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Hormones and fish monosex farming: A spotlight on immunity

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ABSTRACT

Aquaculture is a promising and developing industry worldwide. One of the first step in monosex culturing, particularly in Nile tilapia, is the production of all-male fry; hormones are widely used in this respect. It is known that exogenous treatment with hormones disrupts various systems in the body including the immune and endocrine systems. There has been a growing interest in how hormones shape the biology of the fish. Many researchers all over the world explored how androgen can interact with many of the body systems; however, rarely any of them tried to improve the hormonal method or to find an alternative. The gate is open for research in this field. This review focusses on the potential effects of hormones, particularly androgens on fish immunity, and the up to date solutions (however, they are rare).

1. Introduction

Decades ago, many researchers investigated the role of hormones in modulating the response and function of different body systems in various vertebrates. It is clear that sex hormones shape the different body systems, including the two arms of the immune system (innate and adaptive).

During the early stages of juvenile fish development, genes responsible for sex determination and sex chromosomes through steroidogenesis (Fig. 1) are guiding the gonads towards being male or female. The steroid hormones production and level could be artificially disrupted during this stage, which results in sex change without affecting genotype. Most methods for production of monosex fish target the steroidogenesis during the early stage of development.

This review will discuss the immunological aspects of androgen use in fish with little supportive studies in mammals, and also will describe briefly the old and recent tested methods for all-male production, the impact of androgen on mortality and environment and finally the impact of oestrogens on immunity.

2. Methods for the production of all-males

Monosex production with its pros and cons is needed in aquaculture industry. Because monosex culturing, especially of all-male fish, has many advantages, such as the lack of spawning, rapid growth and uniform size and high body weight [2–5], farmers prefer to use this system in production. One the other hand, some of the methods used in this respect have negative effect on several biological and environmental levels, which will be discussed here.

Many methods (summarised in Fig. 2) were used for this purpose, but one or two methods are still efficiently used. These methods were extensively discussed in Ref. [3], and additionally there are different methods were tested but with less preference and success, such as heat treatment and pulse-electric field induction.

2.1. Manual sexing

Sorting is an old aquaculture method in which the separation and sexing processes are done when the secondary sexual characteristics of the fish are well developed or when the fish are young adults [2]. The grading process acts as a stress for both the fish and the labourers; usually, the results are neither accurate nor satisfactory. Furthermore, females have to be discarded which means fingerling supply goes down to nearly half and farmers loose.

2.2. Inter-specific hybridization

The hybridisation of two specific species, such as *O. niloticus* × *O. viriabilis*, can produce monosex populations. This situation can be seen in tilapia as well as in other species, such as sunfish. This technology was improved by Hulata et al. [6] through the selection of broodstock that produce high YY% progeny.

2.3. Production of all YY male Nile tilapia

Super-males “YY” can be produced by intercrossing between

Abbreviations: 11-KT, 11-ketotestosterone; E2, 17 β-oestradiol; EE2, 17 α-ethinyl oestradiol; DHT, dihydrotestosterone; ifn, interferon; IgG, immunoglobulin G; IgM, immunoglobulin M; il1β, interleukin 1 beta; il8, interleukin 8; MT, 17 α-methyltestosterone; tnfα, tumour necrosis factor α; vtg, vitellogenin

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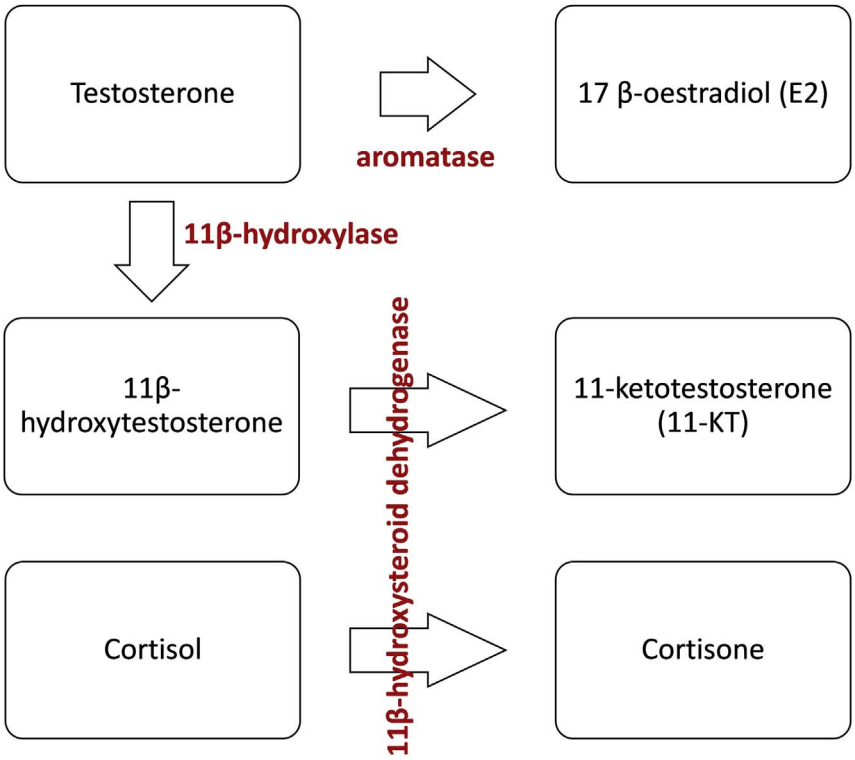

Fig. 1. Simple schematic pathway of fish steroidogenesis. Testosterone is converted to 11-ketotestosterone (11-KT) via the actions of 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase. 11β-hydroxysteroid dehydrogenase converts cortisol to cortisone. Aromatase converts testosterone to 17 β-oestradiol (E2) [1].

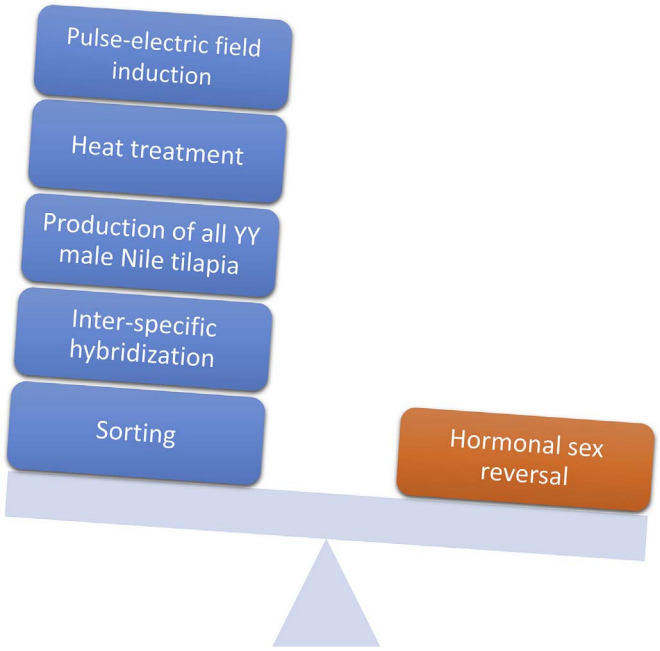

Fig. 2. The different methods used in monosex production, in which the hormones are the widely used technique.

converted females and males. This method depends on the use of oestrogens with a selective progeny testing to get an YY broodstock males or females (see Refs. [3,7] for more details). However, this method takes a long time to obtain an YY broodstock males or females, and the new progenies should be theoretically all-males without any further treatment.

2.4. Heat treatment

Juvenile Nile tilapias (10 days old) were exposed to heat treatment at temperatures ranging from 26 °C to 37 °C. The optimal temperature to achieve the best sex shift towards males and survivability was 36 ± 0.5 °C. The male proportion and fry survival percentages were 86.31% and 65.25%, respectively [8].

2.5. Pulse-electric field induction

Pulse-electric field induction is a newly developed method for monosex production in tilapia. The eggs of Nile tilapia (2-3 days post-fertilisation) were carefully induced in 3 square-wave electric fields of 87.50 kV m^{-1}. This method achieved 89.25% sex reversal of the eggs with less than 25% egg death [9].

2.6. Hormonal sex reversal

Although several methods can be used for monosex production (all-males or -females), hormonal treatment is the method that is preferred and most often used. Hormonal treatment results in monosex, particularly all-male, percentage ranges from 98% to 100% in most cases, however, in the other methods the percentage is less than 90% in the best cases. In addition, it is easy to be used.

In a wide variety of species, sexually undifferentiated fry can be successfully converted to monosex fry if they are treated with hormones or hormone analogues under controlled conditions. Many types of steroids are used in the production of all-male tilapia. For example, 19-norethyltestosterone, fluoxymesterone, ethyltestosterone and 17 α-methyltestosterone (MT) [10], dihydrotestosterone (DHT), androstenedione, trenbolone acetate, mesterolone, testosterone and 17 α-ethynyltestosterone [11] and 17 α-methyldihydrotestosterone [12–14] have all been used. In addition, Khanal, et al. [15] tested a natural source of testosterone (carp testis). Nonsteroidal aromatase inhibitors, such as fadrozole [16,17] have also been used for all-male production. All of these compounds result in a higher percentage of male fish, and they are mostly used via an oral route. However, Nile tilapia fry exposed to 17 α-methyldihydrotestosterone (500 μg/L) on day 10 and day 13 post-fertilisation, for 3 h, were successfully masculinised [18].

MT is the hormone that is most often used in aquacultures, and the

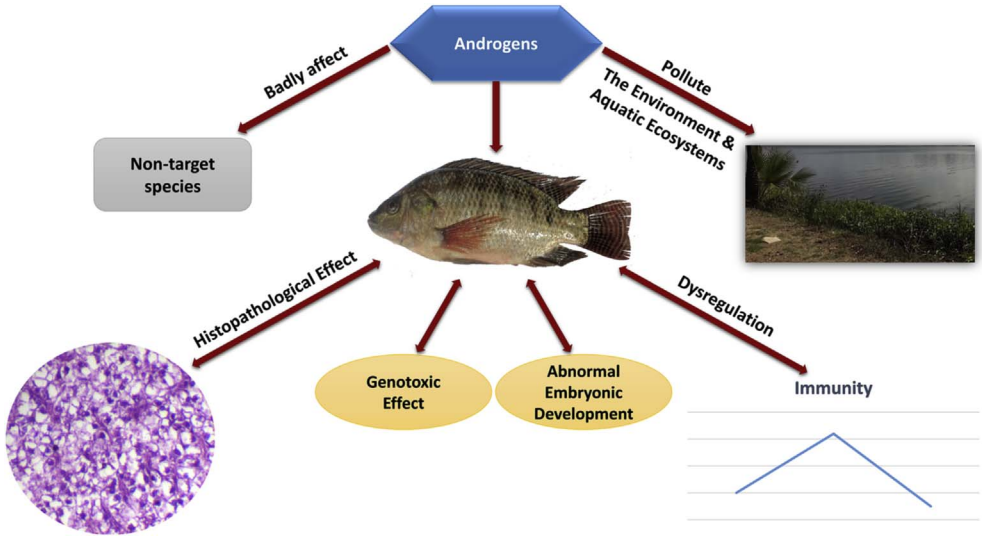

Fig. 3. Diagram illustrating the different aspects of androgens on aquaculture (Nile tilapia as an example) and environment.

only method used in all-male production in many countries, such as Egypt and Philippines. MT has been tested in monosex production in several species, such as spotted scat [19] and medaka [20]. In medaka, goldfish and tilapia, the effective dose of MT required to induce all-male production is 20–30 mg/kg diet; in rainbow trout, the effective MT dose is 0.1–3 mg/kg diet. In zebrafish, the MT dose ranges from l mg/kg diet to 100 mg/kg diet [21]. In Nile tilapia, feeding of dietary MT begins when the larvae have finished yolk sac absorption for 21 days. Different doses of MT (40 mg MT/kg, 50 mg MT/kg, 60 mg MT/kg or 70 mg MT/kg), can efficiently produce all-male fry [22–26].

3. The potential impact of androgen use

There are many concerns about the effect of hormones, particularly androgens, used in aquaculture; such cases are depicted in Fig. 3.

3.1. The impact of androgens on mortality

In general, fish larvae rearing and production are usually hindered by high mortality rates. On the other hand, any disruption in the immunity of fish larvae will threaten their survivability. Synthetic steroids that used in monosex production cause a high mortality rates in several species. In fathead minnows, MT negatively affected the survival rate [27], and caused mortality rates of more than 50% in the treated Sabaki tilapia [28]. Recently, Abo-Al-Ela et al. [29] demonstrated that MT increases the mortality rate of the treated fry in a dose-dependent manner; moreover, this mortality is correlated with immune-related gene expression throughout the treatment. Furthermore, the mortality rate has been shown to increase when increasing the dose of MT in convict cichlid [30].

3.2. The impact of androgens on environment

Environmental detection of potential hazard substances is taking a great interest, which these substances could affect non-target organisms causing undesirable action on the short or long run. Androgens and oestrogens have been detected as pollutants in the environment, especially in drained agroecosystems [31]; androgens effect can negatively extend to non-target species, including early-life stages of fish, which caused cytogenetic toxicity, embryo malformations and hatching delay [32]. Rivero-Wendt et al. [33] suggested the potential environmental risk of MT at environmentally relevant concentrations (0.004 mg/L), which was sufficient to induce vitellogenin (Vtg) alterations as an indicator of environmental stress or pollution. MT could disrupt fish fecundity at environmentally relevant concentrations, and this effect can differ from species to another [reviewd in [34]].

On the other hand and regarding to androgen withdrawal and residues, the adult monosex fish showed high MT concentration levels in the serum and muscle [35]. This finding is in contrast to the results reported by Khalil et al. [36], which did not detect any traces of hormone in the muscle. This is possibly due to the different conditions during samples collection or the various environmental effects or conditions.

3.3. The impact of androgens on immunity

Regarding to the immunity, steroid hormones play a vital role in modulating the immune system [37,38]. However, there is a lot of debate concerning the effect of androgens in suppressing or stimulating the immunity level. In other words, they may positively or negatively affect the health and growth of fish.

On the androgen level, gene networks related to innate immune responses are affected by DHT treatment, as seen in the fathead minnow [39]; and by MT in Nile tilapia [29,40]. MT is a severe endocrine disrupter [41] and has a genotoxic effect on human lymphocytes; it also increases the frequency of sister chromatid exchanges and decreases cell cycle kinetics [42]. In Egypt, Nile tilapia monosex farms have been reported to have low levels of RBCs and lymphocytes [35]. In addition, the disruptive effect of MT was extended to the antioxidant enzyme activities and gene transcription following exposure or dietary intake in Nile tilapia fry [29,43]. Other steroids, such as 11-ketotestosterone (11-KT), have been reported to suppress innate immune responses and respiratory bursts in three-spined sticklebacks and common carp [44,45].

In addition, Chinook salmon's leukocytes incubated with testosterone, MT or mibolerone showed a significant decrease in antibody-producing cells [46,47]. Even in chickens, the feeding of mibolerone during their first 7 weeks caused a regression of the bursa of fabricius [48]. Gilthead seabream that received testosterone microencapsulation implants also showed an early pro-inflammatory but later mixed pro-/anti-inflammatory stimulation [49]. Similarly, MT primed several immune, cellular apoptosis and detoxification transcripts level but suppressed them at the end and after the treatment in Nile tilapia fry [29].

Further reports have showed that at the time of sexual maturation in fish, the immune response was suppressed in response to the high plasma testosterone levels in spring Chinook salmon *in vitro* [50]. Testosterone in high doses causes immunosuppression, including a compromised T-cell immune response [51,52], and was found to inhibit transformation in phytohaemagglutinin and purified protein derivative stimulated lymphocytes *in vitro* [53]. Pre-treatment with testosterone has an immunosuppressive effect before the onset of louse infection in

Atlantic salmon, and this has been supported by the suppression of genes with important roles in the inflammation and immunity [54].

Further support the idea that in mammals, several studies reported that androgen deprivation in males is accompanied with thymus enlargement [37,38,55–58], enhancement of multiple immune responses and increases in the cellularity and size of primary and peripheral lymphoid organs [56]. Interestingly, treatment with testosterone or DHT post-castration results in the inhibition of thymic rejuvenation and the decreased proliferation and apoptosis augmentation of thymic T-cells [37,38,55–57,59–61].

These findings have been confirmed in humans where testosterone treatment in androgen-deficient men caused a decrease in the thymic output of T-cells [62]. Taken together, these results show the suppressive effect of androgens on the development of the thymus and, subsequently, on T-cells. Moreover, it is known that women have a more active response to viral vaccines, including influenza [63–65]. Male mice respond to T-dependent immunizations, but not as well as females [66,67].

In a previous study, neonate mice showed an increase in haemoglobin, lymphocyte count, immunoglobulin M (IgM) antibodies and cell-mediated immune response to grafting; and a decrease in neutrophil granulocytes with divided nuclei and immunoglobulin G (IgG) antibodies after the administration of 1 mg testosterone propionate [68]. Rettew et al. [69] found that castrated (androgen deficient) mice are significantly more susceptible to endotoxic shock. Notably, this could be reversed when the castrated mice were given exogenous testosterone.

In respect to antibodies, IgM is the first that appears following antigen exposure, Suzuki, et al. [70] demonstrated that its plasma level increases simultaneously with the increase of steroid hormones in goldfish. Nevertheless, *in vitro* incubation of IgM-secreting cells with a high dose of testosterone has exhibited tissue-specific functions; it also decreased the number of IgM-secreting cells and suppressed secretion of IgM by cells from the peripheral blood leukocytes, head kidney and spleen of common carp [71]. 11-KT decreases IgM production in many teleost fish species [71–73]. Testosterone, 11-KT and 17 β-oestradiol (E2) decreased IgM secretion from the spleen and head kidney lymphocytes [73].

The egg yolk precursor, Vtg, is a multivalent pattern recognition receptor which has the ability to recognize pathogens via interaction with pathogen-associated molecular patterns receptors [74,75], and it has hemagglutinating and antimicrobial capabilities [76–79]. *In vitro* treatment of male or female tilapia hepatocytes with DHT for 48 h significantly enhanced Vtg protein release [80,81]. Moreover, a short exposure to MT caused a delay and/or inhibition of hatching at 24 h, cardiac oedemas, spine deformities, eye malformations at 48 h and reduced Vtg levels in newly hatched zebrafish [33], but short-term (7 days) treatment of adult male zebrafish with MT (4.5 ng/L) significantly increased the level of Vtg compared to solvent controls. However, previous results revealed that exposure to MT at higher concentrations did not increase Vtg levels [82].

In general and according to Cuesta et al. [83], Abo-Al-Ela et al. [29] and Abo-Al-Ela et al. [40], the administration of androgens negatively modulates the expression of immune-related genes (with earlier up-regulation and late down-regulation), and disrupts the immune system functions (in short-term treatment that ranged from 7 to 10 days), such as phagocytosis (suppressed), lysozyme activity (enhanced), complement and peroxidase activities (enhanced).

3.4. The impact of androgens on phagocytosis

Phagocytosis is the process whereby cells recognize and engulf foreign particles (usually more than 0.5 μm in diameter). It plays an important role in the host's immune defence against invading pathogens [84]. Cytokines, central regulators of the main activities of phagocytes, modulate the phagocytes' interactions with foreign molecules [85]. Interleukin 1 beta (Il1β), interleukin 8 (Il8) and tumour necrosis factor α (Tnfα) can enhance phagocytosis and chemotaxis in trout and bluefin tuna [86–89]. The expression of immune genes, including cytokines can greatly affected by MT treatment [29,40], thus, it might reflected on the phagocytosis process.

Progesterone or 11-KT injections can suppress some immune parameters, including phagocytosis, in carp fish, both *in vivo* and *in vitro* [90,91]. Also, leukocytes' function is negatively correlated with androgen plasma levels [92]. Testosterone causes a significant loss of leukocytes and significant immunosuppression, and, even after adding a supernatant of proliferating lymphocytes not exposed to testosterone, this action cannot be reversed [50]. Short-term treatment with MT significantly depressed the phagocytic process (phagocytic activity and index), but stimulated lysozyme activity in adult Nile tilapia [40]. However, testosterone microencapsulation implants stimulated phagocytic activity and the reactive oxygen species production of leukocytes after 21 days of implantation in gilthead seabream [49]; this can be due to variations in fish species responses, chemicals used, duration of treatment or mode of administration.

3.5. The impact of androgens on histology of immune-related organs

The liver is a vital organ involved in fish defence, and it produces cytokines in healthy and diseased conditions [93]. Androgens are hepatotoxic [94]. Nile tilapia larvae that were fed dietary MT for 28 consecutive days showed a mild nuclear pleomorphism of hepatocytes and moderate to moderately severe vacuolation of the liver. In addition, hepatocytes exhibited mild to moderately severe accumulations of intracellular protein [26]. In salmon, MT injection caused degeneration of the kidney and liver [95]. Prolonged MT treatment had a harmful effect on the kidneys and the livers of juvenile channel catfish as evidenced by marked oedema in the renal corpuscles and tubules and liver weight increases caused by the hepatotrophic response [96].

Massive doses of dietary MT in goldfish caused an extensive proliferation of the rough endoplasmic reticulum, the production of numerous secretory granules and hypertrophy of the Golgi apparatus in the liver [97]. Additionally, anabolic androgenic steroids caused a development of hepatocellular adenomas, peliosis hepatis and hepatocellular hyperplasia [reviewed in [98]].

Electron microscopic examination of rat hepatic tissue from MT-treated animals showed hepatocytic ultrastructural alterations. The most notable changes were swelling of mitochondria, which presented a slightly defined, cristae and electron-lucent matrix and an obvious increase in the number of lysosomes [99].

The spleen and head-kidney are major organs that comprise the fish immune system [100]. MT exposure stimulated kidney hypertrophy in three-spined sticklebacks [101]; increased the height of kidney epithelial cell in the brook stickleback [102]; and caused necrosis and minimal to moderate hyaline droplet degeneration of tubule epithelium, scattered eosinophilic granular cells and a number of regenerating (developing, immature) tubules in the kidney tissue of Nile tilapia [26].

Although the treatment with MT in Nile tilapia showed an increase in melanomacrophage centres in the spleen and the kidney, but MT conjointly administrated with vitamin C showed more increases and accumulation in the melanomacrophage centres in the spleen and the kidney in comparison to MT, vitamin C or control treated groups [40]. According to Abo-Al-Ela et al. [40], vitamin C was able to maintain or enhance the other immune functions, such as phagocytosis in spite of the immunological parameters were negatively affected by MT. This reflects and confirms on the powerful action of vitamin C as potent immunostimulant and antioxidant agent.

4. The impact of oestrogens on immunity

In many cases, oestrogens are used in monosex farming, such as in eel production and in preliminary steps of production of YY super-

males; and oestradiol is one the preferred oestrogen substance in this case. The modulation of gene expression in fish immune system via oestrogens has been reviewed in Ref. [103]; and herein, this section focuses on some studies that may not mentioned in this reference.

Kimble et al. [104] found that oestrogen deficiency caused stimulation of macrophages via increasing IL1 and TNF. In addition, TNFα and interferon gamma (IFNγ) levels declined in oestradiol-treated mice in comparison to non-treated mice [105]. Administration of female hormones, such as oestrogen or progesterone causes a decrease in the lymphocyte count [106]. *In vitro*, oestradiol significantly inhibited the mitogen-induced proliferation of peripheral blood lymphocytes [107]. The effects of oestrogens are mediated through leukocyte-specific oestrogen receptors that located on immune cells, such as leukocytes and this way estrogenic substances can participate in the immune response process [108].

Treatment of T-cells with E2 for 24 h showed a dose-dependent repression of TNFα induced cytotoxicity [109]. E2 suppressed the rainbow trout leukocyte proliferation *in vitro* [110]. In the most cases, the gene expression analysis experienced an early up-regulation. Short exposure to chemical pollutants or E2 induced, in a concentration-dependent manner, antioxidant activity and innate immune gene expression in zebrafish [111,112]. *Vtg* was up-regulated as early as four days post-fertilization after exposure to E2 [113] and also by 17 α-ethinyl oestradiol (EE2) at different periods of early development [114,115].

Japanese medaka exposed to E2 (0.1–10 μg/L) for 28 days showed a variable expression pattern of immune-related genes; complement components (*C3-1*, *C3-2*, *Bf/C2*), lysozyme and ceruloplasmin were down-regulated, while *il-21*, *ifn*, novel immune-type receptor-18 and Ikaros were up-regulated [116]. Alongside after long-term treatment with dietary E2 in rainbow trout, no or significantly low up-regulation of the complement genes, including *C3-1* and *C3-3* was detected following the bacterial challenge in compare to control [117]. These results apparently show that the natural estrogen, E2 harms the ability of immune system to cope with the bacterial infection and lowers the standby defenses.

Furthermore, EE2 may change the capacity of fish to properly respond to infection. The exposure of gilthead seabream to EE2 for 15 days inhibited, in a dose-dependent manner, *il1β* gene expression [118]. Furthermore, detoxification and antioxidant systems are altered by the hormone treatment. EE2 dietary treatment in female largemouth bass affected a set of genes related to oxidative stress and immunity. The hepatic glutathione *S*-transferase mRNA expression was significantly increased while glutathione peroxidase mRNA levels were significantly reduced, indicating a potential oxidative response in the liver after EE2 treatment [119]. In zebrafish, EE2 significantly increased Vtg concentration [120]. In addition, phagocytic indices of juvenile yellow catfish exposed to 1 ng/L EE2 were lower than control group [121].

On the histopathological level, hepatocytes of fish liver exposed to EE2 or E2 were eosinophilic and enlarged, and their nuclei were enlarged with a large prominent nucleolus. The hepatic vessels contained a considerable amount of eosinophilic material (presumed to be Vtg), which accumulated in the trunk kidney and which was pronounced in renal tubules and glomeruli [122].

5. Conclusive remarks and further perspectives

Fish culturing and production is an important investment. Monosex aquaculture, particularly all-male, is critically needed; and, hormones are widely used in this industry. However, hormones alter various body systems, possibly influence the susceptibility of fish to diseases and opportunistic infections and they can pollute the environment. Thus, further studies should be conducted to find alternative more safe ways to ensure all-male aquaculture production, such as using YY males or adding substances, such as vitamin C that can modulate the effects of hormones.

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Conflicts of interest

None.

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