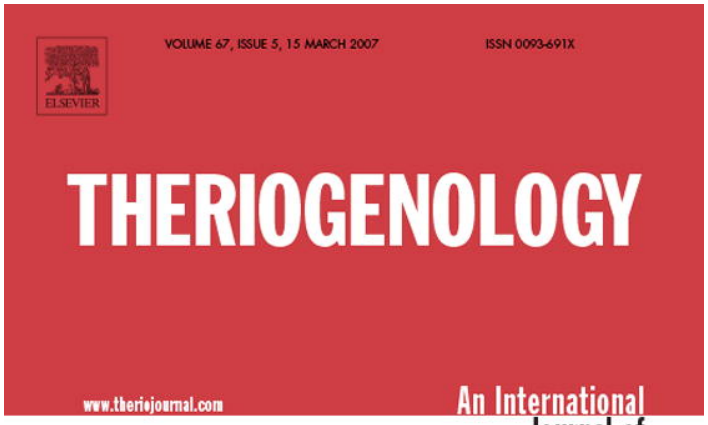

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Sex steroid dynamics during embryogenesis and sexual differentiation in Eurasian perch, *Perca fluviatilis*

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Abstract

It is widely accepted that sex steroid hormones play an important and a specific role during the process of sex differentiation in fish. In order to describe the role of the three main sex steroid hormones (testosterone—T, 17β-estradiol—E2 and 11keto-testosterone—11KT) during embryogenesis and sex differentiation in Eurasian perch, *Perca fluviatilis*, eggs, larvae and juveniles originating from two mixed-sex and two all-female progenies were regularly sampled from fertilization to hatching (D0) and from hatching to day 70 post-hatching (D70). Just after spawning, a significant amount of sex steroids [T (1634.2 pg g^{-1}), E2 (554.4 pg g^{-1}) and 11KT (1513.2 pg g^{-1})] was measured in non-fertilised eggs suggesting a maternal transmission of these steroids. From D2 to D70 post-hatching, E2 levels were significantly higher in mixed-sex progenies (median: 725.7 pg g^{-1}) than in all-female progenies (156.2 pg g^{-1}) and significantly increased after the onset of the histological differentiation of the gonad in both progenies (D35). Levels of 11KT were significantly higher in mixed-sex (median: 431.5 pg g^{-1}) than in all-female progenies (below the limit of assay detection) and significantly increased at D35 in all-female progenies (median value: 343.2 pg g^{-1}). Mean 11KT to E2 ratio was six-fold higher in mixed-sex progenies (1.35) than in all-female progenies (0.24). The data suggest that the 11-oxygenated androgen (11KT) plays a major role in the male differentiation process, and that sex differentiation in Eurasian perch is probably determined by the 11KT to E2 ratio.

Keywords: Embryogenesis; *Perca fluviatilis*; Sexual determination; Sexual differentiation; Sex steroid hormone

1. Introduction

Sex determinism mechanisms in fish are under the control of genetic (sex chromosomes and autosomes), environmental (temperature, density and pH) and physiological factors (sex steroid hormones), which act in synergy to induce the male and female sex differentiation process [1–4]. Yamamoto [5] was the first to observe that treatment with exogenous sex steroids (androgens and estrogens) induced phenotypic sex reversal in medaka (*Oryzias latipes*) and hypothesised that exogenous as well as endogenous sex steroid hormones could play a major role in sex differentiation in fish. Since this observation, it is now well accepted that sex steroid hormones play an important role during the process of sex differentiation in fish [2,3,6].

Although a multitude of non-steroidal hormones are involved in gonadal differentiation, the most investigated hormones are the androgens in particular testosterone (T) and 11-ketotestosterone (11KT), and estrogens mainly 17β-estradiol (E2) [3]. Testosterone is the main androgen in mammals, birds and reptiles,

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while 11KT (11-oxygenated androgen) is often considered as the main androgen involved in testis differentiation in teleosts [3,7–12]. As testosterone is the hormonal precursor of 11KT and E2, its role in sexual differentiation is therefore of great importance and is generally reflected in the processes of sex differentiation [6,13–15]. E2 is considered as a major sex steroid in fish and plays an important role in vertebrate prenatal development, particularly concerning sexual differentiation [16,17].

The presence of sex steroids before and during sexual differentiation was studied in many species and their role during embryogenesis and sex differentiation is considered to be species specific [4,18]. In tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and grey mullet (*Mugil cephalus*) ultrastructural studies of the gonad during the process of sex differentiation have demonstrated the presence of androgens, particularly 11KT and 11-oxygenated androgens, before the onset of sexual differentiation and pointed up their role in sex determination [18–20]. Studies of sex steroid hormones levels and gene expression of 11β-hydroxylase (enzyme involved in the biosynthesis of the 11β-hydroxytestosterone and 11β-hydroxyandrostenedione) during sex differentiation in tilapia and rainbow trout also demonstrated the importance of these steroids in this process [13,21,22]. Similar studies, based on aromatase gene expression (enzyme involved in the biosynthesis of estradiol) and aromatase inhibition during sex differentiation have proven that endogenous oestrogen are responsible for ovarian differentiation in Nile tilapia [11,23,24], Japanese flounder (*Paralichthys olivaceus*, [25,26]) and rainbow trout [11, see also 16]. On the other hand, the lack of steroid-producing cells before the onset of sexual differentiation and the inefficiency of aromatase inhibitor to impair the ovarian differentiation in the medaka, strongly suggest that ovarian formation could be sex steroid independent [4,27]. According to Baroiller et al. [2] and Bogart [28], sex differentiation could also depend on the 11KT to E2 ratio: 11KT excess induces males differentiation while E2 excess induces female differentiation.

The phenotypic sex of Eurasian perch, *Perca fluviatilis*, could easily be controlled by hormonal treatment applied before and during sexual differentiation [29,30]. The use of exogenous 17α-methyltestosterone (masculinizing hormone) induces the production of hormonally sex-reversed males (XX males), which are as fertile as normal XY males in term of fertilization rate, gonadosomatic index, sperm density and motility and plasma sex steroid levels during the breeding period [31]. These results suggested that exogenous sex steroids and therefore endogenous sex steroids could play an important role during sex differentiation in Eurasian perch.

The aim of the present study was to investigate the dynamics of the three main sex steroids (T, E2 and 11KT) during embryogenesis and the course of sexual differentiation in mixed-sex and all-female Eurasian perch progenies, in order to describe and understand their role in sex differentiation mechanisms in this species.

2. Materials and methods

2.1. Fish

Steroid levels during embyogenesis and sexual differentiation were determined on two mixed-sex (theoretically 50% males and 50% females) and two all-female Eurasian perch progenies obtained by artificial reproduction with captive breeders reared in the CEFRA of the University of Liège (Belgium). Mixed-sex progenies were obtained by artificially fertilized eggs with sperm originating from a normal XY male and all-female progenies were obtained by fertilizing eggs with sperm originating from hormonally sex-reversed XX male [30].

2.2. Egg and larval samples

Three grams of eggs were sampled from each egg ribbon just after spawning ($H = 0$) and every 12 h during incubation until hatching (D0). Thereafter, from the second day (D2) until the 70th day (D70) post-hatching, larvae were sampled every 5 days in the morning before feeding. Three grams of larvae were sampled from days D2 to D25 post-hatching. When mean body weight (MBW) of larvae reached 50 mg (D30), the number of larvae sampled per family was fixed at 60 individuals until the end of the sampling period. Samples were stored at −80 °C before steroids extraction. At each sampling date, 20 larvae were individually weighted (±0.1 mg).

According to the observations of Rougeot et al. [30] who determined the onset of gonad differentiation at D30–D35 post-hatching, the sexual differentiation period (from D2 to D70 post-hatching) was split into two sub-periods, the first sub-period ranging from D2 (mean body weight, MBW: 1.2 mg) to D30 (MBW: 79.3 mg), i.e. before the onset of the histological differentiation of the gonad, and the second sub-period ranging from D35 (MBW: 127.8 mg) to D70 (MBW: 1433 mg) after the onset of the histological differentiation of the gonad.

2.3. Sex steroid assays

Steroids were extracted from the whole the eggs or the body [32]. Each eggs or larvae sample were mixed before steroids extraction. For each steroid, 1 g of mixed sample was first homogenized with 1 ml of ethanol 50% and washed with 3 ml of absolute ethanol. After centrifugation of the crude extract (15 min at 4000 × *g* at 10 °C), supernatant was collected and the residue was washed with 1 ml of ethanol 80%. After partial evaporation of all the supernatant, steroid was extracted three times with dichloromethane (v 1: 5) and then conserved in absolute ethanol at −20 °C. Two hundred microliter of this solution were analysed for each sex steroid.

Whole body concentrations of testosterone (T), 17β-estradiol (E2) and 11-ketotestosterone (11KT) were assayed by RIA according to Fostier and Jalabert [33]. All samples and standards were assayed in duplicate. The T and E2 antisera were purchased from the Laboratoire d'Hormonologie de Marloie—Belgium, and the 11KT antiserum was provided by Dr. A. Fostier (INRA). All the radioactive hormones were purchased from Amersham Pharmacia. For each steroid, one assay was performed and 14 samples at various dilutions were run to estimate the intra-assay coefficients of variation (6.33, 6.42 and 8.83% for T, 11KT and E2, respectively). For all three steroid assays, the detection sensitivity was at 4–5 pg ml^{-1}.

2.4. Sex ratio analysis

When fish reached a mean body weight of 20 g, 100 individuals were randomly sampled in each family and killed with an anaesthetic (2-phenoxy-ethanol) overdoses. Gonads were removed for gross morphological examination [30], males having two testes and females displaying a single ovary.

2.5. Statistical analysis

Plasma sex steroid levels in eggs during embryogenesis and sexual differentiation were compared between control and all-female group using the non-parametric Mann–Whitney *U*-test. Sex ratios were compared using the chi(χ^2)-square test. Levels of significance were accepted at $P < 0.05$.

3. Results

3.1. Spawning and embryo dynamics

Just after spawning, a high concentration of sex steroid hormones was accumulated in non-fertilised eggs: testosterone (mean value: 1634.2 pg g^{-1}; median value: 870.2 pg g^{-1}), 11keto-testosterone (mean value: 1513.3 pg g^{-1}; median value: 629.8 pg g^{-1}) and 17β-estradiol (mean value: 550.4 pg g^{-1}; median value: 545.5 pg g^{-1}; Fig. 1). In developing embryos (from 12 to 156 h post-fertilization), testosterone levels were not significantly different ($P > 0.05$) between control and all-female groups (mean values: 1331.0 and 1280.5 pg g^{-1}; median values: 809.0 and 715.1 pg g^{-1} for control and all-female groups, respectively). 11KT levels

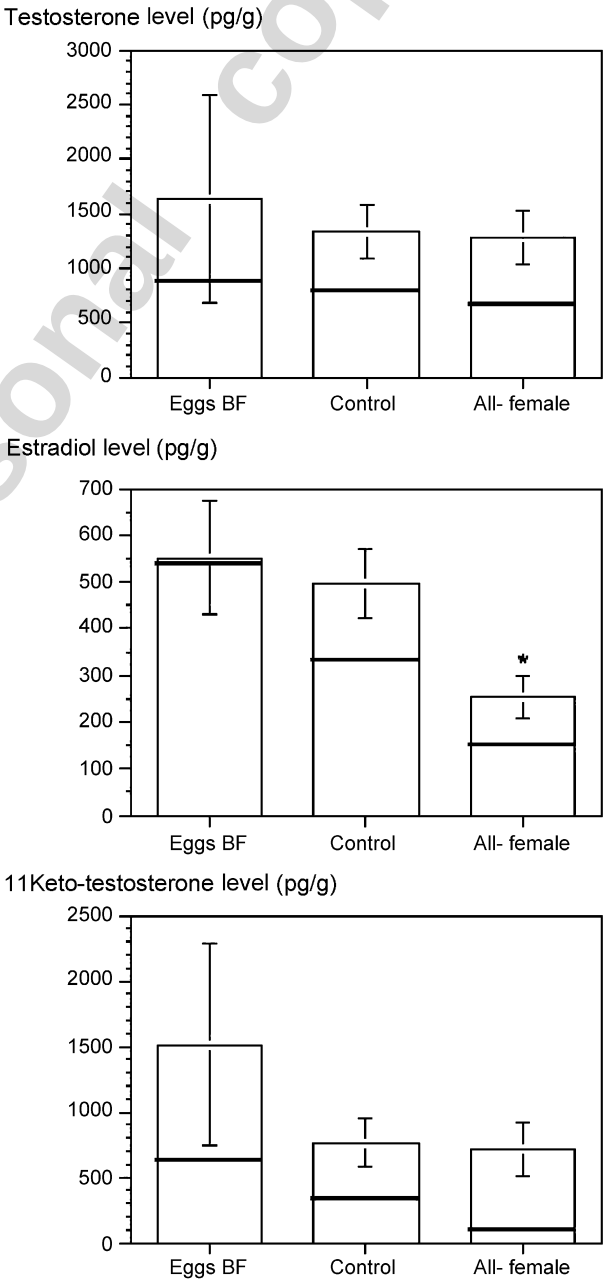

Fig. 1. Sex steroid levels (T, E2 and 11KT) in eggs before fertilization (eggs BF) and in control and all-female developing embryos. Values are mean ± S.E.M., dark trait represent median values. * Significantly different ($P < 0.05$).

ranged from 0.0 (below the limits of assay detection) to 3569.9 pg g^{-1} and were not significantly different between control and all-female embryos (mean values: 760.2 and 717.8 pg g^{-1}; median values: 337.6 and 103.84 pg g^{-1} for control and all-female group, respectively). Surprisingly, all-female embryos displayed a significantly ($U = 612.0$ and $P < 0.05$) lower level of estradiol (mean value: 253.9 pg g^{-1}; median value: 151.5 pg g^{-1}) than the control embryos (mean value 497.4 pg g^{-1}; median value: 335.2 pg g^{-1}).

3.2. Larval and juveniles dynamics

During the entire sexual differentiation period (D2–D70, Fig. 2) testosterone levels ranged between 74.8 and 5531.3 pg g^{-1} and were not significantly different ($U = 257.0$; $P > 0.05$) between control (median value: 542.2 pg g^{-1}) and all-female groups (median value: 727.3 pg g^{-1}; Fig. 3). A peak of testosterone level was observed between D35 and D40 in both progenies (2354.1 and 3076.5 pg g^{-1} for control and all-female family, respectively). Overall, testosterone levels did not changed significantly ($P > 0.05$) between the second sub-period of differentiation for both control and all-female progenies (Fig. 3).

From D2 to D70, estradiol levels were significantly ($U = 550.5$; $P < 0.05$) higher in the control group (median value: 725.7 pg g^{-1}) than in the all-female groups (median: 156.2 pg g^{-1}, Figs. 2 and 3). A significant ($P < 0.05$) increase of estradiol level was observed after the onset of histological differentiation of the gonad (D35–D70) in both control (median value: 920.4 and 519.6 pg g^{-1} for D35–D70 and D2–D30, respectively) and all-female progenies (median values: 178.1 and 99.5 pg g^{-1} for D35–D70 and D2–D30, respectively, Fig. 3).

During the entire sexual differentiation period (D2–D70), 11 keto-testosterone levels were six-fold higher ($U = 521.5$; $P < 0.05$) in mixed-sex progenies (median:

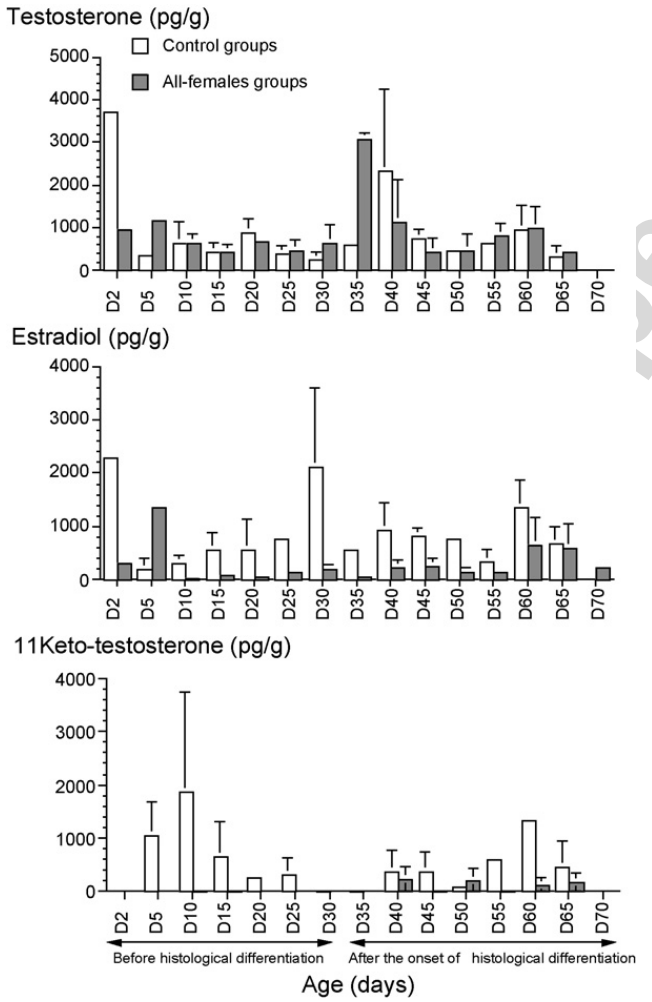

Fig. 2. Whole body levels of testosterone, estradiol and 11keto-testosterone (pg g^{-1}) during larval and juvenile development in mixed-sex and all-female progenies. Values are means ± S.E.

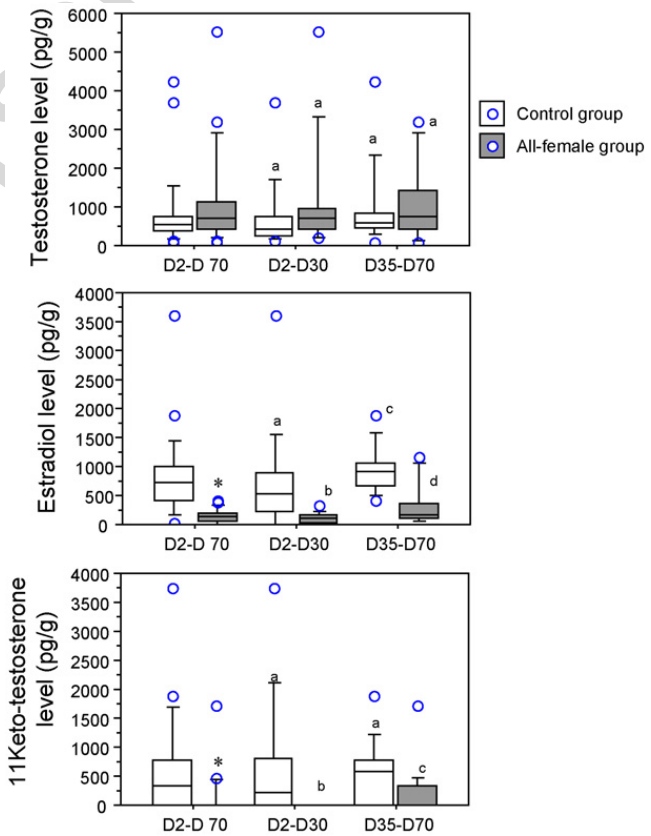

Fig. 3. Box plot graph of the whole body concentration of testosterone, estradiol and 11keto-testosterone (pg g^{-1}) in mixed-sex and all-female progenies before the onset of gonad differentiation (D2–D30) and after the onset of the histological differentiation (D35–D70). Values are mean, median, minimum, maximum, percentile 5 and percentile 95. Values with different superscript letters are significantly different ($P < 0.05$). * Significantly different ($P < 0.05$).

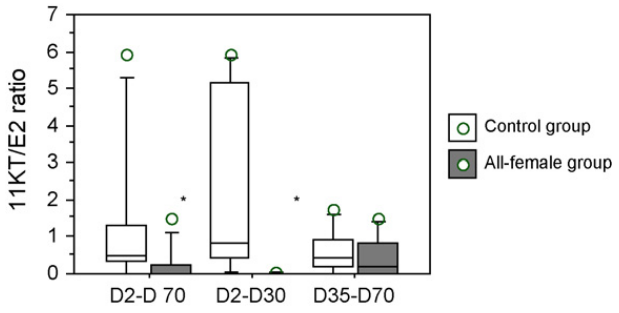

Fig. 4. Box plot graph of the 11 keto-testosterone to estradiol ratio in whole body extract before (D2–D70) and after the onset of the histological differentiation (D35–D70) in Eurasian perch. Values are mean, median, minimum, maximum, percentile 5 and percentile 95. * Significantly different ($P < 0.05$).

431.5 pg g^{-1}) than in all-female progenies (below the limits of assays detection) for which the 11KT level was only measurable from D40 (Fig. 2). In all-female progenies, 11 KT level significantly ($U = 122.0$; $P < 0.05$) increased from D2–D30 (values below the limits of assay detection) to D35–D70 (median value: 343.2 pg g^{-1}). There was no significant change in 11KT levels ($P > 0.05$) observed in mixed-sex progenies (Fig. 3).

From D2 to D70, 11KT to E2 ratio was significantly ($P < 0.05$) higher in mixed-sex groups (1.35) than in all-female groups (0.24, Fig. 4). During the first sub-period (from D2 to D30) this difference was pronounced with a 11KT to E2 ratio 438-fold higher in mixed-sex groups (median value: 0.814; mean value: 2.19) than in all-female ones (median value: 0.00; mean value: 0.005). During the second sub-period (from D35 to D70), after the initiation of the histological differentiation of the gonad, the ratio was not significantly different ($P > 0.05$) between mixed-sex and all-female groups, although it was 1.5-fold higher in mixed-sex groups (median value: 0.456; mean value: 0.631) than in all-female progenies (median value: 0.212; mean value: 0.438).

3.3. Sex ratios

The sex ratio of the mixed-sex groups ranged from 50 to 55% females and therefore did not significantly ($P > 0.05$) deviate from 1: 1. Sex ratios of all-female groups were 100% female.

4. Discussion

The presence of sex steroid hormones in eggs before fertilization supports the idea that some sex steroids were maternally transferred into the eggs [8]. Sex steroids levels in stripped or unfertilised eggs could very be high, as observed in other fish species or in reptiles, suggesting a hormonal transmission from the female to the eggs [8,13,34]. These ''maternal'' sex steroids could be used as precursors to induce the initial sexual differentiation of the brain and/or the gonads. Sex steroid hormones are principally synthesized in gonad, although non-gonadal tissues, such as brain, blood, kidney or liver, can also synthesize these hormones [10,14,19,35]. The detection or presence of significant levels of testosterone, estradiol and 11keto-testosterone in embryos and developing larvae of Eurasian perch before the histological differentiation of the gonad strongly suggest an extra-gonadal synthesis of these sex steroid hormones in Eurasian perch, as has been observed in Coho salmon [8] and tilapia [12].

As for most teleost species [10], the testosterone levels were similar between mixed-sex and all-female batches. In fish, testosterone is not directly implicated in sexual differentiation mechanisms, but plays an intermediate role as precursor of 11-ketotestosterone and 17β-estradiol [2,9]. Nevertheless, in many species, testosterone seems to play an important role in sex differentiation and is closely related to gonadal differentiation [6,8,13,14,20]. In the present study, the testosterone peak observed at D30–D35 could be associated with the occurrence of sex differentiation in Eurasian perch [30], but as for other fish species, it was not possible to conclude if it is a cause or a consequence of gonadal differentiation [8,15,19,20]. The high levels of 11KT (median: 431.5 pg g^{-1}) in mixed-sex groups and the lack of a detectable level in all-female batches suggest that this steroid was particularly important for male sex differentiation in Eurasian perch. The implication of 11KT and 11-oxygenated androstenedione derivatives in male differentiation was previously reported in rainbow trout [7,22,36], Coho salmon [8], Nile tilapia [12] and white sturgeon *Acipenser transmontanus* [37]. Generally the plasma concentrations of 11KT are considerably higher in male than in female teleosts, although in some species, females do have similar of higher blood concentrations of 11KT than males [10,12,38]. Surprisingly, E2 levels were significantly higher in the mixed-sex groups than in all-females groups during the entire course of the study. Although in some species males could display high levels of estradiol [39], this result is difficult to explain. Nevertheless, the general increase of T, 11KT and E2 levels observed after the onset of sexual differentiation (D35–D70) in both progenies suggest an increase of the steroidogenesis and steroid activity after the onset of gonadal differentiation, as it was observed in Nile tilapia and Coho salmon [8,13].

The notable difference of 11KT to E2 ratio between mixed-sex and all-female progenies strongly suggest that sex differentiation in Eurasian perch is closely controlled by this ratio. An excess of estradiol induces the female differentiation process while an excess of 11-KT induces the male differentiation process. This hypothesis, suggested by Baroiller et al. [2] and Bogart [28], seems to concur with the sex steroid differentiation process in Eurasian perch as observed in the present study.

5. Conclusion

Sex steroid hormones, mainly estradiol and 11ketotestosterone, were closely involved in sex differentiation of Eurasian perch. In this species, gonadal differentiation into testis or ovary seem to be controlled by the 11-oxygenated androgen to oestrogen ratio: an excess of 11-oxygenated androgen results in male being produced and an excess of oestrogen produces female perch.

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