Steroid bioaccumulation profiles in typical freshwater aquaculture environments of South China and their human health risks via fish consumption

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Abstract

More attention was previously paid to adverse effects of steroids on aquatic organisms and their ecological risks to the aquatic environment. So far, little information has been reported on the bioaccumulative characteristics of different classes of steroids in cultured fish tissues. The present study for the first time provided a comprehensive analysis of the occurrence, bioaccumulation, and global consumers' health risks via fish consumption of androgens, glucocorticoids and progestanes in typical freshwater cultured farms in South China. The numbers and total concentrations of steroids detected in the tissues of five common species of the cultured fish were in the order of plasma > bile > liver > muscle and plasma > bile, muscle > liver, respectively. The field bioaccumulation factors for the detected synthetic steroids ranged from 450 to 97,000 in bile, 450 to 65,000 in plasma, 2900 to 16,000 in liver, and 42 to 2600 in muscle of fish, respectively. This data suggests that steroids are bioaccumulative in fish tissues. Most importantly, 4-androstene-3,17-dione (AED) and cortisone (CRN) were found to be reliable chemical indicators to predict the levels of steroids in plasma and muscle of the inter-species cultured fish, respectively. Furthermore, the maximum hazard quotients (HQs) of testosterone and progesterone were 5.8 × 10⁻³ and 9.9 × 10⁻⁵, suggesting that human health risks were negligible via ingestion of the steroids-contaminated fish.

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1. Introduction

In recent years, more and more public attentions have focused on the occurrence and fate of steroids in the environment. It has been confirmed that wastewater treatment plants (WWTPs) and livestock farms are two main sources of steroids in the environment (Fan et al., 2011; Liu et al., 2012b). The total estimated contribution of steroids via WWTPs, swine farms and cattle farms in China were up to 140, 66 and 61 t/yr, respectively (Liu et al., 2012b). Furthermore, aquaculture is also an important source of steroids in the aquatic environment (Kolodziej et al., 2004). In the breeding process of aquaculture, steroids come mainly from two ways. One is that fish could excrete metabolized steroids into water like livestock not only via urine, but also via gill or bile (Ellis et al., 2004; Scott and Ellis, 2007; Liu et al., 2012b). The other is that some natural or synthetic steroids were added into the aquaculture environment directly or into the feed to prevent or treat diseases, promote growth, or produce monosex populations (Beardmore et al., 2001).

Although the amount of steroids discharged from a typical aquaculture farm was equal to several hundred animals or a
wastewater treatment plant serving several thousand people (Kolodziej et al., 2004), little is known about occurrence and bioaccumulation of steroids in aquaculture. Only a few studies paid their attention on the occurrence and bioaccumulation of estrogens in fish tissues from pond, reservoir, river, lake, and WWTP effluents (Liu et al., 2012a; Huang et al., 2013; Yang et al., 2014). The concentrations of estrogens including estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2) and estriol (E3) in these aquatic environments were always at very low levels (ng/L) which could still be detected in fish tissues with the bioaccumulation factors up to 40,000 (Liu et al., 2012a; Yang et al., 2014). For other steroids, such as androgens, glucocorticoids and progestagens, there is only one study recently reported the bioaccumulation and human dietary exposure risk of these steroids in marine fish muscle by our group (Liu et al., 2015a).

China is the largest freshwater aquatic product farming country in both production and farming area. In 2015, the output of aquaculture in China was up to 67 million tons (MAPRC, 2016), in which 41.1 million tons of aquatic products were exported to Japan, Association of Southeast Asian Nations, the United States, the European Union, Korea, Hong Kong, Taiwan and other regions (IEAP, 2016). Pond culture is the most common culture mode in China, accounting for 70% of the total output of freshwater aquaculture (Yu, 2015). Most of pond cultured farms are traditionally managed on small scales without effective management measures. According to the guidelines of General Administration of Quality Supervision, Inspection and Quarantine of the People’s Republic of China, synthetic steroids including methyl testosterone, 17α-trenbolone, 17β-trenbolone and megestrol acetate are forbidden to use for any purposes in all food-producing animals in China (QSIQ, 2002). Previous studies have confirmed that synthetic steroids were still being illegally used in the livestock and marine aquaculture farms in China (Liu et al., 2012b, 2015a). Thus, our hypotheses are 1) there might be illegal usage of steroids in feed samples in freshwater pond cultured farms in China, 2) steroids are bioaccumulative in fish tissues and there are linear correlations between single steroid and other individual steroid/total steroids in different tissues, 3) the residual steroids in fish muscle might bring human health risks.

Thus, the aim of this study was to investigate the bioaccumulation of steroids in the bile, plasma, liver and muscle tissues of fish raised in typical freshwater pond cultured farms and human health risks via fish consumption. Moreover, the potential chemical indicators of steroids in fish tissues were explored. To the best of our knowledge, no previous studies have reported the bioaccumulation of androgens, glucocorticoids and progestagens in freshwater pond cultured farms as well as the relationship between single steroid and the rest steroids in different fish tissues. Our study might provide a better understanding of the biological enrichment for different classes of steroids in fish tissues and have implications for appropriate management actions in the aquaculture environment.

2. Materials and methods

2.1. Chemicals and sample collection

Totally, 29 natural and synthetic steroids, including 11 androgens, 4 glucocorticoids, 14 progestagens, and 4 internal standards were selected in this study (Table S1). The detailed information on chemicals and materials is summarized in the Supplementary materials. Three typical freshwater cultured farms (F1, F2 and F3) in Guangzhou, one of the biggest aquatic products farming city as well as a huge aquatic products consumption city in China, were selected as the study areas. The total output of aquatic products in Guangzhou was 480,000 tons in 2015, and 82% of which was freshwater culture (GZSB, 2015). The daily consumption of aquatic products has been estimated up to 1600 tons in Guangzhou, only second to pork (GZRD, 2013). Farm F1 is managed in a swine-chicken-geese-fish polyculture mode including 30,000 tilapias. Farm 2 is managed in a mixed-fish-species pond culture mode, including 90,000 spotted scats and 18,000 tilapias. Both farm F1 and F2 are typically small-scaled aquaculture farms, which are the most common aquaculture breeding mode in South China. Farm F3 is a median-scaled farm with technical staff guidance, including 16,000 crucian carps, 3200 mullets and 1600 mud carps. All influents of these farms are from Zhujiang River, but only farm F3 has a sedimentation tank with 24 h retention time to treat the influent (F3-inf) (Fig. S1).

All samples including four water samples, four suspended particle samples, three sediment samples, three feces samples and five feed samples were collected from three farms in April 2015. Surface water samples were collected from the center of the pond to the pre-cleaned bottles immediately. Three replicate samples were mixed as a composited sample (5 L). To suppress bacterial activity, about 5% (v/v) of methanol was added into each composited water sample and its pH value was adjusted to 3.0 in the field (Zhao et al., 2015a). Surface sediments (0–20 cm, 200 g) were obtained using Petersen grabs sampler and stored in glass bottles. Feeds used in the aquaculture farms (50 g) and feces produced there (100 g) were collected at the same time. All samples were transported back in coolers to the laboratory immediately. The collected water samples were then processed within 48 h. The solid samples (sediment, feed, feces and suspended particles) were freeze dried, ground and homogenized. In total, 37 fish including 6 from farm F1, 13 from farm F2 and 18 from farm F3 were collected from the three ponds in the selected farms. All fish collected in the study were adult and ready for sale. The collected fish species included tilapia (Oreochromis spp), spotted scat (Scatophagus argus), mullet (Mugil cephalus), mud carp (Cirrhinus molitorella), and crucian carp (Carassius auratus), which represent the most common freshwater aquaculture products in South China. Basic information on selected aquaculture farms and sampled fish are listed in Tables S2 and S3. All fish samples were kept alive in ice water aerated by portable air pumps, and sacrificed immediately upon arrival in the laboratory. A pre-rinsed sodium heparin syringe was used to collect the blood of the fish caudal vein, which was then stored in a 2 mL plastic vial for 8 h at 4 °C. The plasma was obtained from the supernatant in the vial which was centrifuged at 10,000 g for 10 min. The bile was obtained from the gall bladder by a syringe needle and stored in a 2 mL cryogenic vial. Approximately 2 g of muscle samples and 0.5 g of liver samples, both in wet weight, were dissected and kept in cryogenic vials independently. All tissues except the plasma were immediately stored at −20 °C until extraction.

2.2. Sample extraction and instrumental analysis

Sample extraction and instrumental analysis were carried out following previously established analytical methods (Liu et al., 2011a, 2014; Zhao et al., 2015a). The detailed method information can be found in the Supplementary materials (Tables S4 and S5). Briefly, water samples were extracted by the solid-phase extraction using Waters Oasis HLB cartridges (500 mg, 6 mL), while the solid samples were extracted with ethyl acetate by ultrasonication. The strong anion exchange/primary-secondary amine (SAX/PSA) cartridges (6 mL, 500 mg) and HLB cartridges (6 mL, 200 mg) were used firstly to remove bile acid from bile and lipid from both liver and muscle tissues, and finally to enrich the steroids in the biota sample extracts, respectively. The plasma and bile samples were extracted by HLB cartridges and SAX/PSA-HLB tandem cartridges, respectively. The dissected liver and muscle samples were
homogenized at 35,000 rpm, extracted with methanol/water-0.1 M acetic acid (50:50, v/v) by ultrasonication, and then purified and enriched by SAX/PSA-HLB tandem cartridges. The target steroid compounds were analyzed by an Agilent 1200 LC-Agilent 6460 QQQ (RRLC-MS/MS) with an electrospray ionization (ESI) source in multiple reactions monitoring (MRM) mode. More information about sample extraction and instrumental analysis was described in the Supplementary materials.

2.3. Data analysis

The bioaccumulation factor (BAF) of a steroid is calculated as the ratio of the concentration of a steroid in a fish tissue to the concentration of the steroid in the corresponding surface water. Multivariate analysis including detrended correspondence analysis (DCA) and redundancy analysis (RDA) was conducted to assess the potential relationship between the concentrations of steroids and fish size (length and weight). DCA was used to calculate the length of the first ordination gradient. If the calculated value is less than 3, RDA should be chosen for analysis. The estimated daily intake (EDI) of a selected steroid via fish consumption for local residents or global consumers is calculated according to the maximum consumption amount of selected fish for an adult. The daily fish consumption amounts in the present study were obtained from a questionnaire-based dietary survey in South China (Guo et al., 2010), and data from Food and Agriculture Organization of the United Nations (FAO, 2011). The hazard quotients (HQs) for steroids to humans are obtained from the ratio of the EDI and the acceptable daily intake (ADI) by adults. An HQ value greater than 1 indicates a high risk of an adverse health effect, and vice versa. For detailed calculation method, please refer to the data analysis part in Supplementary materials.

When calculating the range, median, and mean values, half of the concentration was not counted. One-Way Analysis of Variance (ANOVA) was used to test the statistical significance of the difference among species or tissues with $p < 0.05$. Pearson correlation analysis was conducted to identify the correlation among different steroids in tissues. All data analyses were performed with software SPSS 17.0, Sigma Plot 10.0 and Canoco 4.5.

3. Results and discussion

3.1. Steroids in the aquaculture farms

3.1.1. Steroids in the water samples

Thirteen of 29 steroids were detected in the water samples of aquaculture farms with concentrations ranging from <0.55 (17α-BOL) to 26 ng/L (CRL) (Table 1), which are similar to the levels detected in marine aquaculture farms in China (Liu et al., 2015a), but are apparently higher than those in flow-through systems in USA (Kolodziej et al., 2004) or recirculating aquaculture systems in the Netherlands (Mota et al., 2014). For the water samples, the total concentrations of steroids decreased in the order of farm F1 > farm F2 > farm F3. The total concentrations of steroids detected in the water from farm F1, a swine-chicken-geese-polyculture system, were higher than the other two farms, especially for cortisol. It was most probably due to the following two reasons: steroids excretion pathway and their physiochemical properties. A previous study had confirmed that fish excrete cortisol into the water via the urine, bile or gills (Scott and Ellis, 2007). CRL in the geese feces with concentrations up to 57 ng/g indicated that poultry farming is one of significant sources of steroids. Additionally, glucocorticoids have a lower tendency to be adsorbed onto sediments (log$K_{oc}$: 1.3–1.4) than the other steroids (log$K_{oc}$: 2.2–3.7) (Table S1). Therefore, more CRL was detected in the aqueous phase than in the sediment phase. Three steroids were detected in the influent (F3-inf), indicating that the influent is one of steroids pollution in farm F3. In comparison with influent, the concentrations of two steroids AED and CRL decreased in F3, most likely due to the degradation or dilution. P was relatively unchanged, and the rest of steroids were found to have increased concentrations. Steroids are excreted from fish in

### Table 1

Concentrations of steroids in water, suspended particle, sediment, feces and feed samples from the selected aquaculture farms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Suspended particle (ng/g dw)</th>
<th>Water (ng/L)</th>
<th>Sediment (ng/g dw)</th>
<th>Feces (ng/g dw)</th>
<th>Feed (ng/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1</td>
<td>F2</td>
<td>F3-inf</td>
<td>F1</td>
<td>F2</td>
</tr>
<tr>
<td>ADD</td>
<td>1.7</td>
<td>ND</td>
<td>29</td>
<td>1.3</td>
<td>5.2</td>
</tr>
<tr>
<td>AED</td>
<td>1.3</td>
<td>24</td>
<td>17</td>
<td>7.3</td>
<td>17</td>
</tr>
<tr>
<td>17α-BOL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17β-BOL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>19-NTD</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>T</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17α-TBL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CRL</td>
<td>110</td>
<td>270</td>
<td>190</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>CPTA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.88</td>
</tr>
<tr>
<td>DGT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>7.5</td>
</tr>
<tr>
<td>ET</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.55</td>
</tr>
<tr>
<td>MPA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.31</td>
</tr>
<tr>
<td>NTND</td>
<td>17</td>
<td>ND</td>
<td>30</td>
<td>ND</td>
<td>160</td>
</tr>
<tr>
<td>19-NTD</td>
<td>ND</td>
<td>9.6</td>
<td>17</td>
<td>65</td>
<td>0.83</td>
</tr>
<tr>
<td>P</td>
<td>7.3</td>
<td>12</td>
<td>8.3</td>
<td>4.9</td>
<td>0.93</td>
</tr>
</tbody>
</table>

**Notes:**
- Add: androsta-1,4-diene-3,17-dione; AED, 4-androstene-3,17-dione; 17α-BOL, 17β-boldenone; 17β-BOL, 17α-boldenone; 19-NT, 19-nortestosterone; T, testosterone; 17β-TBL, 17β-trenbolone; CRL, cortisol; CPTA, cyproterone acetate; DGT, dydrogesterone; ET, ethynyl testosterone; MP, medroxyprogesterone; MPA, medroxyprogesterone acetate; NTND, nor ethynodrel; 19-NTD, 19-norethindrone; P, progesterone.
- ND: not detected.
- Below limit of quantitation.
the free and conjugated forms (glucuronides and sulfates) (Mota et al., 2014). When entering the pond of F3, some residual conjugated steroids are likely deconjugated into free forms (Gomes et al., 2009) or converted from some other steroids with similar molecular structures (Egorova et al., 2009; Jenkins et al., 2004).

Ten synthetic steroids were detected in water samples of the aquaculture farms (Table 1). These synthetic ones are used in animal production for the purposes of breeding control and growth promotion (Liu et al., 2012b, 2014, 2015c). Previous studies pointed out that synthetic steroids may induce multiple transcriptional responses to brain and gonads of zebrafish embryos or adults at environmental levels (4.8 ng/L for DGT and 2.0 ng/L for 19-NTD) (Zucchi et al., 2012; Zhao et al., 2015b). Based on the concentration levels of DGT (7.5 ng/L) and 19-NTD (<0.69–3.1 ng/L) detected in the farm water, it is expected that DGT and 19-NTD might cause potentially adverse effects on some sensitive fish species raised in the aquaculture farms.

3.1.2. Steroids in the sediment and suspended particle samples

In the sediment samples, seven steroids were detected with concentrations ranging from <0.41 (DGT) to 6.6 ng/g (P) (Table 1). The most of detected steroids in the sediment samples were also detected in the feces samples or feed samples, demonstrating that steroids in the sediment samples were mainly from the addition of feces and feed. However, CRL was detected in the sediment sample of farm F2 instead of the corresponding feed sample. This result indicated that CRL in the sediment sample should be from the excretion of fish via the urine, bile or gills (Scott and Ellis, 2007). Although the concentrations of adsorbed steroids were low, they were expected to be more recalcitrant to biodegradation in the sediment, which made sediment act as potential sinks (Zhou et al., 2016).

Recently, studies have found that sediment can act as a source of biologically active compounds by direct contact between fish and sediment-associated steroids, although the mechanism of steroids exposure via sediment to aquatic organisms is not well understood (Jessick et al., 2014; Sangster et al., 2014).

Six steroids were detected in the suspended particle samples with concentrations ranging from 1.3 (AED) to 270 ng/g (CRL) (Table 1), which were similar to the levels detected in the livestock farms (Liu et al., 2012c). Among the six detected steroids, three natural steroids AED, P and CRL should be mainly from feces, feed or the excretion of fish. In farm F3, synthetic androgen ADD was detected in the suspended particle sample as well as water sample, but not detected in the water source (F3-inf) or fish feed sample. It is difficult to identify whether ADD comes from transformation from other steroids (Yang et al., 2010) or from artificial addition. In addition, synthetic progestagens NTND and 19-NTD were only detected in the suspended particle samples and water samples, indicating that these two steroids should be mainly from the pollution of water sources. Although the types of steroids detected in the suspended particles were similar to those detected in the sediment, the total concentrations of detected steroids were almost 1–2 orders of magnitude higher than those in the sediment (Table 1). As compared to sediment, suspended particles have a greater tendency to enhance steroids’ bioavailability in fish through ingestion or respiration across gills with smaller particles and better mobility (Sangster et al., 2015).

3.1.3. Steroids in the feces samples

Previous studies paid their attention to the detection of steroids in animal feces in single culture system such as swine farms, cattle farms or chicken farms (Albero et al., 2014; Liu et al., 2012b, 2015c), but few studies reported the concentrations of steroids in poly-culture system as farm F1. As shown in Table 1, seven and six steroids were detected in the swine feces (5.3–3300 ng/g) and chicken feces (4.9–2200 ng/g), respectively, which were lower than those detected in swine feces from livestock farms (<LOQ to 8100 ng/g) (Liu et al., 2012b). Six steroids were detected in geese feces samples (2.0–85 ng/g), which were lower than those detected in poultry litter (ND to 321 ng/g) (Albero et al., 2014). The differences among these feces samples may be due to different excretion ratio and usage of steroids in these animals. ADD is always known to be “pseudo-endogenous” due to its abuse as synthetically produced steroids and endogenous source under certain condition (Scarth et al., 2009). Except ADD, five synthetic steroids (17β-BOL, CPTA, DGT, MP and MPA) were detected in the feces samples (Table 1). These results suggested that there might be usage of synthetic steroids in the farm F1. As nutrient source for fish (Prein, 2002), the animal feces in farm F1 were directly discarded into the fish pond. Animal feces is one of important steroids sources in farm F1, as demonstrated by several detectable steroids at ng/L or ng/g levels in farm water or sediment samples (Table 1).

3.1.4. Steroids in the feed samples

The illegal use of steroids as growth stimulants has been banned in many countries including China for ethics and health reasons in farm animals (EU, 2010; JECFA, 2014; QSIQ, 2002). Eight steroids, including five synthetic steroids (ADD, 17α-BOL, CPTA, DGT and ET), were detected in the five feed samples in this study, with concentrations ranging from <0.19 (17α-BOL) to 16 ng/g (DGT) (Table 1), which were similar to those in our previous report (0.30–21 ng/g) (Liu et al., 2015a). Although synthetic steroids are still being used in the studied aquaculture area, they are not in the forbidden list of steroids in China (QSIQ, 2002). These findings demonstrated that the first hypothesis is not true.

3.2. Steroids in fish tissues

Twenty-one steroids, including eight androgens, four glucocorticoids, and nine progestagens, were detected in at least one type of tissue. Five natural steroids (AED, T, CRL, CRN, and P) and one synthetic steroid (CPTA) were detected in all four tissues. As shown in Fig. 1 and Tables S6-S9, the numbers and total concentrations of steroids detected in the tissues were in the order of plasma > bile > liver > muscle and plasma > bile, muscle > liver (ANOVA, p < 0.05), respectively. For natural steroids, six, six, six and five steroids were detected in the bile, plasma, liver and muscle sample, respectively, and the total concentrations of natural steroids in the tissues were in the same order as the total concentrations of steroids. For synthetic steroids, eight, eleven, six and five steroids were detected in the bile, plasma, liver and muscle sample, respectively. There was no significant difference for the total concentrations of synthetic steroids among the four tissues (ANOVA, p > 0.1).

3.2.1. Bile

Previous studies reported that estrogens showed much higher concentrations in the bile of fish (equal to 4000 μg/L) exposed to sewage treatment effluent than rivers, estuaries, or coastal waters (up to 30 μg/L), especially for synthetic ones (Pettersson et al., 2006; Bizarro et al., 2014; Yang et al., 2014). However, studies concerning other classes of steroids such as androgens, glucocorticoids and progestagens in fish farm environment are very limited. In this study, 14 steroids were detected in the bile samples (Fig. 1 and Table S6). Natural glucocorticoid CRL was the most dominant steroid in the bile samples, with concentrations ranging from 700 to 5900 μg/L (Table S6). Besides CRL, the other three natural steroids AED (86%), T (79%) and P (75%) had high detection frequencies in the bile samples (Table 1 and Table S6). It was difficult to tell from the respective contribution of these natural steroids from...
environment, feed or endogenous existence in the fish species. Eight synthetic steroids including five androgens, one glucocorticoid, and two progestagens were detected in 4–54% of the bile samples with concentrations ranging from 1.1 to 76 mg/L (Table S6). Among these eight synthetic steroids, only ADD and 17α-BOL had relatively high detection frequencies of 54% and 46% in the bile samples, respectively. It was noted that 17α-BOL was detected in tilapia bile samples but not detected in the corresponding environmental samples or feed samples, indicating that 17α-BOL may come from endogenous source or transform from other steroids (Scarth et al., 2009; Yang et al., 2010). For different species, the numbers and total concentrations of steroids detected in the bile samples were in the order of tilapia > crucian carp > mullet > mud carp > spotted scat and tilapia, crucian carp, mullet > mud carp > spotted scat (ANOVA, p < 0.05), respectively. The distribution of steroids in the bile indicated that there were significant differences among different species even at the same cultured environment. For example, less numbers and lower levels of steroids were detected in the bile samples of spotted scat than those of tilapia in farm F2.

3.2.2. Plasma

Seventeen steroids were detected in the plasma samples with concentrations up to 9600 μg/L (CRL) (Table S7), which were rather higher than those exposed to wild environment such as sewage effluents (8.5–12 μg/L) (Fick et al., 2010), estuaries (<0.10–14 μg/L) (Budzinski et al., 2006), or laboratory examination (160–1500 μg/L) (Steele et al., 2013). Except spotted scat, CRL was the most dominant steroid in the plasma samples. CRL is the main glucocorticoid in teleost fish and its plasma concentrations will significantly increase in response to stressors such as handling (Ellis et al., 2004) or high stocking densities (Fanouraki et al., 2008). In this study, synthetic steroids including four androgens, two glucocorticoids, and five progestagens were detected in the plasma samples (Fig. 1). Among the 11 synthetic steroids, 17α-BOL and ADD, may come from endogenous source or transform from other steroids (Scarth et al., 2009; Yang et al., 2010). It was noted that synthetic progestagen CPTA had higher detection frequencies (49%) and detection concentrations (mean 41 mg/L) in the plasma samples (Table S7), indicating that CPTA was more inclined to be bioconcentrated in the fish plasma due to its lipophilicity (log Kow = 4.2). For different species, the numbers and total concentrations of steroids detected in the plasma samples were in the order of tilapia > mullet > spotted scat, crucian carp, mud carp and tilapia, crucian carp, mullet, mud carp > spotted scat (ANOVA, p < 0.05), respectively. Except spotted scat, there was no significant difference in the total concentrations of steroids detected in the plasma samples among different species, suggesting that species might not be an important factor for the distribution of steroids in the plasma. The detection of steroids in the fish plasma could also provide useful means for risk assessment of pharmacological effects by comparison to human therapeutic plasma levels (Fick et al., 2010). The progestagen pharmaceutical MGT was detected in fish plasma with the concentration up to 8.6 μg/L in farm F1, close to the human therapeutic plasma level (10 μg/L) (Miller et al., 1988), which would have a potentially pharmacological effect on the farmed fish (Fick et al., 2010).

Fig. 1. Mean concentrations of steroids in (1) bile (n = 28), (2) plasma (n = 33), (3) liver (n = 37) and (4) muscle (n = 37) of freshwater cultured fish in the study farms.
3.2.3. Liver

Twelve steroids were detected in the liver samples with concentrations up to 970 ng/g (CRL) (Table S8), which were much higher than estrogens in the liver samples of tilapia exposed to the river (1.6–3.3 ng/g) (Chen et al., 2014a) or crucian carp (45 ng/g) and carp (61 ng/g) exposed to sewage effluents (Huang et al., 2013), but lower than the liver samples of common carp exposed to medroxyprogesterone acetate under the laboratory examination (960–6500 ng/g) (Steele et al., 2013). Three natural steroids CRL, T and AED were most frequently detectable steroids in the liver samples. Synthetic steroids including 2 androgens and 4 progestagens were detected in the liver samples (Fig. 1). Higher detection frequency and detection concentrations of synthetic progestagen CPTA occurred in the liver samples (78%) and in the plasma (49%) as well as muscle samples (49%), which demonstrated that CPTA was more inclined to be bioconcentrated in these tissues. Less numbers and lower concentration levels of steroids were detected in the liver samples, as compared to those in the bile and plasma samples. This is probably due to the detoxification of various metabolites by the liver which can break down or modify toxic substances including exogenous steroids (Parkinson, 2001). The numbers and total concentrations of steroids detected in the liver samples were in the order of tilapia, crucian carp > mullet > spotted scat > mud carp and crucian carp > tilapia, spotted scat, mud carp, mullet (ANOVA, p < 0.05), respectively. The significant difference of total concentrations of steroids detected in the liver samples between crucian carp and the other species were mainly due to the contribution from CRL and CPTA.

3.2.4. Muscle

Ten steroids were detected in the muscle samples with concentrations up to 2700 ng/g (CRL) (Table S9), which were higher than those in crucian carp (11 ng/g) and carp (12 ng/g) exposed to sewage effluents (Huang et al., 2013), marine aquaculture products (<0.10–560 ng/g) from Hailing Island (Liu et al., 2015a), or the common carp exposed to MPA under the laboratory examination (150–780 ng/g) (Steele et al., 2013). Natural steroids CRL and CRN displayed the same highest detection frequency of 100% in all the muscle samples (Table S9). CRN was only detected in the muscle samples, but not occurred in the environmental samples or feed samples, which demonstrated that it is endogenous in fish (Liu et al., 2012a). In total, five synthetic steroids were detected in the muscle samples. Among the five synthetic steroids, CPTA displayed the highest detection frequency of 49% and were mainly detected in the muscle samples of fish from farm F3. Considering the undetectable CPTA in the environmental samples from farm F3, the bioaccumulation of CPTA in fish muscles might come primarily from the feed (Fish-F3, 5.7 ng/g). Synthetic androgens 17α-BOL and 19-NT were sporadically detected in the fish muscle samples with the mean concentrations of <0.33 and < 0.55 ng/g, respectively (Table S9). Both synthetic progestagens 17α-HPA and MGT were only detected in the muscle samples of crucian carp rather than in the environmental samples or other species. The reason could be due to the low concentrations (close to or below the limit of detection) of steroids in the environmental samples, which led to be undetectable. These results also indicated that the muscle of crucian carp showed a more bioaccumulative potential for these two synthetic progestagens than the other species. The numbers and total concentrations of steroids detected in the muscle samples were in the order of crucian carp > tilapia, mullet > spotted scat > mud carp and crucian carp, mullet, mud carp > tilapia, spotted scat (ANOVA, p < 0.05), respectively. Lower concentration levels of steroids were detected in tilapia and spotted scat muscle samples, mainly due to relatively lower concentrations of CRL in these two species.

3.2.5. Influence factors

The distribution of endogenous and exogenous steroids in fish tissues are influenced by many factors such as species, life-stage and size (length and weight) (Liu et al., 2011b). Fig. 2 showed that there were clear separations with sample clusters among different fish species (except crucian carp), indicating that fish species had an influence on the distribution of steroids. In this study, RDA analysis was conducted to assess the potential relationship between the concentrations of steroids in the tissues and fish length and weight. The significant variations in steroids concentrations revealed by RDA was 16% (weight, p < 0.01) for the bile samples, 23% (length, p < 0.01) for the plasma samples, 9.0% (weight, p < 0.05) for the liver samples, and 13% (length, p < 0.05) for the muscle samples (Table S10). These results indicated that fish size could influence the bioaccumulation of steroids in different tissues, however, the extent of this influence was relatively small. Besides biological factors, the physicochemical property of a exogenous steroid, for example log Kow, is also an important influence factor (Yang et al., 2014). There are positive linkages of log BAF and log Kow for bile samples (correlation coefficient R = 0.38, p < 0.05) and plasma samples (R = 0.81, p < 0.05) (Fig. S2). For the liver samples, all detected concentrations of CPTA and 19-NT in the water samples or corresponding liver samples were below the LOQs, and half of LOQs were used to calculate the BAFs. As a result, only two values of Log BAF = 4.2 and Log BAF = 3.5 were available for CPTA and 19-NT for different fish species (n = 13). This situation was also found in the muscle samples. Thus, for the liver and muscle samples, it was difficult to evaluate the impact of log Kow on bioaccumulation due to limited data.

3.3. Bioaccumulation of steroids in fish tissues

It is the first study to determine the bioaccumulation of steroids in different tissues of fish in aquaculture farms. Some natural steroids such as P, CRL, E2, 17α-HPA and MGT were used to calculate the BAFs. As a result, only two values of Log BAF = 4.2 and Log BAF = 3.5 are bioaccumulative, in the range of 1000 to 5000 to be bioaccumulative, and greater than 5000 to be very bioaccumulative (Bioaccumulation Criteria, 2012). The median BAFs were higher than 1000 except for ADD in plasma and bile (450), 19-NT (360) and CPTA (42) in the muscle, indicating that the second hypothesis was
true and most of detected synthetic steroids were bioaccumulative or very bioaccumulative in fish tissues. In addition, synthetic steroids are more likely bioaccumulated in tilapia except 17α-BOL and CPTA. These results illustrated that different species can affect the BAF values of steroids in different tissues, and tilapia can be used as a good biological indicator for steroids in aquaculture environment.

3.4. Chemical indicators of steroids in fish tissues

Based on the previous studies, chemical indicators have been found in surface water and sediment environment (Chen et al., 2014b; Liu et al., 2015b). In this study, all detected steroids were used to analyze the correlations between single steroid and the rest steroids in each tissue. Pearson correlation analysis showed there were positive correlations between single steroid and part of individual steroids for bile, plasma, liver and muscle samples based on all fish species (Tables S11-S15). For plasma samples (n = 33), most detected steroids with relatively high detection rates (36–100%) such as T, CRL, CRN, PREL, CPTA and P were statistically related to AED (p < 0.05). In addition, a strong positive linear

![Fig. 2. Redundancy analysis (RDA) ordination plots based on the concentrations of steroids and fish sizes (weight and length) in (1) bile, (2) plasma, (3) liver and (4) muscle samples.](image-url)

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>N</th>
<th>Range</th>
<th>Mean</th>
<th>Median</th>
<th>Species and site for max</th>
</tr>
</thead>
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<tr>
<td><strong>Bile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADD</td>
<td>15</td>
<td>450–9200</td>
<td>2300</td>
<td>1600</td>
<td>Tilapia (Farm F1)</td>
</tr>
<tr>
<td>17α-BOL</td>
<td>6</td>
<td>4300–10000</td>
<td>6500</td>
<td>5900</td>
<td>Crucian carp (Farm F3)</td>
</tr>
<tr>
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<td>8</td>
<td>5800–97000</td>
<td>38,000</td>
<td>30,000</td>
<td>Tilapia (Farm F1)</td>
</tr>
<tr>
<td>17β-TBL</td>
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<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>Tilapia (Farm F2)</td>
</tr>
<tr>
<td>MPA</td>
<td>3</td>
<td>9000–35000</td>
<td>21,000</td>
<td>20,000</td>
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</tr>
<tr>
<td><strong>Plasma</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADD</td>
<td>2</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>Tilapia (Farm F2)</td>
</tr>
<tr>
<td>17α-BOL</td>
<td>4</td>
<td>2400–4300</td>
<td>3000</td>
<td>2600</td>
<td>Mullet (Farm F3)</td>
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<tr>
<td>CPTA</td>
<td>4</td>
<td>3400–65000</td>
<td>20,000</td>
<td>5200</td>
<td>Spotted scat (F2)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>19-NT</td>
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<td>2900</td>
<td>2900</td>
<td>2900</td>
<td>Tilapia (Farm F1)</td>
</tr>
<tr>
<td>CPTA</td>
<td>11</td>
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<td>16,000</td>
<td>16,000</td>
<td>Tilapia and Spotted scat (Farm F2)</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-BOL</td>
<td>1</td>
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<td>2600</td>
<td>2600</td>
<td>Mullet (Farm F3)</td>
</tr>
<tr>
<td>19-NT</td>
<td>3</td>
<td>360</td>
<td>360</td>
<td>360</td>
<td>Tilapia (Farm F1)</td>
</tr>
<tr>
<td>CPTA</td>
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<td>42</td>
<td>42</td>
<td>42</td>
<td>Spotted scat (F2)</td>
</tr>
</tbody>
</table>

*ADD, androsta-1,4-diene-3,17-dione; 17α-BOL, 17α-boldenone; 19-NT, 19-nortestosterone; 17β-TBL, 17β-trenbolone; CPTA, cyproterone acetate; MPA, medroxypregesterone acetate.*
correlation was found between AED and the total steroids including AED (Fig. S3), as well as AED and total steroids not including AED (Table S15). For muscle samples (n = 37), CRL, CPTA, 17α-HPA and MGT were statistically related to CRN (p < 0.05). In addition, a strong positive linear correlation was found between CRN and the total steroids including CRN (Fig. S3), as well as CRN and total steroids not including CRN (Table S15). These results indicated that AED and CRN could be reliable chemical indicators to predict the levels of detected steroids in plasma and muscle for the interspecies cultured fish, respectively. However, for the other tissues, good correlations of different steroids were not obvious. For example, for bile samples (n = 27), among 14 detected steroids, only five steroids AED, 17α-BOL, 19-NT, 17α-TBL and CPTA were statistically related to ADD (p < 0.05) (Table S15). For liver samples (n = 37), although over half of detected steroids (AED, T, 19-NT, CRN, CPTA, 17α-HPA and MGT) were statistically related to NTND (p < 0.05), the correlation was not found between NTND and the total steroids (Table S15). The apparent differences in distribution of steroids among these four tissues were probably due to different chemical properties (Table S1), different exposure concentration (Table 1), uptake rates (Blewett et al., 2013) and metabolic processes (Jurgeila et al., 2006; Scott et al., 2014). As deduced from this study, good correlations between single steroid and other individual steroid/total steroids in fish plasma and muscle samples could give valuable information on distribution of steroids in fish tissues.

3.5. Human health risk from fish consumption

Based on “a worst-case scenario”, the maximum concentrations of steroids detected in fish muscle were used to calculate the daily intake from fish consumption. The estimated daily intake (EDIs) of androgens, glucocorticoids and progestagenas via fish consumption were in the range of 0–100 ng/d, 10,000–180,000 ng/d and 53–9200 ng/d for local residents, respectively (Fig. 3). The EDIs of androgens via freshwater fish consumption were similar to those via estimated marine fish consumption reported in literature (Liu et al., 2015a). However, EDIs of glucocorticoids and progestagens via freshwater fish consumption in this study were found higher than those via marine cultured fish consumption in Hailing Island (Liu et al., 2015a). There were some deviations from the results in the present study, probably due to the different culture environment and species. For the same fish species, the total EDIs of steroids for tilapia in farm F1 was approximately four-fold of that in farm F2, and the gap was mainly caused by CRL, which was only detected in the water sample of farm F1 (Table 1 and Fig. 3). In addition, there was an apparent difference among different species in the same farm. For example, the total EDIs of androgens via crucian carp and mullet in farm F3 were 70 ng/d and 64 ng/d, but no androgen was detected in mud carp muscle. These results were probably due to different bioaccumulation of exogenous androgens as well as different concentration levels via endogenous androgens in the muscle of different species.

According to the International Joint Food and Agricultural Organization’s World Health Organization Expert Committee on Food Additives (JECPA, 2014) and General Administration of Quality Supervision, Inspection and Quarantine of the People’s Republic of China (QSIQ, 2002), only two natural steroids T and P have ADIs with values of 0–2.0 and 0–30 μg/kg bw/d (Table S16). The maximum concentrations of steroids detected in fish muscle were used to calculate the risks to global consumers’ health via consumption of fish exported from Guangzhou of China. The maximum HQs of testosterone and progesterone were 5.8 × 10⁻⁴ and 9.9 × 10⁻⁵ (Table S17), far below 1, suggesting that no human health risk was expected for testosterone and progesterone detected in the fish from selected farms.

The maximum residue limits for steroids in animal tissues have been summarized in Table S16. The use of steroids as growth promoters in animal breeding has been banned in many countries, however, there are only several steroids regulated in the guidelines. For androgens, synthetic ones such as MT, 19-NT, 17α-HPA and 17β-TBL are not allowed to be detected in the edible tissues (QSIQ, 2002). In this study, the synthetic androgen 19-NT was only detected in tilapia muscle with low concentration (<0.55 ng/g) and low detection frequency (8.1%), indicating that it is likely to have certain health risks by the intake of tilapia collected from the aquaculture zones of Guangzhou, South China. For glucocorticoids, CRL was detected in fish muscle with high concentrations up to 2700 ng/g, which was thousands fold higher than other detected steroids (Table S9). Based on the available guidelines, there were no ADIs of natural glucocorticoids and the corresponding potential health risk was difficult to estimate. For progestagens, although the maximum concentrations of synthetic progestagens CPTA, 17α-HPA and MGT were 46 ng/g, 1.7 ng/g, 96 ng/g in the muscle, respectively, which were higher than the MRLs for megestrol acetate and flugestone acetate in the guidelines. Furthermore, many studies have indicated that steroids mixtures and the transformation products can actually exhibit greater toxicity than their parent compounds to fish (Elskus, 2014; Farré et al., 2008). Thus, it is difficult to evaluate the potential health risk of steroids from fish consumption due to limited studies and guidelines. The residual steroids in fish muscle would not only bring human health risk to local residents, but also influence the non-local people due to aquatic product exports. As a consequence, appropriate management actions should be taken to control their use in aquaculture environment.

4. Conclusions

This study demonstrated that natural and synthetic steroids were ubiquitously detected in water, suspended particles, sediment, feed, and animal feces from the selected aquaculture farms. Water sources, feed with illegally added steroids and animal feces were important pollution sources of steroids during the process of aquaculture. Most detected synthetic steroids were bioaccumulative or very bioaccumulative in fish tissues. It was worth noting that AED and CRN could act as reliable chemical indicators to predict the levels of steroids in plasma and muscle of the interspecies cultured fish, respectively. This work provides a context for exploring the bioaccumulation patterns for different classes of steroids, particularly the complex mixtures that cultured fish are exposed to under typical ambient environmental conditions.

![Fig. 3. Estimated daily intakes (ng/d) of steroids via fish consumption for local residents.](image-url)
Further research is required to expand the fish species and tissues, enrich and deduce more generalized bioaccumulation profiles. For local consumers, human health risk was negligible for testosterone and progesterone from the ingestion of steroids-contaminated fish. In addition to the two parent compounds, other steroids mixtures or the transformation products in the edible part might bring potential human health risks. Further work should understand the human health risks due to the presence of steroids mixtures and their transformation products in the edible part, especially in light of increasingly global demand for the security of aquaculture products.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.05.031.

References
