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Cortisol and testosterone accumulation in a low pH recirculating aquaculture system for rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Steroids accumulate in recirculating aquaculture system (RAS), although explanatory factors for such accumulation are still poorly explored. This study investigated the effect of water exchange rate and pH in six replicated RAS on the concentration of the stress hormone cortisol in rainbow trout blood plasma and in the holding water and of the sex steroids testosterone, 11-ketotestosterone (11-KT) and 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) over a 70-day experimental period. Three combinations of water exchange rate and pH were used each treatment, with two replications: (i) high water exchange rate (±1700 L kg^{-1} feed) and neutral pH (±7.3), (ii) low water exchange rate (±500 L kg^{-1} feed) and neutral pH (±7.3) and (iii) low water exchange rate (±500 L kg^{-1} feed) and low pH (±5.8). Plasma cortisol concentrations at day 70 were higher (24.4 ± 9.5 ng mL^{-1}) for fish kept at low pH when compared to fish kept at neutral pH (12.0 ± 0.1 and 8.7 ± 0.2 ng mL^{-1}). Water cortisol and testosterone concentrations at day 35 were higher at low pH than at neutral pH, whereas water 11-KT and 17,20β-P did not differ among treatments. At day 70, there were no significant differences between low and high pH. These results demonstrate that low pH contributes to increased plasma cortisol concentrations and to its accumulation in water, possibly indicating a stress response to low pH. The higher concentration of testosterone but not of the other sex hormones point to unspecified reproductive effects that need further investigation.

Keywords: recirculating aquaculture, steroids, hormones, water, fish

Introduction

Steroids are key mediators of the stress response and reproductive function in fish (Kime 1993; Mommsen, Vijayan & Moon 1999; Oliveira, Silva & Canário 2009). Due to their hydrophobic nature, steroids and their metabolites diffuse through cell membranes and can be released into the fish rearing environment mainly through the gills, urine and faeces (Ellis, James & Scott 2005; Scott, Hirschenhauser, Bender, Oliveira, Earley, Sebire, Ellis, Pavlidis, Hubbard & Huertas 2008). Cortisol is the key hormone produced by the interrenal cells of the head kidney and released into the fish's bloodstream in response to stressful situations (Ellis, Yildiz, López-Olmeda, Spedicato, Tort, Øverli & Martins 2012). Release of cortisol to the water has been measured in several fish species, such as rainbow trout (*Oncorhynchus mykiss*) (Ellis, James, Stewart & Scott 2004), European sea bass, (*Dicentrarchus labrax*) (Fanouraki, Papandroulakis, Ellis, Mylonas, Scott & Pavlidis 2008) and three-spined stickleback (*Gasterosteus aculeatus*) (Sebire, Katsiadaki & Scott 2007). Sex steroids such as testosterone, 11-ketotestosterone (11-KT) and 17,20-β dihydroxypregn-4-en-3-one (17,20β-P) are produced in fish gonads in response to pituitary

gonadotropins and in response to exogenous stimuli (photoperiod, pheromones and reproductive behaviour) (Kime 1993; Scott, Sumpter & Stacey 2010; Keller-Costa, Canário & Hubbard 2015). Their release into the water has also been shown in several species including goldfish (*Carassius auratus*), Nile tilapia (*Oreochromis niloticus*) and rainbow trout (Vermeirssen & Scott 1996; Scott *et al.* 2010; Hubbard, Mota, Keller-Costa, da Silva & Canário 2014; Huertas, Almeida, Canario & Hubbard 2014). Furthermore, steroids and xenobiotics in holding water can be taken into the fish and modify hormone plasma levels (Scott, Pinillos & Huertas 2005; Miguel-Queralt & Hammond 2008). Despite the potential effects of steroid accumulation on fish performance and welfare in aquaculture (Mota, Limbu, Martins, Eding & Verreth 2015), the presence and dynamics of steroids in the rearing water of aquaculture systems has been little studied.

Among the existing aquaculture production systems, steroid concentrations may be higher in systems where water is reused like recirculating aquaculture system (RAS). For instance, steroid concentrations are higher in RAS as compared to flow-through systems (Kolodziej, Harter & Sedlak 2004). Moreover, in the rearing water of RAS, steroids may accumulate to concentrations within the fish olfactory sensitivity range which could influence their behaviour or endocrine status (Mota, Martins, Eding, Canário & Verreth 2014). Ultimately, this could affect early sexual maturation (Budworth & Senger 1993; Scott *et al.* 2005) and could disrupt gamete maturation or spawning interactions (Stacey 2003; Stacey & Sorensen 2006).

Steroid accumulation in RAS varies with steroid hormone, even at low water exchange rates (Good, Davidson, Earley, Lee & Summerfelt 2014). Mota *et al.* (2014) who sampled seven commercial RAS for steroids did not observe correlations between steroid concentrations in the effluent of rearing units and water exchange rate. Therefore, it is not clear whether there is a direct relation between accumulation and water exchange rates. Water pH effect on steroids accumulation is another factor worth to investigate in RAS as production and release of steroids may increase at low water pH as indicated by cortisol plasma concentrations of rainbow trout exposed to acidified water (Iger, Balm & Bonga 1994). Moreover, steroids removal may be reduced at low pH as it inhibits or reduces bacterial activity responsible for degradation of several metabolites including steroids (Eding, Kamstra, Verreth, Huisman & Klapwijk 2006; Eshchar, Lahav, Mozes, Peduel & Ron 2006). Despite the increasing usage of technologies to control pH, alkalinity and CO_2 in RAS (Summerfelt, Zühlke, Kolarevic, Reiten, Selset, Gutierrez & Terjesen 2015), a large variation in water pH is observed in commercial farms, with some fish species being cultured at pH 6.5 or even bellow pH 6 (Dalsgaard, Lund, Thorarinsdottir, Drengstig, Arvonen & Pedersen 2013; Mota *et al.* 2014).

This study aimed at investigating the effect of water exchange rate and pH on circulating cortisol in rainbow trout and the accumulation of cortisol, testosterone, 11-KT and 17,20β-P in the rearing water of RAS over a 70-day experimental period. Rainbow trout was used because its performance and welfare are sensitive to adverse water quality. In addition, farming rainbow trout in RAS is becoming well established (Davidson, Good, Welsh & Summerfelt 2014; Colson, Sadoul, Valotaire, Prunet, Gaumé & Labbé 2015) and to the authors best knowledge, measurement of water steroids in a rainbow trout RAS has never been investigated.

Materials and methods

All procedures involving animals were carried out in accordance with the Dutch law and were approved by the Animal Experiments Committee of Wageningen University, the Netherlands, with the reference number 2010004b.

Experimental design

We tested the effect of water exchange rate and pH on blood plasma and water steroid concentrations in six independent RAS stocked with rainbow trout. During a 70-day experimental period, three combinations of water exchange rate and pH were tested each treatment replicated twice. Two replicates resulted from the compromise between three treatments and the experimental units available, and despite the lower power to detect true effects, it is a statistical valid approach as shown elsewhere (Meriac, Eding, Kamstra, Busscher, Schrama & Verreth 2014). The three treatments were as follows: (i) high water exchange rate ($\pm$1700 L kg^{-1} feed; hydraulic retention time (HRT) 4.5 days) and neutral pH ($\pm$7.3) (H_WN_{pH}),

(ii) low water exchange rate (±500 L kg^{-1} feed; HRT 15.5 days) and neutral pH (±7.3) (L_WN_{pH}) and (iii) low water exchange rate (±500 L kg^{-1} feed; HRT 15.5 days) and low pH (±5.8) (L_WL_{pH}). HRT was calculated as follows:

HRT (days) = total system volume (L)/water exchange average (L day^{-1}).

Recirculating aquaculture systems

Each RAS (Fig. 1) was composed of a fish tank (V = 300 L; hydraulic retention time (HRT) = 15 min), a settling tank (swirl separator, V = 75 L; hydraulic surface load 150 m^3 m^{-2} per day), a UV unit (UV-C 36 W, Phillips, Eindhoven, the Netherlands), a sump (V = 75 L), a cooler–heater (TC20, Teco, Ravenna, Italy) and two trickling filters (i) A = 11.7 m^2, bionet medium, specific surface area = 200 m^2 m^{-3}, Norddeutsche Seekabelwerke, Nordenham, Germany, and, (ii) A = 14.2 m^2, cross-flow medium, specific surface area = 240 m^2 m^{-3}, Fleuren & Nooijen, Nederweert, the Netherlands. Faeces were removed daily from a cooled bottle (4°C) located at the settling tank bottom. The bionet-medium trickling filter was inoculated with the biofilm from the top section of another RAS running at 28°C for over 3 years and was adapted to 16°C by gradually lowering the temperature over a one-week period. The cross-flow medium trickling filter was new and installed 10 days prior to the start of the experimental period. Total water volume of each RAS was ~510 L, and water flow across trickling filters 1 and 2 was approximately 20 and 10 L min^{-1} respectively. The L_WL_{pH} treatment RAS was equipped with a pH control system consisting of an acid storage container (V = 20 L; HCl ± 0.1 M) and a pH controller pump (Endress-Hauser Liquisys M, Endress+Hauser, Burlington, Canada). The treatments H_WN_{pH} and L_WN_{pH} had sodium bicarbonate added when necessary to keep the pH around 7.

Water was exchanged twice daily before feeding at a rate of approximately 1700 L kg^{-1} feed per day (H_WN_{pH}) and 500 L kg^{-1} feed per day (L_WN_{pH} and L_WL_{pH}). The exchange volume was based on the feed load of the previous feeding day.

Fish and feeding

Rainbow trout (N = 250; ±90 g) were obtained from a commercial fish farm (Trout Farm

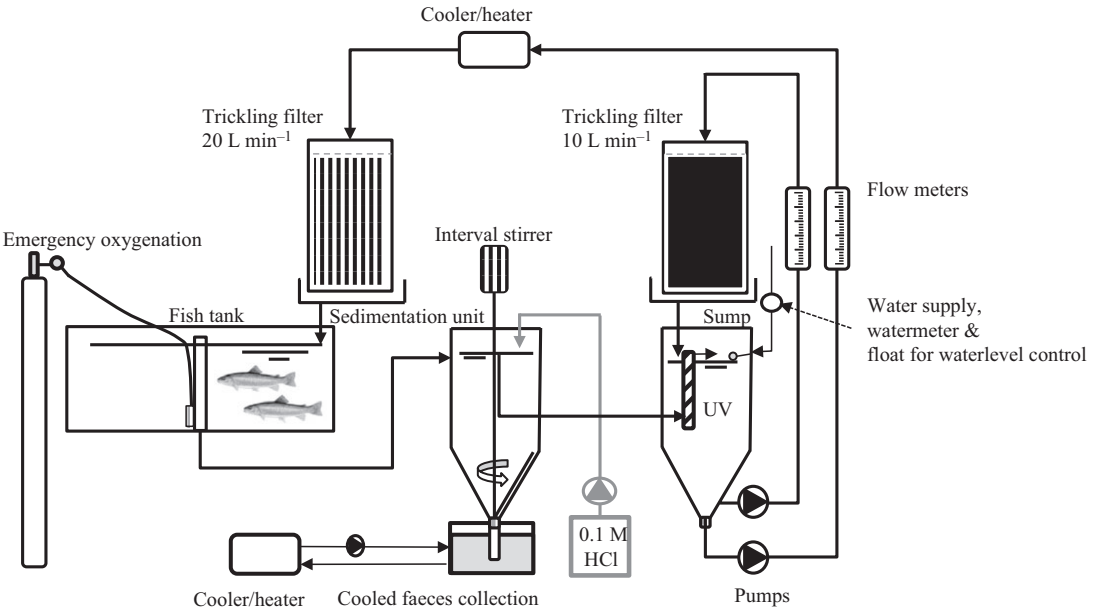

Figure 1 RAS design: fish tank (V = 300 L; hydraulic retention time (HRT) = 15 min), a settling tank (swirl separator, V = 75 L; hydraulic surface load 150 m^3 m^{-2} per day), a UV unit (UV-C 36 W, Phillips, Eindhoven, the Netherlands), a sump (V = 75 L), a cooler–heater (TC20, Teco, Ravenna, Italy) and two trickling filters (1) A = 11.7 m^2, bio-net medium, specific surface area = 200 m^2 m^{-3}, and, (2) A = 14.2 m^2, cross-flow medium, specific surface area = 240 m^2 m^{-3}. *only for L_WL_{pH} (in grey): pH controller pump (Endress-Hauser Liquisys M, Endress+Hauser, Burlington, Canada) – acid storage container (V = 20 L, HCl ± 0.1 M).

Keijzersberg, Blitterswijck, the Netherlands) with an approximate age of 10 months and a sex ratio of three males to seven females. Sex ratio was based on the average ratio in the supplying farm over the past 6 years and was assumed the same in this experiment. After transport to the experimental facilities (De Haar Vissen, Wageningen University), the fish were randomly distributed over the six RAS (41–42 fish per RAS) and allowed to adapt to the rearing and feeding conditions over a period of 4 weeks. During the adaptation period, water quality was maintained within the recommended range for rainbow trout: temperature ±16°C, pH 7–7.5, dissolved oxygen >8 mg L^{-1}, unionized ammonia-N < 0.02 mg L^{-1}, nitrite-N < 0.2 mg L^{-1} and nitrate-N < 100 mg L^{-1}. Photoperiod was maintained at 12L:12D.

During the experimental period, fish were fed an average 1.6% of body weight per day. Twice a day (09:00 and 16:00), fish were fed by hand equal amounts of an experimental (extruded) trout diet (2-mm floating pellets; Research Diet Services, Wijk bij Duurstede, the Netherlands). Analysed feed composition of the experimental diet was as follows: dry matter 971 (g kg^{-1}), crude protein 497 (g kg^{-1} feed dry matter (DM), crude fat 206 (g kg^{-1} DM) and, crude ash 69 (g kg^{-1} DM) and nitrogen-free extract (NFE) 228 g kg^{-1} DM.

At the start of the experiment (day 0), 180 fish (30 fish per RAS) were anaesthetized (0.05 g L^{-1} of MS-222 buffered with 0.1 g L^{-1} of sodium bicarbonate) and individually weighed. The remaining 70 fish were discarded. At day 35, fish were anaesthetized and individually weighed, and half of the initial fish ($N = 90$; 15 fish per RAS) were returned to their RAS. The other half was removed to avoid exceeding system's carrying capacity. At the end of experiment (day 70), fish were euthanized with 0.1 g L^{-1} of MS-222 buffered with 0.2 g L^{-1} of sodium bicarbonate and individually weighed. Growth per metabolic body weight (G_m, g $kg^{-0.8}$ per day) was calculated as $(W_f - W_i)/t/MBW$, where W_f and W_i are final and initial individual bodyweight (in g), t was the duration of experimental period (in days) and MBW is the mean metabolic bodyweight (in $kg^{0.8}$). MBW was calculated as $(W_G/1000)^{0.8}$, where W_G is the geometric mean body weight (in g) calculated as $\sqrt{(W_i \times W_f)}$. Feed intake per metabolic body weight (FI_m, in g $kg^{-0.8}$ per day) was calculated as $FI_{total}/t/MBW$, where FI_{total} is the total feed intake per fish (wet matter) per tank during the experimental period. Feed conversion ratio (FCR) was calculated as FI_m/G_m.

Sampling and analysis

Water samples (10 mL) were taken weekly from the fish tank effluent for total ammonia nitrogen (TA-N), nitrite-N and nitrate-N analyses using a SAN auto-analyser (Skalar, Breda, the Netherlands) (Meriac, Eding, Schrama, Kamstra & Verreth 2013). Dissolved carbon dioxide (CO_2) was measured weekly using a CO_2 portable meter (Oxyguard CO_2 analyser, Oxyguard International A/S, Birkerød, Denmark). Alkalinity was measured weekly in duplicate using and automatic titration with 0.02 M HCl (TIM840 titration manager, Titralab, Radiometer Analytical, Villeurbanne Cedex, France). Temperature, dissolved oxygen and pH (WTW multi 340i; WTW GmbH, Weilheim, Germany) and conductivity (WTW cond 340i; WTW GmbH) were measured daily in the fish tank effluent using portable meters. Nitrile gloves were used during all water sampling and processing activities to prevent cross contamination with steroids.

Blood samples for cortisol measurement were collected at the end of the experiment (day 70), from caudal blood vessels of six fish per treatment replicate using a hypodermic syringe previously flushed with heparin (LEO Pharma BV, the Netherlands) and centrifuged for 10 min at 4000 rpm for plasma collection. Plasma cortisol was measured by enzyme-linked immunosorbent assay (ELISA) kit (Neogen Corporation, Lexington, KY, USA).

Water samples for steroid concentration measurement were collected from the effluent of the fish tank (500 mL) prior to fish feeding (08.30 h) at days 0, 35 and 70. Day 35 sampling was performed prior to fish anesthetization and weighing. Cortisol, testosterone, 11-KT and 17,20β-P were analysed through radioimmunoassay (RIA) as previously described by Mota *et al.* (2014). Water samples were first paper filtered (2 μm; VWR, France) followed by a membrane filter (0.45 μm; Millipore, Ireland). The water sample (±500 mL) was pumped (±12 mL min^{-1}) through an Oasis HLB Plus solid-phase extraction cartridge (Oasis®; Waters, Milford, CT, USA) previously activated with methanol (5 mL) and washed with distilled water (5 mL). Cartridges were eluted (100%

ethanol) and the eluate evaporated in a dry bath (45°C under a gentle flow of nitrogen). The dried residue was redissolved in distilled water and free steroids extracted with diethyl ether, which was evaporated under the same conditions previously described, and the residue was reconstituted in RIA buffer (sodium phosphate 0.05 M, pH 7.6, containing 1% gelatine) and stored (−20°C) until assay. The methodology for steroid RIA is described by Scott, Sheldrick and Flint (1982). Details about the essays are further described in Mota *et al.* (2014). The recommended pH range for the Oasis HLB Plus cartridge utilization is 0–14; additionally, it was confirmed at our laboratory (data not shown) that within the pH levels (5.6–7.4), no differences on steroid retention rate were registered.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA). One-way ANOVA was used to compare survival, feed intake and growth performance, water quality parameters and water steroid concentrations among the three treatments. To account for the variation of fish stock over time (see Fish and feeding), water steroid concentrations were standardized to fish biomass (ng L^{-1} kg^{-1} fish). The logarithmic transformation was applied to the cortisol and testosterone water concentrations to comply with ANOVA assumption of normal distribution. Homogeneity of variances was verified using Levene's test. *Post hoc* multiple comparisons were performed using the Bonferroni test (equal variances assumed) or Games–Howell (unequal variances). A significance level (α) of 0.05 was used. Data are presented as mean ± standard deviation (SD).

Results

Survival, feed intake and growth performance were not significantly affected by water exchange rate or water pH at both sampling periods (Table 1). Fish biomass, respectively, for H_WN_{pH}, L_WN_{pH} and L_WL_{pH} was 3.9, 3.8 and 3.7 kg at day 0; 7.1, 7.0 and 6.8 kg at day 35; and 4.9, 5.2 and 4.7 kg at day 70. The following water quality parameters ranges were found over the 70-day experimental period: temperature (14.9–16.9°C), conductivity (300.1–1428.3 μs cm^{-1}), dissolved oxygen (7.3–9.3 mg L^{-1}), total ammonia nitrogen (TA-N) (0.14–67.87 mg L^{-1}), nitrite-N (0.01–2.63 mg L^{-1}), nitrate-N (10.8–94.2 mg L^{-1}), CO_2 (1–4 mg L^{-1}) and alkalinity (2.4–51.9 mg L^{-1} as $CaCO_3$) (average values presented on Table 2).

The mean plasma cortisol concentration at day 70 was 24.4 ± 9.5 ng mL^{-1} (L_WL_{pH}) for fish kept at low pH and 12.0 ± 0.1 ng mL^{-1} (H_WN_{pH}) and

Table 1 Feed intake and growth parameters (mean ± SD; $N = 2$) of rainbow trout reared in two combinations of water exchange rate (low, L: 500 L kg^{-1} feed and high, H: 1700 L kg^{-1} feed) and pH (low, L: 5.8 and neutral, N: 7.3) in RAS

Parameter	H_WN_{pH}	L_WN_{pH}	L_WL_{pH}	*P*-value
Day 0–35				
Number of fish	30	30	30	
Initial bw (g $fish^{-1}$)	131.0 ± 4.2	126.7 ± 1.6	123.5 ± 2.1	0.160
Final bw (g $fish^{-1}$)	236.3 ± 1.3	233.0 ± 4.3	224.7 ± 1.8	0.052
Survival (%)	100	100	100	n.a.
Feed intake (g $kg^{-0.8}$ per day)	11.44 ± 0.12	11.66 ± 0.15	11.95 ± 0.12	0.063
Growth (g $kg^{-0.8}$ per day)	12.09 ± 0.76	12.43 ± 0.16	12.13 ± 0.15	0.737
FCR	0.95 ± 0.05	0.94 ± 0.02	0.98 ± 0.00	0.414
Day 36–70				
Number of fish	15	15	15	
Initial bw (g $fish^{-1}$)	229.3 ± 1.2	233.3 ± 4.3	217.3 ± 21.7	0.509
Final bw (g $fish^{-1}$)	324.7 ± 5.8	345.9 ± 27.3	314.0 ± 9.5	0.295
Survival (%)	96.7 ± 4.7	90.0 ± 14.1	93.3 ± 9.4	0.931
Feed intake (g $kg^{-0.8}$ per day)	8.93 ± 0.04	8.64 ± 0.32	9.25 ± 0.28	0.191
Growth (g $kg^{-0.8}$ per day)	7.71 ± 0.53	8.78 ± 1.46	8.13 ± 2.84	0.855
FCR	1.16 ± 0.08	1.00 ± 0.20	1.20 ± 0.39	0.732

bw, body weight; FCR, feed conversion rate.

Table 2 Water quality parameters (mean ± SD, except for pH min–max; $N = 2$) of rainbow trout reared in two combinations of water exchange rate (low, L: 500 L kg^{-1} feed and high, H: 1700 L kg^{-1} feed) and pH (low, L: 5.8 and neutral, N: 7.3) in RAS

Parameter	H_WN_{pH}	L_WN_{pH}	L_WL_{pH}	*P*-value
Daily measurements				
Temperature (°C)	16.0 ± 0.1	15.8 ± 0.1	15.9 ± 0.1	0.095
Conductivity (μS cm^{-1})	394.1 ± 0.4[a]	971.3 ± 15.9[b]	1079.7 ± 10.7[c]	<0.001
Dissolved oxygen (mg L^{-1})	8.1 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	0.673
Weekly measurements				
TA-N (mg L^{-1})	0.57 ± 0.02[a]	0.64 ± 0.19[a]	55.01 ± 2.21[b]	<0.001
Nitrite-N (mg L^{-1})	0.42 ± 0.03[a]	0.72 ± 0.14[ab]	0.04 ± 0.00[b]	0.005
Nitrate-N (mg L^{-1})	23.5 ± 0.3[a]	72.9 ± 1.6[b]	21.0 ± 0.7[a]	<0.001
Dissolved CO_2 (mg L^{-1})	3 ± 0	2 ± 0	3 ± 1	0.192
Alkalinity (mg L^{-1} as $CaCO_3$)	29.0 ± 0.2[a]	31.9 ± 0.7[a]	3.9 ± 0.1[b]	<0.001
System management				
Water exchange (L kg^{-1} feed)	1763.2 ± 12.4[a]	482.7 ± 6.5[b]	480.8 ± 12.3[b]	<0.001
pH	7.3–7.4[a]	7.2–7.4[a]	5.6–6.0[b]	<0.001

Superscript alphabets indicates significant differences, $P < 0.05$.

8.7 ± 0.2 ng mL^{-1} (L_WN_{pH}) for individuals kept at neutral pH.

Water cortisol did not differ between the three treatments at day 0 ($P = 0.544$) and at day 70 ($P = 0.120$). It was elevated for L_WL_{pH} compared to H_WN_{pH} and L_WN_{pH} at day 35 ($P = 0.013$) (Fig. 2a). Water testosterone did not differ between the three treatments at day 0 and at day 70

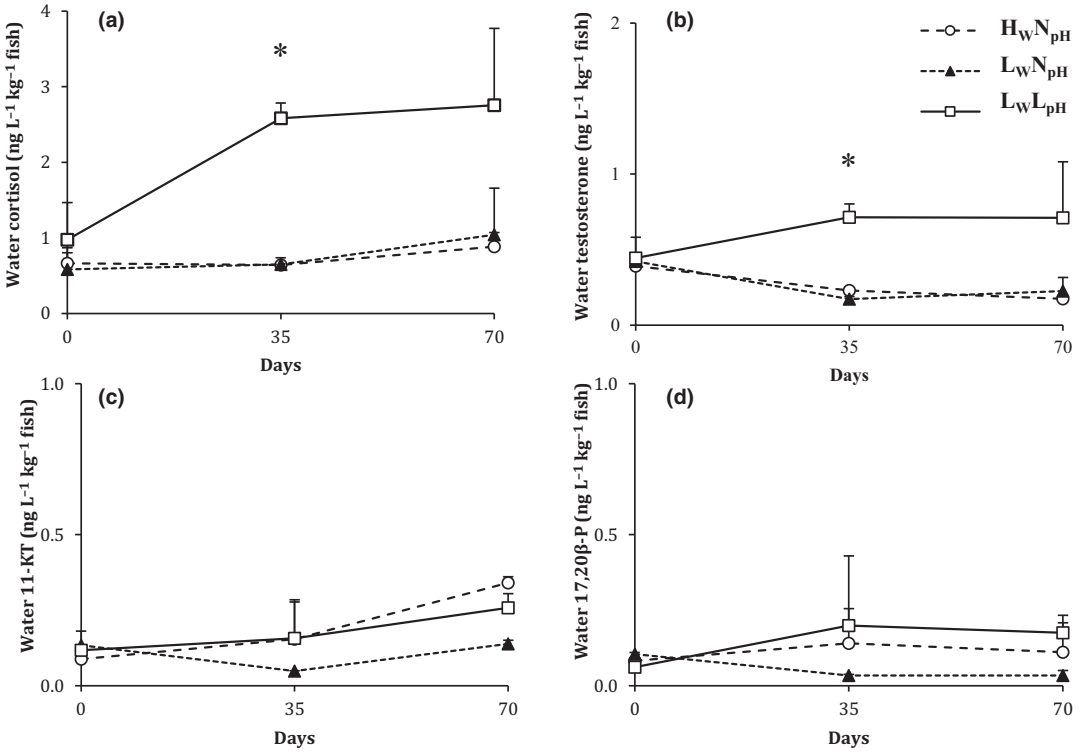

Figure 2 Water cortisol (a), testosterone (b), 11-ketotestosterone (c) and 17, 20β-P (d) levels (ng L^{-1} kg^{-1} fish) in rainbow trout RAS during the 70-day experimental period ($N = 2$). Values are given as mean ± SD. Significant differences are indicated by *($P < 0.05$). Average fish biomass per replicate was 3.6 kg (day 0), 7.0 kg (day 35) and 4.9 kg (day 70).

($P = 0.158$) ($P = 0.802$), whereas it was higher for L_WL_{pH} compared to H_WN_{pH} and L_WN_{pH} at day 35 ($P = 0.004$) (Fig. 2b). Water 11-KT and 17,20β-P were similar for all treatments throughout the experimental period and their concentration ranged between 0.1–0.3 ng L^{-1} kg^{-1} fish and 0.0–0.2 ng L^{-1} kg^{-1} fish respectively (Fig. 2c and d).

Discussion

The present work demonstrates that pH but not water exchange influence steroid concentrations in RAS, as RAS operating at low pH and low water exchange rates (L_WL_{pH}) accumulates higher levels of cortisol and testosterone (Fig. 2). This effect of low water pH was related to a higher plasmatic cortisol concentration for rainbow trout kept at low pH.

Water quality was generally within the recommended levels for rainbow trout, with the exception of the initial elevated nitrite-N concentrations, up to 2.63 mg L^{-1}, (Colt 2006; Timmons & Ebeling 2007) in the systems, which operated at neutral pH (H_WN_{pH} and L_WN_{pH}). The initial accumulation of the intermediate product of nitrification, nitrite, was previously described in RAS with trickling filters (Eding *et al.* 2006), which is related to environmental disturbances or to an immature biofilm. The reduction of nitrite-N levels to 0.1–0.3 mg L^{-1} from week 5 onwards shows a higher nitrite removal capacity by the system, suggesting that the cause of elevated initial nitrite levels was indeed related to a too immature biofilter at the start of the experiment. Nitrite toxicity during the first weeks was counteracted with the addition of NaCl (Colt 2006) to all replicates up to 1200 μS cm^{-1} water conductivity, with no negative consequences for fish survival or performance (Table 1). Elevated concentrations of TA-N (sum of NH_3-N and NH_4-N concentrations) were observed in L_WL_{pH} (Table 2); however, at pH of 5.6–6.0, the toxic ammonia fraction (NH_3-N) only accounted for 0.007–0.016 mg L^{-1} of the ±55 mg TA-N/L observed. Thus, the upper limit of ammonia concentrations measured in the present study is slightly above compared to the recommended concentrations (<0.0125 mg L^{-1}) for salmonids (Timmons & Ebeling 2007). Nevertheless, the acceptable quality of the water was supported by the fact that fish performance was similar for all three treatments and comparable with previously published results (Pedersen, Suhr, Dalsgaard, Pedersen & Arvin 2012; Meriac *et al.* 2013).

The presence of steroids in the rearing water of aquaculture systems was previously described for flow-through systems (Kolodziej *et al.* 2004; Ellis, James, Sundh, Fridell, Sundell & Scott 2007) and RAS (Budworth & Senger 1993; Good *et al.* 2014; Mota *et al.* 2014) with concentrations ranging between 0.1 ng L^{-1} and 217 ng L^{-1}. In the present study, all four steroids measured ranged between 2.2–17.6 ng L^{-1} (cortisol), 0.9–4.9 ng L^{-1} (testosterone), 0.3–1.7 ng L^{-1} (11-KT) and 0.2–1.4 ng L^{-1} (17,20β-P). The low concentration of 11-KT and 17,20-β-P is likely associated with the sexual immaturity of the fish used in the present study, ±16 months of age and 310–346 g of body weight at the end of experiment. Sexual development of rainbow trout usually starts after 2 years of age or, exceptionally, in fish less than a year old but with a body weight above ±320 g (Crandell & Gall 1993). It should be noted, nevertheless, that the levels of steroid concentrations observed for 17,20β-P were still within the olfactory detection range reported for goldfish, carp (*Cyprinus carpio*) and Atlantic salmon (*Salmo salar*) (Scott *et al.* 2010).

The higher concentrations of cortisol and testosterone in the low pH (L_WL_{pH}) treatment at day 35 indicate a possible effect of low pH, on accumulation of these two steroids in water. The accumulation of testosterone (Good *et al.* 2014; Mota *et al.* 2014) was found analogous and positively correlated to TA-N concentration in RAS. This is in agreement with the present study where the highest cortisol and testosterone concentrations (L_WL_{pH}, Table 2) were observed in the treatment with the highest TA-N concentration (±55 mg L^{-1}). TA-N accumulation in RAS may result from lower removal rates of nitrifying bacteria under suboptimal conditions such as low water pH (Eding *et al.* 2006). The relation between bacteria activity in low water pH RAS and the accumulation of steroids should be further investigated.

Rainbow trout subjected to acute stress respond by increased plasma cortisol concentration (Ellis *et al.* 2004). Likewise, rainbow trout subjected to chronic and continuous stress maintain plasma cortisol concentrations elevated for weeks (Pickering 1992). In the present study, plasma cortisol was found to be higher for fish kept at low pH (L_WL_{pH}: 24.4 ± 9.5 ng mL^{-1}) as compared to those kept at neutral pH at the end of the experimental period. This higher plasma cortisol concentration, also associated with higher release rates (Ellis *et al.* 2004), may explain at least partly the

considerable difference (>3x) in the water concentration of cortisol between treatments.

Interestingly, high levels of testosterone accumulate in the water at low pH. The role of testosterone in fish other than acting as substrate for 11-KT and estradiol-17β has been controversial (Magri, Solari, Billard & Reinaud 1985). One of its functions is to provide positive feedback for gonadotropin synthesis during early gametogenesis accelerating gonadal development (Dubois, Florijn, Zandbergen, Peute & Henk 1998). Early sexual maturation of fish results in growth and feed intake reduction and an overall economic loss which is a risk for further development of RAS. Genetic variation (Wolters 2010), feeding and growth (Rowe & Thorpe 1990), light exposure (Imsland, Hanssen, Foss, Vikingstad, Roth, Bjørnevik, Powell, Solberg & Norberg 2013), photoperiod (Good, Weber, May, Davidson & Summerfelt 2015), water temperature and hormonal treatment (Vikingstad, Andersson, Norberg, Mayer, Klenke, Zohar, Stefansson & Taranger 2008) among other environmental parameters are known to influence sexual maturation in fish. Steroids occur and accumulate in RAS water (Good *et al.* 2014; Mota *et al.* 2014), and as demonstrated in the present study, pH but not water exchange influences steroid concentrations in RAS. Whether low pH was promoting early puberty in rainbow trout would need to be examined in a longer study.

An added explanation for the higher cortisol and testosterone concentration at low pH RAS may be related to the physical–chemical properties and possible propensity to be absorbed by sediment or to stay in the aqueous phase that was modified by the water pH (Leszczynski & Schafer 1990).

In conclusion, rainbow trout grown in RAS operating at a low pH (5.6–6.0) as compared to RAS operating at neutral pH (7.2–7.4) display increased blood plasma cortisol concentration suggesting stressful culture conditions. These elevated plasma concentrations are also reflected in water cortisol measurements. Furthermore, high testosterone water concentration is also related to low pH, suggesting alterations in the reproductive endocrinology of the fish. These observations highlight the importance of monitoring steroid hormones in the rearing water of RAS.

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