (TLC). Briefly, the analysis was performed from the dried residue extracts previously obtained and applied to a pre-coated silica gel TLC plates (LK6DF silica gel 60A plates, Whatman Inc., NJ, USA). The plates were developed at room temperature for 50 min, using as mobile phase chloroform:methanol (72:3). Tritiated standard steroids were aliquoted in distinct lanes (cortisol, testosterone, 11-ketotestosterone and 17α,20β-dihydroxy-4-pregnen-3-one) and used as reference. Each water sample lane was divided in 0.5 cm strips that were scraped, eluted with dichloromethane:ethanol (8:2, v/v, 2× 1 mL) and evaporated under vacuum centrifugation at 40 °C. The residue was dissolved in 1 mL RIA assay buffer.

2.3. Data analysis

Homogeneity of variances was tested using Levene's test. Steroid analysis of make-up water, influent and effluent of the rearing unit was done using the average from the two sampling periods followed by an Independent Students *t*-test. Analysis of rearing conditions, i.e., standing stock, stocking density, total volume (rearing unit + water treatment unit), make-up water, temperature, pH, conductivity, TA-N, Nitrite-N, Nitrate-N, orthophosphate-P on effluent of rearing unit steroid concentrations was tested

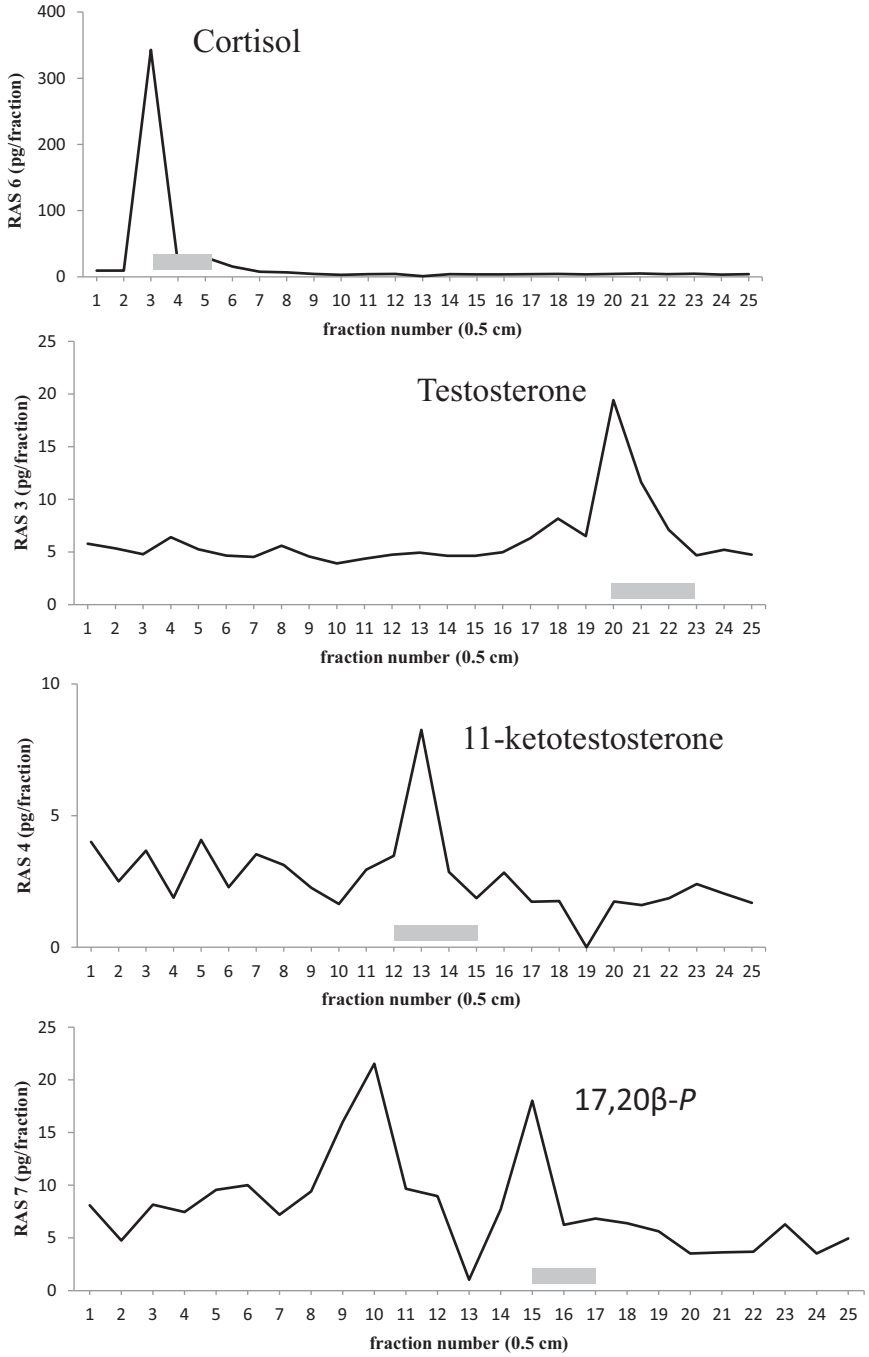

Fig. 2. Thin-layer chromatography scan of immunoreactive steroids in four random water samples from the effluent of a rearing unit (RAS 3, 4, 6 and 7). Grey areas show elution positions of the tritiated standards (1) cortisol, (2) testosterone, (3) 11-ketotestosterone and (4) 17,20β-*P*.

by Pearson's correlations. For Pearson's correlations the α was recalculated after Bonferroni correction for multiple tests (initial $\alpha = 0.05/16 = 0.003$). Statistical analysis was performed with IBM SPSS Statistics 19 (IBM Corp., USA). A significance level (α) of 0.05 was used. Data are presented as mean ± standard deviation (s.d.).

3. Results

3.1. Steroid concentration and variation

Analysis of water extracts on TLC showed a single peak for each steroid, except for 17,20β-*P* which cross-reacted also with a secondary less polar compound (Fig. 2). All steroid concentrations were higher in the influent and effluent of rearing unit when compared to make-up water (Fig. 3). Overall total cortisol concentration was 15.7% higher in the effluent when compared with the influent (Table 2). Sex steroids showed a smaller non-significant increase in concentration between influent and effluent. Also notable is the 53.8% overall increase of cortisol sulphate between influent and effluent that contrasted with the overall decrease of the other steroid sulphates (Table 2). Total cortisol concentrations (max. 217.0 ng/L) were higher when compared to total testosterone (max. 13.7 ng/L), total 11-ketoteststerone (max. 6.4 ng/L) and total 17,20β-*P* (max. 12.8 ng/L) (Tables 3a and 3b).

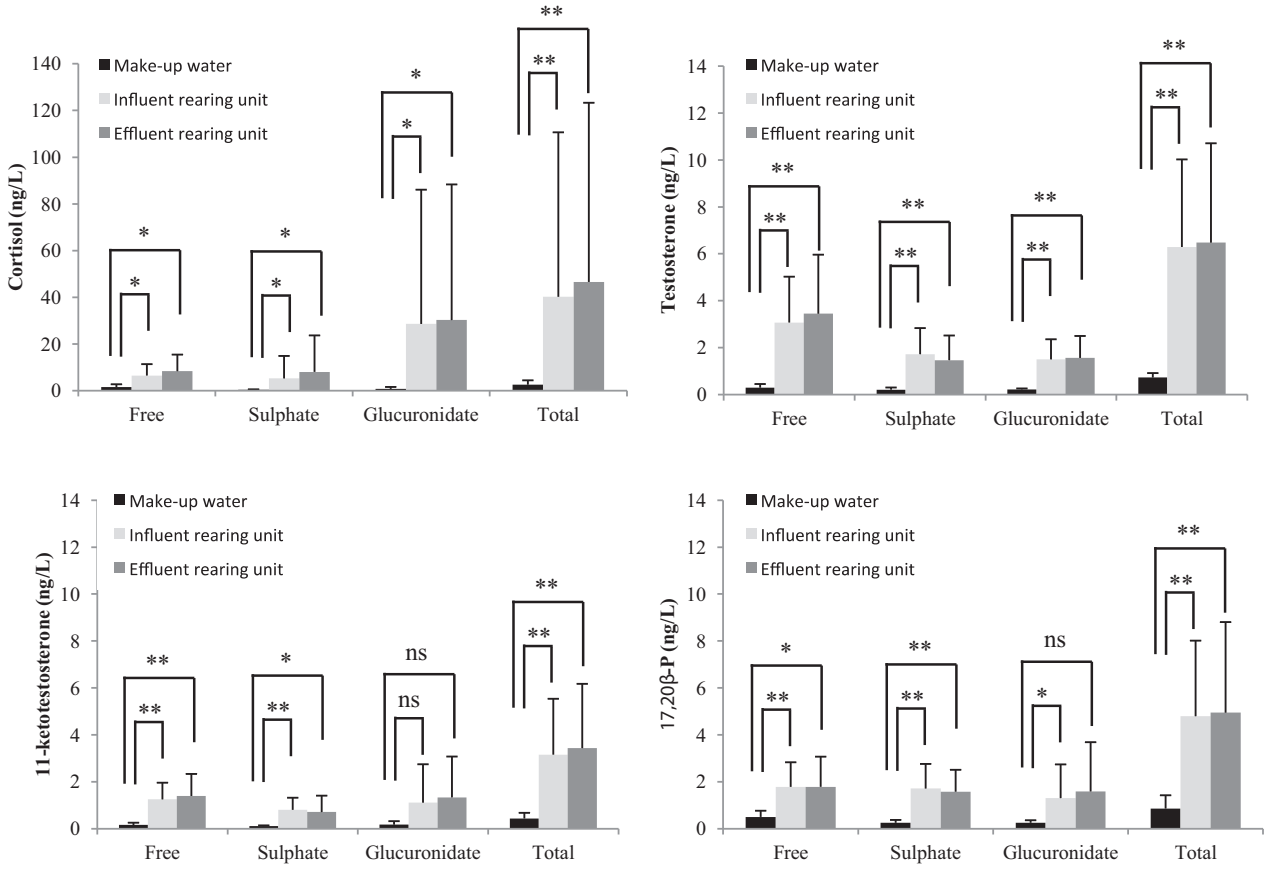

Fig. 3. Mean ± s.d. steroid concentrations in the make-up water, influent and effluent of the rearing unit for the seven sampled RAS ($N = 7$). *p-Value <0.05; **p-value <0.01; ***p-value <0.001; ns – not significant.

Total cortisol also exhibited the highest variation among the different RAS (CV 163.0%) when compared to the sex steroids (total testosterone = 68.2%, total 11-ketotestosterone = 80.4%, total 17,20β-P = 78.3%). Higher variation in cortisol concentration was also observed between the two sampling periods, particularly in RAS 4 (CV = 94.6%; Table 3b).

3.2. Steroid concentration and rearing condition

Table 4 shows the correlations between water steroid concentrations and rearing conditions. Testosterone and 11-ketotestosterone showed a significant and positive correlation with stocking density, TA-N and orthophosphate-P. None of the other

Table 2
Mean ± s.d. steroid concentrations in the influent and effluent of the rearing unit for the seven sampled RAS ($N = 7$).

Steroids (ng/L)	Influent of rearing unit	Effluent of rearing unit	p-Value	Overall increase (%)
Cortisol				
Free	6.4 ± 4.9	8.3 ± 7.1	0.343	29.7
Sulphate	5.2 ± 9.6	8.0 ± 15.6	0.103	53.8
Glucuronide	28.5 ± 57.5	30.2 ± 58.1	0.334	6.0
Total	40.2 ± 70.5	46.5 ± 76.8	0.047*	15.7
Testosterone				
Free	3.1 ± 2.0	3.4 ± 2.5	0.296	9.7
Sulphate	1.7 ± 1.1	1.5 ± 1.0	0.070	−11.8
Glucuronide	1.5 ± 0.9	1.6 ± 0.9	0.711	6.7
Total	6.3 ± 3.7	6.5 ± 4.2	0.825	3.2
11-Ketotestosterone				
Free	1.2 ± 0.7	1.4 ± 0.9	0.327	16.7
Sulphate	0.8 ± 0.5	0.7 ± 0.7	0.520	−12.5
Glucuronide	1.1 ± 1.6	1.3 ± 1.7	0.054	18.2
Total	3.2 ± 2.4	3.4 ± 2.7	0.646	6.3
17,20β-P				
Free	1.8 ± 1.0	1.8 ± 1.3	0.605	0.0
Sulphate	1.7 ± 1.0	1.6 ± 0.9	0.514	−5.9
Glucuronide	1.3 ± 1.4	1.6 ± 2.1	0.500	23.1
Total	4.8 ± 3.2	4.9 ± 3.9	0.959	2.1

* Indicates significant effect (p-value < 0.05).

Table 3a
Mean ± s.d. steroid concentrations in the influent of the rearing unit for the seven sampled RAS ($N = 2$, except when there is no s.d. where $N = 1$).

Steroids (ng/L)	RAS 1	RAS 2	RAS 3	RAS 4	RAS 5	RAS 6	RAS 7	Overall CV (%)
Cortisol								
Free	13.7	8.2 ± 7.9	0.8 ± 0.0	6.6 ± 4.0	3.8 ± 0.3	11.9 ± 7.3	1.7 ± 0.4	73.8
Sulphate	27.0	1.4 ± 0.6	1.2 ± 0.3	2.6 ± 0.6	1.2 ± 0.6	1.4 ± 0.6	1.7 ± 1.2	184.2
Glucuronide	157.7	12.0 ± 3.9	1.6 ± 1.7	23.8 ± 19.6	1.8 ± 0.3	1.3 ± 0.8	2.6 ± 2.0	200.5
Total	198.4	21.6 ± 12.4	3.6 ± 2.0	33.0 ± 23.1	6.9 ± 0.5	14.6 ± 7.0	6.0 ± 0.5	173.3
Testosterone								
Free	2.7	6.9 ± 3.1	1.0 ± 0.2	1.6 ± 0.2	5.1 ± 0.8	3.4 ± 0.2	1.5 ± 0.0	67.8
Sulphate	0.8	3.0 ± 2.6	0.8 ± 0.1	0.6 ± 0.7	3.5 ± 3.3	2.6 ± 0.3	1.5 ± 0.4	65.8
Glucuronide	1.6	3.1 ± 2.6	0.7 ± 0.2	0.4 ± 0.1	2.3 ± 1.6	2.1 ± 1.7	0.8 ± 0.6	63.0
Total	5.0	13.0 ± 8.2	2.4 ± 0.5	2.6 ± 1.0	11.0 ± 5.7	8.1 ± 1.6	3.8 ± 0.2	63.7
11-Ketotestosterone								
Free	2.0	1.8 ± 0.6	0.3 ± 0.1	1.1 ± 0.4	2.1 ± 0.7	1.0 ± 0.6	0.6 ± 0.2	57.4
Sulphate	0.8	1.3 ± 0.2	0.4 ± 0.4	0.1 ± 0.0	1.3 ± 0.2	1.4 ± 1.4	0.4 ± 0.2	64.8
Glucuronide	4.4	2.8 ± 2.4	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.3	0.2 ± 0.0	0.2 ± 0.0	144.7
Total	7.2	5.9 ± 3.2	0.9 ± 0.6	1.3 ± 0.5	3.8 ± 1.1	2.6 ± 1.9	1.1 ± 0.1	76.7
17,20β-P								
Free	0.5	3.1	0.8	1.6	3.0	2.4	1.1	58.8
Sulphate	1.7	3.3	0.6	0.5	2.8	1.6	2.2	58.8
Glucuronide	0.4	4.4	0.4	0.9	1.5	1.0	0.5	110.9
Total	2.6	10.8	1.9	2.9	7.3	5.0	3.7	65.1

Table 3b
Mean ± s.d. steroid concentrations in the effluent of the rearing unit for the seven sampled RAS ($N = 2$, except when there is no s.d. where $N = 1$).

Steroids (ng/L)	RAS 1	RAS 2	RAS 3	RAS 4	RAS 5	RAS 6	RAS 7	Overall CV (%)
Cortisol								
Free	14.2	7.3 ± 7.0	0.8 ± 0.0	15.3 ± 15.4	3.0 ± 1.7	17.5 ± 16.9	1.7 ± 0.9	82.2
Sulphate	43.4	1.6 ± 0.2	1.7 ± 1.2	2.3 ± 1.4	1.2 ± 0.4	1.5 ± 0.2	4.4 ± 4.0	195.4
Glucuronide	159.5	14.3 ± 6.5	1.3 ± 1.0	32.2 ± 30.4	2.1 ± 0.3	1.1 ± 0.6	2.6 ± 1.7	190.6
Total	217.0	23.2 ± 13.3	3.8 ± 2.1	49.9 ± 47.2	6.3 ± 1.0	20.1 ± 16.5	8.6 ± 3.2	163.0
Testosterone								
Free	1.9	8.6 ± 2.3	1.3 ± 0.0	1.8 ± 0.2	5.5 ± 1.5	3.8 ± 0.8	1.7 ± 0.3	76.2
Sulphate	0.5	2.7 ± 2.0	0.7 ± 0.2	0.6 ± 0.7	3.4 ± 2.7	1.6 ± 1.8	1.3 ± 0.1	73.0
Glucuronide	1.3	2.4 ± 0.8	0.7 ± 0.3	0.5 ± 0.3	3.2 ± 0.9	1.7 ± 1.2	1.3 ± 0.6	60.5
Total	3.7	13.7 ± 5.1	2.7 ± 0.5	3.0 ± 1.1	12.1 ± 5.1	7.1 ± 0.2	4.2 ± 0.2	68.2
11-Ketotestosterone								
Free	2.0	1.8 ± 0.6	0.2 ± 0.0	1.0 ± 0.1	3.0 ± 1.4	1.2 ± 0.9	0.6 ± 0.0	67.1
Sulphate	0.5	1.4 ± 0.1	0.4 ± 0.3	0.2 ± 0.0	2.0 ± 0.7	0.3 ± 0.0	0.3 ± 0.0	98.1
Glucuronide	4.7	3.2 ± 2.2	0.3 ± 0.2	0.2 ± 0.1	1.0 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	129.3
Total	7.1	6.4 ± 2.9	0.9 ± 0.6	1.4 ± 0.2	6.1 ± 2.4	1.7 ± 0.9	1.2 ± 0.1	80.4
17,20β-P								
Free	0.4	3.9	0.6	1.5	2.6	2.5	0.9 ± 0.1	72.3
Sulphate	2.6	2.7	0.5	0.5	2.2	1.5	1.0 ± 0.3	60.1
Glucuronide	0.5	6.2	0.7	0.8	2.1	0.6	0.3 ± 0.1	132.0
Total	3.4	12.8	1.8	2.7	6.9	4.7	2.3 ± 0.5	78.3

rearing conditions (standing stock, total volume, make-up water, temperature, pH, conductivity, Nitrite-N, Nitrate-N) investigated showed a significant correlation with steroid concentrations (data not shown).

Table 4
Pearson's correlations between steroid concentrations in the effluent of rearing unit and orthophosphate-P, TA-N and stocking density ($N = 13$). Only the statistical significant correlations are shown.

Coefficients of Pearson correlation and significance	
Orthophosphate-P × free testosterone	0.853 ($p = 0.001$)
Stocking density × free testosterone	0.835 ($p = 0.001$)
Stocking density × 11-ketotestosterone sulphate	0.791 ($p = 0.002$)
Stocking density × total 11-ketotestosterone	0.801 ($p = 0.002$)
TA-N × free testosterone	0.890 ($p < 0.001$)
TA-N × testosterone glucuronide	0.810 ($p = 0.001$)
TA-N × total testosterone	0.875 ($p < 0.001$)
TA-N × free 11-ketotestosterone	0.891 ($p < 0.001$)
TA-N × 11-ketotestosterone Sulphate	0.882 ($p < 0.001$)
TA-N × total 11-ketotestosterone	0.899 ($p < 0.001$)

4. Discussion

Cortisol is the principal glucocorticoid in teleost fish and its plasma concentrations notably rise when fish are exposed to stressors (Mommsen et al., 1999) such as handling (Ellis et al., 2004, 2007) or high stocking densities (Fanouraki et al., 2008). Total cortisol concentration in the water increased 15.7% with water flowing through the rearing units (Table 2), suggesting that fish release large amounts of cortisol into the water. Commercial fish diets were suggested to be an important source of steroids: cortisol has been reported to range between 35 and 67 ng/g feed (Feist and Schreck, 1990) and sex steroids between 0.4 and 11 ng/g feed (Pelissero et al., 1989; Sower and Iwamoto, 1985). However, estimations using the average feed load (152 kg/d) and standing stock (22,464 kg) in this research (Table 1) and cortisol release rates (0.5–5 ng/g/h) reported by Fanouraki et al. (2008) suggests that fish diets only account for 0.5% of cortisol input in RAS (diets 0.008 g/d vs. fish 1.483 g/d). Thus fish account for >99% of steroids in RAS.

Notably cortisol sulphate increased between influent and effluent (53.8%) compared to cortisol and cortisol glucuronide (Table 2).

This result was not expected as in rainbow trout free steroids account for 40% of the total excretion (Vermeirssen and Scott, 1996). Steroid clearance in fish seems to depend on steroid lipophilicity; free steroids (lipid-soluble) diffuse into the water across the gills whereas sulphate and glucuronide steroids (lipid-insoluble steroids) are excreted via kidney (Maren et al., 1968). In rainbow trout clearance of 17,20β-*P* and its conjugates follows three preferred pathways: (1) the free form via the gills, (2) the sulphate form via the urine, and (3) the glucuronide form via the bile (Ellis et al., 2005; Vermeirssen and Scott, 1996). The unexpected high cortisol sulphate increase may be related to species specificities and/or to sulphate conjugation in the rearing units. The dynamics of steroid conjugation and release has been little studied.

Sex steroids occurred at almost 10-fold lower concentrations compared to cortisol (Fig. 3). Yet, even at these low concentrations sex steroids can be detected by the olfactory system and in some species sex steroids can act as pheromones (Sorensen et al., 1990). For instance, male goldfish can detect free 17,20β-*P* and 17,20β-*P* sulphate at concentrations as low as 0.3 ng/L (10^{-12} M) and 3.0 ng/L (10^{-11} M) (Sorensen et al., 1995). Testosterone is also able to elicit odorant responses in fish at concentrations as low as 0.003 ng/L (Moore and Scott, 1991). The 17,20β-*P* and testosterone concentrations measured in RAS were in the range of 0.4–6.2 ng/L and 0.5–8.0 ng/L, respectively (Tables 3a and 3b). This shows that RAS rearing water contains sex steroid concentrations, which can affect fish. However, there are large differences in olfactory sensitivity to steroids between species and some may be insensitive to steroid odours (Stacey, 2010). Of the species studied, only African catfish is known to produce steroid glucuronides from the seminal vesicles which function as pheromones and that females are particularly sensitive to 3α,17α-dihydroxy-5β-pregnan-20-one-3α-glucuronide (Lambert and Resink, 1991). To our best knowledge for the other species no information is available in literature.

RAS seems to be very efficient in removing steroids. Using Table 1 mean values (standing stock: 22,464 kg; make-up water: 575 L/kg feed; feed load: 152 kg/d) and a (low) cortisol release rate of 0.5 ng/g/h Fanouraki et al. (2008) we would expect to find cortisol concentrations of approximately 3084 ng/L. However, the steroid concentrations measured here were orders of magnitude lower (mean concentration: 10 ng/L; Tables 3a and 3b), indicating a treatment efficiency of >99%. Treatment efficiencies of this order have been reported before for wastewater treatment plants (Chang et al., 2007) and variation on steroid concentrations were found to be dependent on numerous factors such as treatment type (e.g. trickling filter), optimization (e.g. hydraulic time) and competing compounds for sorption sites (Gomes et al., 2009). In wastewater treatment plants adsorption of steroids in sludge plays an important role. However, adsorption is not equal for all steroids – glucocorticoids have lower tendency to adsorb onto sludge than androgens and progestins (Liu et al., 2012). This lower adsorption of glucocorticoids, i.e. cortisol to sludge, together with its high production in the rearing units may explain the high levels of cortisol in RAS. Another possible reason for the low concentrations of sex steroids might be that all water samples were collected from grow-out systems containing sexually immature fish, except for Nile tilapia which were sexually mature.

Cortisol displayed a remarkably high variation among the seven RAS (CV: 163%) (Table 3b). Differences in systems configuration and species responsiveness to high stocking densities could be explanatory factors. However, other factors such as ozonation and UV could also be involved as these systems had the lowest cortisol values (RAS 3, 5, 7). The exception was RAS 1 containing Dover sole which showed high cortisol in RIA. However, thin-layer chromatography analysis revealed that most of this cortisol immunoreaction was cortisone (data not shown). That, together with the measurement of cortisol in sole blood plasma and bile (not shown), indicates that cortisol was likely converted to cortisone in RAS.

Sex steroid concentrations were positively correlated with TA-N. TA-N (NH_3-N + NH_4-N) is a fish metabolite converted by bacteria into Nitrite-N and then in Nitrate-N in RAS bio-reactors (Eding et al., 2006). Similarly, steroids may be degraded by bacteria present in RAS as is observed in aerobic (Horinouchi et al., 2004) and anaerobic (Fahrbach et al., 2010) waste water treatments. Fish culture conditions may not be optimal for bacteria as shown by TA-N accumulation in RAS 2 and 5. Therefore this correlation suggests that a culture factor is affecting bacteria quantity or activity in RAS. Based on Table 1 and current knowledge about the nitrification process, pH (low) is likely affecting bacteria on steroids removal. Sex steroid concentrations were also positively correlated with orthophosphate-P and stocking density. Orthophosphate-P water concentrations are a measure of the degree of new make-up water in RAS (Martins et al., 2009). Therefore, these results suggest that an intensification of fish production through a decrease of make-up water supply per unit of fish produced and increase of stocking density will lead to a build-up of steroids in the water. Although intensification is critical for the success of RAS, this ultimately could affect fish performance. However, whether this is the case and in what way needs to be further investigated.

In conclusion, the present study shows that the measured steroids (a glucocorticoid, two androgens and a progestin) in their free and conjugated forms are present in significant concentrations in the rearing water of commercial RAS. Cortisol displayed the highest concentrations and largest variability among RAS and over time. Furthermore, steroid concentrations in RAS are higher than those reported for flow-through systems (Kolodziej et al., 2004), which suggests that steroids tend to accumulate in closed systems at levels susceptible to be detected by fish, at least in some species. TA-N and stocking density were the rearing condition most relevant to explain the variation of steroid concentrations in the water of RAS. Whether, the observed levels impair fish welfare and growth performance in RAS, remains to be investigated.

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