Steroids accumulation in recirculating aquaculture systems

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Thesis

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Prof. Dr A.P.J. Mol,

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to be defended in public

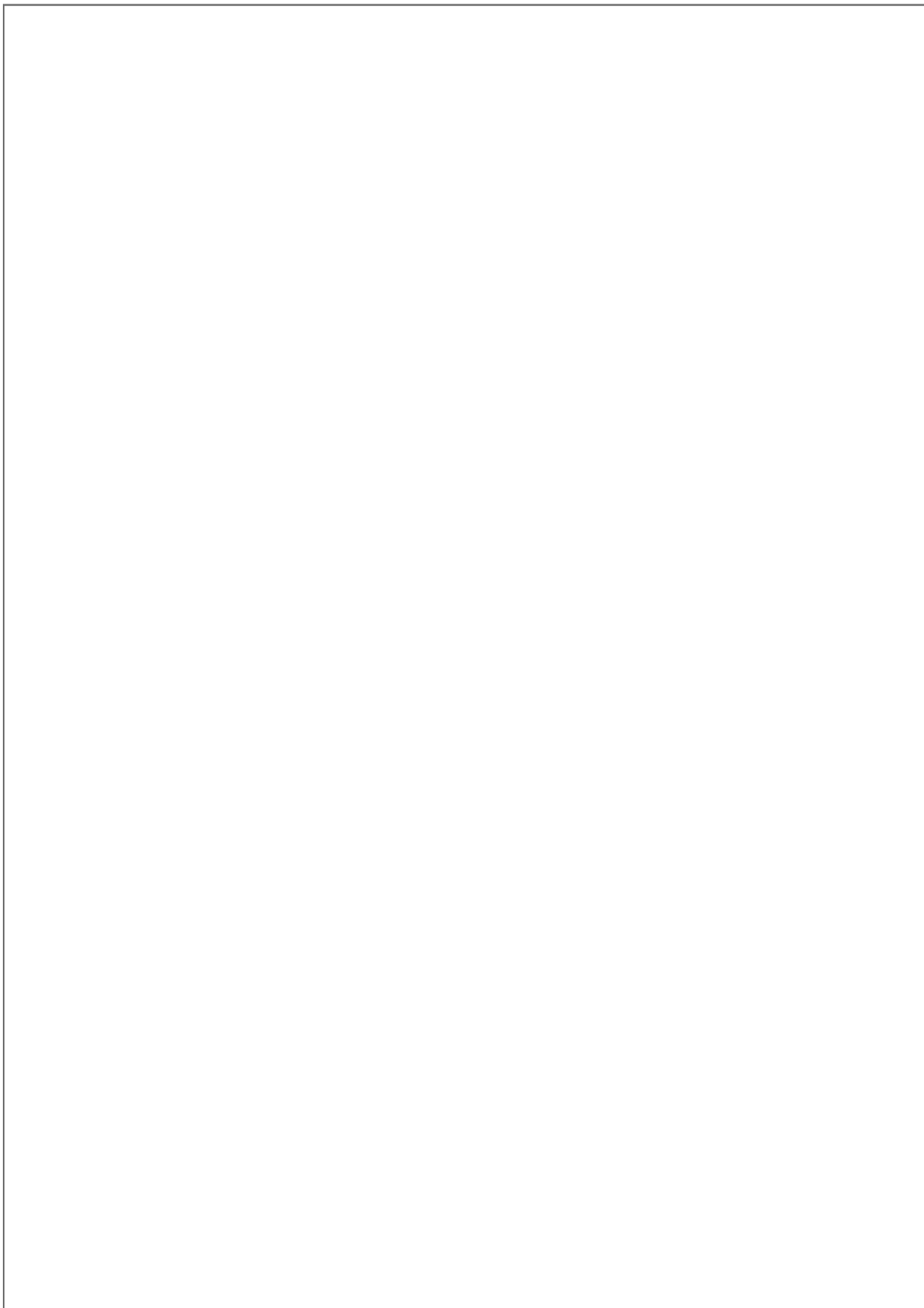on Monday 23 November 2015

at 4 p.m. in the Aula.

To my family and Sofia

Abstract

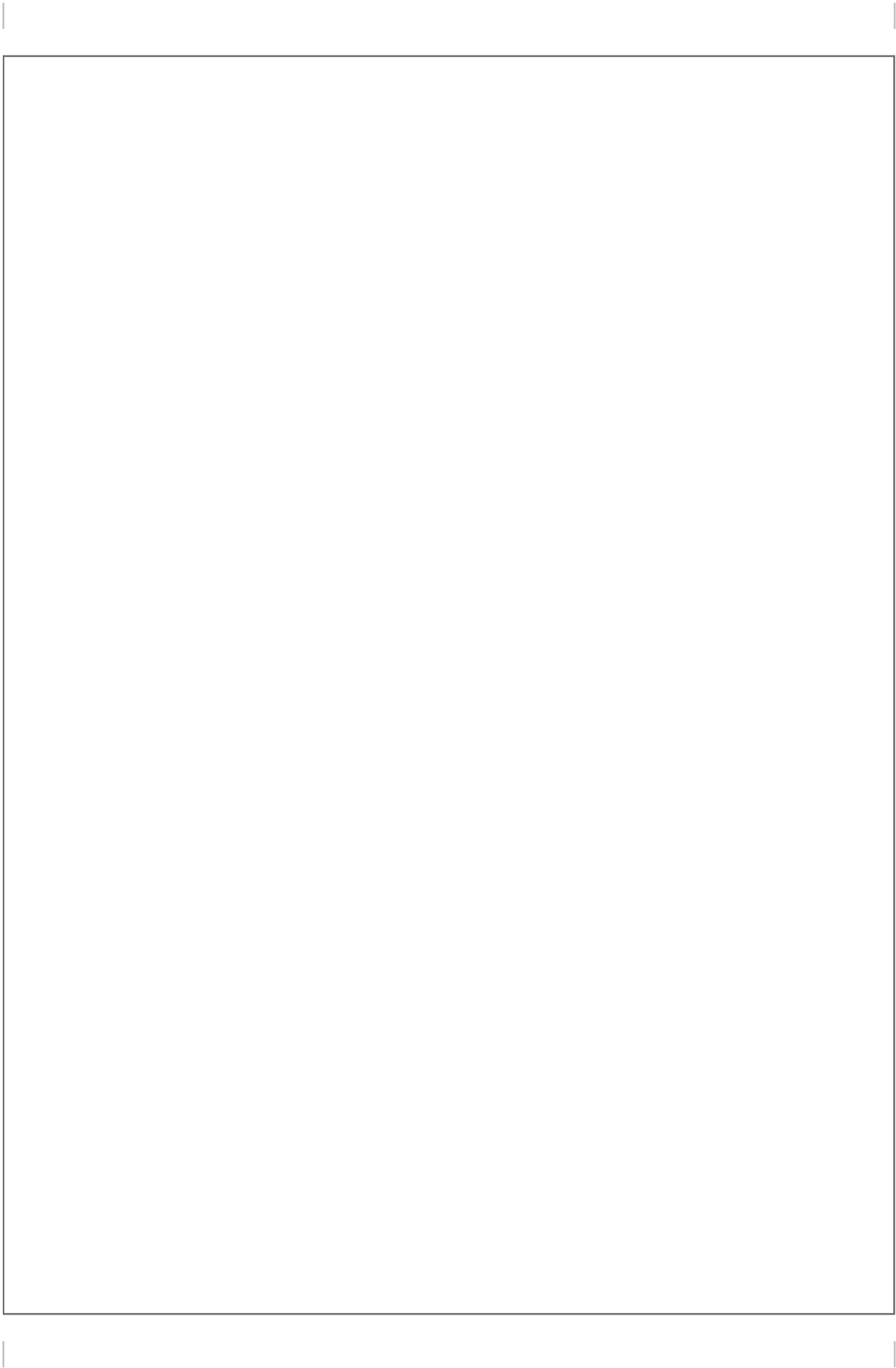**Mota, V.C.** (2015): Steroids accumulation in recirculating aquaculture systems.

PhD thesis. Wageningen University, The Netherlands.

Recirculating aquaculture systems (RAS) are intensive land-based systems in which water is re-used after treatment and fish are grown under controlled conditions. It is well known that several substances accumulate in RAS water that may negatively affect fish performance. Steroidal hormones and their metabolites, responsible for vital processes in fish, such as sexual maturation and stress response, are released into the rearing water. The general aim of this thesis was to determine the occurrence of steroids in the rearing water of RAS and how different culture conditions may contribute to their accumulation. The first study showed reduced feed intake and growth by Nile tilapia cultured in a RAS designed for high accumulation of substances from feed, fish and bacteria. This reduced performance was likely associated with the production and release of fish-related substances such as steroids. Moreover, the second study showed that steroids occurrence in RAS is widespread as indicated by the presence of cortisol, testosterone, 11-ketotestosterone and 17,20β-P in their free and conjugated forms in seven commercially operating RAS. The results obtained in the RAS sampled for this study, i.e. freshwater (African catfish) vs. seawater (turbot), cold water (Dover sole) vs. warm water (Nile tilapia), and small dimension (7 m^3) vs. large dimension (2500 m^3) suggest that steroids are probably present in the water of any RAS farm. The third and fourth studies showed that several factors contribute to the presence and accumulation of steroids in RAS water, particularly fish stocking density, occurrence of stressor events and decrease of water pH. The fifth study assessed chemical communication of Nile tilapia and Mozambique tilapia and showed that these species have high olfactory sensitivity to steroidal pheromones, to the point of being able to detect these molecules at concentrations as low as 511 ng/L (10^{-9}M). These results show that steroids concentrations present in RAS are within the threshold of detection of Nile and Mozambique tilapia. Overall, steroidal hormones and pheromones may accumulate in RAS water at concentrations potentially detected by some fish species. Furthermore, the current work showed that RAS cleaning devices cannot completely remove steroids from water, which may have implications for fish chemical in RAS.

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CHAPTER

General introduction

Aquaculture

The global population is likely to reach 9 billion people by 2050. Consequently, one of the major challenges of this century is, as defined by the World Food Summit of 1996, food security i.e., for all people to have access at all times to sufficient, safe, nutritious food to maintain a healthy and active life (FAO, 1996). Seafood has an important role to play in food security and is the world's most traded food commodity in value, even surpassing those from agriculture such as rice, wheat, coffee and sugar (Asche, 2014). Fish, in particular, is an essential food item providing high quality protein, omega-3 fatty acids, vitamins and minerals. The world food fish consumption more than doubled from 9.9 to 19.2 kg of fish per capita between the 1960s and 2012 (FAO, 2014). For this progress, aquaculture production has been playing an increasingly greater role. While fish capture production has been steady since the 1990s, the contribution of aquaculture production grew from 13 percent to 26 percent in 2000 and to 42 percent in 2012, from a total of 158 million tons. It is now clear that the expected increase in demand for food fish can only be met by aquaculture production, and this requires sustainable intensification (Godfray et al., 2010). The sustainable intensification in aquaculture lies on producing more food fish from the same land area and from the same water quantity while reducing the environmental impacts such as eutrophication and fish escapes. The need for aquaculture intensification together with increasing environmental regulations led to the development of water re-use systems (Colt, 2006; Martins et al., 2010). Therefore, increased efficiency and optimization of recirculating aquaculture systems (RAS) will play an important role in aquaculture intensification and sustainably meet the foreseen food fish demand.

Recirculating aquaculture systems

RAS are intensive land-based systems in which water is re-used after treatment and fish are grown under controlled environmental conditions. RAS are generally composed of three units combined sequentially: i) fish rearing unit; ii) mechanical filtration unit; and iii) biological filtration unit (Figure 1) (Timmons and Ebeling, 2007). Fish rearing units may display various shapes and depths, but circular tanks are usually used due to improved water mixing and maximization of solids removal compared to polygonal shapes (Summerfelt et al., 2004). The mechanical filtration unit is designed to remove

solid waste, which mainly consists of feed and fecal waste. There are two main processes for solids removal from recirculating water: i) by gravity, such as sedimentation in settling tanks, or ii) by particle size, such as microfiltration with microscreen drum filters (van Rijn, 2013). Solids removal from the water column is an essential step to avoid the proliferation of heterotrophic bacterial activity in biological filtration. The biological filtration unit is installed in RAS to convert ammonia excreted by fish, which is highly toxic, into less toxic substances such as nitrate, through a biological process called nitrification (Eding et al., 2006). Although different biofilters are used (e.g. trickling filters, moving bed, among others), in all cases their design is intended to maximize surface area (substrate) and to provide oxygen to the autotrophic nitrifying bacteria (Blancheton et al., 2007). Other components in more sophisticated RAS designs can include additional treatments such as the injection of oxygen (Colt, 2006), ultraviolet (UV) irradiation, ozonation (Summerfelt et al., 2009) and denitrification (van Rijn et al., 2006).
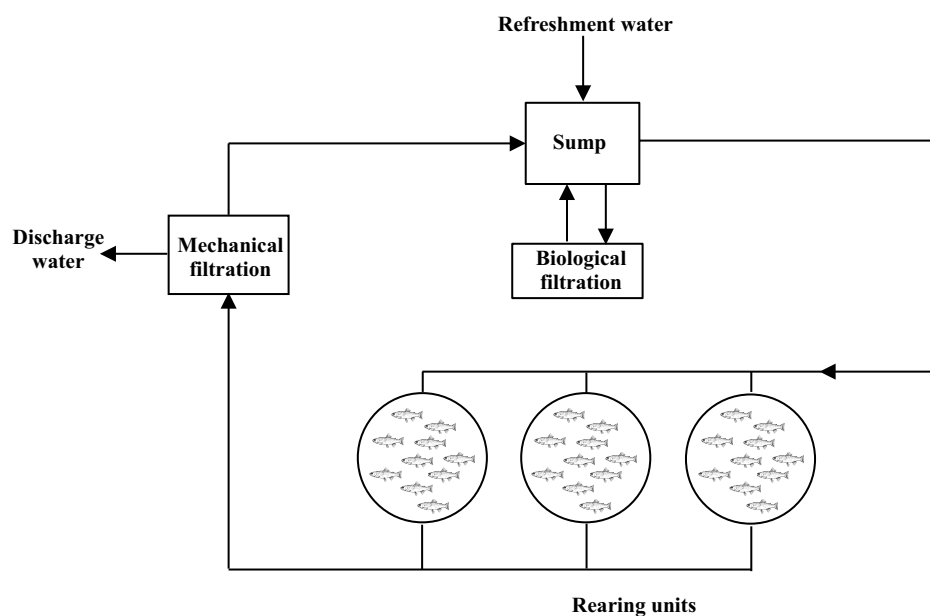

Figure 1. Recirculating aquaculture systems design

Another distinct feature of RAS is the possibility to control environmental parameters and, consequently, customize the culture conditions to the target species. For instance, water temperature can range between 14ºC and 30ºC depending whether the culture species is adapted to cold water, such as rainbow trout *Oncorhynchus mykiss* (Colson et al., 2015), or adapted to warm water such as Nile tilapia *Oreochromis niloticus* (Shnel et al., 2002). Water pH is typically maintained between 7 and 8 for most species, such as in yellowtail kingfish *Seriola lalandi* culture (Abbink et al., 2012), but pH can also be maintained below 6 as applied in European eel *Anguilla anguilla* culture (Heinsbroek and Kamstra, 1990; Eding and Kamstra, 2001). Salinity can be controlled in RAS allowing the culture of freshwater species, e.g. Nile tilapia (Martins et al., 2010) and marine species, e.g. turbot *Scophthalmus maximus* (Labatut and Olivares, 2004). While several other environmental parameters can be manipulated in RAS, the option to control the amount of water that is re-used, i.e. the water exchange rate, is the most distinctive characteristic. Water exchange rates can range between 30 - 300 L/kg feed/day, as in the cases of Nile tilapia, African catfish *Clarias gariepinus* and European eel in nearly closed RAS (Shnel et al., 2002; Martins et al., 2009a), or 9000 L/kg feed, as observed in rainbow trout semi-closed RAS (Roque d'orbcastel et al., 2009).

RAS are highly adaptable systems and their use is becoming more widespread throughout fish lifecycle, including larval rearing, broodstock and ongrowing, for both freshwater and marine species (Blancheton et al., 2007; Martins et al., 2010). Maintaining high water quality is a critical factor for RAS success and monitoring water quality is particularly relevant as fish performance has been shown to be impaired by the accumulation of various substances in the rearing water (Deviller et al., 2005; Martins et al., 2009a; Martins et al., 2009b; Davidson et al., 2011b; van Bussel et al., 2012). Deviller et al. (2005) reported that European sea bass *Dicentrarchus labrax* reared in RAS for one year exhibited a reduction of 15% in growth when compared to fish reared in flow-through systems (FTS). Additionally, common carp *Cyprinus carpio* eggs incubated with water from RAS operated at low water exchange rates showed reduced egg hatching, higher larval mortality and reduced larval development (Martins et al., 2009b). Large individuals of Nile tilapia (± 300g) showed a trend towards growth retardation in RAS while small individuals (± 80g) did not show this response (Martins

et al., 2009a). These examples of poor fish performance illustrate the importance to further investigate the accumulation of substances in RAS.

Accumulation of substances in the water

The discharge of RAS effluent may be 10-100 times lower compared to FTS (Blancheton et al., 2007). Thus, the old quote "the solution for pollution is dilution" can be hardly applicable to RAS and therefore the "pollution" needs to be eliminated or converted within RAS, otherwise it will accumulate in the fish rearing water (Table 1). Three sources of substances that cause "pollution" can be considered in RAS: i) feed, ii) system/bacteria, and iii) fish.

Feed often contains plant material, part of which is indigestible. This fraction passes the fish intestinal tract, and ends up in the water. In RAS, most of this undigested fraction will be removed by mechanical filtration (generally > 30 - 100 μm particles) but the smaller organic compounds will accumulate in the water. Some of these organic compounds are converted to humic substances, which are responsible for the yellow–brownish coloration of RAS water (Hubbard et al., 2002). However, if the presence of these substances is undesired, they can be degraded through ozonation (Kusakabe et al., 1990). Other important feed components are fishmeal and fish oil, which are sources of metals and minerals (Choi and Cech, 1998; Berntssen et al., 2010). Several heavy metals accumulate in low water exchange rate RAS at levels that can be potentially toxic for fish (Davidson et al., 2009; Martins et al., 2011a). It is important to note that heavy metals present in RAS can also result from the erosion of system materials and the refreshment water. Presently, the best solution to avoid high levels of heavy metals in water is to increase the water exchange rate, i.e. dilution.

Bacteria are in many ways a critical component of RAS. The heterotrophic bacteria consume oxygen, release metabolite by-products and compete with nitrifying bacteria in the biofilters (Leonard et al., 2002; Michaud et al., 2006). The autotrophic nitrifying bacteria are responsible for the conversion of ammonia excreted by fish into nitrite and nitrate (Eding et al., 2006). Nitrate at high concentrations, despite being far less toxic (300 - 400 times) than ammonia or nitrite (Person-Le Ruyet et al., 1995) can impact fish performance, health and welfare (van Bussel et al., 2012; Davidson et al., 2014).

Table 1. Substances that accumulate in the water of recirculating aquaculture systems

Substance	Example	Reference
Nitrogen	Total ammonia nitrogen, Nitrite, Nitrate	(Eding et al., 2006; van Bussel et al., 2012; Davidson et al., 2014)
Phosphate	Orthophosphate	(Shnel et al., 2002; Davidson et al., 2009; Martins et al., 2009b)
Heavy metals	Arsenic, Iron, Nickel, Zinc, Boron, Copper, Lithium, Magnesium, Phosphorous	(Deviller et al., 2005; Davidson et al., 2009; Martins et al., 2009b; Martins et al., 2011a)
Off-flavor compounds	2-methylisoborneol (MIB), Geosmin	(Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010)
Dissolved organic substances	Humic acids (yellow–brownish coloration of water)	(Hirayama et al., 1988; Leonard et al., 2002)
Carbon dioxide	Dissolved carbon dioxide	(Blancheton et al., 2007; Good et al., 2010)
Hormones	Testosterone, 11-ketotestosterone, progesterone, estradiol, unknown crowding factors	(Francis et al., 1974; Pfuderer et al., 1974; Budworth and Senger, 1993; Good et al., 2014a)

Nitrate accumulation is generally controlled through dilution resulting from increased water exchange (Eding et al., 2006), but additional denitrifying filters can be incorporated in RAS to convert nitrate into nitrogen gas (N_2) (van Rijn et al., 2006). Other bacterial by products that accumulate in RAS, such as geosmin and 2-methylisoborneol (MIB), are responsible for the off-flavors found in fish fillet, (Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010). Units providing anaerobic water treatment may reduce their accumulation in RAS (Guttman and van Rijn, 2008).

Fish are known to excrete a wide variety of metabolic by-products into the water, including nitrogenous wastes (e.g. ammonia) and carbon dioxide. Nowadays, both can be easily removed from the water using biological filtration or stripping devices, which prevents their accumulation up to toxic levels (Colt, 2006; Eding et al., 2006; Santos et al., 2012). However, the presence and possible effect of other metabolites in RAS in particular hormones metabolites has been little studied. For example, the stress hormone cortisol has been shown to be released into the water by rainbow trout (Ellis et al., 2004), Atlantic salmon *Salmo salar* (Ellis et al., 2007) and European sea bass *Dicentrarchus labrax* (Fanouraki et al., 2008). Likewise, testosterone and 11-ketotestosterone (11-KT), two important sex hormones, are released by several cichlids species during territorial disputes (Hirschenhauser et al., 2008; Keller-Costa et al., 2015). To the author's best knowledge, only two studies reported the presence of hormones in RAS water (Budworth and Senger, 1993; Good et al., 2014a). Whether steroids in RAS occur at concentrations that can be sensed or taken up by fish remains to be investigated.

Steroids

Chemical messengers play a vital role in exchanging information within and between individuals. The class of messengers within individuals is called hormones and their chemical origin varies from peptides, proteins, amino acid derivatives and steroids. Steroidal hormones have a particular importance in teleost fish as they modulate homeostasis, stress response, reproduction, courtship and aggressive behaviours (Borg, 1994; Scott et al., 2008). Steroidal hormones are synthetized from cholesterol in gonads and adrenal glands (head kidney) and are grouped into five categories according to their action: i) glucocorticoids, ii) mineralocorticoids, iii) progestogens, iv) androgens and v)

estrogens (Figure 2). Glucocorticoid and mineralocorticoid action in teleost fish are generally carried out by cortisol, which accounts for stress response and welfare status (McCormick et al., 2008), whereas androgens, estrogens and progestogens, are related to fish sexual maturation and reproduction (Scott et al., 2008). The other class of chemical messengers is associated with exchange of information between individuals. These messengers are called pheromones and are crucial for interspecific communication (Keller-Costa et al., 2015).
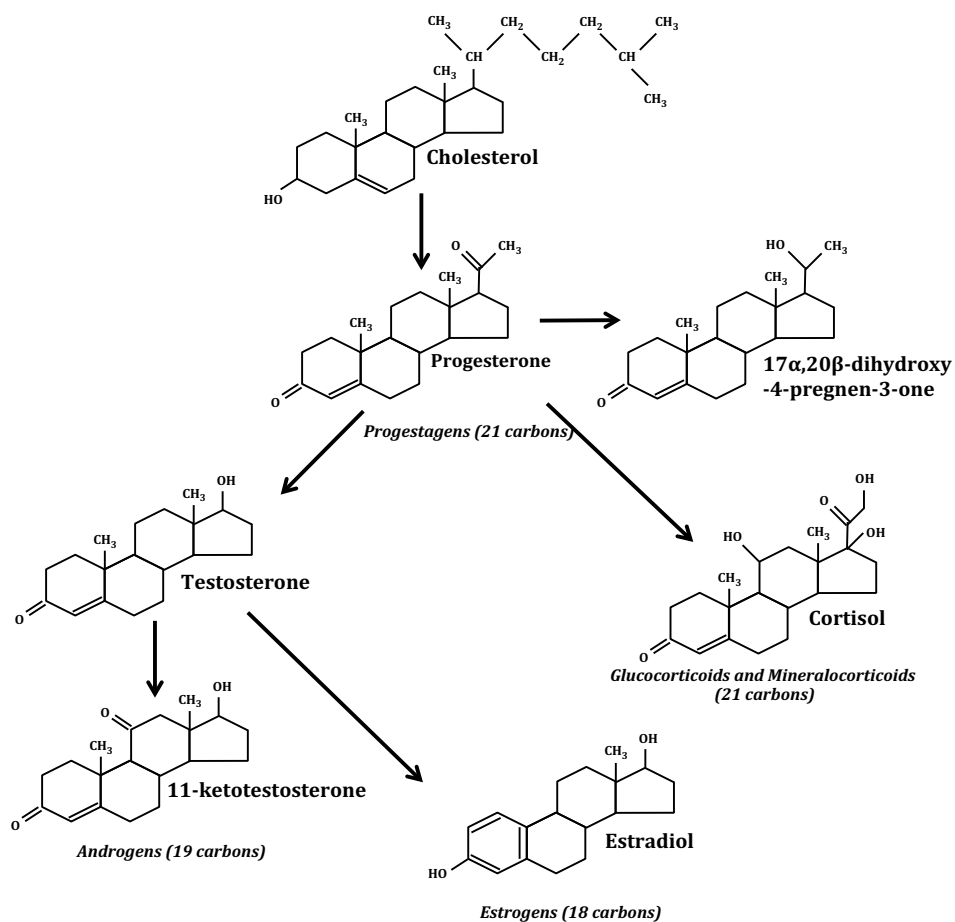

Figure 2. Steroidogenesis

Stress and welfare

Cortisol is the main corticosteroid and mineralocorticoid in teleost fish and is produced in head kidney. Metabolic regulation of carbohydrates, protein and amino acids, glucose regulation during stress, osmoregulation, growth and reproduction are among the many known roles for cortisol. Plasma cortisol levels rise immediately in response to an external stressor (Mommsen et al., 1999) and for this reason one of the most important applications of cortisol measurement is its use as an indicator of fish stress (Ellis et al., 2012). For instance, fish crowding and fish confinement lead to an increase in basal plasma cortisol levels from less than 50 ng/ml to 800 ng/ml in European sea bass (Fanouraki et al., 2008; Lupatsch et al., 2010; Santos et al., 2010) although the amplitude of endocrine response varies with species. Plasma cortisol clearance takes place in the liver but also through passive diffusion via renal and branchial routes (Mommsen et al., 1999). Several authors reported the release of cortisol in fish culture water (Ruane and Komen, 2003; Ellis et al., 2004; Scott and Ellis, 2007; Sebire et al., 2007; Fanouraki et al., 2008). However, it remains to be tested whether situations that elicit the production of blood plasma cortisol and release into the water will ultimately lead to its accumulation in RAS.

Sexual maturation and reproduction

Fish sexual maturation and reproduction are regulated by three groups of steroid hormones: androgens, estrogens and progestagens (Kime, 1993; Borg, 1994). Male fish androgen are produced in testis and the major biologically active androgens are testosterone and 11-KT. 11-KT promotes germ cell proliferation and is involved in agonistic and reproductive behaviour (Kime, 1993). Estrogens are produced in the female ovary and the most important steroid is 17β-estradiol, which promotes vitellogenesis in the liver. Progestogens, such as 17,20β-dihydroxypregn-4-en-3-one (17,20β-P), are produced both in ovary and testis and exert important actions especially during final gamete maturation. Blood circulating levels of sex steroids vary through the annual reproductive cycle according to the stage of gonad development (Kime and Manning, 1982). For instance, blood plasma concentration of 17β-estradiol throughout the reproductive cycle of female Senegalese sole *Solea senegalensis* ranges between 0.5 ng/ml and 4.5 ng/ml, and that of testosterone between 0.1 ng/ml and 0.5 ng/L in (García-López et al., 2007). Likewise, in male Senegalese sole, testosterone blood plasma

concentration ranges between 0.2 ng/ml and 1.5 ng/ml and 11-KT between 2 ng/ml and 20 ng/ml (García-López et al., 2006). Like cortisol, sex steroids and metabolites are released into the water (Vermeirssen and Scott, 1996; Scott and Ellis, 2007; Hirschenhauser et al., 2008) including in RAS (Budworth and Senger, 1993; Good et al., 2014a). However, a comprehensive understanding of the dynamics of steroid release in the water and its effects is still missing.

Pheromones

Pheromones can be defined as biological active substance or a mixture of substances released by an individual that induce a specific and adaptive response in conspecifics (Karlson and Lüscher, 1959). Several biological active substances known to be pheromones in fish include bile acids, prostaglandins, amino acids and steroids (Stacey and Sorensen, 2009). Pheromones originating from steroids are widespread throughout teleosts, including several economically important fish families like Cyprinidae (e.g. carps), Salmonidae (e.g. salmon and trout), Cichlidae (e.g. tilapias) (Sorensen and Stacey, 2004; Stacey and Sorensen, 2009; Hubbard, 2014; Keller-Costa et al., 2015). Steroidal pheromones can be unmodified hormones or hormone metabolites. An example of an unmodified hormone acting as pheromone is 17,20β-P, which induces final oocyte maturation in females when present in blood plasma and can induce sperm production when detected by males in the water (Sorensen et al., 1995; Scott et al., 2010). Steroidal metabolites are the result of hormone deactivation. These metabolites are generally more hydrophilic than the parent compound, rendering them more water soluble, and can be conjugated as glucuronide or as sulphate (Figure 3). Some of these conjugated steroid metabolites are potent pheromones. Male tilapia are known to release the urinary sex pheromone conjugate (5β-pregnanetriol 3α-glucuronide) that stimulates females to increase the production of 17,20β-P and therefore accelerate oocyte maturation (Keller-Costa et al., 2014). Similarly, the steroid glucuronide 3α,17α -dihydroxy-5β-pregnan-20-one 3α-glucuronide is a powerful male pheromone in the reproduction of the African catfish (Lambert and Resink, 1991). The effect of pheromones is not restricted to gender interaction, since pheromones released by sexually mature European eel males also stimulate gonadal development in immature males (Huertas et al., 2006). The implication of the accumulation of hormone metabolites in RAS on chemical communication between fish is potentially significant,

considering that the fish olfactory system is extremely sensitive and that these substances can be detected in water at concentrations as low as 0.3 ng/L (10^{-12} M) (Moore and Scott, 1991; Sorensen et al., 1995).
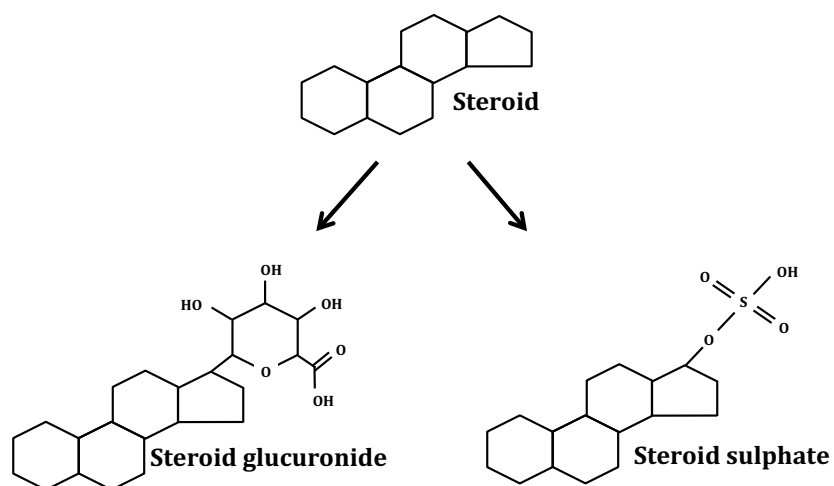
Figure 3. Steroids conjugation

General aim and research questions

In summary, systems that re-use water such as RAS may potentially accumulate a wide range of substances in the water. Steroidal hormones and pheromones are responsible for vital processes in fish, and their occurrence in RAS may impact its environmental and economic feasibility. However, the accumulation of fish steroids in the water is little studied. Therefore, a general aim of this thesis is to determine the occurrence of steroids in the rearing water of RAS and how different culture conditions may contribute to their accumulation. Such information is important to define strategies in RAS management in order to prevent negative effects on fish welfare, reproduction and growth performance.

This thesis aims at answering the following research questions:

1. Do steroids accumulate in the rearing water of RAS?

2. Which culture factors may be responsible for steroids accumulation in RAS?

3. How specific is the fish olfactory system to steroids?

Thesis outline

This thesis is composed of a general introduction (**chapter 1**), five experimental chapters (**chapter 2, 3, 4, 5 and 6**) and a general discussion (**chapter 7**). **Chapter 2** tested whether RAS that have high and low potential to accumulate substances from feed, fish and bacteria affect feed intake and growth of Nile tilapia, African catfish, and European eel. The next step was to determine the concentration of substances originated by fish in commercial scale RAS, especially stress and sex steroid hormones (**chapter 3**). When operating RAS for commercial purposes multiple variables may potentially contribute to the accumulation of steroids. Therefore, in **chapter 4**, we examined the release rate of steroids by Nile tilapia under experimental conditions, particularly cortisol and testosterone, and their accumulation in RAS at different water exchange rates. Moreover, the effect of an acute stress event on the accumulation of these hormones was also tested. In **chapter 5** we investigated the effect of water exchange rate and pH on rainbow trout blood plasma cortisol and the accumulation of cortisol, testosterone, 11-KT and 17,20β-P in the rearing water of RAS. Once the standard steroid concentrations present in RAS were determined, we focused on whether fish could detect these substances and how specific their olfactory system for steroids was. Therefore, **chapter 6** tested whether two tilapia species (Mozambique tilapia, *Oreochromis mossambicus* and Nile tilapia) have the same olfactory sensitivity to the male urine (a strong odorant) of each other as to their own and to a dominant-male pheromone. Finally in **chapter 7**, the main results from all experimental chapters are discussed in the context of the existing literature. Overall conclusions and practical implications are presented.
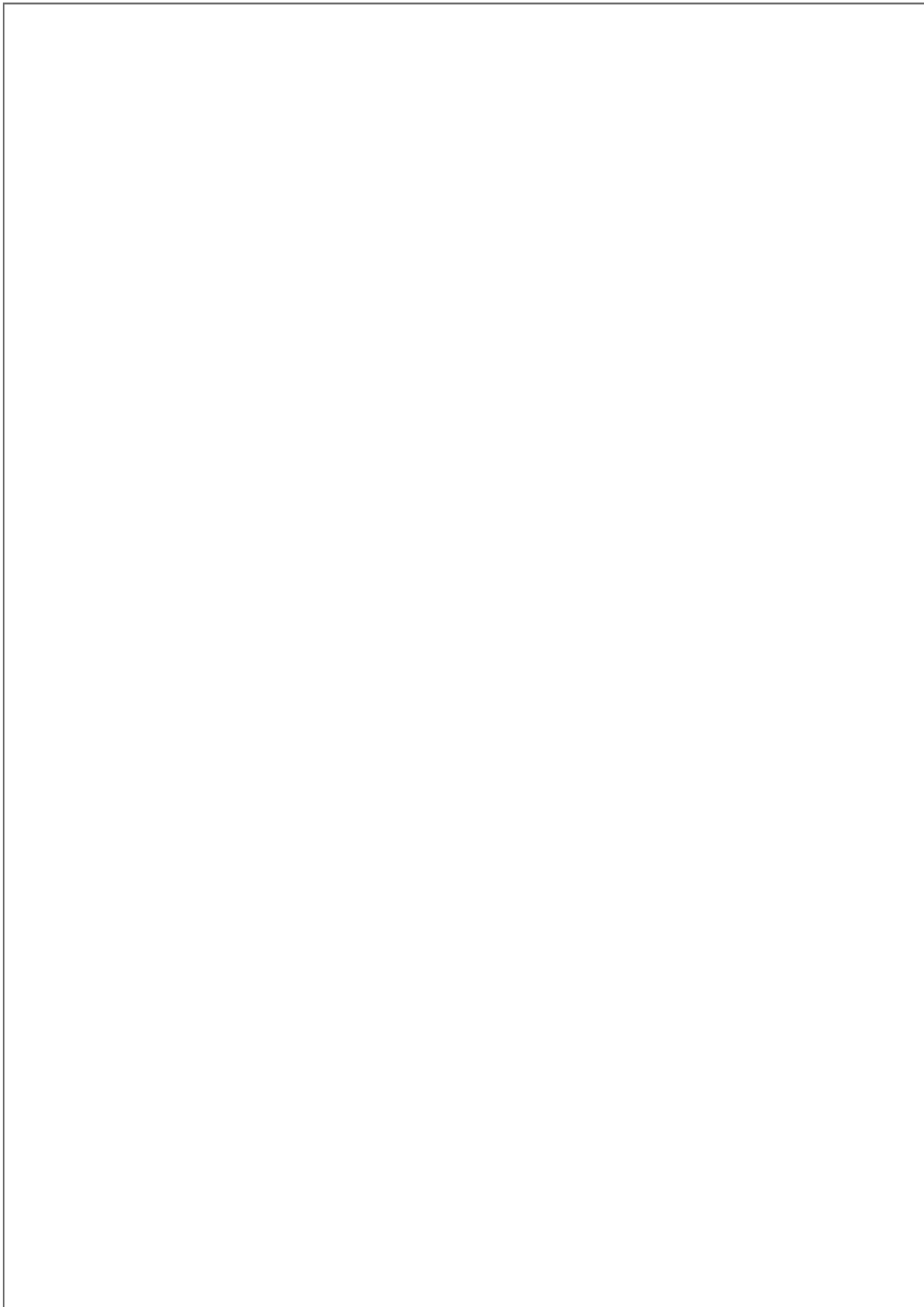
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The effect of nearly-closed RAS on the feed intake and growth of Nile tilapia (*Oreochromis niloticus)*, African catfish (*Clarias gariepinus*) and European eel (*Anguilla anguilla*)

V.C. Mota

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Abstract

One of the challenges that Recirculating Aquaculture Systems (RAS) are still facing is the risk that in RAS fish grow less than in flow-through systems due to the accumulation of substances originating from feed, fish or bacteria associated with the water re-use. The present study investigated whether RAS with high and low accumulation levels of these substances affect feed intake and growth of Nile tilapia *Oreochromis niloticus*, African catfish *Clarias gariepinus*, and European eel *Anguilla Anguilla*. One-hundred and twenty individuals of each species were used (start body weights: Nile tilapia 264.8±8.3 g; African catfish 253.2±2.1 g and European eel 66.6±1.3 g). For a period of 39 days, growth and feed intake were compared between high and low accumulation RAS. HIGH accumulation RAS was designed for maximal accumulation of substances in the water by operating the system at nearly-closed conditions (30 L/kg feed/d), using mature biofilters and high feed loads; and (2) LOW accumulation RAS was designed to be a proxy for flow-through systems by operating at high water exchange rates (1500 L/kg feed/d), new biofilters and low feed load. HIGH accumulation RAS induced a reduction in feed intake (42%) and growth (83%) of Nile tilapia, as compared to systems that are a proxy for flow-through conditions. This effect was not observed in European eel and African catfish. The cause of this reduced feed intake and growth rate of Nile tilapia is still unclear and should be addressed in further studies.

Introduction

Recirculating aquaculture systems (RAS) allow the combination of increased productivity with reduced water usage and effluent discharge (Martins et al., 2010). As a result of increased stocking densities and reduced water exchange rates, bacterial metabolites, such as nitrate and others (Schrader et al., 2010; Davidson et al., 2014), or heavy metals originating from the feed, (Deviller et al., 2005; Martins et al., 2011a), and hormones or pheromones produced by the fish (Mota et al., 2014; Keller-Costa et al., 2015), may be released into the water and impair fish feed intake and growth.

Recent studies show that these substances may lead to lower feed intake by the fish and ultimately inhibit their growth. Deviller et al. (2005) reported that European sea bass (*Dicentrarchus labrax*) reared in RAS for one year exhibited a reduction of 15% in growth when compared to fish reared in flow-through systems. Common carp (*Cyprinus carpio*) eggs incubated with water from RAS operated at low water exchange rates showed reduced egg hatching, higher larval mortality and reduced larval development (Martins et al., 2009b). However, other studies have suggested that fish can be successfully grown at highly intensive RAS (Shnel et al., 2002) and that denitrifying reactors can convert several substances into other products (Martins et al., 2011b). Despite the increasing use of RAS to culture fish, the effect of substances accumulation in RAS water on fish feed intake and growth is still poorly explored. Therefore, the present study aimed at testing whether RAS with high and low levels of substances accumulation induce a reduction in feed intake and growth in Nile tilapia (*Oreochromis niloticus)*, African catfish (*Clarias gariepinus*) and European eel (*Anguilla anguilla*). These three species were selected as experimental species since their farming in Europe is mainly performed in RAS (Martins et al., 2010).

Material and Methods

The present experiment was approved by the Ethical Committee for Animal Experiments of Wageningen University, The Netherlands and is filed under the reference number 2008014b.

Experimental animals and feeding

Nile tilapia (initial body weight of 264.8±8.3 g, all male, Swansea silver strain, TilAqua, The Netherlands), African catfish (253.2±2.1 g, Fleuren & Nooijen BV, The Netherlands) and European eel (66.6±1.3 g, Mondi-Aal BV, The Netherlands) were stocked at the experimental facilities of De Haar Vissen, Wageningen University.

Each species was stocked in groups of 20 fish per tank (6 tanks per species) and allowed to adapt to experimental facilities and feed for 13 days. During the adaptation period,

each experimental tank was connected to a RAS operated at a high water exchange rate (1500 L/kg feed/d). When the experiment started, 3 tanks per species were uncoupled from the original RAS system and started receiving water from another RAS which was operated at 30 L/kg feed/d.

Fish were fed *ad libitum* during the adaptation and experimental period, twice a day (08:00 and 15:00) using a commercial tilapia diet (3 mm floating pellets; 44% crude protein, 10% fat, 25% carbohydrate and 11.5% ash; Skretting, France). The feeding procedure started by manual feeding of 5 pellets to a floating ring that was placed in each tank. New pellets were added to maintain continuously 5 pellets within the ring for 90 min. The same feed and feed procedure was used for all species.

Recirculating Aquaculture Systems (RAS)

Two RAS were used (Fig. 1) during a 39-day experimental period: (1) a RAS with low water exchange (30 L/kg feed/d, termed HIGH accumulation RAS), which was designed for maximal accumulation of substances; and (2) a RAS with high water exchange (1500 L/kg feed/d, termed LOW accumulation RAS) in which the avoidance of the accumulation of substances was attempted (Table 1).

HIGH and LOW accumulation RAS contained 9 experimental tanks of 0.06 m^3 (3 tanks per species) each. All experimental tanks included an air stone to ensure oxygen supply and removal of carbon dioxide from the water. The water flow rate across each experimental tank was monitored daily and was approximately 6 L/min, which resulted in a hydraulic retention time (HRT) of 10 min. Alkalinity was supplied in form of sodium bicarbonate ($NaHCO_3$) to maintain pH above 6, whenever necessary.

Additionally to the 9 experimental tanks, HIGH accumulation RAS contained 4 culture tanks (0.450 m^3 each; flow rate of 23 L/min.) stocked with group-housed Nile tilapia (± 184 g; 155 ind/m^3). These culture tanks had the objective to load up this system and therefore contribute to the release of fish-related substances. In addition, these fish were fed a high amount (mean feed intake: 0.73 kg/day; source of feed-related substances) of the feed described earlier, through a 24-hour automatic belt feeder. This system also contained a 5-year-old mature biofilter and an up-flow sludge blanket-denitrification reactor (0.48 m^3) both functioning as the potential source of system-related substances.

Experimental fish (Nile tilapia, African catfish and European eel) in the HIGH accumulation RAS received the same water as the culture tanks containing Nile tilapia while experimental fish in the LOW accumulation RAS did not (no culture tanks were used).
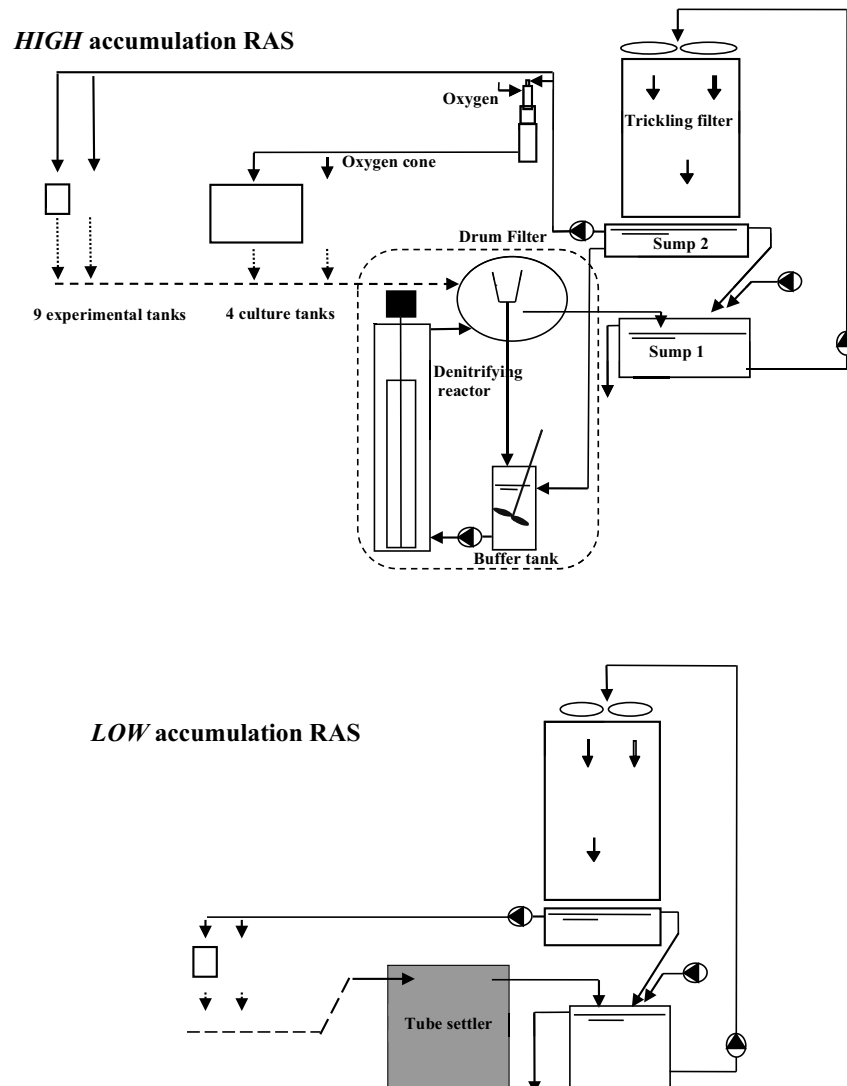
Figure 1. Scheme of the Recirculating Aquaculture Systems (RAS) used. At HIGH accumulation RAS (30 L/kg feed/d) water flows from 4 culture tanks (0.450 m³), 9 experimental tanks (0.060 m³) – drum filter (Hydrotech HDF 501-1P, 0.02 m³, mesh size 90 μm) – sump 1 (0.600 m³) – denitrifying reactor (0.480 m³) – trickling filter (0.070 m³, water flow 151.2 L/min.; ventilator air flow: 1.39 m/s; media Bionet 200 m²/m³, media volume 1.200 m³) – sump 2 (0.300 m³) – oxygen cone (0.050 m³) – experimental tanks. At LOW accumulation RAS (1500 L/kg feed/d) water flows from 9 experimental tanks (0.060 m³) – tube settler (1.31 m³) – sump 1 (0.740 m³) – trickling filter (0.070 m³) – sump 2 (0.300 m³) – experimental tanks. Flow across experimental tanks was ± 6 L/min.

Table 1. Characteristics of the RAS differing in the level of substances accumulation.

	HIGH accumulation RAS	_LOW accumulation_ RAS
Water Exchange rate (L/kg feed/day)	30	1500
Feed related substances		
Cumulative feed burden (mg/L) [a]	33 333	667
Mean feed intake (kg/day)	1.22	0.59
System related substances		
Biofilter age	5-year-old	1-month-old
Denitryfing reactor	present	not present
Fish related substances (culture tanks)		
Mean biomass (kg) [b]	16.0	not present
Mean density (kg/m^3) [b]	35.6	not present

[a] Calculated as mg of feed per day divided by liters of water discharge per day, during the 39 days of experimental growth period (Colt et al. 2006).

[b] During the 39 day experimental growth period.

LOW accumulation RAS was operated at high water exchange rate (1500 L/kg feed/d) with a young biofilter (in use for one month and fully acclimated based on TA-N < 0.3 mg/L and Nitrite-N < 0.1 mg/L water concentration during the adaptation period) and had no culture tanks. Therefore, it could be considered as a proxy for flow-through systems.

Daily water exchange was performed from sump 1 (Fig. 1). The water exchange volume (L) was calculated based on the feed load of the previous day and the exchange rate of each RAS: HIGH accumulation RAS (30L/ kg feed/day) or LOW accumulation RAS (1500 L/kg feed/day). The total volume of water to be replaced was removed every day from the system which had its inlet tap closed. Only after the water removal was completed the new well water supply was allowed in the system.

Measurements

Water quality measurements were performed using the common effluent of the experimental tanks. Temperature, pH, conductivity and dissolved oxygen were measured daily, using portable meters. Once a week, water from each RAS was analysed for TA-N (Total Ammonia-Nitrogen), nitrite-N, nitrate-N, ortho-phosphate-P, and total bicarbonate using a SAN auto-analyser (Skalar, Breda, The Netherlands).

The mean individual initial (Wi) and final (Wf) body weight of fish was determined by individually weighing the fish to the nearest 1.0 gram at the beginning and end of the experimental period (t). Fish were anesthetized with 0.3 g/L of Tricaine methanesulfonate (TMS, Crescent Research Chemicals, Phoenix, Arizona, USA) buffered with sodium bicarbonate ($NaHCO_3$, 0.6 g/L) before body weight measurements. Growth per metabolic body weight (G_m, $g/kg^{0.8}/d$) was calculated as (Wf - Wi)/t/MBW, where t was the duration of experimental period (t). Mean metabolic body weight (MBW, in $kg^{0.8}$) was calculated as $(W_G/1000)\char`^0.8$, where the geometric mean body weight (W_G, in g) was calculated as $\sqrt{}$ (Wi × Wf). Feed intake per metabolic body weight (FI_m, in $g/kg^{0.8}/d$) was calculated as feed given (in g) t/MBW, where feed given is the total feed intake per fish per tank during the experimental period. Feed conversion ratio (FCR) was calculated as FI_m/ G_m.

Statistical analysis

Statistical analyses were performed using SPSS (version 15.0). Data in percentage were transformed using arcsin [$\sqrt{(x/100)}$]. Homogeneity of variance was tested using Levene's F-test. All analyses were conducted using a t-test to assess differences between treatments. A significance level (α) of 0.05 was used. Data are presented as mean ± standard deviation (SD).

Results

Water quality

Water quality parameters measured in HIGH and LOW accumulation RAS during the 39-day experimental period are summarized in Table 2. Conductivity (t_{10} = 36.12, P < 0.01), nitrate-N (t_8 = 27.25, P < 0.01) and ortho-phosphate-P (t_8 = 38.59, P < 0.01) were higher in HIGH accumulation RAS than in LOW accumulation RAS, whereas pH (t_{10}=-4.94, P < 0.01), total bicarbonate (t_8 = -4.27, P < 0.01) and Ammonia-N (t_8 = -3.29, P < 0.01) was lower in HIGH accumulation RAS than in LOW accumulation RAS. Temperature, dissolved oxygen, TA-N and nitrite-N were similar in both RAS.

Feed intake and growth

Feed intake and growth parameters are summarized in Table 3. Initial body weight of Nile tilapia was similar in HIGH and LOW accumulation RAS (t_4 = 1.64, P = 0.18), whereas final body weight was lower in HIGH accumulation RAS than in LOW accumulation RAS (t_4 = -4.36, P = 0.01). Nile Tilapia cultured in HIGH accumulation RAS exhibited significantly lower feed intake (t_4 = -4.05, P = 0.02) and growth (t_4 = -5.15, P < 0.01) than Nile Tilapia cultured in LOW accumulation RAS. African catfish and European eel cultured in HIGH and LOW accumulation RAS exhibited similar feed intake (P > 0.05)

and growth (P > 0.05) at the end of the experimental period.

Discussion

This study showed that RAS operated at conditions that maximize the accumulation of substances in the water induce a significant reduction in feed intake (41.9%) and growth (83.2%) of Nile tilapia, as compared to systems that are a proxy for flow-through conditions. This effect was not observed in European eel and African catfish during the 39 days experimental period.

Fish feed intake is influenced by a number of factors including water quality (Kestemont and Baras, 2001). In the present study, some water quality parameters (pH, conductivity, Ammonia-N, nitrate-N, ortho-phosphate-P and total bicarbonate) differed between HIGH and LOW accumulation RAS. The use of different water exchange rates was the primary cause for such differences. However, the measured values of pH, conductivity, nitrate-N, ortho-phosphate-P and total bicarbonate in both RAS remained within the optimal range for growth of Nile tilapia (Popma and Lovshin, 1996), African catfish (Eding and Kamstra, 2001) and European eel (Tesch, 2003). Therefore, the water quality parameters measured are unlikely to be responsible for the lower feed intake and growth of Nile tilapia cultured in HIGH accumulation RAS. Nevertheless one cannot exclude the effect of other water quality parameters that were not measured, such as heavy metals (Deviller et al., 2005; Davidson et al., 2009; Martins et al., 2011a), dissolved CO_2 (Colt, 2006) or suspended solids (Davidson et al., 2011a). The measurement of these parameters would have been beneficial in ruling out additional variables that could have impacted feed intake.

Feed intake might also have been impaired by the presence of social hierarchies. One of the major differences between African catfish, European eel and Nile tilapia is that the first two species do not develop strong social hierarchies as compared to Nile tilapia.

Nile tilapia shows strong, frequently agonistic interactions to establish dominance hierarchies (Oliveira and Almada, 1996). The social stress imposed by the dominant fish on the subordinates is known to affect feed intake in Nile tilapia (Fernandes and Volpato, 1993). In the present study, all tanks containing Nile tilapia clearly exhibited a dominant fish, which kept control over the feeding location forcing the majority of the remaining individuals into a corner of the tank. Despite the potential impact of social hierarchy on feed intake, one might assume that this effect would be equal for both RAS containing the experimental Nile tilapia as the fish number, body weight and density were kept similar.

Table 2. Water quality parameters measured in HIGH and LOW accumulation RAS during the 39-day experimental period.[a]

	HIGH accumulation RAS	*LOW* accumulation RAS	P - value
Temperature (ºC)	26.0 ± 0.7	25.9 ± 0.6	0.09
pH	6.2 – 7.0	7.2 – 7.5	< 0.01*
Conductivity (µS/cm)	1752.8 ± 66.0	341.9 ± 69.3	< 0.01*
Dissolved oxygen (mg/L)	7.0 ± 0.4	6.0 ± 0.5	0.06
TA-N (mg/L)	0.20 ± 0.19	0.16 ± 0.03	0.63
Ammonia-N (mg/L)	0.001 ± 0.001	0.002 ± 0.001	< 0.01*
Nitrite-N (mg/L)	0.09 ± 0.03	0.12 ± 0.01	0.05
Nitrate-N (mg/L)	88.35 ± 5.21	17.02 ± 2.67	< 0.01*
Ortho-phosphate-P (mg/L)	17.5 ± 1.0	1.1 ± 0.3	< 0.01*
Total bicarbonate (mg/L)	9.7 ± 3.9	33.4 ± 11.8	< 0.01*

[a] Values are given as mean ± SD. n = 6 for temperature, pH, conductivity and dissolved oxygen; n = 5 for TA-N, Ammonia-N, Nitrite-N, Nitrate-N, Ortho-phosphate-P and Total bicarbonate. Significant differences are indicated by * ($p<0.05$).

Another aspect that may explain the difference in feed intake and growth observed for Nile tilapia in HIGH and LOW accumulation RAS is the accumulation in the water of system, feed- and fish-related substances. Bacteria located in different system units such as the biofilter, may also contribute to growth retardation due to the production of metabolic by-products. Nitrifying bacteria are responsible for the conversion of ammonia excreted by fish into nitrite and nitrate. Nitrate, despite far less toxic (300 - 400 times) than ammonia or nitrite (Person-Le Ruyet et al., 1995), can impact fish performance, health and welfare (van Bussel et al., 2012; Davidson et al., 2014). Substances responsible for the off flavors found in fish fillet, such as geosmin and 2-methylisoborneol (MIB) are other bacteria by-products that accumulate in RAS (Schrader and Summerfelt, 2010).

Feed-related substances such as heavy metals can accumulate in the water of low water exchange RAS (Martins et al., 2011a) and might lead to lower fish growth due to their toxicity (Deviller et al., 2005). Another study also found an increased swimming speed and greater incidence of side swimming behaviour in rainbow trout cultured in near-zero water exchange recirculating aquaculture systems (Davidson et al., 2011b). Fish-related substances such as alarm pheromones caused by skin damage might be released

and trigger behaviour changes in other conspecifics sharing the same water (Keller-Costa et al., 2015).

It is important to note that the only species exhibiting growth retardation (i.e. Nile tilapia) was the same species used to create the fish related-substances (i.e. fish used in the culture tanks in HIGH accumulation RAS). Although social hierarchy data were not gathered in the culture tanks, a large number of fish scales were collected from culture tanks effluents, suggesting that aggression had occurred among these fish. The production and release of fish metabolites and excretory substances into the water was likely to occur in such crowding conditions. For instance, stress (e.g. cortisol) and sex steroids (e.g. 11-ketotestosterone) were found to accumulate in RAS water (Good et al., 2014a; Mota et al., 2014). In addition, Martins et al. (2009a) using a similar experimental set-up to the one used in the present study found that large (± 300g) Nile tilapia showed a growth retardation pattern in low water exchange RAS. In their study, experimental tilapia also received water from culture tanks containing group-housed Nile tilapia; however, the experimental animals were individually housed. In the present study, experimental tilapia were group-housed and after being exposed to water from other tanks also containing group-housed Nile tilapia, growth retardation was detected. Therefore, the possibility that such growth retardation is potentiated by some mechanism involving social interactions is reinforced. The culture tanks connected to HIGH accumulation RAS contained all-male Nile tilapia of a large weight class. Despite the gonadsomatic index (GSI) was not assessed in the present study, Nile tilapia with this weight are known to be sexually mature (Popma and Masser, 1999) and consequently release sexual steroids into the water, which could facilitate the occurrence of aggressive behaviour (Keller-Costa et al., 2015). Thus, it is possible that the culture tanks containing Nile tilapia in HIGH accumulation RAS functioned as a source of fish-related substances and resulted in a shift from energy away from growth to reproductive development. However, whether these fish-related substances are responsible for the low feed intake and growth observed in Nile tilapia remains to be tested in a LOW accumulation RAS with culture tanks containing Nile tilapia.

In conclusion, males of Nile tilapia with approximately a weight of 300 g exhibit a reduction in feed intake (42%) and growth rate (83%) in HIGH accumulation RAS when compared with LOW accumulation RAS. The growth performance of African catfish and European eel was not significantly reduced in HIGH accumulation RAS. To what extent the performance of African catfish and European eel would have been affected if the species used in the culture tank (i.e. source of fish-related substances) had matched those of the experimental tanks remains to be investigated.

Table 3. Feed intake and growth of fish in HIGH and LOW accumulation RAS over a 39-day experimental period.[1]

	HIGH accumulation RAS	LOW accumulation RAS	P - value
Nile tilapia			
Initial bw (g/fish)	270.6 ± 8.0	258.9 ± 9.4	0.18
Final bw (g/fish)	280.0 ± 7.3	317.0 ± 12.8	0.01 *
Survival (%)	100.0 ± 0.0	96.3 ± 6.4	0.42
Feed intake (g/kg$^{0.8}$/d)	3.75 ± 0.80	6.45 ± 0.83	0.02 *
Growth (g/kg$^{0.8}$/d)	0.68 ± 0.53	4.04 ± 0.99	< 0.01 *
FCR	9.28 ± 7.75	1.63 ± 0.19	0.16
African catfish			
Initial bw (g/fish)	254.7 ± 7.1	251.7 ± 7.6	0.64
Final bw (g/fish)	503.6 ± 15.3	549.7 ± 47.6	0.23
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	1.00
Feed intake (g/kg$^{0.8}$/d)	13.98 ± 0.21	14.47 ± 1.42	0.59
Growth (g/kg$^{0.8}$/d)	14.51 ± 0.78	16.77 ± 2.17	0.21
FCR	0.97 ± 0.05	0.87 ± 0.06	0.10
European eel			
Initial bw (g/fish)	65.7 ± 0.5	67.5 ± 0.5	0.10
Final bw (g/fish)	74.7 ± 3.3	82.6 ± 4.0	0.06
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	1.00
Feed intake (g/kg$^{0.8}$/d)	6.08 ± 0.55	6.96 ± 0.74	0.18
Growth (g/kg$^{0.8}$/d)	1.94 ± 0.66	3.06 ±0.82	0.14
FCR	3.36 ± 1.03	2.36 ± 0.49	0.20

[1] Values are given as mean ± SD. n = 3 for all parameters. Significant differences are indicated by * (p<0.05).

As these results were obtained using an experimental period of 39 days, further studies should test whether growth retardation would persist or even increase using a longer experimental period. Further studies should aim to discriminate between the different sources that may cause the growth retardation in nearly-closed RAS.

Acknowledgements

3

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 accumulate in the water of RAS at levels that can potentially be detected by some fish species.

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 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.

Material and methods

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).
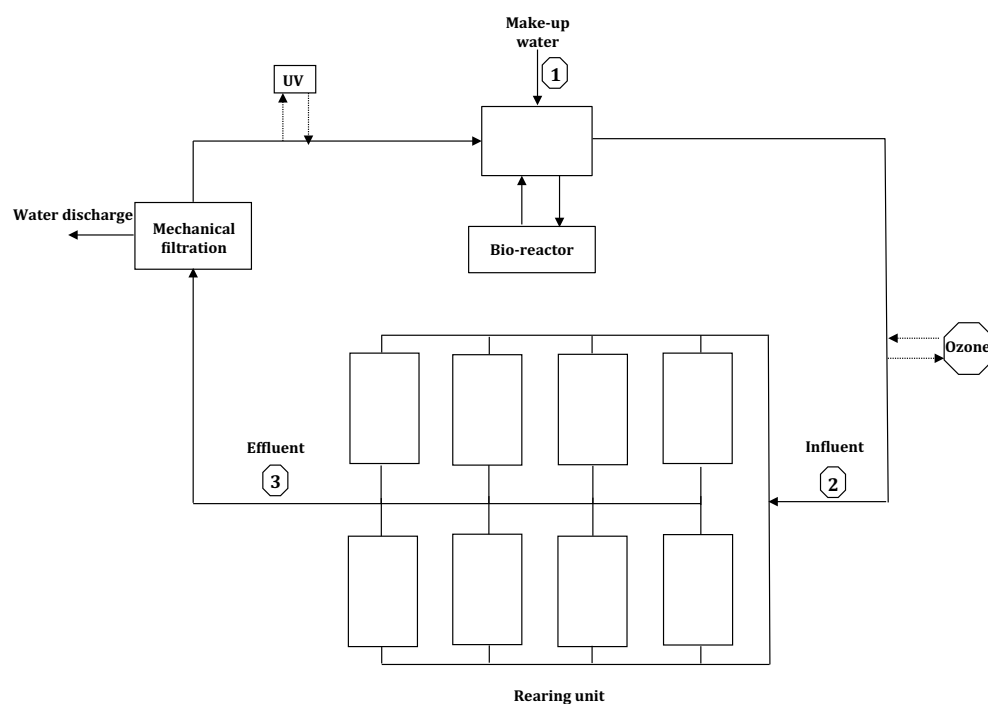

Figure 1. Schematic design of a general recirculating aquaculture system. Numbers represent sampling points: 1) make-up water; 2) influent of rearing unit and; 3) effluent of rearing unit. Arrows indicate the direction of the water flow. Not all components may be present in a specific recirculating system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

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[1]							
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)	20000	35000	65000	7500	6000	4750	19000
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[1]							
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)[1]	19	24	17	25	26	24	21
pH[1]	5.9	5.5	7.7	6.2	6.6	7.5	7.7
Conductivity (µs/cm)[1]	40600	2500	40000	1900	4000	4200	36300
TA-N (mg/L)[2]	5.7	63.5	0.3	1.4	48.8	5.9	1.0
Nitrite-N (mg/L)[2]	0.14	0.15	0.05	0.22	4.6	1.3	0.31
Nitrate-N (mg/L)[2]	64.5	92.3	27.0	91.1	53.5	72.3	73.6
Orthophosphate-P (mg/L)[2]	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.[1] value or information provided by the facility manager.[2] value measured

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 ºC. 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.

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 ºC under a gentle flow of nitrogen. The dried residue was re-dissolved in 0.1 ml distilled water, 3 x 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 ºC) 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 ºC under a gentle nitrogen flow and the dried residue was re-dissolved in 0.5 ml sodium 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 3x with diethyl ether as described above, radioimmunoassay buffer added and stored at -20ºC 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) and Scott et al., (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., New Jersey, 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.

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 by Pearson's correlations. For Pearson's correlations the α was recalculated after Bonferroni correction for multiple tests (initial α = 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.).

Results

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) (Table 3a and Table 3b). 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).

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 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).
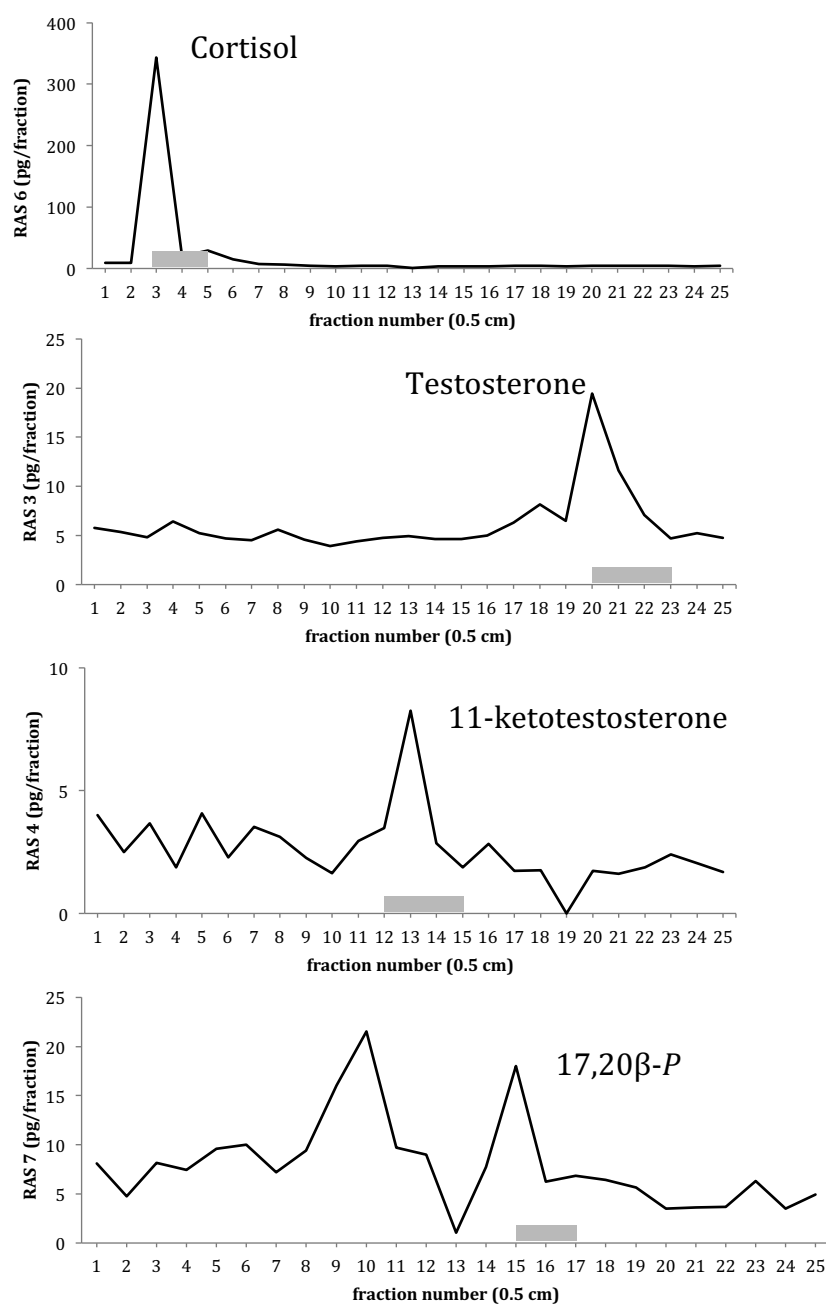
Figure 2. Thin-layer chromatography scan of immunoreactive steroids in four random water samples from the effluent of a rearing unit (RAS 3, 4, 6 and 7). Grey areas show elution positions of the tritiated standards 1) cortisol, 2) testosterone, 3) 11-ketotestosterone 4) 17,20β-P.
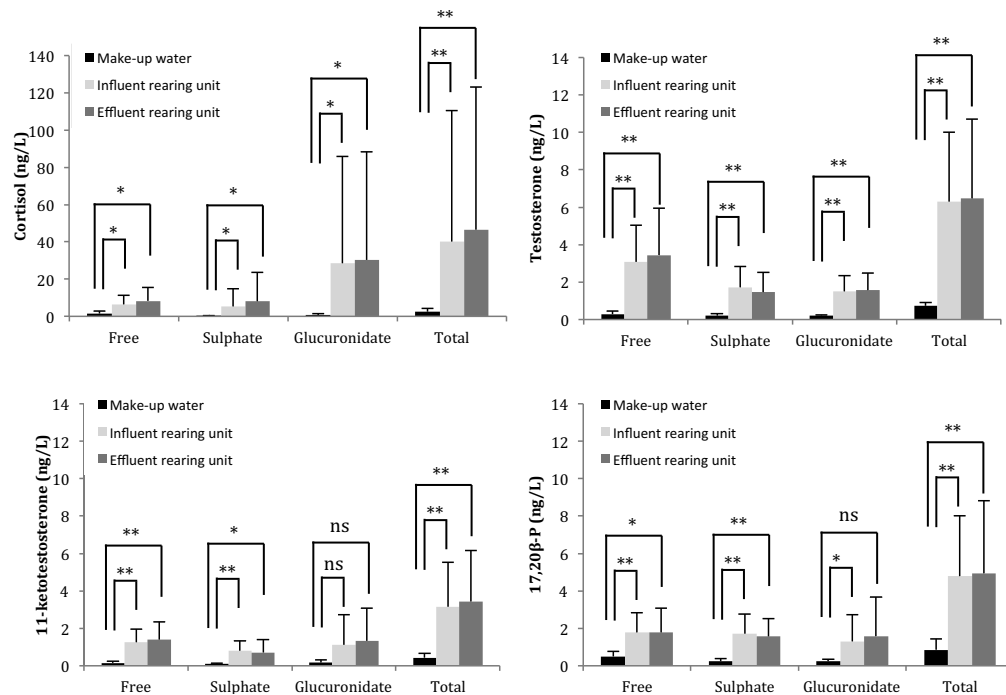
Figure 3. Mean ± s.d. steroid concentrations in the make-up water, influent and effluent of the rearing unit for the seven sampled RAS (N=7). *p-value <0.05; **p-value <0.01; ***p-value <0.001; ns – not significant.

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; Ellis et al., 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 – 67 ng/g feed (Feist and Schreck, 1990) and sex steroids between 0.4 – 11 ng/ g feed (Sower and Iwamoto, 1985; Pelissero et al., 1989). 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 (Vermeirssen and Scott, 1996; Ellis et al., 2005). 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 (Table 3). 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, 2010a). 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.

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

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; Table 3ab), 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.

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.
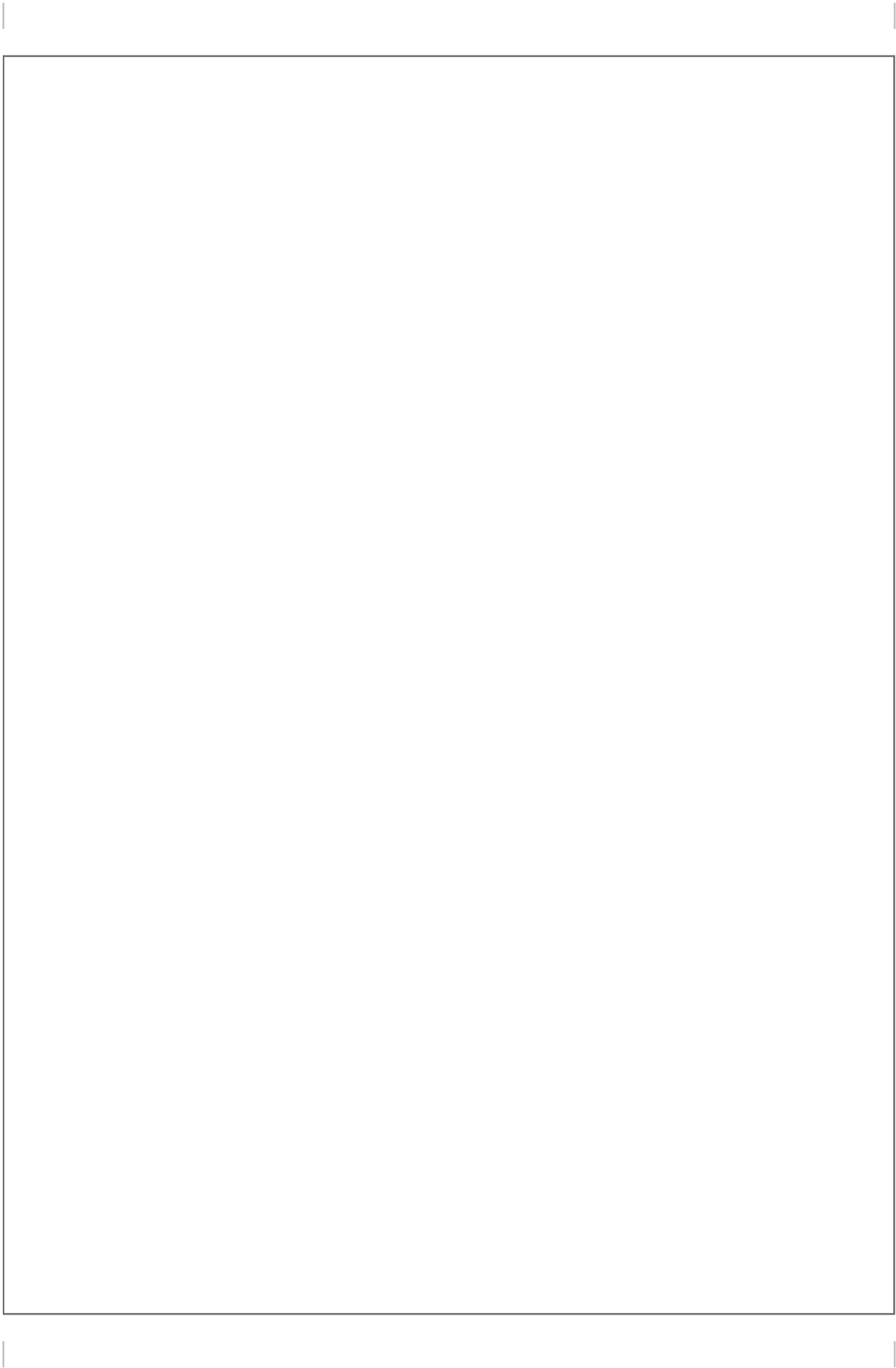
Coefficients of Pearson correlation and significance	
Orthophosphate-P x Free Testosterone	0.853 ($p = 0.001$)
Stocking density x Free Testosterone x	0.835 ($p = 0.001$)
Stocking density x 11-ketotestosterone Sulphate	0.791 ($p = 0.002$)
Stocking density x Total 11-ketotestosterone	0.801 ($p = 0.002$)
TA-N x Free Testosterone	0.890 ($p < 0.001$)
TA-N x Testosterone Glucuronide	0.810 ($p = 0.001$)
TA-N x Total Testosterone	0.875 ($p < 0.001$)
TA-N x Free 11-ketotestosterone	0.891 ($p < 0.001$)
TA-N x 11-ketotestosterone Sulphate	0.882 ($p < 0.001$)
TA-N x Total 11-ketotestosterone	0.899 ($p < 0.001$)

Orthophosphate-P water concentrations are a measure of the degree of new make-up water in RAS (Martins et al., 2009a). 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.

Acknowledgments

4

Water cortisol and testosterone in Nile tilapia (*Oreochromis niloticus*) recirculating aquaculture systems

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Abstract

The accumulation of steroids released by fish in recirculating aquaculture systems (RAS) may potentially influence their physiology and behaviour. However, information about steroid release, accumulation and removal rates is still scarce. The present study examined the release rate of cortisol and testosterone by Nile tilapia, *Oreochromis niloticus*, and their accumulation in six identical lab scale RAS operated at different water exchange rates (150 L/kg feed/day, *LowRAS* and 1500 L/kg feed/day, *HighRAS*) and how steroids accumulation reflect the stress induced by grading and weighing. Water cortisol and testosterone concentrations during the experimental period ranged between 1.0 and 5.1 ng/L and between 1.4 and 9.4 ng/L, respectively. These corresponded to 38% and 10% higher concentrations of cortisol and testosterone in *LowRAS* than in *HighRAS*, respectively. Overall steroid release rates were 0.02 ± 0.02 and 0.04 ± 0.02 ng/g bw/hour for cortisol and testosterone, respectively. Two hours after grading and weighing, water cortisol concentration increased 30% in the *LowRAS* whereas water testosterone concentration remained unchanged. Cortisol and testosterone discharge from the system were, respectively, 87% and 89% lower in *LowRAS* than in *HighRAS*. Cortisol and testosterone treatment efficiency by the biofilter was 18% and 8% lower in *LowRAS* than in *HighRAS*, respectively. These findings show that reduced water usage and acute stressors can induce significant accumulation of hormones in the rearing water at levels close to physiological response. Accumulation of physiological metabolites, in particular steroids, should be taken in consideration when designing and managing RAS to prevent exceeding allowable concentrations.

Introduction

Steroids have multiple roles in growth, stress response, reproduction and behaviour in all vertebrates. Fish release large amounts of steroids to culture water (Vermeirssen and Scott, 1996) that may accumulate to high concentrations in recirculation aquaculture systems (RAS). Hormonal steroids and their metabolites released by fish may play an important role in chemical communication and behaviour (Hubbard et al., 2014; Keller-Costa et al., 2015; Sorensen and Wisenden, 2015). Furthermore, some steroids can be taken up from the water mostly through the gills, potentially modifying the endocrine environment (Scott et al., 2005). Yet, steroid dynamics in aquaculture is poorly understood. In flow-through systems (FTS) two studies report the presence of steroids: Kolodziej et al. (2004) on water testosterone in rainbow trout *Oncorhynchus mykiss*, Nimbus Chinook salmon *Oncorhynchus tshawytscha* and steelhead trout *Salmo gairdneri* hatcheries, and Ellis et al. (2007) report water cortisol in Atlantic salmon, *Salmo salar*. Accumulation of steroids is expected to occur in RAS due to the continuous re-use of water in these systems. For example, testosterone was found to accumulate in RAS (Budworth and Senger, 1993; Good et al., 2014a). Whether the rate of water exchange is responsible for steroids accumulation in RAS is not clear (Mota et al., 2014).

RAS are designed to control and prevent accumulation of metabolites, such as carbon dioxide, ammonia and fecal waste (Timmons and Ebeling, 2007). The following design parameters are needed to estimate metabolite accumulation: the metabolite release rate by the fish, the removal rate of the metabolite by the bio-filter or treatment device, and the maximum allowable concentration of the metabolite in the rearing water. We hypothesize that steroid accumulation can be controlled in RAS using the same design parameters. Steroids release rates are only available for some fish species (Scott et al., 2008) but do not include Nile tilapia *Oreochromis niloticus*, a major species cultivated worldwide (FAO, 2014). The scarce information that exists on steroid removal rates in biofilters shows that removal rates will change depending upon the type of steroid (Liu et al., 2012; Good et al., 2014a). Therefore, the present study was intended to quantify cortisol and testosterone concentration, release rates and discharge, removal and treatment efficiency in RAS. Cortisol and testosterone were chosen because they differ (1) in their physiological function in fish (Kime, 1993; Ellis et al., 2012), which may result in different release rates, and (2) in their degradability (Liu et al., 2012), which may lead to different removal efficiencies and the resulting water concentration for both steroids. Cortisol is a corticosteroid hormone produced as an adaptation to stressful situations (Ellis et al., 2012), and high cortisol concentrations in culture water are usually an indicator of fish stress status (Fanouraki et al., 2008). Testosterone is a sex steroid present at high concentrations in both males and females. Although its specific

function in fish is a matter of debate, it is the common precursor of estrogens (females) and 11-oxygenated androgens (males) with key physiological roles in gametogenesis (Borg, 1994; Lubzens et al., 2010). In addition, testosterone is a potent odorant in precocious male Atlantic Salmon parr (Moore and Scott, 1991). As the accumulation of steroids in culture water of RAS is not known for Nile Tilapia, results were compared to the accumulation of metabolites of fish (total ammonia nitrogen, TA-N) and bacteria (nitrate). The effect of water exchange rate on cortisol and testosterone concentrations was also tested using two water exchange rates over a 4-week experimental period: 1500 L/kg feed, mimicking a high water exchange recirculating system, and 150 L/kg feed, mimicking a conventional RAS. In addition, a 24 h stress test was performed to evaluate fish grading and weighing effect on water cortisol and testosterone concentrations in RAS.

Material 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 2010048.e.

Experimental animals

Nile Tilapia (N=84) obtained from a commercial farm (Twentevis, Visverkoop B.V., The Netherlands) were randomly distributed over six RAS located at the experimental facilities De Haar Vissen, Wageningen University, and allowed to adapt to the rearing and feeding conditions over two weeks. After the adaptation period, fish were individually weighed (under sedation; 0.3 g/L of tricaine methane sulfonate (TMS) buffered with 0.6 g/L sodium bicarbonate) to the nearest 1.0 g and randomly re-distributed by gender over the same six RAS (14 fish /tank). Fish body weight was 344.2 ± 5.7 g (mean ± s.d.), sex ratio was 8/9 males to 6/5 females and initial fish biomass per RAS was 4818.7 ± 70.5 g.

During the adaptation and experimental periods fish were fed twice a day (09:30h and 17:30h) at a rate of 0.64 % body weight (BW) per day using a commercial diet (5 mm floating pellets; 35% crude protein, 8% crude fat, 3.2% crude fiber, 1% calcium and 1% phosphorus; Skretting, France). Fish were fed restrictively to maintain feed load within the trickling filter carrying capacity and high fish stocking densities ($\sim$ 67 kg/m^3) to mimic commercial conditions. Water quality parameters were maintained within the recommended range for tilapia (Timmons and Ebeling, 2007): temperature 28 ˚C, oxygen >4 mg/L, TA-N < 3mg/L, NH$_3$-N < 0.06mg/L and Nitrite-N < 0.06 mg/L. Photoperiod was maintained at 12L:12D.

Experimental systems

Each of the six identical experimental RAS (Fig. 1) was composed of a rectangular fish tank (V = 72 L; hydraulic retention time (HRT) = 24 min.), a suspended solids removal unit (V = 66 L; HRT = 9 min.) and a trickling filter (A=11.7 m², bio-net medium, specific surface area = 200m²/m³, Norddeutsche Seekabelwerke, Nordenham, Germany). Feaces were removed daily from the suspended solids removal unit. The trickling filter was inoculated with the biofilm from the top section of another RAS running at 28 ˚C for over 3 years. Total water volume of each RAS was ~138 L, water flow across the trickling filter and fish tank was approximately 12.5 L/min. and 5 L/min, respectively.

Experimental design

Cortisol and testosterone concentration

The effect of water exchange rate supply on cortisol and testosterone concentration was assessed in a four-week experimental period using six independent identical RAS as experimental units and a mono factorial design with 2 levels (treatments) and 3 replicates per treatment: 1500L/kg feed (*HighRAS*, mimicking a high water exchange recirculating system) and 150 L/kg feed (*LowRAS*, mimicking a conventional recirculating system). During the adaptation period all six RAS were operated at a water exchange rate of approximately 675 L/kg feed (mid-point between the two experimental water exchange rates). At the start of the experiment, the water exchange rate was adjusted to the values of the experimental treatments, i.e. 150 L/kg feed/day or 1500 L/kg feed/day. Water was exchanged daily before feeding from the suspended solids removal unit at a rate of approximately 1500 L/kg feed /day (*HighRAS*) and 150 L/kg feed/day (*LowRAS*). The exchange volume was based on the feed load of the previous feeding day.

Water samples for cortisol and testosterone analysis were collected weekly from the fish tank effluent in 1 L containers at 08:30 h and stored at -20 ˚C. Additional water samples (10 ml) were taken every week for TA-N, nitrite-N and nitrate-N analysis using a SAN auto-analyzer (Skalar, Breda, The Netherlands)(Meriac et al., 2013). Temperature, pH, conductivity and dissolved oxygen were measured daily using portable meters. Nitrile gloves were used during all water sampling and processing activities to prevent cross contamination with steroids.

Cortisol and testosterone release rate

Weekly water samples were collected over the 4-week experimental period to determine cortisol and testosterone release rates (ng/g BW/hour). Every week, for each RAS, the fish tank water inlet was closed (08:30h) and water bypassed during 1h from the sump to the sedimentation unit to mimic static conditions in the fish tank. Water

samples per RAS were collected from the fish tank effluent at 08:30h and 09:30h (before feeding) into a 1 L container via siphoning to avoid fish disturbance.
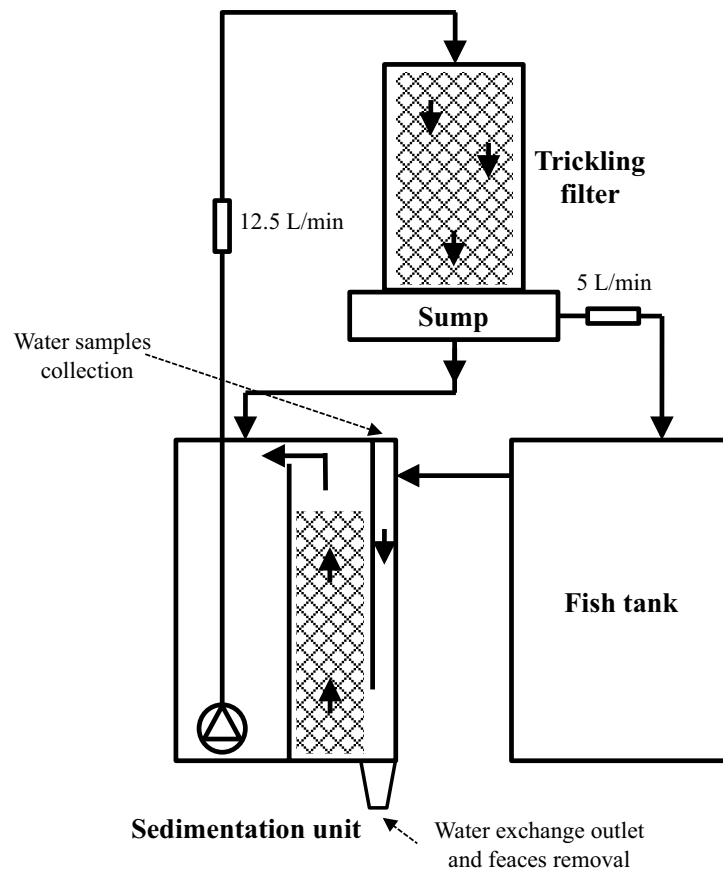

Figure 1. RAS scheme: water flows from fish tank (V = 72 L; hydraulic retention time (HRT) = 24 min.) – sedimentation unit (V = 66 L; HRT = 9 min.) – trickling filter (A = 11.7m²; media bio-net, 200 m²/m³ specific surface area). Total system volume was 138 L.

Twenty-four hour stress test

The effect of an acute stressor on cortisol and testosterone concentration in the water was assessed the day after the four-week experimental period. No effects were expected for testosterone; nevertheless, it was included as a negative control. At the end of the experimental period, fish were harvested from the fish tanks, individually weighed, held

out of the water for a total of 60s (Barcellos et al., 1999) and placed in one of the four 120 L containers filled with clean aerated water: 1) males *LowRAS*; 2) females *LowRAS*, 3) males *HighRAS* and 4) females *HighRAS*. Immediately thereafter (within 10 min), eleven fish (6 males and 5 females) were randomly taken from either the *LowRAS* or *HighRAS* containers and randomly assigned to the three *LowRAS* or three *HighRAS*, respectively. Fish were kept within the same treatment as before (*LowRAS* or *HighRAS*) to avoid confounding variables. For the 24h stress test body weight was 339.9 ± 6.2 g, sex ratio was 6 males to 5 females and initial fish biomass per RAS was 3738.7 ± 30.9 g.

Water samples from the fish tank effluent were taken at 0, 2, 4, 6, 8, 16 and 24 h after fish exposure to the stressor. Water was conveyed from the fish tank into 1 L containers by siphoning to avoid fish disturbance. Water removed during sampling was replaced by water with adjusted cortisol and testosterone levels following concentration calculations. Water exchange was interrupted during this 24 h period.

Steroids discharge, removal rates and treatment efficiencies

Treatment efficiencies and removal rates for cortisol and testosterone were determined using the release rates observed for each steroid in the present study. Calculations for steroid release rate, steroid production per day, treatment efficiency and steroid removal rate are summarized in Table 1.

Cortisol and testosterone analysis

Cortisol and testosterone were measured by radioimmunoassay (RIA) as previously described by Mota et al. (2014). Briefly, water samples were first paper filtered (2 μm; VWR, France) followed by a membrane filter (0.45 μm; Millipore, Ireland). The sample (± 500 ml) was 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 (100% ethanol) and the eluate evaporated in a dry bath (45 °C under a gentle flow of nitrogen). The dried residue was re-dissolved in distilled water and free steroids extracted with diethyl-ether, which was evaporated under the same conditions previously described and the residue was re-constituted in RIA 1 ml buffer (sodium phosphate 0.05 M, pH 7.6, containing 1% gelatin) and stored (-20 ºC) until assay. Cortisol antiserum was made with 20-CR50 (Fitzgerald Industries International, Concord, USA) and tritiated cortisol (GE Healthcare Europe GmbH, Carnaxide, Portugal), and the testosterone antiserum was kindly donated by Dr. David Kime (University of Sheffield, UK). The mean recovery efficiency of the extraction and RIA of spiked water samples with cortisol (Hydrocortisone H4001, Sigma-Aldrich, The Netherlands) was 84%. RIA detection limit was 36 pg/L for cortisol and 60 pg/L for testosterone.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 19 (IBM Corp., USA). Two-way repeated-measures ANOVA was used to compare the water exchange rate and time effect through the experimental period. Sphericity was tested using Mauchly's test and when this assumption was violated Greenhouse-Geisser correction was applied. When the time effect was not statistically significant, independent Student's t-test was used to compare the water exchange rate for a specific time point, and paired Student's t-test used to compare results between different time-points of the same RAS treatment. Homogeneity of variances for independent Student's t-test was tested previously using Levene's test. A significance level (α) of 0.05 was used. Data are presented as mean ± standard deviation (s.d.).

Results

Water quality

Water quality conditions measured throughout the experimental period in the fish tanks were: temperature (27.8 ± 0.2 ℃), dissolved oxygen (7.0 ± 0.2 mg/L), pH (6.9 – 7.3), conductivity (3109 ± 101 μs/cm), TA-N (< 1 mg/L), nitrite-N (< 1 mg/L) and nitrate-N (< 150 mg/L). TA-N and nitrate-N water concentration over the 4-week experimental period is presented in Figure 2.

Cortisol and testosterone concentration

Water cortisol concentration was 34% and 43% significantly higher in *LowRAS* than in *HighRAS* at week 3 (p = 0.007) and week 4 (p = 0.013), respectively (Fig. 3). The average cortisol concentration throughout the experimental period was 3.6 ± 0.1 ng/L for *LowRAS* and 2.6 ± 0.4 ng/L for *HighRAS*. Significant differences were observed for water testosterone between weeks (p = 0.002). Although the water testosterone ranged between 1.4 and 9.4 ng/L, no significant effect of water exchange rates (*LowRAS* vs. *HighRAS*) was observed (p = 0.599).

Cortisol and testosterone release rate

Cortisol and testosterone release rates were 0.02 ± 0.02 and 0.04 ± 0.02 ng/g BW/hour, respectively (Fig. 4). These release rates were not significantly affected by water exchange rates (cortisol: p = 0.557; testosterone: p = 1.000).

Table 1. Calculations.

Parameter	Unit	Formula
Steroid release rate (R_X)[1]	[ng/g/hour]	$R_X = (V_{Fish\ tank} * (C_{X,t} - C_{X,0}) * t)/(W_{total})$
Steroid production (P_X)	[ng/day]	$P_X = R_{X,\ average} * W_G * N * 24\ hours$
Steroid discharge (D_X)	[ng/day]	$D_X = C_{X,\ Fish\ tank\ out} * Q_{water\ exchange}$
Flow across the fish tank ($Q_{Fish\ tank}$)[2]	[L/day]	$Q_{Fish\ tank} = (P_X - D_X)/(C_{X,\ Fish\ tank\ out} - C_{X,\ Fish\ tank\ in})$
Treatment efficiency bio-reactor (TE)[2]	[%]	$TE = 100\% * (C_{X,\ bio\text{-}reactor\ out} - C_{X,\ bio\text{-}reactor\ in})/(C_{X,\ bio\text{-}reactor\ in})$
Fish tank influent concentration ($C_{X,Fish\ tank\ in}$)	[ng/L]	$C_{X,Fish\ tank\ in} = C_{X,\ trickling\ filter\ out}$
Steroid removal rate bio-reactor unit (r_X)	[ng/m²/day]	$r_X = (P_X - D_X)/11.7m^2$

[1] adapted from (Scott and Ellis, 2007), steroid release rate was determined weekly for a period of 1 hour. [2] adapted from (Timmons and Ebeling, 2007). W_{total} is the fish biomass per fish tank (in g), fish biomass was determined by weighing at the start and the end of the experiment and for week 2 and 3 by linear interpolation; W_G is the fish body weight; N: number of fish in tank (N=14); X is cortisol or testosterone; $V_{Fish\ tank}$ is fish tank volume in L; $C_{X,t}$ and $C_{X,0}$ are the concentrations (ng/L) of steroid X at t (t=1 hour) and t=0; R_X = the average testosterone or cortisol release rate (N=12 per steroid); $C_{X,Fish\ tank\ out}$ is the average measured concentration of X in the fish tank outlet in ng/L (N=12); $Q_{water\ exchange}$ is 150 L/kg feed or 1500 L/kg feed for the *LowRAS* treatment or *HighRAS* treatment respectively (4.3 L/day *LowRAS* and 43 L/day *HighRAS*); $Q_{Fish\ tank}$ is 5L/min; $C_{X,\ Fish\ tank\ in}$ is the average calculated concentration of X (ng/L) in the inflow water of the fish tank; $C_{X,\ trickling\ filter\ in}$ and $C_{X,\ trickling\ filter\ out}$ is the concentration of X (ng/L) in the water going in and out the trickling filter respectively; r_X is the removal rate for X in the trickling filter assuming no removal in the solids removal unit and for the biofilter configuration and operational conditions in this research.
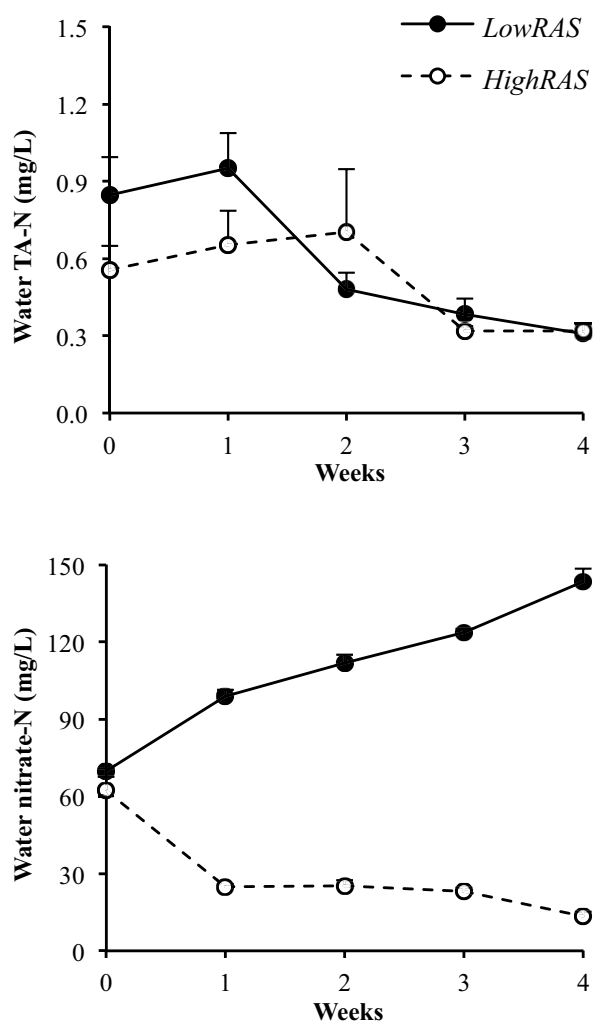
Figure 2. TA-N and nitrate-N concentration (mg/L) in *LowRAS* (150 L/kg feed) and *HighRAS* (1500 L/kg feed) treatment over the 4-week experimental period. (N = 3). Values are given as mean ± s.d.
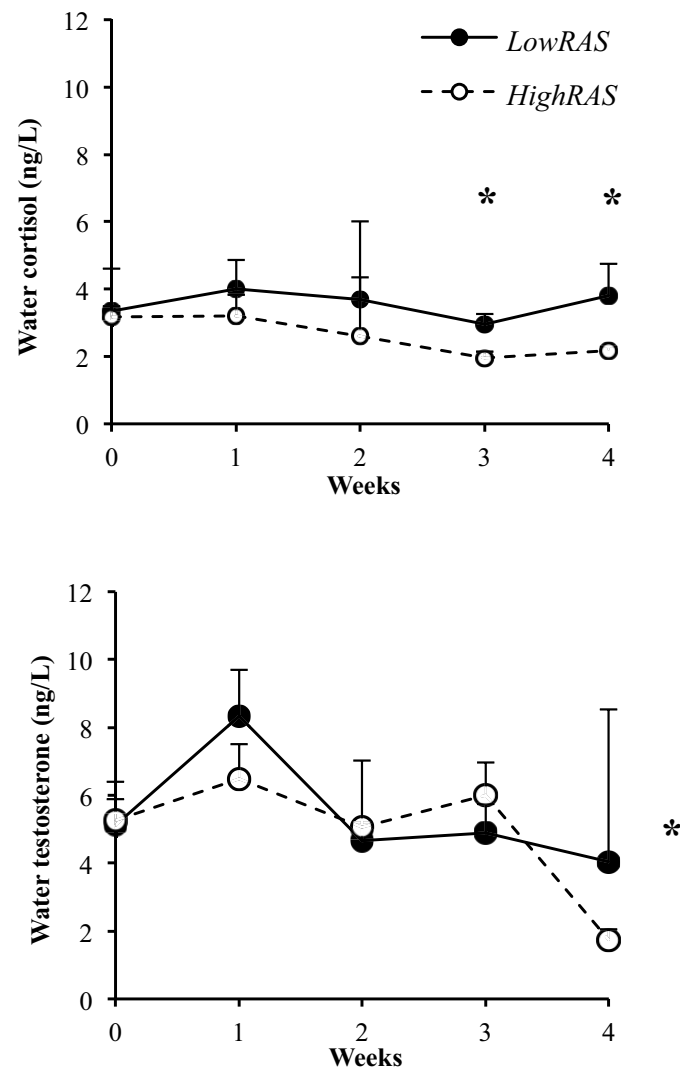
Figure 3. Cortisol and testosterone concentration (ng/L) in *LowRAS* (150 L/kg feed) and *HighRAS* (1500 L/kg feed) treatment over the 4-week experimental period. (N = 3). Values are given as mean ± s.d. Significant differences are indicated by * (p<0.05).
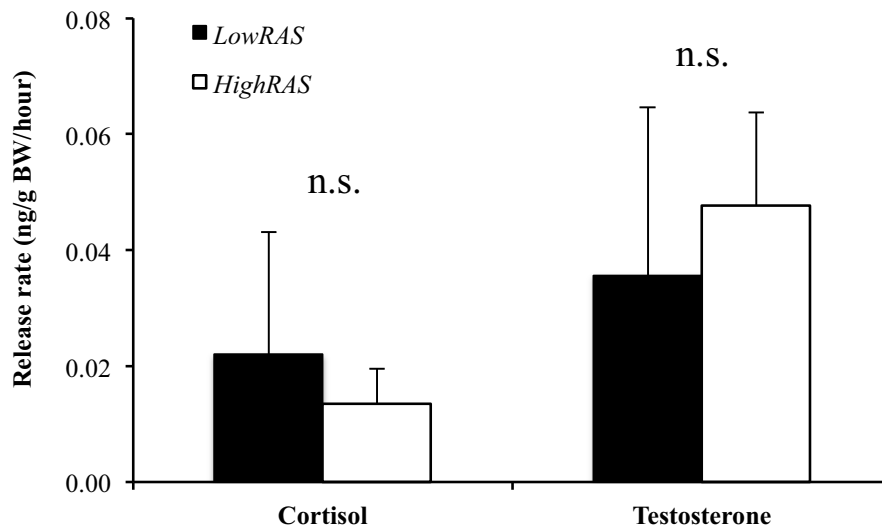
Figure 4. Average cortisol and testosterone release rates (ng/gBW/hour) into the water by fish in *LowRAS* (150 L/kg feed) and *HighRAS* (1500 L/kg feed) treatment over the 4–week experimental period. Values are given as mean ± s.d. (N = 3).

Twenty-four hour stress test

Two hours after the application of the acute stressor, water cortisol concentration increased 30% in *LowRAS* as compared to its initial concentration (0 h: 5.02 ± 0.89; 2 h: 6.53 ± 0.74; p = 0.162) and decrease thereafter to the levels observed at 0 h (Fig. 5). *LowRAS* mean cortisol concentration was 47 % higher at 2 h (*LowRAS*: 6.53 ± 0.74 vs. *HighRAS*: 4.43 ± 0.80; p = 0.023) and 27% higher at 24h (*LowRAS*: 4.98 ± 0.90 vs. *HighRAS*: 3.92 ± 0.62; p = 0.180) when compared with the *HighRAS* treatment. No effect of the acute stressor on testosterone concentration was observed within and between water exchange rates over the 24 h measurement period (water exchange rate: p = 0.323; time: p = 0.086).

Steroids discharge, removal rates and treatment efficiencies

Steroids discharge from the system was lower in *LowRAS* than in *HighRAS* (Table 2). Discharge rate for cortisol and testosterone was 87% and 89% lower in *LowRAS*, respectively. Cortisol and testosterone treatment efficiency by the biofilter was 18% and 8% lower in *LowRAS* than in *HighRAS*, respectively (Table 2). Removal rates for testosterone were approximately twice those of cortisol regardless of the water exchange rate. Although the concentration of cortisol and testosterone in discharge water was higher in *LowRAS*, removal rate was also higher in *LowRAS* for both hormones (Table 2).

Table 2. Cortisol and testosterone production, discharge, treatment efficiency and removal rate for the *LowRAS* and *HighRAS* treatment.

Parameter		*LowRAS*		*HighRAS*	
		Cortisol	Testosterone	Cortisol	Testosterone
Production (P_X)[1]	(ng/day)	2241	4489	2151	4317
Flow ($Q_{Fish\ tank}$)[2]	(L/day)	7200	7200	7200	7200
Water exchange ($Q_{water\ exchange}$)[3]	(L/day)	4.3	4.3	43	43
$C_{fish\ tank\ in}$ [4]	(ng/L)	3.25	4.80	2.34	4.34
$C_{fish\ tank\ out}$ [5]	(ng/L)	3.56	5.42	2.62	4.91
Discharge from systems (D_X)[6]	(ng/day)	15	23	112	210
Treatment efficiency (TE)[7]	(%)	9	11	11	12
Removal rate (r_X) [8]	(ng/m²/day)	206	413	189	380

[1] Steroid release rates used to calculate cortisol and testosterone production (P_X) per day are 0.02 ng/g BW/h and 0.04 ng/g BW/h respectively. Fish biomass used to calculated P_X was 4700 g (*LowRAS)* and 4716 g (*HighRAS).*[2] the flow rate across the fish tank (this study),[3] the daily applied water exchange (this study),[4] the calculated average influent steroid concentration,[5] the average of the measured steroid concentrations in the fish tank effluent over the four week period (N= 12), [6] steroid discharge calculated from the daily water discharge in this study and the average of the weekly measured steroid concentration in the fish tank effluent (N=3) (this study), [7] calculated, [8] calculated.

Discussion

This study found different accumulation patterns and release rates for cortisol and testosterone in RAS and that only cortisol accumulation was affected by the water exchange rates. Results also suggested that fish handling affects steroid concentration in the water.

The accumulation of steroids in aquaculture systems has been previously reported. Kolodziej et al. (2004) detected the endogenous steroids estrone, testosterone, and androstenedione at concentrations near 1 ng/L in the raceways and effluents of three fish hatcheries operating as FTS. Those concentrations were lower than results observed in the present study (Fig. 3) and reported for commercial RAS (cortisol: 3.8–217.0 ng/L; testosterone: 3–12.5 ng/L) (Mota et al., 2014). Good et al. (2014a) also detected cortisol and testosterone at 1.8 and 3.1 ng/L, respectively, in Atlantic salmon cultured in RAS.
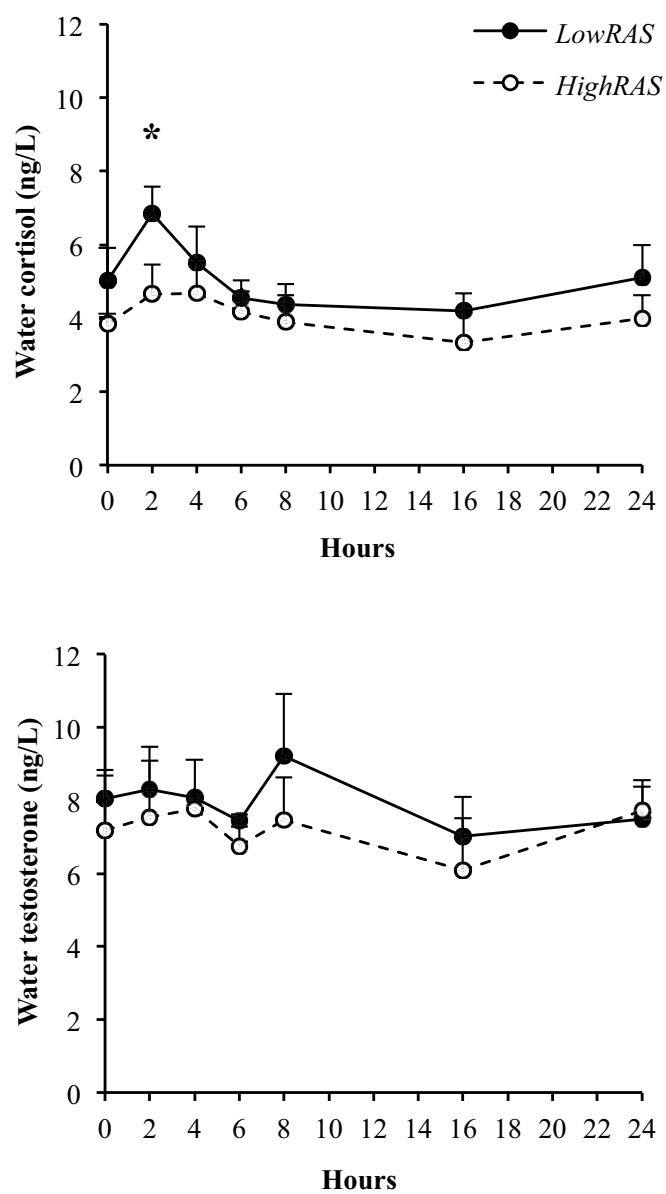
Figure 5. Twenty-four hour water cortisol and testosterone concentration in *LowRAS* (150 L/kg feed) and *HighRAS* (1500 L/kg feed) treatment after fish were exposed to an acute stressor. Values are given as mean ± s.d (N = 3). Significant differences are indicated by * ($p<0.05$).

These different concentrations of steroids observed in FTS and RAS systems are likely associated with water recirculation and exchange rate.

RAS systems are operated at different water exchange rates to control abiotic variables, such as temperature and pH, and, more importantly, to avoid the accumulation of waste metabolites released by fish (Colt, 2006). RAS with low water exchange rate are prone to accumulate harmful molecules such as TA-N, and differences in the concentration of water nitrate-N between systems is usually a consequence of different water exchange rates (Martins et al., 2010). Water nitrate-N concentration depends mostly on the dilution rate of the water exchange, and its production results from autotrophic bacterial conversions in RAS bio-reactors, i.e. nitrification, which takes place in two sequential steps: (1) the conversion of TA-N (NH_3-N + NH_4-N) into nitrite-N by ammonia oxidizing bacteria, and (2) the conversion of nitrite-N into nitrate-N by nitrite oxidizing bacteria (Eding et al., 2006). Consequently, as expected, nitrate-N concentration was significantly higher in the RAS treatment with low water exchange rate supply (*LowRAS*) (Fig. 2). Whether water steroid concentrations are compared to TA-N or nitrate is unknown. Cortisol concentration was significantly higher in the RAS treatment with low water exchange rate (*LowRAS*); however, its concentrations curve throughout the experiment was similar in both RAS treatments and followed the removal kinetics of TA-N instead of the nitrate-N (Fig. 2). The present study shows that the average cortisol concentration throughout the experimental period was 38% higher for *LowRAS* as compared to *HighRAS*, and significantly higher at week 3 and week 4, thus suggesting that water cortisol concentration is, to some extent, associated to the dilution rate resulting from water exchange. Contrasting results were observed for testosterone (Fig. 3) where no differences were observed in testosterone concentration between water exchange rates. On the other hand, water testosterone concentration showed remarkable variations over the 4-week experimental period. Water testosterone concentration may result from the intense fish interactions observed during the experimental period, which has been previously associated to an increase of testosterone production and release in the closely related *O. mossambicus* (Oliveira et al., 2001). It is also possible that the different concentrations of these two steroids are associated with their physical-chemical properties and consequent propensity to be absorbed by sediment or to stay in the aqueous phase. Such propensity is usually indicated by the ratio log K_{ow}, octanol-water partition coefficient; the lower the ratio, the more likely it is for a molecule to remain in the aqueous phase (Leszczynski and Schafer, 1990). Cortisol has a higher tendency to stay in RAS water than testosterone (log K_{ow} cortisol 1.86 vs. log K_{ow} testosterone 3.27). This property of cortisol may explain the

higher influence of water exchange rate on water cortisol concentrations. The same pattern was observed in waste water treatment where androgens (e.g. testosterone), estrogens and progestagens were mainly removed by degradation and sorption, while the removal of glucocorticoids (e.g. cortisol) was primarily due to degradation (Liu et al., 2012; Good et al., 2014a).

Accumulation of steroids in RAS, as of any other molecules, is inherently associated to its release rate (production) and treatment efficiency. Treatment efficiencies for cortisol and testosterone were lower in *LowRAS* biofilter (Table 2). The lower removal rate recorded in *LowRAS* treatment may be associated with a different biofilm thickness or a different bacteria composition in the biofilter (Eding et al., 2006). It is also important to note that *LowRAS* have higher concentrations of organic carbon in the water (Martins et al., 2009a), particularly humic acids, which may contribute to reduce steroids availability in water as these molecules adsorb onto the surface of the humic acid microvesicles (Hubbard et al., 2002).

Steroid release rates of 0.02 and 0.04 ng/g BW/hour were recorded in the present study for cortisol and testosterone, respectively (Fig. 4). Moreover, release rates were similar for both water exchange rates (Fig. 4). These results are in the same range as those previously reported for Atlantic salmon, where basal cortisol release rates were estimated to be in the region of 0.02–0.04 ng/g BW/hour (Ellis et al., 2007). Higher cortisol release rates have also been reported for stressed fish. Three-spined stickleback (*Gasterosteus aculeatus*) showed a cortisol release varying from 0.20 to 1.00 ng/g BW/hour when stressed (Sebire et al., 2007), whereas the maximum cortisol release rate (1.31 ng/g BW/hour) in European sea bass was observed 0.5 to 1 h post stress (Fanouraki et al., 2008).

Isolated events that affect animal welfare and induce stress may also affect the accumulation of steroids in RAS. For instance, handling and weighing of fish causes an increase of the production and release of cortisol (Foo and Lam, 1993; Ellis et al., 2004). Fanouraki et al. (2008) tested the acute stress response of European sea bass to a handling stressor by placing the fish in a clean water tank operating as FTS. Results of the latter study showed an abrupt increase in water cortisol concentration that reached its maximum 4h after the stress event, and was followed by a constant decrease accordingly to the dilution factor. A similar result was observed when rainbow trout were exposed to handling stress (Ellis et al., 2004). While the present study also recorded an increase of cortisol concentration after the handling stress, the return of cortisol to the basal levels was faster (4h after the stress event).

In conclusion, in RAS containing Nile Tilapia, cortisol (a glucocorticoid) and testosterone (an androgen) are released to the culture water. Water cortisol concentration depends

on the water exchange rate that is used and higher concentrations are expected in lower water exchange rates. Fish grading and weighing has a short-term effect on the water cortisol concentrations (30% increase after 4 h), after which concentrations return to the basal levels. These findings show that reduction in water usage and fish handling may lead to a significant increase of hormones in the rearing water, which emphasizes the importance of accounting for steroids in the design and management of RAS.

Acknowledgments

5

Accumulation of cortisol and testosterone in low pH recirculating aquaculture systems indicates stressing conditions and reproductive dysfunction

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Abstract

Steroids are key mediators of the fish stress response and reproductive functions. Although it is known that steroids accumulate in recirculating aquaculture systems (RAS), 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 stress hormone cortisol concentration in rainbow trout *Oncorhynchus mykiss* blood plasma, and the holding water concentration of cortisol and 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 in each treatment and replicated twice: 1) high water exchange rate (±1700 L/kg feed) and neutral pH (±7.3), 2) low water exchange rate (±500 L/kg feed) and neutral pH (±7.3), and 3) low water exchange rate (±500 L/kg feed) and low pH (±5.8). Plasma cortisol concentrations at day 70 were on average 142% higher for fish kept at low pH when compared to individuals kept at neutral pH. Water cortisol and testosterone concentrations were 225% and 237% higher at low pH than at neutral pH, respectively, whereas water 11-KT and 17,20β-P did not differ among treatments. The low concentration of 11-KT and 17,20-β-P are likely associated with the sexual immaturity of the fish used in the present study. These results demonstrate that low pH contributes to increased plasma cortisol concentration 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 points to unspecified reproductive effects that need further investigation. These results emphasize the importance to monitor stress and reproductive hormones in the rearing water.

Introduction

Steroids are key mediators of the stress response and reproductive function in fish (Kime, 1993; Mommsen et al., 1999; Oliveira et al., 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 feaces (Ellis et al., 2005; Scott et al., 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 et al., 2012). Release of cortisol to the water has been measured in several fish species, such as rainbow trout *Oncorhynchus mykiss* (Ellis et al., 2004), European sea bass, *Dicentrarchus labrax* (Fanouraki et al., 2008) and three-spined stickleback *Gasterosteus aculeatus* (Sebire et al., 2007). Sex steroids such as testosterone, 11-ketotestoterone (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 et al., 2010; Keller-Costa et al., 2015). The release of these hormones into the water has also been shown in several species including goldfish *Carassius auratus*, Nile tilapia *Oreochromis niloticus* and rainbow trout (Vermeirssen and Scott, 1996; Scott et al., 2010; Hubbard et al., 2014; Huertas et al., 2014). Furthermore, steroids and xenobiotics in holding water can be taken into the fish and modify hormone plasma levels (Scott et al., 2005; Miguel-Queralt and Hammond, 2008). Despite the potential effects of steroid accumulation on fish performance and welfare in aquaculture (Mota et al., 2015), the presence and dynamics of steroids in the rearing water of aquaculture systems have been little studied.

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

Steroid accumulation in RAS varies with steroid hormone, even at low water exchange rates (Good et al., 2014a). On the other hand, 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 straightforward relation with water exchange rates. Water pH, which tends to be lower in RAS, may be another factor that influences steroid accumulation. Water pH in RAS can drop below 6 as a result of carbon dioxide (CO_2) released by fish (Eshchar et al., 2006) and ammonia removal by biological filtration (nitrification produces acid) if these two acidifying processes are not counteracted by a degassing unit (CO_2) or the addition of alkalinity to the water (Eding et al., 2006). In RAS farms pH can vary between 5 and 8.5 (Dalsgaard et al., 2013; Mota et al., 2014). 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 et al., 1994). Moreover, low pH inhibits or reduces bacterial activity responsible for the degradation of several metabolites (Eding et al., 2006; Eshchar et al., 2006).

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 is sensitive to adverse water quality. In addition, farming rainbow trout in RAS is becoming well established (Davidson et al., 2014; Colson et al., 2015). Additionally, to the author's best knowledge, the measurement of water steroids in rainbow trout RAS has not been performed yet.

Material 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, as previously described by Meriac et al. (2014). During a 70-day experimental period, three combinations of water exchange rate and pH were tested and each treatment replicated twice. The three treatments were: i) high water exchange rate (±1700 L/kg feed) and neutral pH (±7.3) ($H_W N_{pH}$), ii) low water exchange rate (±500 L/kg feed) and neutral pH (±7.3) ($L_W N_{pH}$), and iii) low water exchange rate (±500 L/kg feed) and low pH (±5.8) ($L_W L_{pH}$).

Recirculating aquaculture systems

Each RAS (Fig. 1) was composed of a fish tank (V = 300L; hydraulic retention time (HRT) = 15 min), a settling tank (swirl separator, V = 75L; Hydraulic surface load 150m^3/m^2/day), a UV unit (UV-C 36 W, Phillips, Eindhoven, The Netherlands), a sump

(V = 75L), a cooler–heater (TC20, Teco, Ravenna, Italy), and two trickling filters (1) A=11.7 m^2, bio-net medium, specific surface area = 200m^2/m^3, Norddeutsche Seekabelwerke, Nordenham, Germany and, (2) A=14.2 m^2, cross-flow medium, specific surface area = 240m^2/m^3, Fleuren & Nooijen, Nederweert, The Netherlands. Feaces were removed daily from a colled bottle (4ºC) located at the settling tank bottom. The bio-net 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 filter 1 and 2 was approximately 20 L/min and 10 L/min, respectively. The L_WL_{pH} treatment RAS were 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 added sodium bicarbonate necessary to keep the pH around 7.

Water was exchanged twice daily before feeding at a rate of approximately 1700 L/kg feed /day (H_WN_{pH}) and 500 L/kg feed/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 Keijzersberg, Blitterswijck, The Netherlands) with an approximate age of 10 months and a sex ratio of 3 males to 7 females. 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, un-ionized ammonia-N < 0.02mg/L, Nitrite-N < 0.2 mg/L and Nitrate-N < 100 mg/L. Photoperiod was maintained at 12L:12D.

During the experimental period fish were fed an average 1.6% of body weigh 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). Analyzed feed composition of the experimental diet was as follows: dry matter 971 (g/kg); crude protein 497 (g/kg feed dry matter (DM); crude fat 206 (g/kg DM) and; crude ash 69 (g/kg DM) and nitrogen free extract (NFE) 228 g/kg DM.
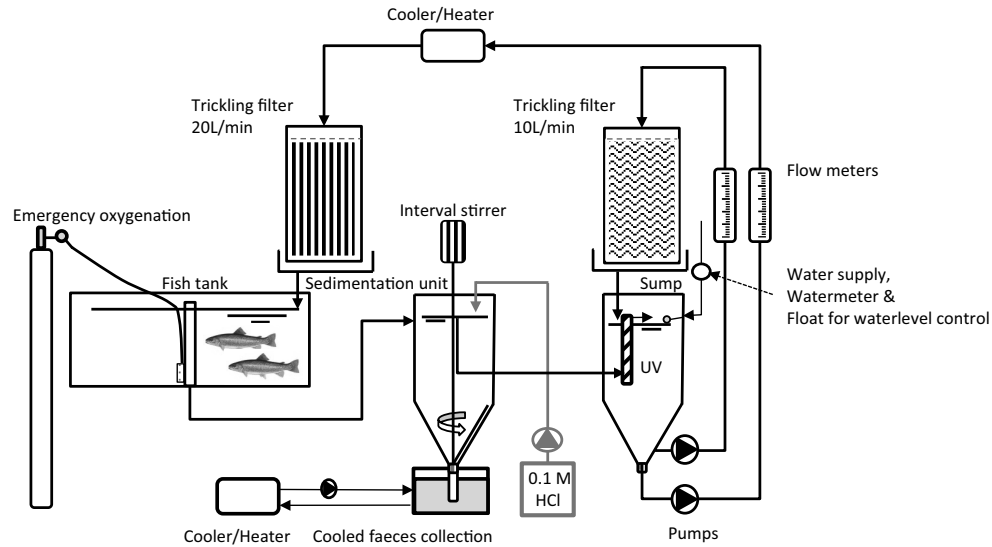
Figure 1. RAS design: fish tank (V = 300L; hydraulic retention time (HRT) = 15 min), settling tank (swirl separator, V = 75L; Hydraulic surface load 150 m^3/m^2/day), UV unit (UV-C 36 W, Phillips, Eindhoven, The Netherlands), sump (V = 75L), cooler– heater (TC20, Teco, Ravenna, Italy), and two trickling 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 ± 0.1M).

At the start of the experiment (day 0), 180 fish (30 fish per RAS) were anaesthetized (0.05g/L of MS-222 buffered with 0.1g/L of sodium bicarbonate) and individually weighed. The remaining 70 fish were discarded. At day 35, fish were anaesthetized, individually weighed and half of the initial fish (N = 90; 15 fish per RAS) was 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 sacrificed with 0.1g/L of MS-222 buffered with 0.2g/L of sodium bicarbonate and individually weighed. Growth per metabolic body weight (G_m, g/kg$^{0.8}$/d) was calculated as (W_f - W_i)/t/MBW, where W_f and W_i are final and initial individual bodyweight (in g), t is 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$^{0.8}$/d) 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 analysis using a SAN auto-analyzer (Skalar, Breda, The Netherlands)(Meriac et al., 2013). Temperature, dissolved oxygen and pH (WTW multi 340i, WTW GmbH, Weilheim, Germany) and conductivity (WTW cond 340i, WTW GmbH, Weilheim, Germany) were measured daily from the fish tank effluent water 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 Immune Sorbent Assay (ELISA) kit (Neogen Corporation, USA).

Water samples for steroid concentration measurement were collected from the effluent of the fish tank (500 ml) prior to fish feeding (08.30h) 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 analyzed 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) 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 (100% ethanol) and the eluate evaporated in a dry bath (45 ˚C under a gentle flow of nitrogen). The dried residue was re-dissolved in distilled water and free steroids extracted with diethyl-ether, which was evaporated under the same conditions previously described and the residue was re-constituted in RIA buffer (sodium phosphate 0.05 M, pH 7.6, containing 1% gelatin) and stored (-20 ºC) until assay. The methodology for steroid RIA is described by Scott et al. (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. 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., 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, water steroid concentrations were standardized to fish biomass (ng/L/kg 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 (s.d.).

Results

Survival, feed intake and growth performance were not significantly affected by water exchange rate or water pH at the two sampling periods (Table 1). Fish biomass, respectively for H_WN_{pH}, L_WN_{pH} and L_WL_{pH} was 3.9 kg, 3.8 kg and 3.7 kg at day 0; 7.1 kg, 7.0 kg and 6.8 kg at day 35; and 4.9 kg, 5.2 kg 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), dissolved oxygen (7.3 – 9.3 mg/L), TA-N (0.14 – 67.87 mg/L), nitrite-N (0.01 – 2.63 mg/L), nitrate-N (10.8 – 94.2 mg/L) (Table 2).

Plasma cortisol concentration at day 70 was on average 142% higher for fish kept at low pH (L_WL_{pH}: 24.4± 9.5 ng/ml) as compared to individuals kept at neutral pH (H_WN_{pH}: 12.0 ± 0.1 ng/ml and L_WN_{pH}: 8.7 ± 0.2 ng/ml).

Water cortisol did not differ between the three treatments at day 0 (p = 0.544) and at day 70 (p = 0.120). It was significantly elevated for L_WL_{pH} compared to H_WN_{pH} and L_WN_{pH} at day 35 (p = 0.013)(Figure 2A). Water testosterone did not differ between the three treatments at day 0 and at day 70 (p = 0.158) (p = 0.802), whereas it was significantly higher for L_WL_{pH} compared to H_WN_{pH} and L_WN_{pH} at day 35 (p = 0.004) (Figure 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/kg fish and 0.0 - 0.2 ng/L/kg fish, respectively (Figure 2C and 2D).

Discussion

The present work demonstrates that pH but not water exchange influence steroid concentrations in RAS systems, since RAS operating at low pH and low water exchange rates (L_WL_{pH}) accumulate higher levels of cortisol and testosterone (Fig. 2). This effect of low water pH was related to a higher plasmatic cortisol concentration, which was on average 142% higher for rainbow trout kept at low pH.

Table 1. Fish performance (mean ± s.d., N = 2).

Parameter	$H_W N_{pH}$	$L_W N_{pH}$	$L_W L_{pH}$	*P - value*
Day 0 to 35				
Number of fish	30	30	30	
Initial bw (g/fish)	131.0±4.2	126.7±1.6	123.5±2.1	0.160
Final bw (g/fish)	236.3±1.3	233.0±4.3	224.7±1.8	0.052
Survival (%)	100	100	100	n.a.
Feed intake (g/kg$^{0.8}$/d)	11.44±0.12	11.66±0.15	11.95±0.12	0.063
Growth (g/kg$^{0.8}$/d)	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 to 70				
Number of fish	15	15	15	
Initial bw (g/fish)	229.3±1.2	233.3±4.3	217.3±21.7	0.509
Final bw (g/fish)	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$^{0.8}$/d)	8.93±0.04	8.64±0.32	9.25±0.28	0.191
Growth (g/kg$^{0.8}$/d)	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 and management (mean ± s.d., except pH min–max, N = 2).

| Parameter	H_WN_{pH}	L_WN_{pH}	L_WL_{pH}	*P - value*
Daily measurements				
Temperature (ºC)	16.0 ± 0.1	15.8 ± 0.1	15.9 ± 0.1	0.095
Conductivity (µS/cm)	394.1 ± 0.4 [a]	971.3 ± 15.9 [b]	1079.7 ± 10.7 [c]	<0.001
Dissolved oxygen (mg/L)	8.1 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	0.673
Weekly measurements				
TA-N (mg/L)	0.57 ± 0.02 [a]	0.64 ± 0.19 [a]	55.01 ± 2.21 [b]	<0.001
Nitrite-N (mg/L)	0.42 ± 0.03 [a]	0.72 ± 0.14 [ab]	0.04 ± 0.00 [b]	0.005
Nitrate-N (mg/L)	23.5 ± 0.3 [a]	72.9 ± 1.6 [b]	21.0 ± 0.7 [a]	<0.001
System management				
Water exchange (L/kg 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
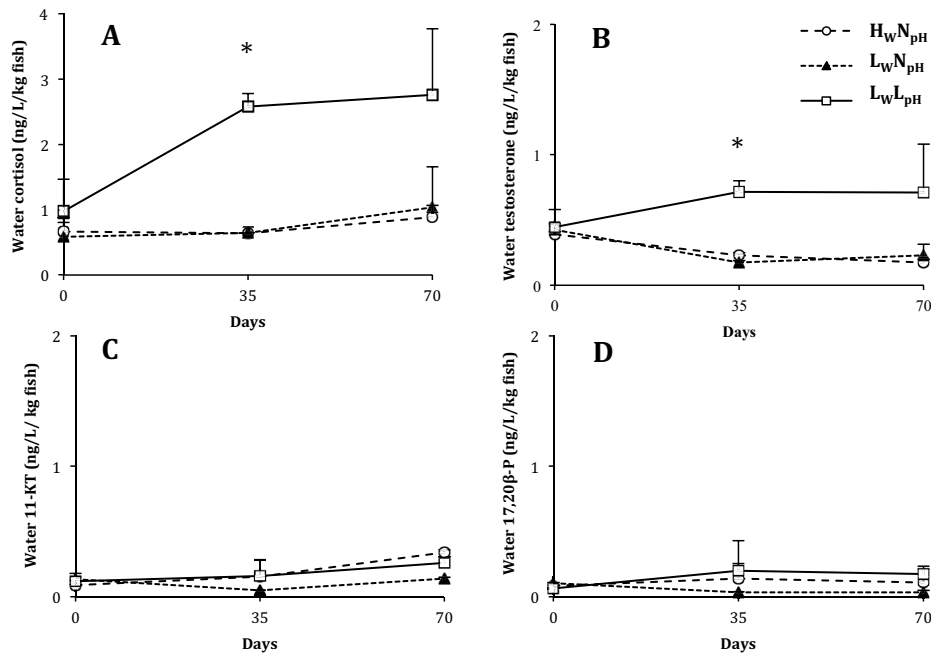
Figure 2. Water cortisol (A), testosterone (B), 11-ketotestosterone (C) and 17, 20β-P (D) levels (ng/L/kg fish) in rainbow trout RAS during the 70-day experimental period (N = 2). Values are given as mean ± s.d. Significant differences are indicated by * (p<0.05). Average fish biomass per replicate was 3.6 kg (day 0), 7.0 kg (day 35) and 4.9 kg (day 70).

Water quality was generally within the recommended levels for rainbow trout, with the exception of the elevated nitrite-N concentrations (Colt, 2006; Timmons and Ebeling, 2007) in the systems which operated at neutral pH (H_WN_{pH} and L_WN_{pH}). Nitrite toxicity was counteracted with the addition of NaCl (Colt, 2006), with no negative consequences for fish 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 of the ± 55 mg TA-N/L observed, which was still within the recommended concentrations. The NH_4-N form accounted for >99.9% of the ±55 mg TA-N/L, and is thought to be 300 - 400 times less toxic for fish compared to NH_3-N (Thurston et al., 1981). 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 et al., 2012; Meriac et al., 2013).

The presence of steroids in the rearing water of aquaculture systems was previously described for FTS (Kolodziej et al., 2004; Ellis et al., 2007) and RAS (Budworth and Senger, 1993; Good et al., 2014a; Mota et al., 2014) with concentrations ranging between 0.1 ng/L – 217 ng/L. In the present study, all four steroids measured ranged between 2.2 ng/L – 17.6 ng/L (cortisol), 0.9 ng/L – 4.9 ng/L (testosterone), 0.3 ng/L – 1.7 ng/L (11-KT) and 0.2 ng/L – 1.4 ng/L (17,20β-P). The low concentration of 11-KT and 17,20-β-P are likely associated with the sexual immaturity of the fish used in the present study, ±16 months of age and 310 g – 346 g of body weight at the end of the experiment. Sexual development of rainbow trout usually starts after two years of age or, exceptionally, in fish less than a year old but with a body weight above ±320 g (Crandell and 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 other species, such as 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 indicate a possible effect of low pH on the accumulation of these two steroids in water. The accumulation of 11-KT (Good et al., 2014a) and testosterone (Mota et al., 2014) were found analogous and positively correlated, respectively, 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). TA-N accumulation in RAS may result from lower removal rates of nitrifying bacteria under sub-optimal conditions such as low water pH (Eding et al., 2006). The finding that steroid levels were elevated in the low pH treatment suggests that the activity of bacteria is reduced at low pH conditions, which result in cortisol and testosterone accumulation in the rearing water.

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 142% higher for fish kept at low pH (L_WL_{pH}: 24.4± 9.5 ng/ml) 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 et al., 1985). One of its functions is to provide positive feedback for gonadotropin synthesis during early gametogenesis accelerating gonadal development (Dubois et al., 1998). Fish early sexual maturation results in growth and feed intake
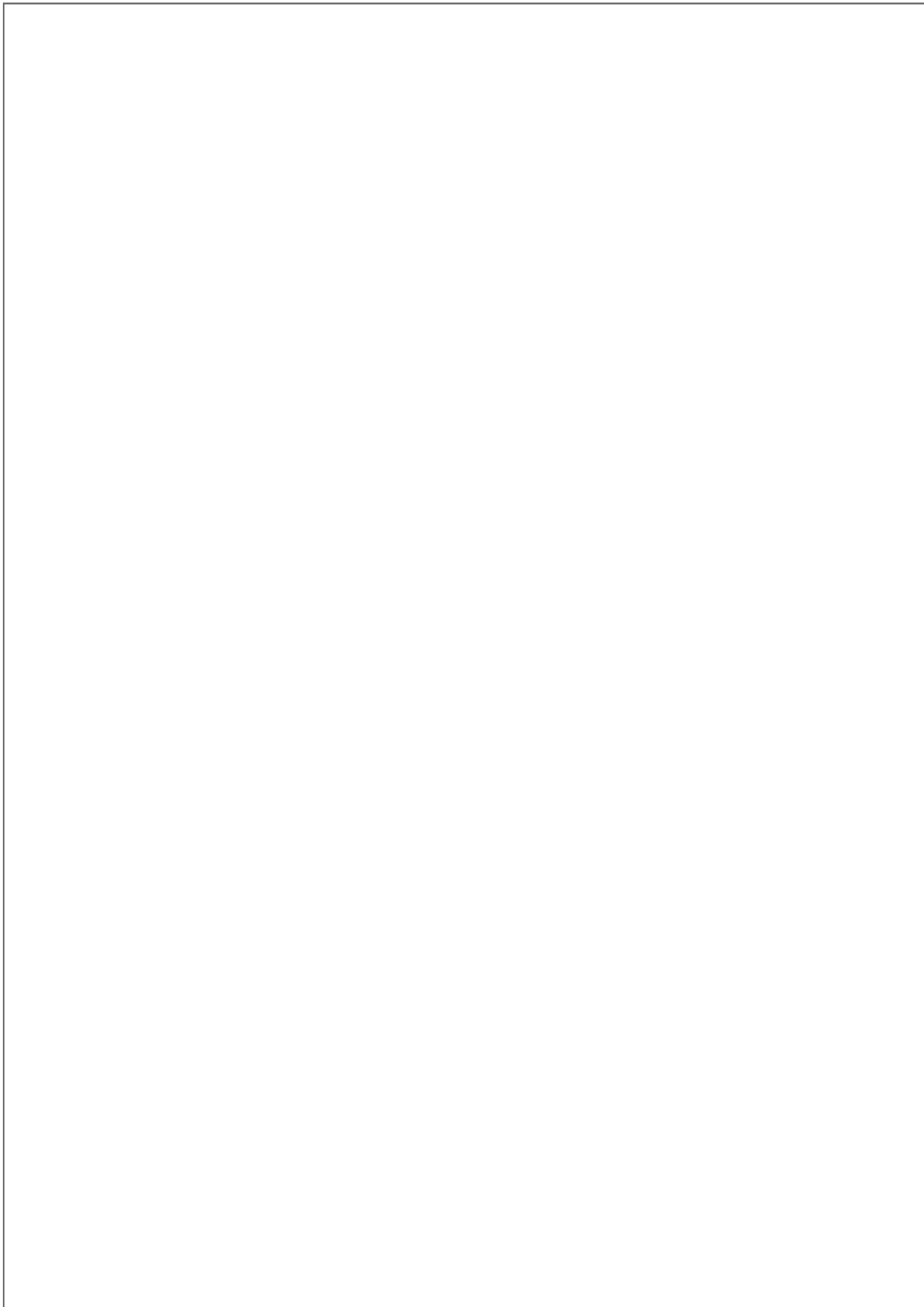
reduction and an overall economic loss, which is a risk for further development of RAS. Genetic variation (Wolters, 2010), feeding and growth (Rowe and Thorpe, 1990), light exposure (Imsland et al., 2013), photoperiod (Good et al., 2015), water temperature and hormonal treatment (Vikingstad et al., 2008) are known to influence fish sexual maturation. Steroids occur and accumulate in RAS water (Good et al., 2014b; Mota et al., 2014) and, as demonstrated in the present study, pH but not water exchange influences steroid concentrations in RAS systems. Whether low pH promoted early puberty in rainbow trout would need to be examined in a longer study.

An added explanation for the higher cortisol and testosterone concentrations at low pH RAS may be related with 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 and 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) displays 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 systems.

Acknowledgments

6

Chemical communication in tilapia:

A comparison of *Oreochromis mossambicus* with *O. niloticus*

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Abstract

In allopatric speciation species differentiation generally results from different selective pressures in different environments, and identifying the traits responsible helps to understand the isolation mechanism(s) involved. Male Mozambique tilapia (*Oreochromis mossambicus*) use urine to signal dominance; furthermore, 5β-pregnane-3α,17α,20β-triol-3α-glucuronide (and its a-epimer, 5β-pregnane-3α,17α,20α-triol-3α-glucuronide), in their urine is a potent pheromone, the concentration of which is correlated with social status. The Nile tilapia (*Oreochromis niloticus*) is a close relative; species divergence probably resulted from geographical separation around 6 million years ago. This raises the question of whether the two species use similar urinary chemical cues during reproduction. The olfactory potency of urine, and crude extracts, from either species was assessed by the electro-olfactogram and the presence of the steroid glucuronides in urine from the Nile tilapia by liquid-chromatography/mass-spectrometry. Both species showed similar olfactory sensitivity to urine and respective extracts from either species, and similar sensitivity to the steroid glucuronides. 5β-pregnane-3α,17α,20β-triol-3α-glucuronide was present at high concentrations (approaching 0.5 mM) in urine from Nile tilapia, with 5β-pregnane-3α,17α,20α-triol-3α-glucuronide present at lower concentrations, similar to the Mozambique tilapia. Both species also had similar olfactory sensitivity to estradiol-3-glucuronide, a putative urinary cue from females. Together, these results support the idea that reproductive chemical cues have not been subjected to differing selective pressure. Whether these chemical cues have the same physiological and behavioural roles in *O. niloticus* as *O. mossambicus* remains to be investigated.

Introduction

The Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*Oreochromis niloticus*) are maternal mouth-brooding African cichlids of enormous scientific and economic importance, both in aquaculture and – as a direct consequence – as invasive species (particularly in Asia, Australia and North and South America) (Lowe et al., 2012; Russell et al., 2012; Sanches et al., 2012). The Mozambique tilapia has also proven to be an excellent model species for teleost reproduction, due to its widespread availability, robustness and its highly developed courtship and dominance behaviours (Baerends and Baerends-van Roon, 1950). During the spawning season, the males congregate in 'leks' wherein they establish a social hierarchy, and dig and defend pits in the substrate; the more dominant males occupy the pits closer to the centre of the lek. Ripe females then visit these leks, choose one or more males with which to spawn, and incubate the fertilized eggs in their mouths away from the males (Turner, 1986). Despite – or, perhaps, because of – the clear importance of vision in cichlid behaviour and speciation (Seehausen et al., 1999; Kocher, 2004; Seehausen et al., 2008), little work has addressed the possible role of chemical cues in these processes.

We have previously shown that male Mozambique tilapia urinate at high frequency immediately before aggressive male–male encounters and during courtship, and that the urine from dominant males is more potent an odorant than that from subordinate males (Miranda et al., 2005; Barata et al., 2007; Barata et al., 2008). The urinary bladders of dominant males are larger and more muscular than those of subordinate males, and females; an apparent adaptation to allow storage of larger volumes of urine for release in the appropriate social context (Keller-Costa et al., 2012) Furthermore, exposure to male urine evokes an increase in 17,20β-P (the oocyte maturation-inducing steroid) (Nagahama, 1987, 1997) metabolism in females (Huertas et al., 2014), whereas prevention of urination results in higher aggression in male–male encounters (Keller-Costa et al., 2012). Together, this evidence strongly suggests that males are signaling both to rival males and potential female mates via (a) urinary pheromone(s). This hypothesis has been strengthened by the recent identification of 5β-pregnane-3α,17α,20β-triol-3α-glucuronide and 5β-pregnane-3α,17α,20α-triol-3α-glucuronide in the urine of males, the concentration of which depends on social status of the donor and which also act as potent pheromones on females (Keller-Costa et al., 2014). Steroid glucuronides have been shown to play pheromonal roles in the reproduction of several fish species (reviewed by; Stacey and Sorensen (2006); Stacey and Sorensen (2009)). However, how – or even if – species specificity is conferred to the pheromonal message largely remains unclear (Stacey, 2010b; Levesque et al., 2011; Lim and Sorensen, 2011). The African cichlids have generated great interest in evolutionary biologists because of

the speciation 'explosion' that occurred in this group in the East African lakes around two million years ago (for example, see; (Kocher, 2004; Seehausen et al., 2008; Schwarzer et al., 2009). Given their often dazzling colouration and patterning, much of the focus has been on visual signaling as part of speciation and reproductive isolation mechanisms (Seehausen et al., 1999). However, growing attention has recently been paid to the role of olfactory cues in reproductive isolation (for example, see (Plenderleith et al., 2005; Blais et al., 2007; Smadja and Butlin, 2009)). The Mozambique and Nile tilapia are thought to have diverged, presumably through geographical separation, around 6 million years ago (Genner et al., 2007). It is therefore reasonable to hypothesize that selective pressure to evolve different communication strategies during reproduction must have been weak or absent, and the chemical cues used by the two species are likely to be the same. Specifically, here we wished to test: (i) whether both species have similar olfactory sensitivity to male urine from the other species as their own; (ii) whether urine from male Nile tilapia contains the same steroids as those previously identified in Mozambique tilapia and; (iii) if so, whether the two species have the same olfactory sensitivity to these steroids.

Materials and methods

Fish

Fish care and experimentation complied with the guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals under a ''Group-1'' license issued by the Veterinary General Directorate of the Ministry of Agriculture, Rural Development and Fisheries of Portugal. Mozambique tilapia (60–200 g) were taken from a self-propagating population kept in the fish-holding facilities at the University of the Algarve. Nile tilapia (200–500 g) were transported from the experimental hatchery of Wageningen University 'De Haar Vissen' (Wageningen, The Netherlands) and kept in similar conditions at the University of the Algarve. Both species were kept at 27 °C under a 12L:12D photoperiod and fed daily with commercial cichlid feed (Sparos Lda., Portugal).

Urine collection

Social groups of either species (three males and six to eight females) were established. Regular observations (three times per week for two weeks prior to urine collection) were taken to identify the dominant male in each group – black colouration in the Mozambique tilapia, white colouration in the Nile tilapia, occupation and defence of a nest or floor area in both species. Urine samples were then taken from the dominant male from each tank by gently squeezing the abdomen immediately above and anterior to the genital papilla, and collecting the urine directly into a glass vial. Successive

samples obtained from each male were frozen until at least 1.0 ml had been taken. A pool of 6 ml was then made using equal volumes from each male, and 3 ml subjected to solid-phase extraction (C18 cartridges Waters 'Sep-Pak®', Waters Corporation, Milford, MA, USA). Retained substances were eluted with 3 ml methanol and both unretained '(aqueous fraction') and retained ('eluate') were aliquotted and stored at -20 °C until use. The remaining pool of 3 ml of untreated urine from each species was also aliquotted and frozen. Immediately prior to use in EOG recording (see below), samples were thawed and diluted in charcoal-filtered tap-water.

Steroid glucuronides

5β-Pregnane-3α,17α,20α-triol-3α-glucuronide (20α-P-3-G) and 5β-pregnane-3α,17α,20β-triol-3α-glucuronide (20β-P-3-G) were synthesized from the precursor 3α,17α-dihydroxy-5β-pregnan-20-one as described in (Keller-Costa et al., 2014). 3α,17α-dihy-droxy-5β-pregnan-20-one-3α-glucuronide (20one-P-3-G) and 17β-estradiol-3-glucuronide were bought from Steraloids Inc. (Newport, RI, USA). All steroids (10^{-3} M) were dissolved in ethanol or ethanol:water (50:50) and stored at -20 °C until use. Steroids were diluted to the appropriate dilution in charcoal-filtered tap-water immediately prior to use in electro-olfactogram (EOG) recording (see below). A solution of 10^{-5} M L-serine was similarly prepared from 10^{-3} M aliquots stored at -20 °C.

Recording the electro-olfactogram

Mature tilapia, of both sexes, were anaesthetized with NaHCO3- buffered MS222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich) in water (200 mg l^{-1}), immobilized with 3 mg kg $^{-1}$ gallamine triethiodide (Sigma–Aldrich) and the EOG recorded as previously described in detail (Frade et al., 2002). All odorants were presented as a 4 s pulse in order of increasing concentration with at least 1 min between stimuli. The EOG amplitude was measured (in mV) from the baseline to the peak of the initial downward deflection of the trace. This was blank-subtracted (blank water – the same water used to dilute the stimuli – given as a stimulus) and normalized to the response to 10^{-5} M L-serine, similarly blank-subtracted. For the urine and respective fractions, linear regression was applied to a plot of normalized EOG amplitude against log(dilution), using only concentrations giving responses significantly greater than blanks. The calculated thresholds of detection (intercept on the x axis) and slopes were compared by Student's t test (paired within species and unpaired between species) and corrected for multiple testing using the False Discovery Rate method (Q < 0.05) of Benjamini and Hochberg (1995). For the steroids, normalized data were fitted to a three-parameter Hill plot, and the derived Imax (maximal response amplitude) and EC50 ('half maximal effective concentration', or concentration of odorant required to give a response 50% of the maximum) values compared by Student's t test (paired within species and unpaired

between species). A P value of less than 0.05 was taken as significant. Data are shown as mean ± S.E.M.

Liquid-chromatography/mass-spectrometry

The LC–MS system was an Agilent Technologies 1200 Series LC coupled to a Bruker Daltonics HCT ultra (ion trap), able to carry out MSn, n = 11. The spray and ion optics conditions were the follow- ing: ionization, negative polarity; capillary voltage, 3.5 kV; drying gas (nitrogen), 330 °C at 10 L/min; nebulizer gas pressure, 50 psi; capillary exit voltage, 130 V; skimmer voltage, 40 V. A Hamilton PRP-1 reversed phase LC column (15.0 cm length, 2.1 mm internal diameter, 5 lm average particle diameter), stabilised at 25 °C was used. The eluent system was acetonitrile (A) and water (B), both with 0.1% of formic acid. The gradient started with 20% of A, followed by a linear increase up to 80% in 20 min. In a second gradient step an increase up 100% took place in 5 min. A final cleaning step using 100% of A during 5 min was made after each run. The eluent was then allowed to recover the initial conditions (20% of A and 80% of B) in 1 min and then stabilise for an additional 6 min before the next run.

Results

Olfactory responses to conspecific and heterospecific urine

Consistent with previous studies, the urine of dominant male Mozambique tilapia evoked strong EOG responses in males of the same species (Fig. 1), with an estimated threshold of detection of $1:10^{6.04\pm0.10}$ (Fig. 1C). However, urine from Nile tilapia evoked similar-sized responses, resulting in a similar concentration–response curve with similar slopes and threshold of detection ($1:10^{6.00\pm0.06}$). Conversely, Nile tilapia were slightly less sensitive to conspecific urine than that from Mozambique tilapia (P < 0.05); the threshold of detection for conspecific urine was $1:10^{5.16\pm0.04}$, whereas that of heterospecific urine was $1:10^{5.98\pm0.08}$ (Fig. 1D). The slopes could not be compared between species, as the relatively smaller response to L-serine in Nile tilapia resulted in larger (approximately twofold) normalized responses than in Mozambique tilapia.

In the Mozambique tilapia, there were no significant differences of EOG responses to the eluate of conspecific urine and those of the eluate of Nile tilapia urine (Fig. 2A); thresholds of detection were $1:10^{6.07\pm0.15}$ for conspecific urine and $1:10^{-5.94\pm0.11}$ for heterospecific urine, and slopes were similar.
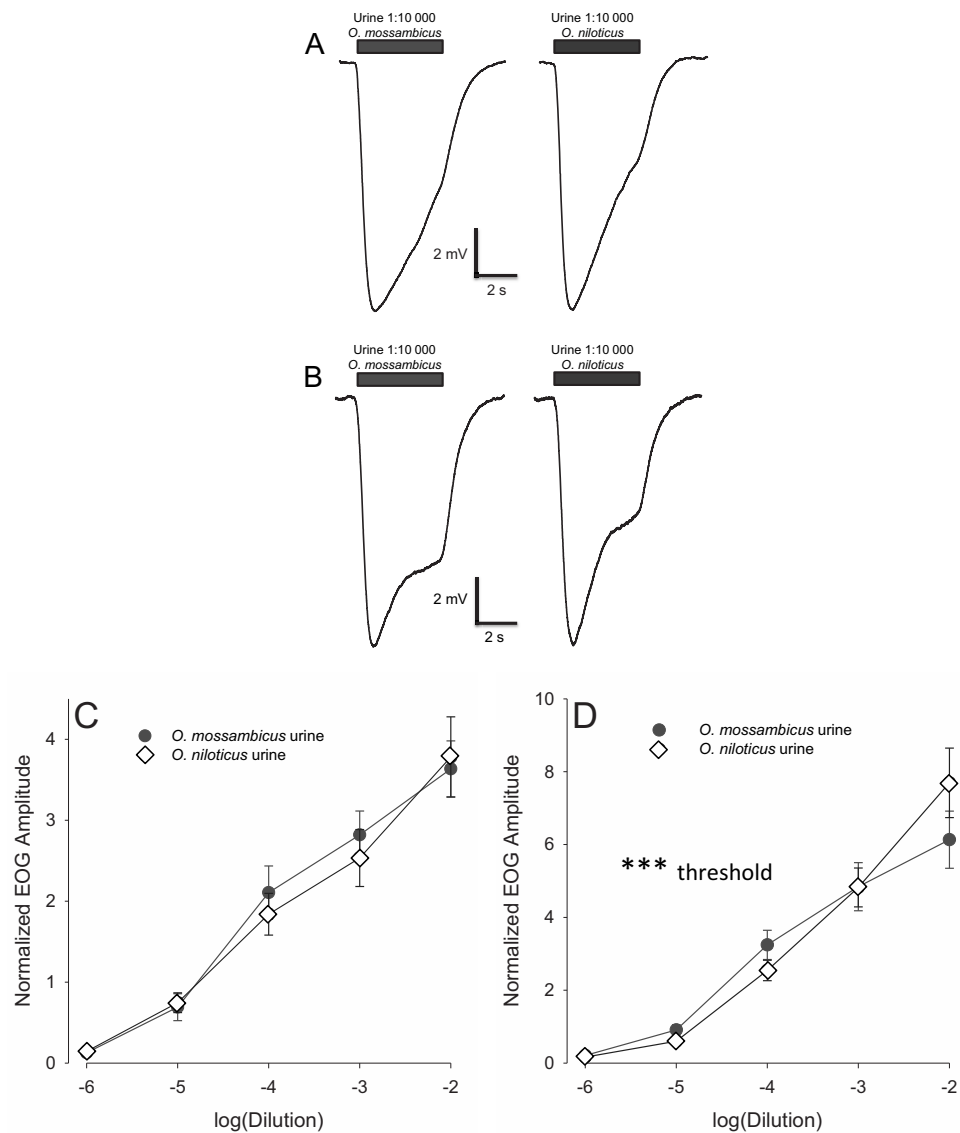
Figure 1. Olfactory responses of tilapia to conspecific and heterospecific urine. Typical electro-olfactogram (EOG) responses of (A) Mozambique and (B) Nile tilapia in response to dilutions of urine pool (diluted 1:10,000) from male Mozambique (*O. mossambicus*) and Nile (*O. niloticus*) tilapia. Semi-logarithmic plots of normalised EOG responses of the Mozambique tilapia (C) and Nile tilapia (D) to dilutions of untreated male urine from Mozambique tilapia (grey circles) and Nile tilapia (white lozenge). Data are shown as mean ± S.E.M. (N = 7); ***P < 0.001 comparing thresholds calculated from linear regression of individual semi-logarithmic plots.

In the Nile tilapia – in contrast to the whole urine – the eluates of both species proved to be equally potent (Fig. 2B); thresholds of detection were $1:10^{5.76\pm0.04}$ for conspecific eluate and $1:10^{5.83\pm0.09}$ for heterospecific eluate, and slopes were equal (whereas, again, the inter-specific difference was maintained).

However, in the Mozambique tilapia, the aqueous fraction of conspecific urine proved to be slightly more potent than that of the heterospecific aqueous fraction (Fig. 3A); the threshold of detection was $1:10^{5.77\pm0.013}$ compared to $1:10^{5.39\pm0.05}$ for the aqueous fraction from Nile tilapia urine, although this just failed to reach significance. The slopes were again equal. Interestingly, this pattern was repeated in the olfactory responses from Nile tilapia (Fig. 3B); the aqueous fraction of urine from Mozambique tilapia was significantly more potent than that of conspecifics. Thresholds of detection were $1:10^{4.79\pm0.03}$ for the aqueous fraction of conspecific urine and $1:10^{5.21\pm0.11}$ for heterospecific ($P < 0.01$).

Liquid chromatography/mass spectrometry

The urine pool from both species showed a major peak at 9.37 min, showing m/z 511 under negative polarity (Fig. 4). Both species also showed a minor peak at 8.94 corresponding to an isomer compound (also m/z 511). Based on previous studies with Mozambique tilapia and on the analysis of authentic reference compounds, we assign these signals to 20β-P-3-G and 20α-P-3-G, respectively (Fig. 4). The estimated concentration for 20β-P-3-G in both species approached 0.5 mM, consistent with previously published data for the Mozambique tilapia (Keller-Costa et al., 2014). This strongly suggests that both stereoisomers are also present in the urine of male Nile tilapia, at a similar ratio, and at similar concentrations. Although other, minor, peaks were seen in both species, none of these coincided with that of the standard for 20one-P-3-G; indicating this compound is not, therefore, a normal constituent of tilapia urine (Fig. 4).

Olfactory sensitivity to 20α-P-3-G and 20β-P-3-G

Consistent with our previous study Keller-Costa et al. (2014), Mozambique tilapia had olfactory sensitivity to both 20α-P-3-G and 20β-P-3-G (Fig. 5A). Both steroids evoked sigmoidal concentration–response curves, with thresholds of detection around 10^{-9} M, and reaching a plateau at 10^{-6} M. In Nile tilapia, similar sigmoidal concentration–response curves were evoked (Fig. 5B), with similar thresholds and plateaus. In both species, there was a tendency for 20α-P-3-G to evoke a slightly higher apparent Imax than 20β-P-3-G (Fig. 5C), but this failed to reach statistical significance. As with urine, the normalized responses were larger in Nile tilapia than in Mozambique tilapia.
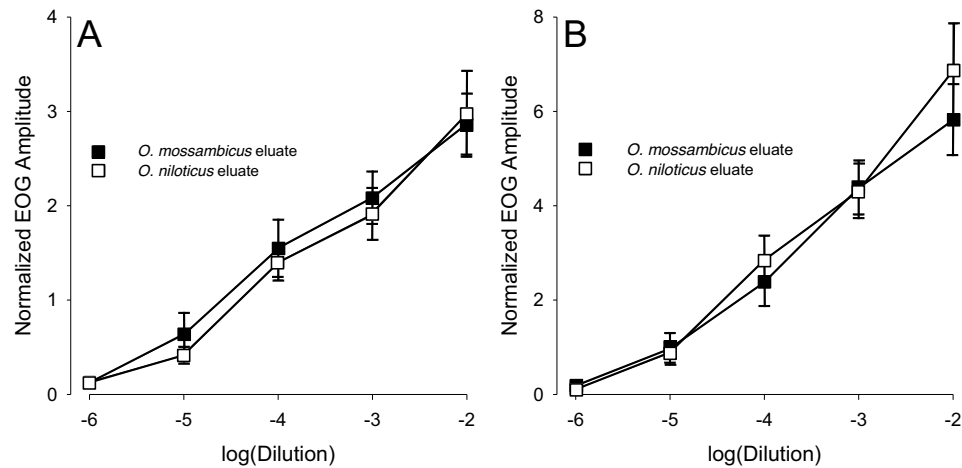
Figure 2. Semi-logarithmic plots of normalised EOG responses of the Mozambique tilapia (A) and Nile tilapia (B) to dilutions of the eluate of solid-phase extracts of male urine (non-polar/hydrophobic fraction) from Mozambique tilapia (black squares) and Nile tilapia (white squares). Data are shown as mean ± S.E.M. (N = 7); there are no statistical differences between the two stimuli in either species.
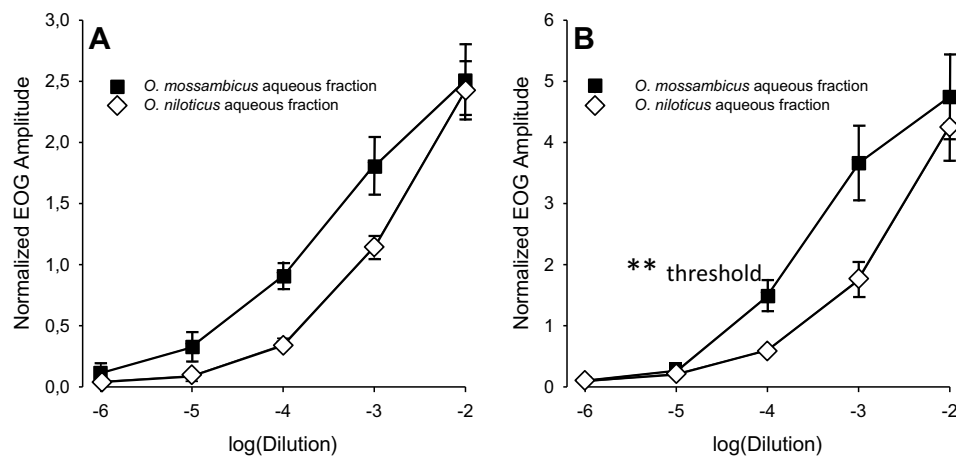

Figure 3. Semi-logarithmic plots of normalised EOG responses of the Mozambique tilapia (A) and Nile tilapia (B) to dilutions of the aqueous fraction of male urine from Mozambique tilapia (black squares) and Nile tilapia (white lozenges). Data are shown as mean ± S.E.M. (N = 7); $^{**}P < 0.01$ comparing thresholds calculated from linear regression of individual semi-logarithmic plots.

More importantly, however, the apparent EC50 values were similar in both species (Fig. 5D); in both the Mozambique tilapia, the apparent EC50 for 20β-P-3-G (21.8 ± 6.1 nM) was significantly lower than that of 20α-P-3-G (153.3 ± 49.1 nM) and in the Nile tilapia the apparent EC50 for 20β-P-3-G (46.1 ± 11.8 nM) was significantly lower than that of 20α-P-3-G (158.2 ± 31.1 nM).

Olfactory sensitivity to 20α-P-3-G and 20β-P-3-G

Consistent with our previous study Keller-Costa et al. (2014), Mozambique tilapia had olfactory sensitivity to both 20α-P-3-G and 20β-P-3-G (Fig. 5A). Both steroids evoked sigmoidal concentration–response curves, with thresholds of detection around 10^{-9} M, and reaching a plateau at 10^{-6} M. In Nile tilapia, similar sigmoidal concentration–response curves were evoked (Fig. 5B), with similar thresholds and plateaus. In both species, there was a tendency for 20α-P-3-G to evoke a slightly higher apparent Imax than 20β-P-3-G (Fig. 5C), but this failed to reach statistical significance. As with urine, the normalized responses were larger in Nile tilapia than in Mozambique tilapia. More importantly, however, the apparent EC50 values were similar in both species (Fig. 5D); in both the Mozambique tilapia, the apparent EC50 for 20β-P-3-G (21.8 ± 6.1 nM) was significantly lower than that of 20α-P-3-G (153.3 ± 49.1 nM) and in the Nile tilapia the apparent EC50 for 20β-P-3-G (46.1 ± 11.8 nM) was significantly lower than that of 20α-P-3-G (158.2 ± 31.1 nM).

Olfactory sensitivity to 20one-P-3-G and estradiol-3-G

Although 20one-P-3-G is not present in male urine of either Mozambique or Nile tilapia, it is commercially available, whereas 20α-P-3-G and 20β-P-3-G are not. Nevertheless, the Mozambique tilapia had olfactory sensitivity to it, giving sigmoidal concentration–response curves (Fig. 6A). Estradiol-3-G, another 3-glucuronide steroid, also evoked sigmoidal concentration–response curves, but never as large amplitude EOGs as the other steroid glucuronides tested. Similar olfactory sensitivity to 20one-P-3-G and estradiol-3-G was seen in the Nile tilapia (Fig. 6B); both evoked sigmoidal concentration–response curves, but the normalized amplitudes of EOG responses were much larger for 20one-P-3- G than estradiol-3-G. In both species, the Imax evoked by 20one-P-G was similar to that of 20α-P-3-G and 20β-P-3-G (Fig. 6C), whereas that of estradiol-3-G was significantly lower. Nevertheless, the ratio between the two was similar in the two species. Despite the relatively low amplitude of responses evoked by estradiol-3- G, this steroid was detected with the lowest apparent EC50 values (Mozambique, 0.25 ± 0.12 nM; Nile, 0.44 ± 0.16 nM; Fig. 6D). Apparent EC50 values for 20one-P-3-G and estradiol-3-G were similar between the two species. The apparent Hill coefficients for all steroids were around one in both species.
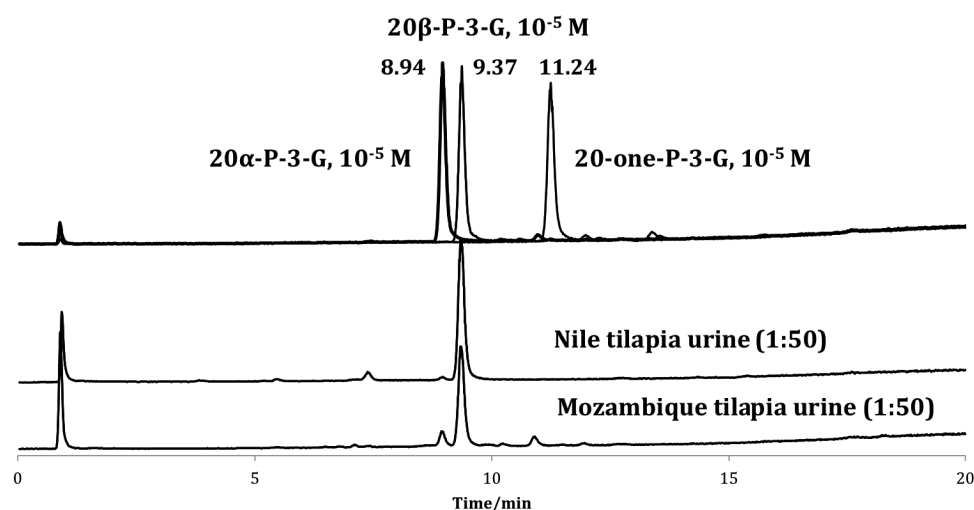
Figure 4. Representative LC/MS traces of male urine (diluted 1:50) from Mozambique tilapia (red) and Nile tilapia (blue) showing the major peaks which coincide with 5β -pregnan-3α,17α,20α -triol-3β -glucuronide (upper pink trace) and minor peaks that coincide with 5β -pregnan-3α,17α,20α -triol-3α -glucuronide (upper green trace) standards. The chromatogram for the 5β -pregnan-3α,17α,20one-triol-3α -glucuronide (upper purple trace) is also shown; no equivalent peaks are seen in the urine from either species. Numbers in black refer to retention times (min).

Discussion

Olfactory responses to male urine

The current study shows that urine taken from dominant males of either Mozambique or Nile tilapia is a potent odorant for conspecifics. For the Mozambique tilapia, this agrees with our previous studies (Frade et al., 2002; Barata et al., 2007; Barata et al., 2008; Keller-Costa et al., 2014). However, this is a novel observation for the Nile tilapia. Furthermore, we have shown that, despite geographic isolation, the urine from one species is equally potent, if not more so, to the other. Solid-phase extracts (the non-polar/ hydrophobic components) of male urine from either species evoked similar responses in both. This does not necessarily mean, however, that the active compounds are the same. Conversely, the polar/hydrophilic components remaining in the filtrate proved to be more potent in the urine of the Mozambique tilapia than the Nile tilapia, irrespective of the species of the receiver. This may not mean that the odorants involved are the same, but it is suggestive that urinary odorants released by the two species may differ significantly in this fraction; could this be the fraction wherein cues concerning species identity are found?

Steroid glucuronides in tilapia urine

We have previously identified 20α-P-3-G and 20β-P-3-G as components of the urinary pheromone in male Mozambique tilapia. 20β-P-3-G is more abundant than the 20α-P-3-G, at a ratio of approximately 15:1 (although there is considerable inter-individual variation; (Keller-Costa et al., 2014). In dominant males, the urinary concentration can reach as high as 0.5 mM, an exceptionally high concentration for any steroid in any fluid, suggesting an active transport and/or concentrating mechanism in the renal system of both species. The current study has shown that both steroids are present at similar concentrations and at a similar ratio in the urine from dominant Nile tilapia, suggesting that the olfactory potency of the eluate fraction of both species may be due mainly to these two steroids. The 20keto form is not, apparently, present in the urine from either species, although both species have high olfactory sensitivity to it. This steroid, however, has been identified as a component of the male pheromone of the African catfish (van den Hurk and Resink, 1992).

Olfactory sensitivity to steroid glucuronides in tilapia

20α-P-3-G and 20β-P-3-G are potent odorants for the Mozambique tilapia (Keller-Costa et al., 2014). Both evoke sigmoidal concentration–response curves when olfactory activity is assessed by EOG; this is unusual, as most 'conventional' fish odorants, such as amino acids or bile acids, evoke linear or exponential semi-logarithmic concentration–response curves (for example, see; (Hara, 1994; Zhang and Hara, 2009; Hubbard et al., 2011). Nevertheless, the dynamic range of olfactory sensitivity to these steroids in tilapia lies approximately between 10^{-9} and 10^{-6} M, corresponding to a dilution of 1:500–1:500,000 of crude urine. This fits well with the observed olfactory sensitivity to untreated urine, and its corresponding C18 eluate, and can explain behavioural and physiological pheromonal effects (both during courtship/reproduction and male–male aggression; (Barata et al., 2007; Barata et al., 2008; Huertas et al., 2014; Keller-Costa et al., 2014), which typically take place at close range. However, the olfactory sensitivity is insufficient to propose a long-range role for this urinary pheromone, such as that proposed for the sea lamprey (Li et al., 2002; Sorensen et al., 2005). Nevertheless, it is also clear that there are other components in the urine that both species can smell. What are these components, and what is their role?

Given that the two steroid glucuronides are present in similar concentrations in the urine of both species, it is interesting to note that the aqueous filtrate fractions evoke different responses; the urine filtrate from Mozambique tilapia has higher olfactory activity than that of the Nile tilapia, irrespective of the receiver species.
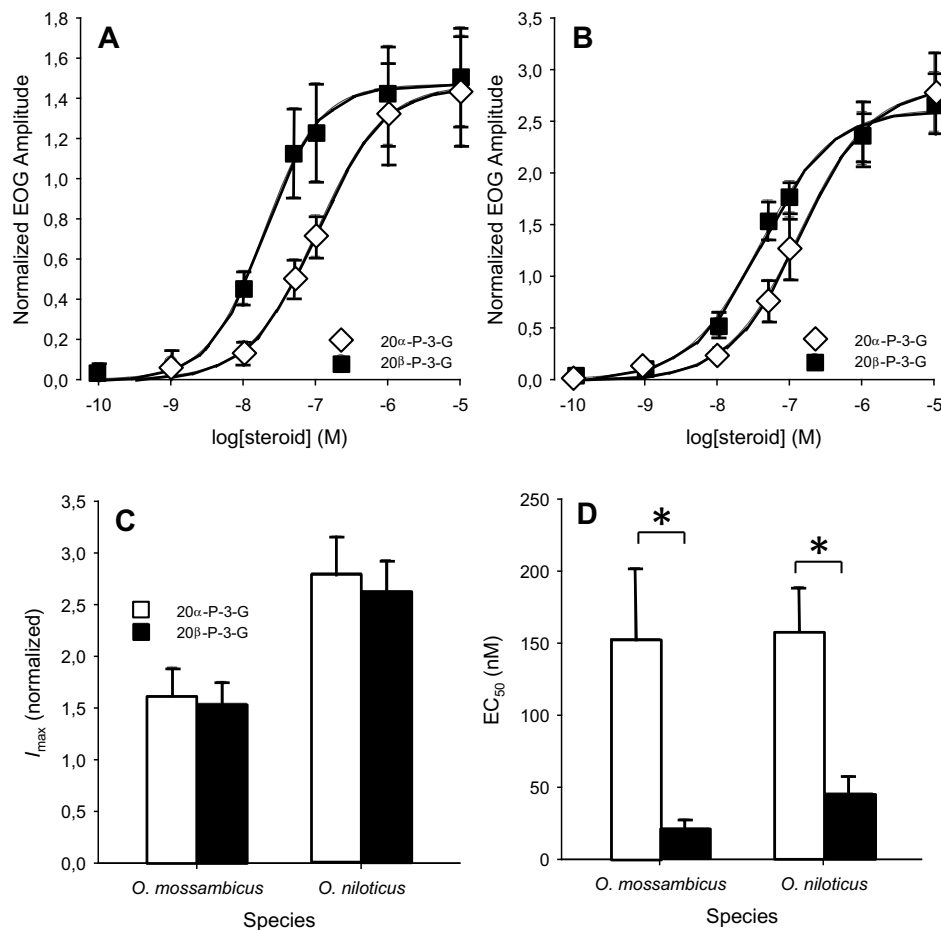
Figure 5. Olfactory sensitivity to urinary steroid glucuronides in the Mozambique and Nile tilapia. Semi-logarithmic plot of normalised EOG amplitude against concentration of 20α -P-3-G (white) and 20β -P-3-G (black) in the Mozambique tilapia (A) and Nile tilapia (B). The apparent I_{max} values are similar for the two steroids, independently of species but larger in the Nile than Mozambique tilapia (C), whereas the apparent EC_{50} values are significantly lower for 20β -P-3-G than 20α -P-3-G in both species, but similar between species (D). Data are shown as mean ± S.E.M. (N = 7); *P < 0.05.
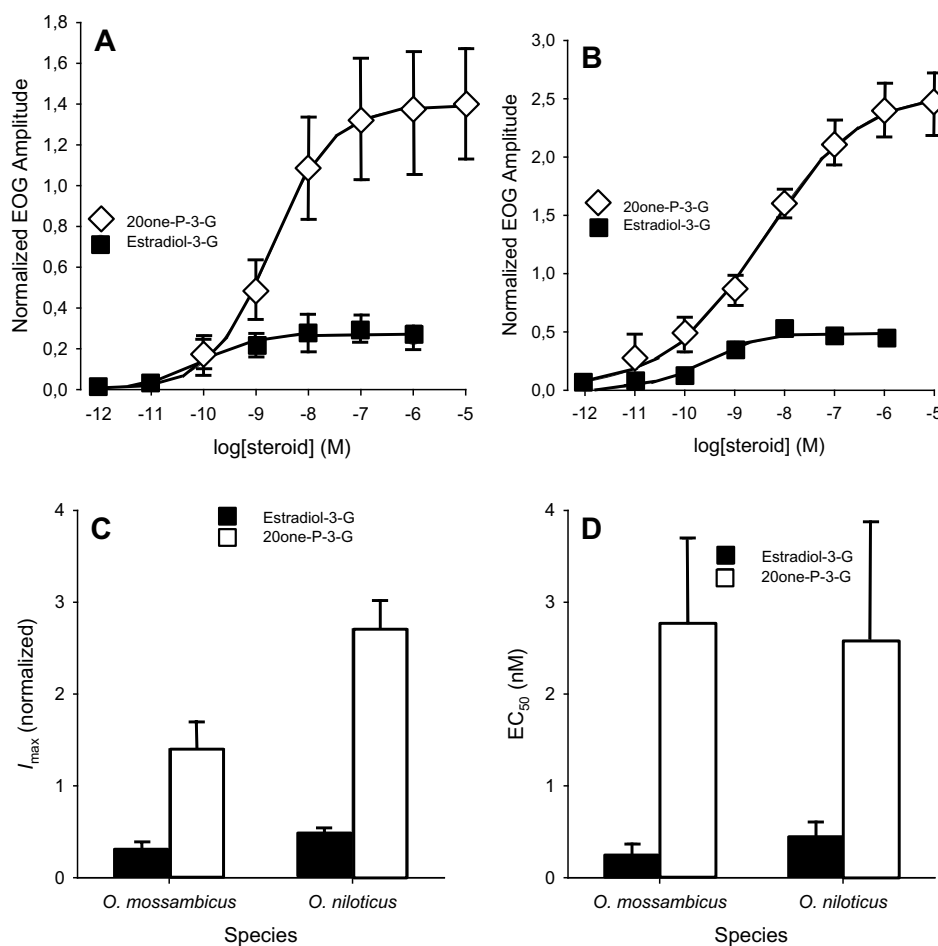
Figure 6. Olfactory sensitivity to steroid glucuronides in the Mozambique and Nile tilapia. Semi-logarithmic plot of normalised EOG amplitude against concentration of 20one- P-3-G (white) and estradiol-3-G (black) in the Mozambique tilapia (A) and Nile tilapia (B). The apparent Imax values markedly different for the two steroids, independently of species but, again, larger in the Nile than Mozambique tilapia (C), whereas the apparent EC_{50} values are significantly lower for estradio-3-G than 20one-P-3- G in both species, but similar between the two species (D). Data are shown as mean ± S.E.M. (N = 7); *P < 0.05.

It is possible that the two species are sensitive to different components in this fraction, but – given the similarity of the concentration– response curves between the two species – it is more likely that they are detecting the same compounds, and that these compound differ in concentration between the two species. This suggests that species-specificity may be conferred to the pheromonal message by odorants in this fraction (as shown in cyprinids; (Levesque et al., 2011; Lim and Sorensen, 2011). Clearly, the identities of these compounds need to be established before this can be tested. However, evidence also suggests that hydrophilic urinary components – possibly trimethylamine – play a role in the communication of social status in the fathead minnow (Martinovic-Weigelt et al., 2012).

Since the work of Crapon de Caprona (1980), chemical cues have been known to be important to cichlids. For example, urination rates increase in different social contexts in male *Astotilapia burtoni* (a mouth-brooding cichlid from Lake Tanganyika) in a similar way to the Mozambique tilapia (Maruska and Fernald, 2012). However, the identity of the odorant(s) involved is not yet known. Using a different approach, Cole and Stacey (2006) showed that *A. burtoni* had olfactory sensitivity to some conjugated steroids (both glucuronides and sulphates at the 3 and 17 positions) and the authors suggest five distinct olfactory receptor mechanisms to account for this. Given that we putatively have identified only two olfactory receptor mechanisms in the Mozambique tilapia, both detecting 3-glucuronide steroids, it is interesting to speculate that the species radiation in Lake Tanganyika (and other African lakes) involved the evolution of olfactory sensitivity to a greater range of steroid conjugates. Thus, investigations into the role(s) of chemical communication in reproductive isolation and species radiation in African cichlids would be of interest.

In conclusion, the current study has shown that the same urinary steroid glucuronides are present in the urine of male Mozambique and Nile tilapia, and that both species have similar olfactory sensitivity to these steroid glucuronides. Whether the two tilapia species interpret these chemical messages in the same way, however, remains to be investigated. Furthermore, the role of chemical communication in cichlid species radiation should be addressed.

Acknowledgments

7
CHAPTER

General discussion

Water quality is critical for aquaculture systems to attain fish good health conditions and maximize fish growth. This is especially relevant for systems where water is filtered and re-used, such as recirculating aquaculture systems (RAS), where closely monitoring the accumulation of substances in the water is of utmost importance. Indeed, it was first shown in this thesis that substances accumulate in nearly-closed RAS with negative consequences for fish performance, particularly feed intake and growth (**Chapter 2**). While toxic substances such as ammonia and nitrite are known to accumulate in RAS systems and are, consequently, routinely monitored (Timmons and Ebeling, 2007), the presence of other substances such as steroids is still poorly studied. This motivated the experiment performed in **Chapter 3**, which demonstrates that hormonal steroids and metabolites accumulate in the rearing water of commercial RAS. Although it was shown that increasing fish stock density might lead to a build-up of steroids in water, information on steroids concentration was still scarce. This overlooked subject was addressed in **Chapters 4 and 5**, which suggest that reducing water exchange and low pH increase steroid concentration. Moreover, acute stressor events may lead to a significant release of steroids by fish that take up to 24 hours to be removed from the rearing water. Lastly, **Chapter 6** demonstrates that fish are olfactory sensitive to chemical cues present in the urine of other fish, particularly steroids released by closely related species. In summary, this thesis shows that substances accumulate in RAS, particularly steroids, up to levels that can be detected by fish, and with consequences for fish performance.

The work here described addressed some poorly explored questions regarding steroid accumulation in RAS, while its side effects remain to be investigated. This final Chapter explores the results of the previous Chapters and their implications for RAS operation, particularly to maximize treatment efficiency and its consequences for fish physiology. Its implications to aquaculture research and industry are also discussed together with suggestions for future research. Lastly, this chapter summarizes the main conclusions drawn from this thesis' research.

Accumulation of substances in RAS

Major developments on RAS technology during the last decades enabled culturing the wide range of species that are currently observed (Martins et al., 2010; Dalsgaard et al., 2013). Ongoing research on RAS treatment efficiency (denitrification reactors and ozone) and on better culture conditions for new species will foster RAS development in the near future. However, there are still challenges to overcome. Poor fish performance related to the accumulation of various substances in the rearing water of RAS (Deviller et al., 2005; Martins et al., 2009a; Martins et al., 2009b; Davidson et al., 2011b; van Bussel et al., 2012) is a major bottleneck that needs to be addressed. Unpublished

observations show that fish growth retardation becomes more severe with time, i.e. the longer a RAS is in operation, the stronger the effect. For instance, a study comparing European sea bass *Dicentrarchus labrax* cultured in RAS with flow-through systems (FTS) found that fish grown in RAS exhibited a 15% reduction compared to FTS after one year of culture (Deviller et al., 2005). More recently, it was found that common carp *Cyprinus carpio* eggs incubated with water from RAS operated at low water exchange rates reduced egg hatching, increased larval mortality and reduced larval development (Martins et al., 2009b). The same authors of this latter study also found that large Nile tilapia *Oreochromis niloticus* exhibit growth retardation in nearly closed recirculating systems (Martins et al., 2009a).

Despite evidence of fish growth retardation in RAS, the cause of this poor performance remains an open question. In the current thesis, only Nile tilapia, one of the three species tested in **Chapter 2** (Nile tilapia, African catfish *Clarias Gariepinus* and European eel *Anguilla anguilla*) was affected by the RAS designed for maximal accumulation of system, feed- and fish-related substances as it showed reduced feed intake (42%) and growth (83%). As the conventional water quality parameters measured were within the recommended levels for all three species, it is likely that other water quality parameters that were not measured were differently affecting the three species. It is known that substances such as heavy metals (Deviller et al., 2005; Davidson et al., 2009; Martins et al., 2011a), dissolved CO_2 (Colt, 2006), suspended solids (Davidson et al., 2011a), and bacterial by-products that accumulate in RAS (Schrader and Summerfelt, 2010) can affect fish performance. It is also known that the use of low water exchange RAS increased the swimming speed of rainbow trout *Oncorhynchus mykiss* and resulted in a greater incidence of side swimming behaviour (Davidson et al., 2011b). Fish components, such as alarm pheromones can be released triggering behaviour changes in conspecifics sharing the same water (Smith, 1992; Keller-Costa et al., 2015). African catfish increases the swimming activity in response to chemical alarm cues in RAS water (van de Nieuwegiessen et al., 2009). Likewise, chemical alarm cues induced antipredator behaviours and stress responses in Nile tilapia, including decreased feeding activity and increased ventilation rate and cortisol level (Sanches et al., 2015).

This information is critical for the interpretation of the results observed in **Chapter 2**, as Nile tilapia the only species exhibiting growth retardation was also the species used to mimic water conditions with accumulation of fish related-substances (i.e. fish used in the culture tanks in the RAS designed for maximal accumulation of substances). The production and release of fish metabolites and excretory substances into the water was likely to occur in such crowding conditions. It is, therefore, possible that the culture tanks containing Nile tilapia in the RAS designed for maximal accumulation of

substances functioned as a source of fish-related substances and resulted in a poor performance of experimental Nile tilapia, but without negative consequences for feed intake and growth of the other tested species; could this mean that hormones and pheromones accumulate in RAS water? Are the consequences of hormones and pheromones accumulation species-specific?

It is not new that hormones and pheromones may accumulate in RAS. Initial studies pointed out that "unknown crowding factors" might accumulate in the water of RAS and trigger inhibition of spawning, changes in growth rate and slower heart rates (Yu and Perlmutter, 1970; Francis et al., 1974; Pfuderer et al., 1974). Although the modern technologies for substances identification (e.g. mass-spectrometry, nuclear magnetic resonance) were not as developed at the time those studies were performed (Yu and Perlmutter, 1970; Francis et al., 1974; Pfuderer et al., 1974), it was already pointed out that substances associated with crowding factors could be removed from water using organic solvents and activated charcoal and, therefore, would be of lipid nature, likely steroids. More recently, testosterone was measured in RAS water and it was observed that testosterone could be transferred from fish-to-fish (Budworth and Senger, 1993). It is important to note that the origin of testosterone in the latter study (Budworth and Senger, 1993) was exogenous, i.e. a portion of the fish were injected with testosterone and the others also responded to treatment. Nevertheless, this study showed for the first time that steroids could remain long enough in RAS water to allow their measurement. Additionally, it was demonstrated that steroids can be measured at very low concentrations (< 1 ng/L) from the effluent of flow-through systems (FTS); testosterone was detected in hatcheries of rainbow trout, Nimbus Chinook salmon *Oncorhynchus tshawytscha* and steelhead trout *Salmo gairdneri* (Kolodziej et al., 2004), and cortisol was measured in Atlantic salmon *Salmo salar* tanks (Ellis et al., 2007). Thus, knowing that artificially added steroids can stay long enough in RAS to be measured, and that fish produce enough steroids to be measured in FTS effluent, we asked ourselves whether fish related steroids accumulate in RAS.

Seven commercially operating RAS were sampled for cortisol, testosterone, 11-ketostestosterone (11-KT) and 17,20β-P in their free and conjugated forms and all hormones were detected (1 – 217 ng/L) in the rearing water of RAS. Note that this result is extremely robust since data came from a wide diversity of RAS from freshwater (e.g. African catfish) and seawater (e.g. turbot *Scophthalmus maximus*), as well as cold water (e.g. Dover sole *Solea solea*) and warm water (e.g. Nile tilapia), and from RAS of small dimension (system total volume: 7 m^3) and large dimension (2500 m^3). This provides notable evidence that steroids are present in the water of any RAS farm. However,

whether the source of steroids was fish, feed or make-up water still needed demonstration at this stage of work.

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 (Sower and Iwamoto, 1985; Pelissero et al., 1989). However, our estimations (**Chapter 3**), using the average feed load (152 kg/d) and standing stock (22,464 kg), together with cortisol release rates (0.5–5 ng/g/h) reported by Fanouraki et al. (2008), suggest that fish diets only account for 0.5% of cortisol input into RAS (diets 0.008 g/d vs. fish 1.483 g/d). Furthermore, measurements of water supply showed that steroids presence is negligible (<1 ng/L) (**Chapter 3**). These calculations lead to the conclusion that fish account for >99% of steroids in RAS. This is strongly supported by the notable increase on steroid concentration when water passes through the rearing units (**Chapter 3**), which shows that fish are excreting large amounts of free and conjugated (sulphate and glucuronide) steroids. Steroid excretion in fish depends 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 renal operation (Maren et al., 1968). Clearance of 17,20 β-P and its conjugate forms in rainbow trout take the following 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 (Vermeirssen and Scott, 1996; Ellis et al., 2005). Independently on the culture species, i.e. Nile tilapia, turbot, African catfish, pike-perch *Stizostedion lucioperca*, Dover sole, European eel and yellowtail amberjack *Seriola lalandi* free steroids, sulphate- and glucuronide-steroids were measured, which shows that the steroids clearance pathway is probably similar for all species. Our results are in line with findings from other authors where, for instance, the excretion of cortisol to the water was measured in several fish species, such as rainbow trout (Ellis et al., 2004), European sea bass, (Fanouraki et al., 2008) and three-spined stickleback (*Gasterosteus aculeatus*) (Sebire et al., 2007). The release of sex steroids such as testosterone, 11-KT and 17,20β-P into the water has also been shown in several species including goldfish (*Carassius auratus*), Nile tilapia and rainbow trout (Vermeirssen and Scott, 1996; Scott et al., 2010; Hubbard et al., 2014; Huertas et al., 2014).

It is now evident that steroids occur and accumulate in RAS which raises the question: which factors are responsible for steroids accumulation in RAS? It is known from wastewater literature that concentrations of steroids are associated with their physical-chemical properties and consequent propensity to be absorbed by sediment or to stay in the aqueous phase. Such propensity is usually indicated by the ratio log K_{ow}, octanol-water partition coefficient; the lower the ratio, the more likely it is for a molecule to

remain in the aqueous phase (Leszczynski and Schafer, 1990). Cortisol has a higher tendency to stay in water than testosterone (log K_{ow} cortisol 1.86 vs. log K_{ow} testosterone 3.27). This property of cortisol may explain the higher cortisol concentrations found in **Chapter 3, 4 and 5** compared to the sex steroids. The same pattern was observed in waste water treatment where androgens (e.g. testosterone), estrogens and progestagens were mainly removed by sorption and degradation, while the removal of glucocorticoids (e.g. cortisol) was primarily due to degradation (Liu et al., 2012). But how do RAS culture factors affect steroid accumulation in RAS? This motivated the work performed in **Chapter 3, 4 and 5**, where we correlate culture factors with steroid concentrations, and test the effect of water exchange rate, acute stress and water pH.

The most distinctive characteristic of RAS is the control of water re-used. Water exchange rate can range between 30 - 9000 L/kg feed (Shnel et al., 2002; Martins et al., 2009a; Roque d'orbcastel et al., 2009). This rate consequently affects the degree of accumulation of several substances in RAS, such as nitrate (Eding et al., 2006) and heavy metals (Davidson et al., 2009), among others. Therefore, one would also expect water exchange rate to affect steroid concentration in RAS. However, no correlations were observed between steroid concentrations in the effluent of rearing units and water exchange rate (**Chapter 3**). This may be due to low precision and stability of the water exchange rates that are used in commercial conditions. Nevertheless, testosterone concentrations were positively correlated with orthophosphate-P **(Chapter 3)**. Orthophosphate-P water concentrations are a measure of the degree of new refreshment water in RAS (Martins et al., 2009a). High testosterone concentration was also associated with low exchange rate RAS, whereas the concentration of others steroids, such cortisol and 11-KT, did not reveal the same pattern (Good et al., 2014a). In contrast to the latter study, **Chapter 4** shows that water cortisol concentrations were associated with water exchange rate, whereas such effect was inexistent for testosterone in RAS operating under the same conditions. In **Chapter 5**, neither cortisol nor testosterone were related with water exchange rate. Although the results outlined above clearly suggest that steroids are present in RAS water in higher concentrations as compared to refreshment water and FTS, the influence of water exchange rate on steroid concentration remains unclear. The higher organic load in the water of RAS, particularly humic acids, can contribute to increase steroid treatment efficiency as steroids are adsorbed onto the surface of the humic acid microvesicles (Hubbard et al., 2002).

Steroid concentration was significantly correlated with total ammonia nitrogen (TA-N) concentration (**Chapter 3**). This result is in accordance with the described association between testosterone and 11-KT with TA-N concentration in RAS (Good et al., 2014a).

TA-N (NH_3 -N + NH_4 -N) is a fish metabolite converted by bacteria into nitrite-N and then into nitrate-N in RAS biofilters (Eding et al., 2006). TA-N accumulation in RAS results from lower removal rates of nitrifying bacteria under sub-optimal conditions. This positive correlation between TA-N and steroids suggests that an unknown culture factor is affecting bacteria quantity or activity in RAS. Low water pH inhibits the action of nitrifying bacteria. In **Chapter 5**, RAS operating at low pH (5.6 – 6.0) displayed increased cortisol and testosterone as compared to RAS operating at neutral pH (7.2 – 7.4). The same low RAS also presented high TA-N (**Chapter 5**, **Table 2**), which suggests a possible effect of low pH on the accumulation of steroids in the rearing water. It is possible that the activity of bacteria is reduced at low pH conditions, which may consequently result in steroids accumulation in the rearing water. This raises the question whether bacteria have an active role removing steroids from RAS water. This is particular relevant since steroids are degraded by bacteria that are likely present in RAS, as is observed in aerobic (Horinouchi et al., 2004) and anaerobic (Fahrbach et al., 2010) wastewater treatments. Microbial transformation of testosterone was observed to occur in soil, stream sediments and within wastewater treatment units. Several bacteria can metabolize the steroid molecule into carbon dioxide and water. A denitrifying bacteria strain (*Steroidobacter denitrificans*) able to grow on testosterone as the sole source of carbon and energy and with nitrate as the electron acceptor has been identified (Fahrbach et al., 2008). It is expected that bacteria can remove a large part of the steroids produced by fish when favorable environmental conditions occur together with the high nitrate concentrations often observed in RAS (over 50 mg/L, **Chapter 3, Table 1**). Other rearing factors may also explain variation in water steroids concentration. For instance, higher stocking density was positively correlated with higher steroid concentrations in the water (**Chapter 3**). European sea bass kept at stocking density of 50 kg/m^3 increased both blood plasma concentrations and cortisol release rates into the water as compared to fish kept at 20 kg/m^3 (Fanouraki et al., 2008). Acute stressors such as fish handling and exposure to air may also lead to a temporary but significant increase of cortisol in RAS water (**Chapter 4**). While several culture factors may explain the presence of steroids in RAS, it is still unknown whether fish detect the observed steroid concentrations.

Fish detection of steroids

Steroids occur in RAS and their concentration depends on numerous variables. But what does it mean to the fish reproductive cycle, behaviour and ultimately growth performance? In summary, steroids in the water may interact with fish in two ways: i) re-uptake and ii) olfactory detection.

Fish can re-uptake steroids present in holding water which ultimately affects their hormone plasma levels (Scott et al., 2005; Miguel-Queralt and Hammond, 2008). Testosterone, 11-KT and, estradiol-17β administered to rainbow and brown trout *Salmo trutta* affected differently the plasma levels of adrenocorticotropic hormone (ACTH) and cortisol: estradiol-17β elevated ACTH and cortisol whereas 11-KT suppressed ACTH (Pottinger et al., 1996). Also, rainbow trout injected with testosterone, excreted testosterone into RAS water, which was later re-uptaken by other rainbow trout (not injected) that shared the same system (Budworth and Senger, 1993). The re-uptake mediated by steroid binding proteins (SBP) present in fish plasma was shown in common carp *Cyprinus carpio* (Chang and Lee, 1992), goldfish (Van Der Kraak and Biddiscombe, 1999) and tench *Tinca tinca* (Scott et al., 2005). However, not all steroids hold the same affinity to be taken up from the water. Tench has a high affinity to uptake androstenedione, testosterone, 17,20 β-P from the water, whereas no affinity was observed for cortisol (Scott et al., 2005). Thus, steroid re-uptake is selective and elevates the plasma levels of the uptake steroid but also affects the plasma levels of others steroids. However, whether fish can re-uptake steroids at the observed concentrations in RAS (1 – 217 ng/L, **Chapter 3, 4 and 5**) is questionable. It is not likely that in RAS fish are exposed to steroid concentrations as high as an implant containing 1 – 20 mg per fish (Budworth and Senger, 1993; Pottinger et al., 1996). Steroids in water are more likely to interact with fish through olfactory detection.

Fish reproductive cycle is regulated by a cascade of hormones along the brain–pituitary–gonad axis (Kime, 1993). Gonadotropin-releasing hormone (GnRH) stimulates the release of follicle stimulation hormone (FSH), which stimulates gametes growth (spermatogenesis and vitellogenesis) and the production of the luteinizing hormone (LH), which regulates gametes maturation (spermiation and oocyte maturation) (Mylonas et al., 2010). FSH and LH action stimulates synthesis of sex steroid hormones (androgens, estrogens and progestogens) in fish gonads. A good example of olfactory detection and physiological consequence is the role of the maturation inducing hormone, 17,20β-P, in goldfish (Scott et al., 2010). Ovaries of female goldfish produce 17,20β-P in response to LH stimulation, which promotes final oocyte maturation (Stacey and Sorensen, 2006). Subsequently, 17,20β-P conjugates (sulphate and glucuronide) are released into the water via urine (Scott and Sorensen, 1994). Male goldfish has extreme olfactory sensibility to 17,20β-P conjugates and these conjugates act as reproductive pheromones to male goldfish (Sorensen et al., 1990) by inducing LH production and thereafter spermiation (Stacey and Sorensen, 2006; Scott et al., 2010).

In **Chapter 6**, we found that Nile tilapia, females and males, detect a potent steroidal pheromone (5β-Pregnane-3α,17α,20α-triol-3α-glucuronide) (and its α –epimer) at

concentrations as low as ± 511 ng/L (10^{-9}M). These steroids were previously identified in the urine of dominant males of Mozambique tilapia, *Oreochromis mossambicus*. It was shown that females exposed to it develop a rapid, 10-fold, increase in the production of 17,20β-P (Keller-Costa et al., 2014). The perception of these steroids by the females primes their endocrine system to accelerate oocyte maturation and possibly promote spawning synchrony (Keller-Costa et al., 2015). It is likely that the two steroid conjugates (5β-Pregnane-3α,17α,20α-triol 3α-glucuronide and its α –epimer) are potentially present in RAS at the range of detection and capable to modulate fish behaviour and reproductive cycle. Considering that Nile and Mozambique tilapia, and goldfish and Atlantic salmon can detect steroids at least 0.3 ng/L (10^{-12} M) (Moore and Scott, 1991; Sorensen et al., 1995) we can assume that other fish species not yet studied have similar sensitivity to detect steroids at the concentrations found in RAS: 3.8–217.0 ng/L for cortisol, 3–12.5 ng/L for testosterone, 0.9–7.1 ng/L for 11-KT and 1.8–12.8 ng/L for 17,20 β-P (**Chapter 3, 4 and 5**).

Additionally, in **Chapter 6** Nile tilapia and Mozambique tilapia urine was filtrated into two fractions: i) an aqueous fraction containing hydrophilic substances, and ii) an eluate fraction containing hydrophobic substances, including the steroid two pheromones described above. Both tilapia species evoked similar olfactory responses to the eluate fraction, whereas aqueous filtrate fractions evoked different responses. Whether species-specificity may be conferred to the pheromonal message by odorants in this aqueous filtrate fraction remains to be tested, i.e. whether species-specific chemical communication may also be encountered in hydrophilic urine compounds. While chemical communication has been widely described for Mozambique tilapia (Frade et al., 2002; Barata et al., 2008; Hubbard et al., 2014; Keller-Costa et al., 2014; Keller-Costa et al., 2015), this information is new and limited for Nile tilapia, and its knowledge can bring some light to improve the culture of this important species.

Research implications and future directions

Water quality is a common and critical factor usually addressed when studying different factors to improve aquaculture production (Colt, 2006). Poor water quality limits fish feed intake (Timmons and Ebeling, 2007) thereby reducing growth (Paterson et al., 2003; Deviller et al., 2005; Colt, 2006). Consequently, several cleaning systems have been developed for RAS to improve water quality and maximize fish growth and conditions. Such cleaning approaches including mechanical filtration, biological filtration and more sophisticated filtration such as ultraviolet (UV) irradiation, ozonation (Summerfelt et al., 2009) or denitrification (van Rijn et al., 2006), are now commonly applied in RAS design. These cleaning systems can remove, or significantly reduce, several fish metabolites such as feaces, ammonia, nitrite, nitrate, CO_2, among

others. Further improvements in water cleaning should focus on additional metabolites that may impact fish performance under rearing conditions, such as steroids. Namely the biofiter type and its hydraulic retention time impact on treatment efficiency and steroids removal.

Interestingly, high levels of testosterone accumulate in 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 et al., 1985). One of its functions is to provide positive feedback for gonadotropin synthesis during early gametogenesis accelerating gonadal development (Dubois et al., 1998). Fish early sexual maturation results in growth and feed intake reduction, aggressive behaviour, reduced fillet quality, and an overall economic loss which is a risk for further development of RAS (Paaver et al., 2004; McClure et al., 2007; Taranger et al., 2010). Genetic variation (Wolters, 2010), feeding and growth (Rowe and Thorpe, 1990), light exposure (Imsland et al., 2013), photoperiod (Good et al., 2015), water temperature and hormonal treatment (Vikingstad et al., 2008) are known to influence sexual maturation in fish. Steroids occur and accumulate in RAS water (**Chapter 3, 4 and 5**) and as demonstrated in **Chapter 5,** pH but not water exchange influences steroid concentrations in RAS systems. Whether low pH was promoting early puberty in rainbow trout would need to be examined in a longer study.

RAS seem to have a treatment efficiency of >99%, i.e. over 99% of steroids produced by fish are likely degraded by system bacteria, and fixed in sludge and humic acids. However, a significant concentration of steroids can still be detected. Further research should focus on removing this 1% from larval rearing and fish ongrowing RAS, which may be solved by application of ozone (Summerfelt et al., 2009) or by activated sludge treatment (Liu et al., 2012). On the other hand, the presence of sex hormones and pheromones in RAS for broodstock may be useful for successful fish reproduction in captivity. However, critical steroid threshold is likely species-specific and is yet to be unraveled.

Main conclusions

The following main conclusions were drawn from this thesis as regards to the presence and accumulation of steroids in RAS:

- Intensification of fish production in RAS can result in sub-optimal rearing conditions and thus reduced fish performance;

- Steroids occur at higher concentration in RAS compared to FTS and refreshment water, independently on the fish species, water treatment units and water quality;

- Increased fish stocking density, occurrence of stressor events and decreased water pH are associated with high steroid concentrations and, reducing water exchange rates may induce accumulation of steroids in specific conditions;

- The fish olfactory system is extremely sensitive to steroids in the water, and the threshold of detection is within the range of steroid concentrations present in RAS water

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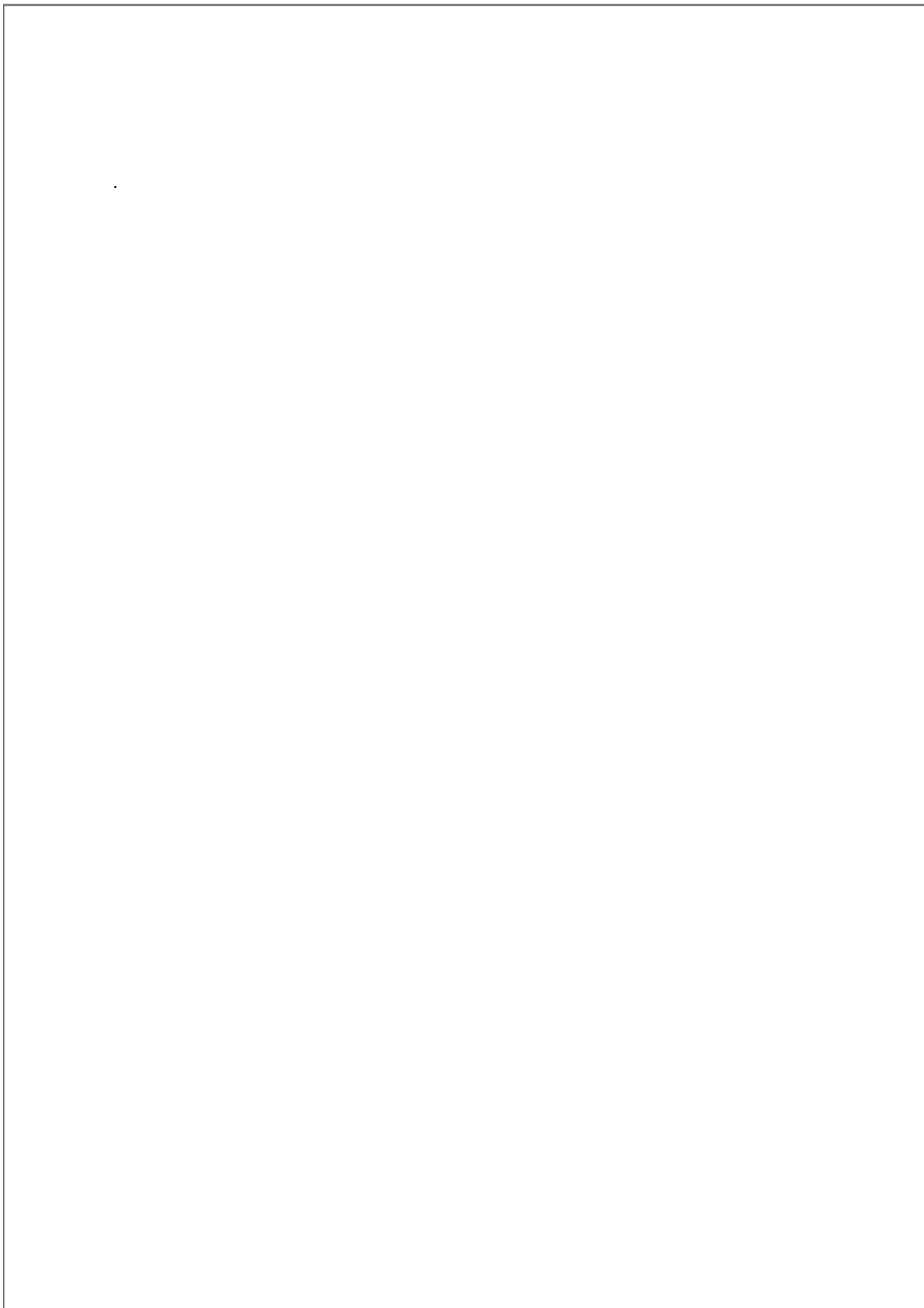
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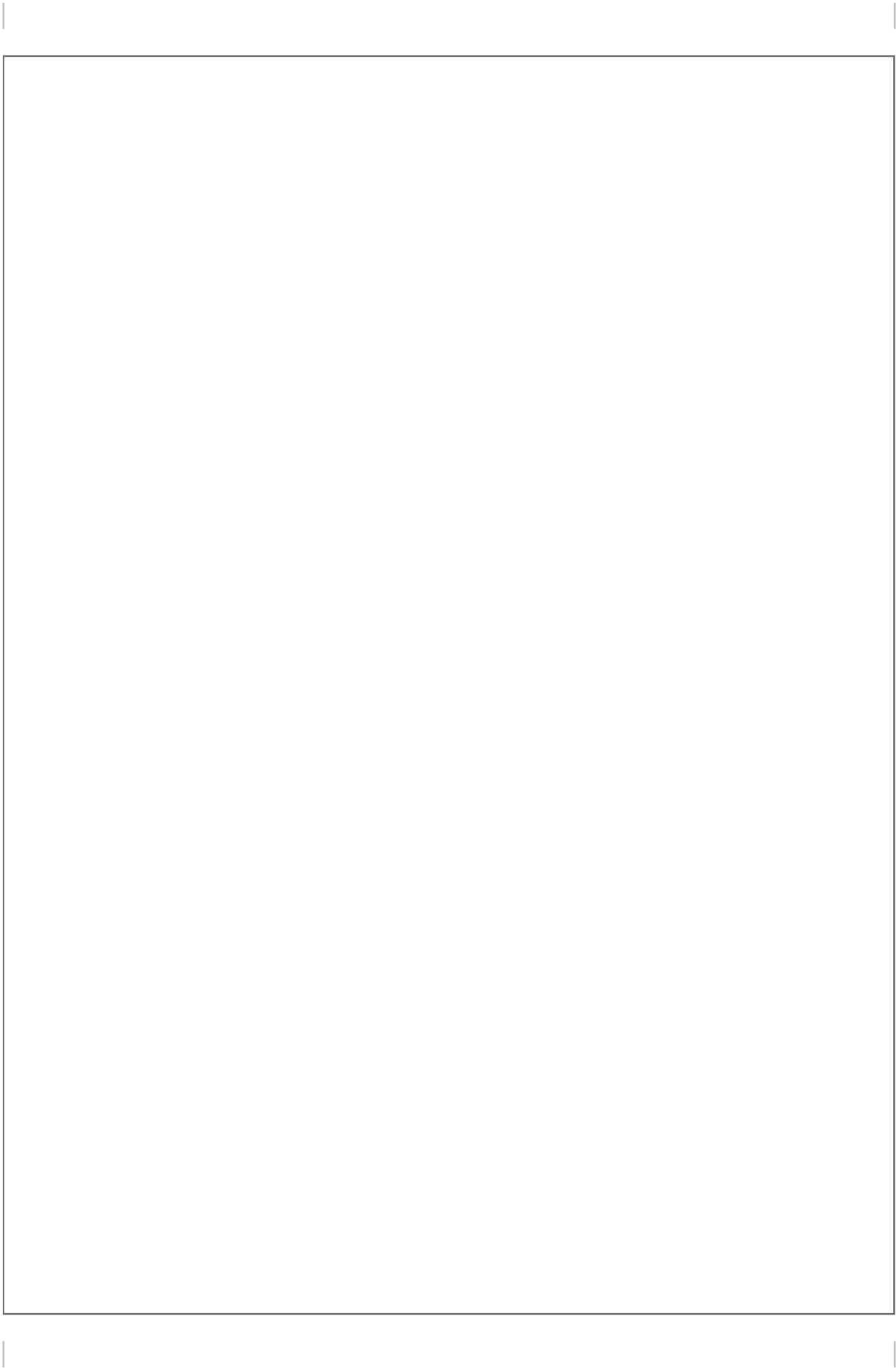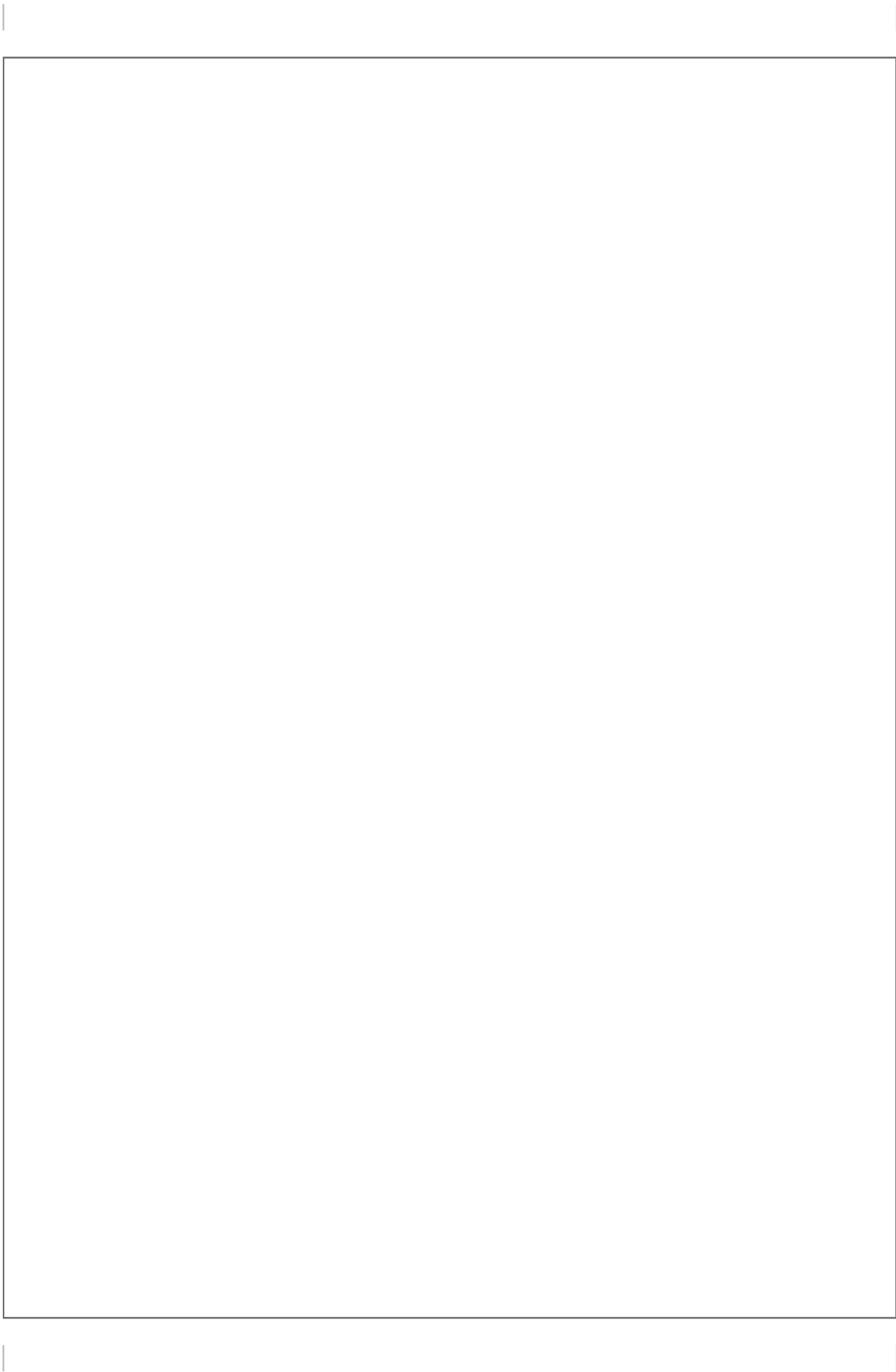
Appendices

Summary (English)

Samenvatting (Dutch)

Acknowledgements

Curriculum vitae

Training and supervision Plan

Summary

One of the major challenges of the century is how to feed the world's human population which by 2050 is expected to reach the 9 billion mark. The expected increasing demand for products of aquatic origin, including fish can only be addressed by aquaculture. The need for aquaculture industrialization together with increasing environmental limitations and regulations motivated the development of water re-use systems. Recirculating aquaculture systems (RAS) are intensive land based systems where water is re-used after treatment and where fish are grown under controlled environmental conditions. The need for increased production efficiency and for optimizing of RAS is critical to meet the targets fish food demand. Consequently, maintaining high water quality is a critical factor for RAS success, particularly monitoring the accumulation of substances that may impair fish performance. Steroidal hormones and its metabolites are responsible for vital processes in fish such as sexual maturation and stress response and, are released into the rearing water in a continuous or pulsatile manner. However, the accumulation of fish steroids in the water of RAS and its implications for fish is poorly understood. The general aim of this thesis is to determine the occurrence of different classes of steroids in RAS and how culture conditions may contribute to its accumulation or elimination and ultimately how they may affect fish performance. Such information is important to define strategies in RAS management and design to prevent negative effects on fish growth, welfare and reproduction. The background and rationale to the problem is introduced in **Chapter 1.**

Chapter 2 investigates whether RAS with high and low accumulation of substances originating from feed, fish or bacteria affect feed intake and growth of Nile tilapia *Oreochromis niloticus*, African catfish *Clarias gariepinus*, and European eel *Anguilla anguilla*. For a period of 39 days, growth and feed intake were compared between high and low accumulation RAS. High accumulation RAS was designed for maximal accumulation of substances in the water by operating the system at nearly-closed conditions (30 L/kg feed/d), using mature biofilters and high feed loads. Low accumulation RAS was operated to bea proxy to flow-through systems (FTS) by operating at high water exchange rates (1500 L/kg feed/d), with new biofilters and low feed load. High accumulation RAS caused a reduction in feed intake (42%) and growth (83%) of Nile tilapia, as compared to low accumulation RAS. This effect was not observed in European eel and African catfish.

Chapter 3 aims to determine (1) the concentrations and variation of cortisol and sex steroids in RAS, and (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. Results

showed significantly higher steroid concentrations in the inflow and effluent when compared with the make-up water. On average cortisol concentration was 15.7% higher in the effluent when compared to the inflow. 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 were orders of magnitude lower than initial estimations indicating a water treatment efficiency of >99%. 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.
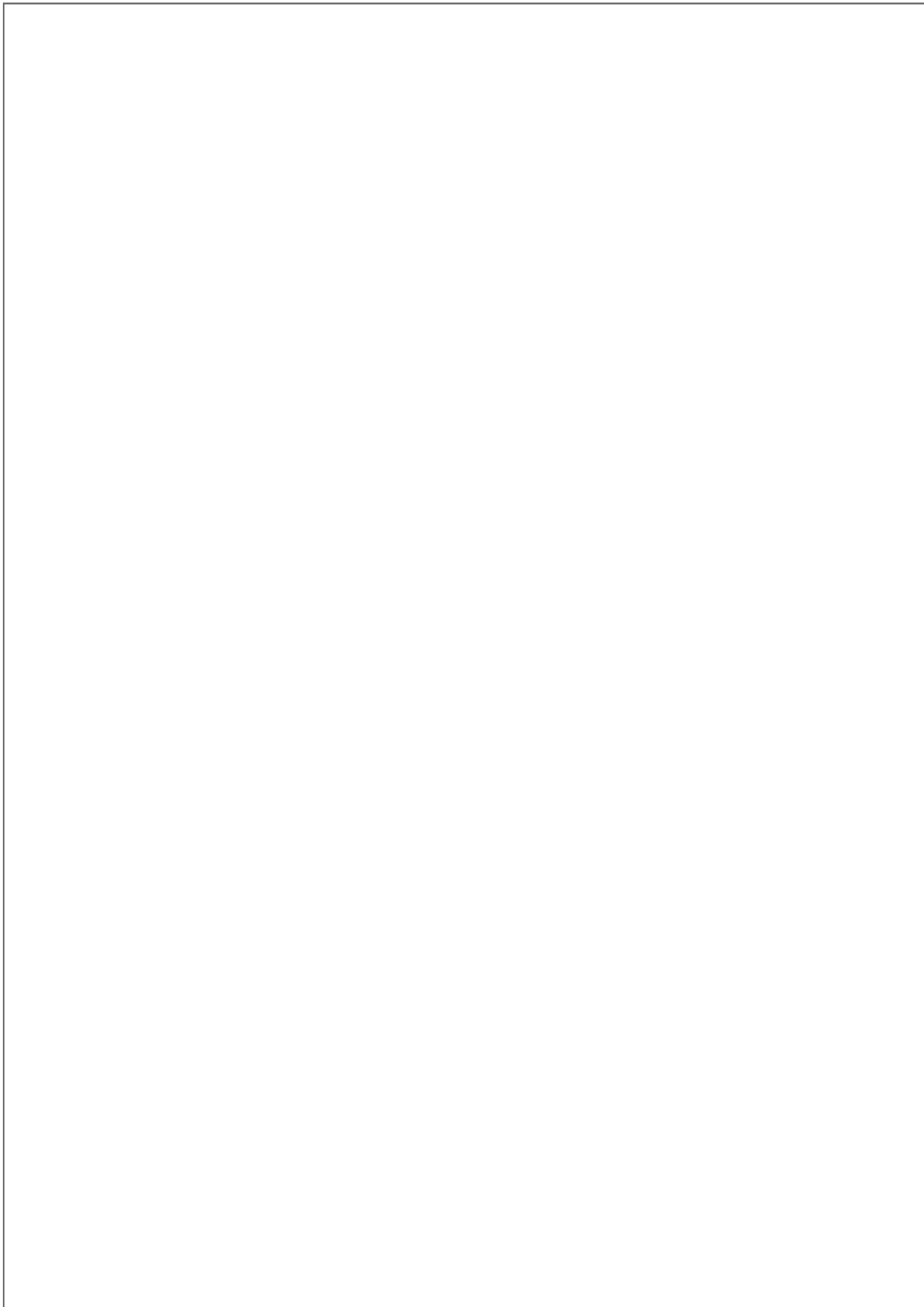
Chapter 4 examines the release rate of cortisol and testosterone by Nile tilapia, and their accumulation in RAS with different water make-up-levels (150 L/kg feed, *LowRAS* and 1500 L/kg feed, *HighRAS*) and how release rate is affected by a stress event. Water cortisol and testosterone concentrations during the experimental period ranged between 1.0 and 5.1 ng/L and between 1.4 and 9.4 ng/L, respectively. Two hours after grading and weighing, water cortisol concentration increased 30% in the *LowRAS* whereas water testosterone concentration remained unchanged. Cortisol and testosterone discharge from the system were, respectively, 87% and 89% lower in *LowRAS* than in *HighRAS*. Cortisol removal efficiency by the biofilter was 25% lower in *LowRAS* than in *HighRAS*, whereas no differences in treatment efficiency were detected for testosterone. These findings show that reduced water usage and acute stressors can induce significant accumulation of hormones in the rearing water.

Chapter 5 investigates the effect of water exchange rate and pH on rainbow trout *Oncorhynchus mykiss* blood plasma cortisol and water concentration of cortisol, testosterone, 11-ketotestosterone and the maturation-inducing hormone 17,20β-dihydroxypregn-4-en-3-one over a 70-day experimental period. Three combinations of water exchange rate and pH were used: 1) high water exchange rate (±1700 L/kg feed) and neutral pH (±7.3), 2) low water exchange rate (±500 L/kg feed) and neutral pH (±7.3), and 3) low water exchange rate (±500 L/kg feed) and low pH (±5.8). Water cortisol and testosterone concentrations were higher at low pH compared to neutral pH at day 35, whereas at day 0 and 70 were no differences. Water 11-KT and 17,20β-dihydroxypregn-4-en-3-one did not differ among treatments. Plasma cortisol concentrations at day 70 for fish kept at low pH was 24.4± 9.5 ng/ml (L_WL_{pH}) whereas for individuals kept at neutral pH was 12.0 ± 0.1 ng/ml (H_WN_{pH}) and 8.7 ± 0.2 ng/ml (L_WN_{pH}). The results demonstrate that low pH contributes to the accumulation of steroids in RAS and highlight the importance to monitor stress and reproductive hormones in the rearing water.

Chapter 6 investigated the specificity of bodily fluids as olfactory stimuli in two related species. The olfactory potency of urine and crude extracts of urine from Mozambique tilapia *Oreochromis mossambicus* and Nile tilapia was assessed by the electro-olfactogram and the concentration of the urinary steroid glucuronides 5β-pregnane-3α,17,20β-triol 3α-glucuronide and 5β-pregnane-3α,17,20α-triol 3α-glucuronide measured by liquid-chromatography/mass-spectrometry. Both species showed similar olfactory sensitivity to urine and respective extracts from either species, and similar sensitivity to the steroid glucuronides. 5β-pregnane-3α,17,20β-triol-3α-glucuronide was present at high concentrations (approaching 0.5 mM) in urine from Nile tilapia, with 5β-pregnane-3α,17,20α-triol 3α-glucuronide present at lower concentrations, similar to the Mozambique tilapia. Both species also had similar olfactory sensitivity to estradiol-3-glucuronide, a putative urinary cue from females. Together, these results support the idea that reproductive chemical cues have not been subjected to differing selective pressure.

In the final **chapter 7**, the main results from all experimental chapters are discussed in the context of the existing literature. The following main conclusions were drawn from this thesis:

- Intensification of fish production in RAS can result in sub-optimal rearing conditions and thus reduced fish performance;

- Steroids occur at higher concentration in RAS compared to FTS and refreshment water, independently of the fish species, water treatment units and water quality;

- Increased fish stocking density, occurrence of stressor events and decreased water pH are associated with high steroid concentrations and, reducing water exchange rates may induce accumulation of steroids in specific conditions;

- The fish olfactory system is extremely sensitive to steroids in the water, where they may function as odorant signals with a threshold of detection in the range of steroid concentrations present in RAS water.

Samenvatting

Eén van de grote uitdagingen in deze eeuw is het voeden van de wereldbevolking, waarvan verwacht wordt dat deze in 2050 9 miljard mensen zal bedragen. De daardoor verwachte toename in de vraag naar producten van aquatische oorsprong, waaronder vis, kan alleen worden bereikt via aquacultuur. De noodzaak het productieproces in de aquacultuur te industrialiseren was samen met de toename in beperkingen opgelegd ten aanzien van het milieu en door regelgeving, de motivatie voor het ontwikkelen van recirculatie aquacultuur systemen (RAS). RAS zijn intensieve op het land gevestigde viskweeksystemen waarin het water na zuivering wordt hergebruikt en waarin de vissen worden gekweekt onder volledig gecontroleerde milieu omstandigheden. Verhoogde productie-efficiëntie en het vervolmaken van RAS zijn essentieel om aan de toekomstige vraag naar vis te kunnen voldoen. Een cruciale factor voor het succesvol kweken van vis in RAS is de handhaving van een uitstekende waterkwaliteit. Met name de controle over de accumulatie van stoffen die kweekresultaten van vis kunnen beïnvloeden is daarbij van groot belang. Stoffen die door vis op een continue of pulserende wijze aan het water worden afgegeven zijn de steroïde hormonen en hun metabolieten. Deze stoffen zijn verantwoordelijk voor vitale processen in vis zoals bijvoorbeeld de seksuele rijping en de stressrespons. Echter, de factoren die de accumulatie van vis steroïden in het water van RAS bepalen en de gevolgen van accumulatie voor de vis worden nog niet volledig begrepen. Het algemene doel van dit proefschrift was de aanwezigheid van verschillende klassen van steroïden in RAS te bepalen, hoe kweekomstandigheden kunnen bijdragen aan hun accumulatie of eliminatie en hoe ze uiteindelijk de vis kunnen beïnvloeden in zijn functioneren. De beschikbaarheid van deze informatie is van belang voor het ontwikkelen van management strategieën en nieuw te ontwerpen RAS ter voorkoming van negatieve effecten op groei, welzijn en voortplanting van vis. De achtergrond en de gedachten die ten grondslag liggen aan het hier bestudeerde probleem worden ingeleid in **hoofdstuk 1**.

In **hoofdstuk 2** is onderzocht of in een recirculatie aquacultuur systeem met hoge en lage accumulatie van stoffen, afkomstig van visvoeder, vissen en bacteriën, de voeropname en groei van Nijl tilapia *Oreochromis niloticus*, Afrikaanse meerval *Clarias gariepinus* en Europese paling *Anguilla anguilla* van groei worden beïnvloed. De 'hoge accumulatie RAS' behandeling was ontworpen om maximale ophoping van stoffen in het water te realiseren door gebruik te maken van een lage waterverversing (30 L/kg voeder/dag), een volgroeid biofilter en een hoge voerbelasting. De 'lage accumulatie RAS behandeling' was een benadering van systemen met een hoge waterverversing (1500 L/ kg voer/dag), een nieuw biofilter en een lage voerbelasting. De experimentele periode

bedroeg 39 dagen. Het recirculatie systeem met een hoge accumulatie aan stoffen veroorzaakte een verminderde voeropname (42%) en groei (83%) bij Nijl tilapia in vergelijking met het recirculatie systeem met een lage accumulatie aan stoffen. Dit effect werd niet waargenomen bij Europese paling en Afrikaanse meerval die in hetzelfde RAS waren gehuisvest.

Het onderzoek in **hoofdstuk 3** is gericht op het bepalen van: (1) de concentratie van en variatie in cortisol en geslachtshormonen in het water van commerciële RAS en (2) de invloed van viskweekfactoren op steroïden concentraties in zeven commerciële RAS. Elk RAS werd tweemaal bemonsterd op drie verschillende punten: (1) het verversingswater; (2) het influent van de viskweek unit en (3) het effluent van de viskweek unit. Resultaten toonden significant hogere steroïden concentraties in het influent en het effluent van de viskweekunit in vergelijking met het verversingswater. Gemiddeld was de cortisol concentratie 15,7% hoger in het effluent ten opzichte van het influent van de viskweek unit. De bezettingsdichtheid, de totaal ammoniakale stikstof (TA-N) concentratie en de orthofosfaat-P concentratie (een indicatie voor de mate van waterverversing) lieten een positieve correlatie met de concentraties aan geslachtshormonen in het water zien. De gemeten steroïden concentraties lagen ordes van grootte lager dan de initieel geschatte concentraties, en wezen op een waterzuiveringsefficiëntie >99%. De resultaten in dit onderzoek suggereren dat intensivering van de visproductie door verlaging van de waterverversing en verhoging van de bezettingsdichtheid zal leiden tot accumulatie van steroïden in het viskweekwater.

In **Hoofdstuk 4** wordt verslag gedaan van de afgiftesnelheid van cortisol en testosteron door Nijl tilapia, de accumulatie van deze steroïden in RAS bij twee verschillende waterverversingen (150 L/kg voer/dag, *LowRAS* en 1500 L/kg voer/dag, *HighRAS*) en na afloop van een gestandaardiseerde stress-gebeurtenis (sorteren en wegen). Water cortisol en testosteron concentraties varieerden tijdens de experimentele periode tussen respectievelijk 1,0 en 5,1 ng/L en 1,4 tot 9,4 ng/L. Dit kwam overeen met een 38% en 10% hogere concentratie voor respectievelijk cortisol en testosteron in respectievelijk de *LowRAS* en *HighRAS* behandeling. Twee uur na sorteren en wegen, steeg de cortisol concentratie 30% in de *LowRAS* behandeling, terwijl de testosteron concentratie in het water ongewijzigd bleef. De lozing van cortisol en testosteron uit het systeem waren, respectievelijk 87% en 89% lager in de *LowRAS* dan in de *HighRAS* behandeling. De cortisol verwijderingsefficiëntie van het biofilter was 25% lager in de *LowRAS* behandeling wanneer vergeleken met de *HighRAS* behandeling terwijl er geen verschil werd waargenomen in de treatment efficiency voor testosteron. Deze

bevindingen toonden aan dat een vermindering in waterverbruik en acute stressoren significante accumulatie van hormonen in het water kunnen veroorzaken.

In **Hoofdstuk 5** is in een experiment met regenboogforel *Oncorhynchus mykiss* het effect van waterververversing en pH op het cortisol gehalte in bloedplasma en de concentratie van cortisol, testosteron, 11-ketotestosterone (11-KT) en het rijping inducerende hormoon 17,20β- dihydroxypregn-4-en-3-on (17,20β-P) in het water bepaald. Drie combinaties van water verversing en pH werden gebruikt: 1) hoge waterververversing (± 1700 L/kg voeder) en neutrale pH (± 7.3), 2) lage waterververversing (± 500 L/kg voeder) en neutrale pH (± 7.3), en 3) lage waterververversing (± 500 L/kg voeder) en lage pH (± 5.8). Het experiment besloeg een proefperiode van 70 dagen. Water cortisol en testosteron concentraties waren op dag 35 hoger bij lage pH in vergelijking met neutrale pH, terwijl 11-KT en 17,20β-P concentraties in het water niet verschilden tussen de behandelingen. Bloedplasma cortisol concentraties op dag 70 waren voor vissen gehouden bij een lage pH 24.4 ± 9.5 ng/ml (L_WL_{pH}) en voor vissen gehouden bij een neutrale pH 12.0 ± 0.1 ng/ml (H_WN_{pH}) en 8.7 ± 0.2 ng/ml (L_WN_{pH}). De resultaten toonden aan dat een lage pH bijdraagt aan de accumulatie van steroïden in RAS en benadrukt het belang om stress- en geslachtshormonen in viskweekwater te monitoren.

In **Hoofdstuk 6** wordt de specificiteit van lichaamsvloeistoffen als olfactorische stimuli in twee verwante vissoorten onderzocht. De olfactorische potentie van ruwe urine extracten van Tilapia mossambica *Oreochromis mossambicus* en Nijl tilapia *Oreochromis niloticus* werd beoordeeld aan de hand van het electro-olfactogram en door de concentratie van steroïde-glucuroniden 5β-pregnaan-3α, 17,20β-triol 3α-glucuronide en 5β- pregnaan-3α, 17,20α-triol 3α-glucuronide in de urine te bepalen. De steroïden concentratie in de urine werd gemeten door middel van vloeistof-chromatografie / massaspectrometrie. Beide vissoorten toonden een vergelijkbare olfactorische gevoeligheid voor de urine en de ruwe urine extracten van beide soorten, en een zelfde gevoeligheid voor de steroïden-glucuroniden. 5β-pregnaan-3α, 17,20β-triol-3α-glucuronide was in hoge concentraties aanwezig (benaderde 0,5 mM) in de urine van Nijl tilapia terwijl 5β-pregnaan-3α, 17,20α-triol 3α-glucuronide in lagere concentraties aanwezig was, vergelijkbaar met die van Tilapia Mossambica. Beide vissoorten hebben eenzelfde olfactorische gevoeligheid voor estradiol-3-glucuronide, een vermeend urine signaal van vrouwtjes. De resultaten samengenomen ondersteunen de opvatting dat chemische signalen in relatie tot voortplanting niet zijn onderworpen aan een verschil in selectiedruk.

In het laatste hoofdstuk, **hoofdstuk 7**, worden de belangrijkste resultaten van alle experimentele hoofdstukken in het kader van de bestaande literatuur besproken. De belangrijkste conclusies die uit dit proefschrift kunnen worden getrokken zijn:

- intensivering van de visproductie in RAS kan resulteren in sub-optimale kweekomstandigheden, en dus verminderd functioneren van de vis;
- steroïden komen in hogere concentraties voor in RAS wanneer vergeleken met concentraties in doorstroomsystemen en verversingswater en zijn onafhankelijk van de vissoort, de waterbehandeling en de waterkwaliteit;
- een verhoogde visbezettingsdichtheid, het optreden van stressvolle gebeurtenissen en een lage water pH zijn geassocieerd met hogere steroïden concentraties in RAS, terwijl het verminderen van de waterverversing in bepaalde omstandigheden accumulatie van steroïden kan veroorzaken;
- het olfactorisch systeem van vis is zeer gevoelig voor steroïden in water en de detectiedrempel valt binnen de range van steroïden concentraties gemeten in RAS water.

Acknowledgements

Revisiting that memory back on a white and cold January day of 2009, when I first arrived in Wageningen, it feels it was a long, long time ago. That first passage during my ERASMUS programme together with my studies at the University of Algarve would build up the pillars of what would become my PhD project. The persons I then met and the conversations we held were vital for the coming years. From that early winter to this closing autumn of 2015, many colleagues and friends have helped and contributed to the completion of my thesis. To all of you that have encouraged and enlightened me into making this thesis see the light of day my most sincere gratitude.

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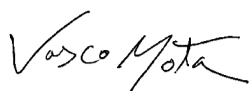
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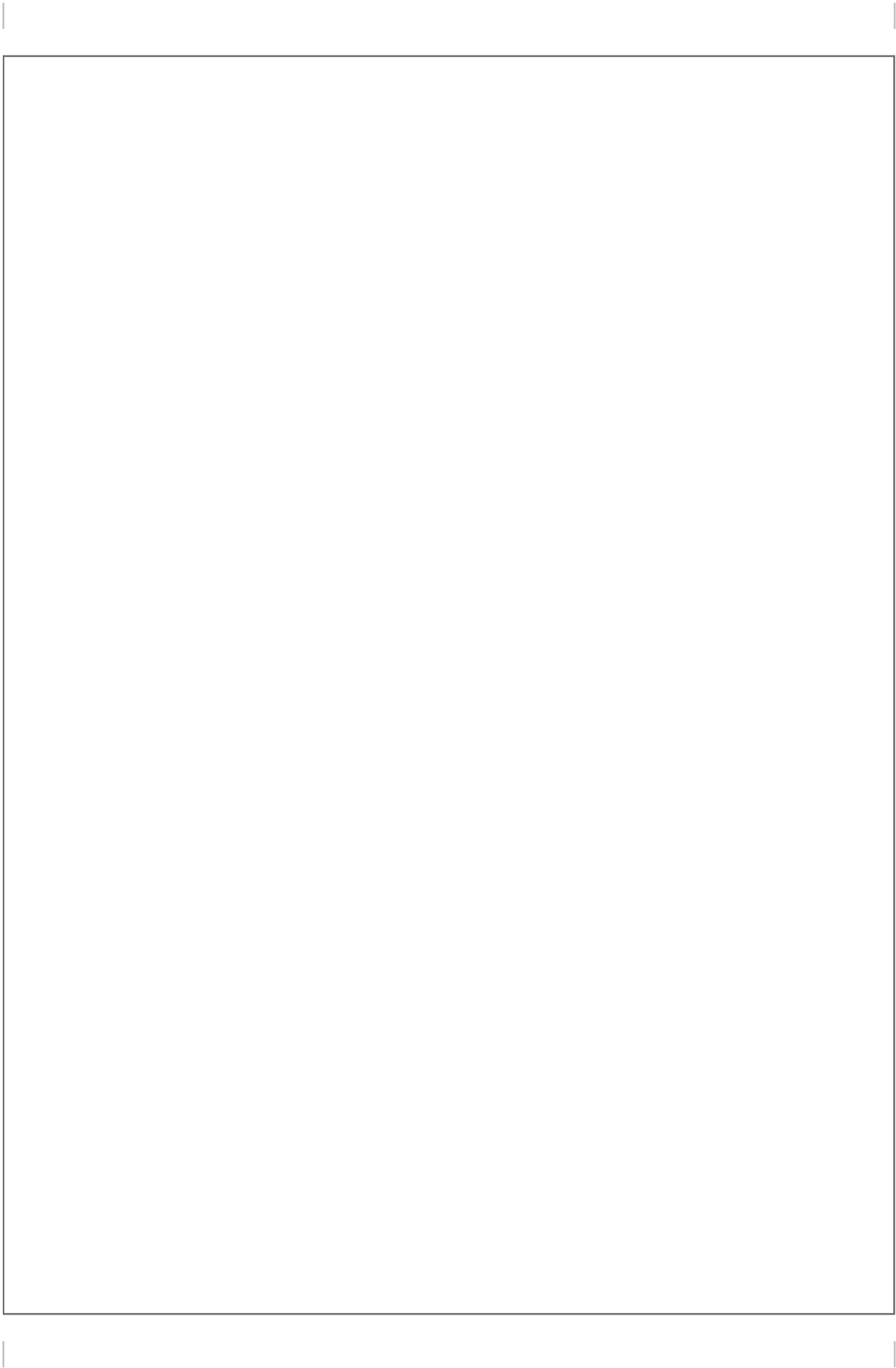
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Biography

Vasco Mota, born on the 8th of August in 1986 in the city of Lisbon, Portugal, moved permanently to the region of Algarve, where he finished his Secondary Education in the area of Natural Sciences. As a secondary school student he was exceptionally focused, often adopting a curious and adventurous approach.

In 2004 he moved back to Lisbon, where he undertook a Bachelor of Science in Marine Sciences, achieving a final mark of 15 out of 20, at the University of Lisbon.

In 2007, he enrolled in a master programme at the University of Algarve in Faro, Portugal. After completing the first year of the master's, during summer holidays, he ventured into a 2-month international post-graduation in the Lehigh University, entitled "The Global Village for Future Leaders of Business and Industry" in Bethlehem, Pennsylvania, U.S.A. Afterwards, he moved to The Netherlands to develop his master thesis during a 6-month Erasmus programme at the Wageningen University. His thesis *Effects of low pH and high TA-N on performance of turbot (Psetta maxima) in recirculating aquaculture systems* earned him an award for Best Master Thesis in the field of Animal Science by the Animal Science Study Group NZV. In 2009, he completed his Master in Sciences in Aquaculture and Fisheries, with specialization in Aquaculture, achieving a final mark of 17 out 20 and receiving a Merit Grant from the Algarve University.

In January 2010, after receiving a PhD Grant by the Portuguese Foundation for Science and Technology, he started his PhD research at the Aquaculture and Fisheries Group at the Wageningen University, The Netherlands, in cooperation with the Centre for Marine Sciences (CCMAR), Faro, Portugal, which included the attendance of 13 scientific and professional courses in The Netherlands, Spain and Norway, teaching and lecturing in the Wageningen University and 8 communications in international conferences. For the communication presented in Rhodes, Greece, the European Aquaculture Society awarded him the Ibrahim Okumus Award for the Best Scientific Poster. During the same period, he lectured 3 Bachelor of Sciences and Master of Sciences courses and supervised 3 Bachelor of Sciences students undergoing their thesis. After finishing his experimental work in The Netherlands, he ventured alone in a bicycle journey back to Portugal and continued the thesis writing from Lisbon.

From early 2014 to the Autumn of 2015 he worked as a Medical Sales Representative for the EFFIK pharmaceutical group, Lisbon, Portugal. In early January 2016, he shall become a researcher in closed-containment aquaculture at Nofima, the Norwegian Institute of Food, Fishery and Aquaculture.

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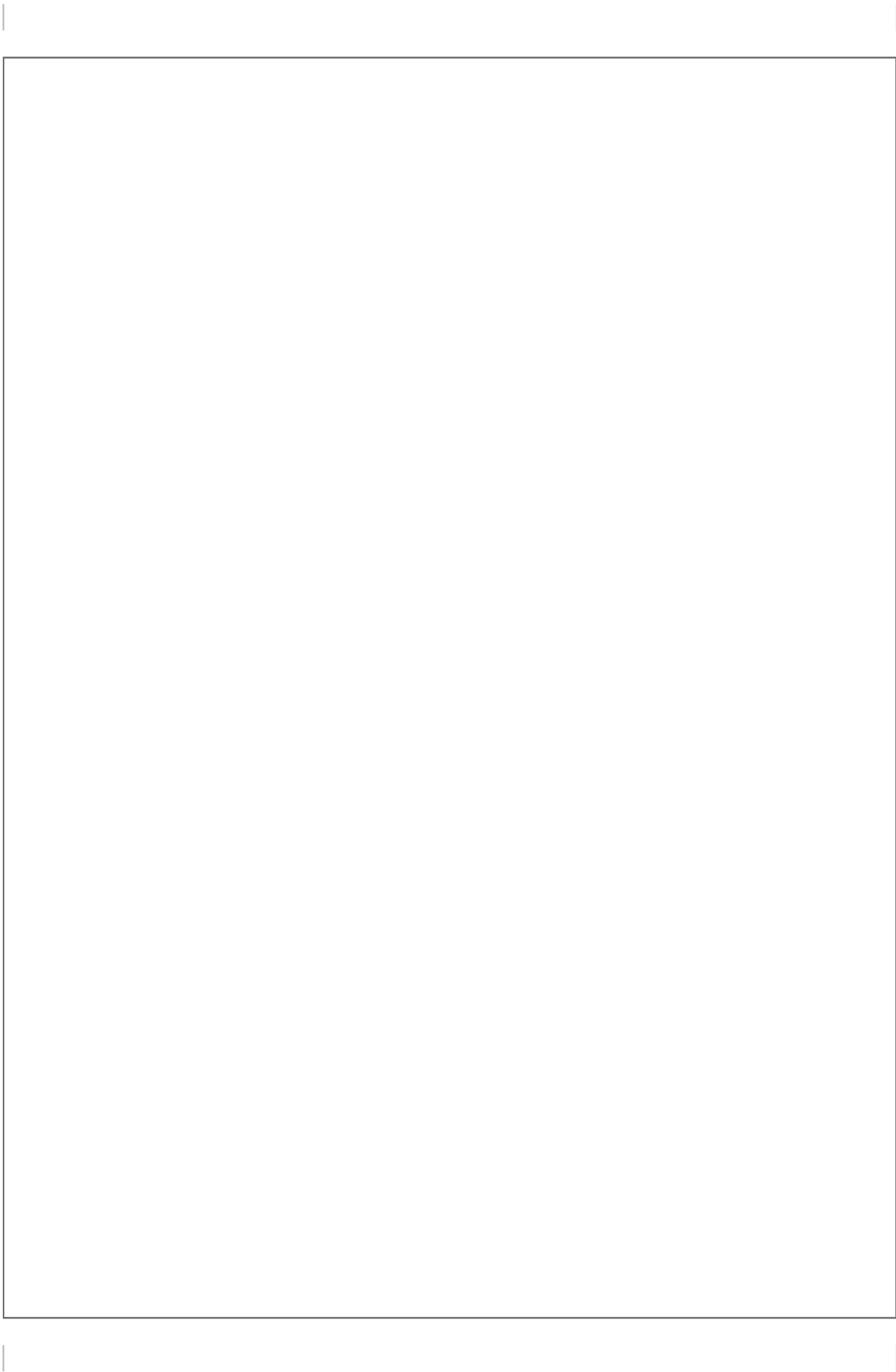
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Notes

Notes