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Effects of aromatase inhibitors on *in vitro* steroidogenesis by Atlantic salmon (*Salmo salar*) gonadal and brain tissue

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Abstract

In order to assess the efficacy of selected aromatase inhibitors on Atlantic salmon (*Salmo salar*) ovarian and brain tissue, *in vitro* systems were developed for measuring 17β-estradiol (E_2) production by these tissues. Isolated vitellogenic follicles, or homogenised whole brains were incubated at 10 °C in complete Cortlands solution for 18 or 42 h respectively, and E_2 levels in the medium were determined by RIA. The addition of testosterone to the medium increased E_2 production in all preparations. E_2 production by whole brain homogenate was reduced by co-incubation with the aromatase inhibitors 1,4,6-androstatriene-3,17-dione (ATD), 4-androstene-4-ol-3,17-dione (OHA), aminoglutethimide, fadrozole or miconazole. Fadrozole, ATD, and OHA reduced E_2 production by vitellogenic follicles at a medium concentration of 0.1 μg mL^{-1}, whereas miconazole was only effective at 10 μg mL^{-1}. This study demonstrates a simple and rapid screening method for assessing the efficacy of aromatase inhibitors on fish tissues, and that the aromatase inhibitors ATD, OHA and fadrozole are potent inhibitors of both brain and gonadal aromatase *in vitro*, in Atlantic salmon.

Keywords: Aromatase inhibitor; P450 aromatase; *Salmo salar*; Follicle; Brain; Steroidogenesis; *In vitro*

1. Introduction

Concomitant with increased development of, and reliance on, aquaculture of finfish, has been an increase in the need to control and manipulate fish reproduction (Piferrer, 2001; Devlin and Nagahama, 2002). A frequent demand on culture systems has been for the production of monosex or sterile populations, for reasons of production efficiency. In many species, males are preferred for reasons of faster growth (*eg.* tilapia) or enhanced marketability (*eg.* ornamental species). However, in some species, such as salmonids, females are preferred for their reduced propensity to early maturation, with an associated growth benefit (Piferrer, 2001).

Yamamoto (1969) first demonstrated that androgens and estrogens respectively are the inducers of male and female sex differentiation in fishes. Subsequently, the application of steroid hormones, during the labile period of gonadal development, has been used for the production of monosex fish stocks (Piferrer, 2001; Devlin and Nagahama, 2002). However, an alternative to the use of androgens to promote sex differentiation as males, is the use of compounds that inhibit estrogen synthesis, typically via inhibition of the activity of aromatase. Aromatase (cytochrome P450arom) is the enzyme responsible for the conversion of androgens to estrogens, and in fish, is most active in the gonad and brain tissue; however, other tissues may also have aromatase activity at lower levels (Callard et al., 1978; Zhao et al., 2001; Moore et al., 2002). Recently, studies have determined that unlike mammals, some teleost species, including goldfish, *Carassius auratus*, (Tchoudakova and Callard, 1998) and zebrafish, *Danio rerio*, (Kishida and Callard, 2001) carry multiple copies of the

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aromatase gene. Molecular studies have demonstrated differential expression of these genes in different tissues (Callard et al., 2001; Tchoudakova et al., 2001). Notably, different genes are predominantly expressed in the brain, and the ovary (Callard et al., 2001; Kishida and Callard, 2001). Tissue-specific gene expression and enzyme activity also vary during development, with activity in brain tissue preceding that in the gonads (Kishida and Callard, 2001).

Aromatase inhibitors, largely developed for human therapeutic use in the treatment of estrogen-dependent tumours, have been shown to be effective in studies on all major classes of non-mammalian vertebrates (Wozinak et al., 1992; Piferrer et al., 1994; Chardard and Dournon, 1999). In salmonids, aromatase inhibitors have been shown to affect circulating steroids, aromatase activity and response to gonadotropin (Antonolopou et al., 1995; Afonso et al., 1999, 2000). Piferrer et al. (1994), Guiguen et al. (1999), Kitano et al. (1999) and Kwon et al. (2002) demonstrated the effectiveness of aromatase inhibitors for the control of sex determination in fish. In a study on goldfish, Zhao et al. (2001) showed that the gonad and brain isozymes responded differently to a range of aromatase inhibitors, and that generally, the ovarian isozyme was more sensitive to the aromatase inhibitors tested, than was the brain isozyme.

Previous studies of the role of aromatase in Atlantic salmon, *Salmo salar* (Andersson et al., 1988; Antonolopou et al., 1995, 1999) have focussed primarily on its effects on hormonal regulation in relation to male maturation. These studies assessed the functional role of aromatase in the brain–pituitary–gonadal axis, and focussed, by necessity, on whole animal models; however, in their investigations of Atlantic salmon aromatase, Antonopolou et al. (1995, 1999) also investigated several aromatase inhibitors, administered by slow-release implants and *in vitro*.

The use of the whole animal model in studies of steroidogenesis, although having some advantages, is resource intensive and time consuming, and can ultimately impact on sample sizes and statistical power (Shilling et al., 1999). The alternative *in vitro* approach has proven to be an informative tool for investigating steroidogenesis in fishes; however, *in vitro* systems need to be optimised with respect to key variables. Incubation conditions, including temperature, duration and medium components can all influence *in vitro* tissue steroidogenesis (Tyler et al., 1990; Rahman et al., 2002). Medium concentrations of steroids tend to increase with increasing incubation time (Zhao and Wright, 1985; González and Piferrer, 2002); however, extended incubation can also lead to the production of secondary metabolites that may not be measured by the assay techniques used, or result in the uptake of steroids secreted, by the components of tissue itself (Haddy and Pankhurst, 1998). As enzymatic activity is strongly dependent on temperature, so incubation temperature affects the rate of steroidogenesis (Haddy and Pankhurst, 1998; González and Piferrer, 2002). Steroidogenesis may also be enhanced by the provision of steroid substrates (Pankhurst, 1997; Rahman et al., 2002) or hormones that promote the activity of steroidogenic enzymes (Zhao and Wright, 1985), or be disrupted by inhibitors of enzyme activity (Pelissero et al., 1996; Afonso et al., 1997). Consequently it is necessary to calibrate the performance of incubations against the major incubation variables involved.

The development of non-steroidal methods for sex manipulation is considered to be of strategic value to the aquaculture industry, as public aversion to the use of steroid hormones in animal production is increasing. The present study formed the first step in the development of a commercially appropriate protocol for the masculinisation of Atlantic salmon using aromatase inhibitors. Gonad and brain tissue was incubated either alone or with testosterone (T) as a precursor, over a range of incubation times and temperatures previously demonstrated to span the optimal range in other salmonids (Afonso et al., 1997; Haddy and Pankhurst, 1998), and 17β-estradiol (E_2) released into the medium measured by RIA. Optimised protocols were then used to assess the *in vitro* efficacy of the aromatase inhibitors 1,4,6-androstatriene-3,17-dione (ATD); 4-androstene-4-ol-3,17-dione (OHA); 4-[5,6,7,8-tetra-hydroimidazo-[1,5-*a*]-pyridin-5-yl benzonitrile HCl] (fadrozole); 1-[2,4-dichloro-*b*-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole (miconazole) and 3-[*p*-aminophenyl]-3-ethyl-piperidine-2,6-dione (aminoglutethimide). Measurement of aromatase activity was achieved indirectly, by measurement of E_2 synthesis, as the use of radioisotopes, necessary for direct measurement, was not permitted in the hatchery where the experimental work was conducted.

2. Materials and methods

2.1. Fish

Vitellogenic 3+ year old female Atlantic salmon broodstock produced by Salmon Enterprises of Tasmania Pty. Ltd. (Saltas), at Wayatinah, Tasmania were used in all experiments. Broodstock were reared either at Saltas' Florentine (Experiment 1) or Wayatinah (Experiments 2–4) facilities. Fish used in Experiments 1, 3 and 4 were maintained in 66,000 L capacity tanks, while those for Experiment 2 were maintained in a 25,000 L raceway. All tanks and raceways were run as flow through systems at natural temperature and photoperiod. Fish were fed a commercial diet (Skretting, Australia) to satiation.

2.2. Follicle preparation and incubation

Fish were netted from the holding tank, quickly euthenased by a blow to the head, and stored on ice prior to dissection.

In both Experiments 1 and 2, ovaries were removed, rinsed and maintained in chilled complete Cortlands solution (CCS) (Wolf, 1963). Follicles were manually dissected from a central section from each ovary, rinsed and stored for up to 1 h in chilled CCS until incubation. Incubations were carried out in the incubation media described below, in 24 well tissue culture plates (Iwaki). Either five or ten follicles per well were used, as described below. At the end of the incubation period, the culture medium was aspirated to 1.5 mL micro-centrifuge tubes and frozen (−18 °C) until assay. Oocyte diameter of 30 follicles was determined to 0.01 mm using digital vernier callipers. Each experiment was performed twice using follicles from two different fish.

For experiments using gonadal tissue, follicles were in mid-vitellogenesis and the size range (mean ± S.E.) for the two fish was 1.90 ± 0.05 and 3.34 ± 0.11 mm and 4.29 ± 0.07 and 4.89 ± 0.06 mm for Experiments 1 and 2 respectively.