Control of sex inversion in female Atlantic salmon (*Salmo salar*).

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requirements for the Degree of
Doctor of Philosophy

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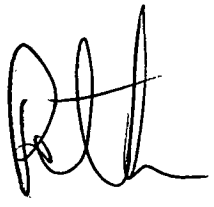
Declaration

Except as stated herein this thesis contains no material which has been accepted for the award of any higher degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text.

Peter S. Lee



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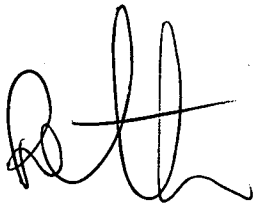

Peter S. Lee

Abstract

Commercial production of Atlantic salmon, *Salmo salar* (L.), in Tasmania is becoming increasingly reliant on female stocks for year round production. Hatchery production of female monosex stocks uses milt from sex inverted females ("neomales") to produce all female offspring. This study was undertaken investigate the effects of aromatase inhibitors (AI) on Atlantic salmon and to facilitate the development of techniques for their commercial application.

Dietary treatment for 800 °C days from first feeding, with methyltestosterone at doses of 1 or 3 mg kg^{-1} food or methyldihydrotestosterone (MDHT) at 1 mg kg^{-1} food resulted in 100% masculinisation of female Atlantic salmon. Masculinisation followed 2 h immersion of alevins in MDHT at 400 μg l^{-1} during the period 0 to 28 days post median hatch (DPMH) and complete masculinisation was achieved after immersion at 14 and 21 or 14 and 28 DPMH. More sex inverted males with patent sperm ducts were produced by immersion than dietary treatments.

The capacity of five AI: 1,4,6-androstatriene-3,17-dione (ATD); 4-androstene-4-ol-3,17-dione (OHA); 1-[2,4-dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole (miconazole); 3-[p-aminophenyl]-3-ethyl-piperidine-2,6-dione (aminoglutethimide) and 4-[5,6,7,8-tetra-hydroimidazo-(1,5-α)-pyridin-5-yl] benzonitrile monohydrochloride (fadrozole) to reduce estradiol (E_2) production by Atlantic salmon brain and gonadal tissue was assessed *in vitro*. Fadrozole was the most potent, effective at doses of 0.1-10.0 μg ml^{-1}. ATD, OHA and miconazole were also effective on brain and gonadal tissue, but aminoglutethimide was only effective on brain tissue.

Immersion of Atlantic salmon alevins in ATD at a dose of 0.5 mg L^{-1} on 14 or 14 and 28 DPMH produced 13% neomales. This incidence was increased to 47 and 22% following treatment on 7 and 14, or 14 and 21 DPMH respectively and to 51-54% with a dose of 5 mg L^{-1} ATD. Miconazole, aminoglutethimide, fadrozole and OHA were all ineffective for sex inversion. Precocious maturation was induced by photoperiod manipulation, and 67-88 % of mature neomales had patent sperm ducts and fertility (~70%) comparable to normal males.

ATD was taken up by alevins more readily than OHA; however, tissue levels of both declined by 50%, after 24 h and by >90% after 7 days. Uptake levels declined with alevin age, but retention rates were similar. Reducing the inter-treatment interval to 7 days resulted in increased androgen levels following the second treatment with ATD. Immersion in fadrozole at doses of 10 and 50 mg L^{-1} resulted in tissue levels of 300 and 1000 ng $fish^{-1}$ which declined by 80% after 3 days.

These studies demonstrate the commercial feasibility of androgens and AI for sex inversion of Atlantic salmon. The efficacy of immersion treatment with AI is dependent on both enzyme inhibition and the uptake and retention characteristics of AI.

DEDICATION

To Imogen and Isaac

who provided the inspiration

and

Sue

who gave me the courage to start

and the strength to finish.

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Above all I thank my family. To my children, Imogen and Isaac, for reminding me what is truly important in life, and yes, I will get a real job when I grow up! To my wife Sue, without whom this would never have come to pass, and who has sacrificed much over the past four years to help me reach my goal, this belongs to you as much as me. Your love, support and generosity of spirit has seen us all through an extraordinary few years and helped me get through this and many other challenges. I love you deeply.

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List of Abbreviations

17,20-βP	17α-20β-dihydroxy-4-pregen-3-one
17HSD	17-hydroxysteroid-dehydrogenase
AGT	aminoglutethimide
AI	aromatase inhibitor
ATD	1,4,6-androstatriene-3,17-dione
DD	°C days
DPMH	days post-median hatch
ESD	environmental sex determination
FAD	fadrozole
GSD	genetic sex determination
MCZ	miconazole
MDHT	17α-methyldihydrotestosterone
OHA	4-androstene-4-ol-3,17-dione
OSS	out-of-season smolt
TSD	temperature sex determination

Chapter 1

General Introduction

Of all the animals, the boy is the most unmanageable.

Plato

1. General Introduction

1.1 The Tasmanian Atlantic salmon farming industry

The first introductions of Atlantic salmon, *Salmo salar* (L.), to Tasmania occurred in the 1860s, with the importation of ova collected from Scotland, England and Wales. The first release of Atlantic salmon occurred in 1865, but populations failed to establish in Tasmanian waters from this, and a number of subsequent releases, and it would be 120 years before a breeding population of Atlantic was established in Tasmania, albeit in captivity (Jungalwalla, 1991a). In 1981 the Tasmanian Fisheries Development Authority commenced a program to establish a salmonid farming industry in the state. Owing to the existence, at the time, of a ban on importation of fresh salmonid products into Australia, ova for the Tasmanian industry were sourced from an established landlocked population of Atlantic salmon in Gaden, New South Wales. This population originated from ova imported from Nova Scotia in the 1960s, and had been maintained through a hatchery assisted breeding program since that time (Jungalwalla, 1991a). Ova from Gaden were transferred to Tasmania in 1984, 1985 and 1986, and these were ongrown to become the original Tasmanian Atlantic salmon broodstock cohorts, of 1987,1988 and 1989 (Jungalwalla, 1991a).

The Tasmanian state government established the Atlantic salmon farming industry with a high level of regulation, under the auspices of the Salt-Water Salmonid Culture Act, in 1985. The act established a company, Salmon Enterprises of Tasmania Pty. Ltd. (Saltas), formed as a partnership between the Tasmanian government and commercial sector. Among the objectives of Saltas were the establishment and operation a hatchery capable of producing 1 million Atlantic salmon smolts per annum, and the responsibility to undertake research to improve breeding stocks and to benefit the developing marine farming sector (Jungalwalla, 1991a). Currently Saltas operates two hatcheries, utililising state-of-the-art recirculation systems (Anonymous, 1999) to produce in excess of 3 million smolts per annum (H.R. King, personal communication). The Tasmanian Atlantic salmon farming industry now comprises 5 hatchery companies, supplying 8 independent marine farming companies, (Tasmanian Salmonid Growers Association,

unpublished data). Industry production has increased from 53 T in 1985/86 to 15000 T, valued at A$170 million in 2001/02 (Battaglene, 2002)

Farmed Tasmanian Atlantic salmon reach full maturity over autumn (March to May), with spawning occurring in May. Most (97%) Tasmanian Atlantic salmon smoltify as 'S1', that is in the second spring after hatching, and are transferred to sea during the spring, September-November (Jungalwalla, 1991a). A high proportion of farmed Atlantic salmon in Tasmania reach maturity after their first year in sea water, a phenomenon termed 'grilsing' in the northern hemisphere; however, unlike typical northern hemisphere grilse which fail to reach a suitable size for harvest prior to maturation, Tasmanian grilse reached a viable harvest size of 3-5 kg within 12 months of transfer to sea water (Jungalwalla, 1991b, a). The onset of maturation does, however, impose a limit on production, with a requirement to harvest stock before the development over austral summer (December – February) of undesirable changes associated with the onset of secondary sexual characteristics, such as uneven flesh colour, poor flesh texture and darkened skin (Jungalwalla, 1991b). Harvesting of fish transferred as smolts in spring is therefore limited to a 6 to 8 month period (Jungalwalla, 1991b). In 1988 Saltas commenced a series of research programs aimed at extending the harvest period of farmed Atlantic salmon in Tasmania by developing production methods for non-maturing stocks and methods for early smolt transfer (Jungalwalla, 1991a). The introduction of non-maturing, triploid stocks has proven to be an effective means of extending the harvest season (Jungalwalla, 1991b). Subsequently, the introduction of out-of-season smolts (OSS), which have been photo-manipulated to be able to be transferred to sea in the Austral autumn (March – May) has made possible a program of year round harvesting (King, 1995)

The success of triploid and OSS production strategies is; however, predicated upon the use of monosex female populations to overcome issues of maturation. Triploid male salmonids develop testes and secondary sexual characteristics (Lincoln and Scott, 1984; Krisfalusi and Cloud, 1999). Similarly, male salmonids have a greater tendency to enter maturation at a smaller size than females (Bye and Lincoln, 1981), and high proportions of precociously mature males have been

recorded elsewhere in mixed sex populations of photo-manipulated Atlantic salmon smolt (Duston and Saunders, 1992). In view of this, Saltas has maintained a production strategy whereby all triploids and OSS are produced as monosex female populations, and monosex female stocks now account for approximately 70% of Saltas' total smolt production (H.R. King, personal communication).

1.2 Sex determination and sex differentiation in fishes

The processes of sex determination and sex differentiation in fishes have been the subject of much discussion in the literature and a number of comprehensive reviews covering all aspects of fish sex determination and differentiation have been produced in recent years (Nakamura et al., 1998; Baroiller et al., 1999; Piferrer, 2001; Devlin and Nagahama, 2002). There is general agreement among authors as to the definitions of these terms, namely that sex determination is the sum of genetic or environmental processes governing sex differentiation, whereas sex differentiation refers specifically to developmental events whereby undifferentiated gonadal tissue develops to form testes, ovaries or a hermaphroditic gonad (Nakamura et al., 1998; Baroiller et al., 1999; Piferrer, 2001; Devlin and Nagahama, 2002) and these definitions have been applied in the present study.

In his seminal studies of medaka, *Oryzias latipes*, Yamamoto (synthesised in Yamamoto, 1969) recognised that steroid hormones play a key role in sex differentiation of fish, and proposed steroids as the natural inducers of sex differentiation. Accepting this premise, the development of cells producing sex steroids should precede gonadal differentiation (Nakamura et al., 1998). Although there is a substantial amount of data supporting this hypothesis, there is no clear evidence of differences between sexes in either the presence of sex steroid-producing cells or steroid levels (Devlin and Nagahama, 2002). This may be due to an inability to measure steroids at sufficiently low levels (Devlin and Nagahama, 2002) or that the origin of the steroids is not gonadal. Molecular data has indicated differences between individuals, possibly reflecting sex differences, in steroidogenic enzyme gene activity in the brain of the zebrafish, *Danio rerio* (Trant et al., 2001). Prior to the development of steroidogenic cells in the gonad, both androgen and estrogen receptors may be detected, and it is through this

uncoupling of steroid synthesis and reception that exogenous steroids may be applied to manipulate sex differentiation (Devlin and Nagahama, 2002). The period during which exogenously applied steroids may affect sex differentiation has been termed the labile period, and this may be regarded as commencing prior to the onset of sex differentiation, and extending to a time after which gonadal tissue no longer displays developmental bipotency (Piferrer, 2001). The onset of the labile period may not be synchronous for androgens and estrogens, with both teleosts (Piferrer and Donaldson, 1989) and amphibians (Petrini and Zaccanti, 1998) exhibiting sensitivity to estrogens at developmentally earlier stages than sensitivity to androgens, and this may be reflected in the onset of ovarian differentiation prior to that of testis (Laird et al., 1978; Piferrer and Donaldson, 1989)

Teleosts are known to have both genetic and environmental control of sex determination (Devlin and Nagahama, 2002). Genetic sex determination (GSD) is the most prevalent in the fishes, known to occur in 24 families, compared to environmental sex determination (ESD), in 12 families (Devlin and Nagahama, 2002). The most common from of ESD; temperature-dependent sex determination (TSD), is a well recognised phenomenon among reptiles, but it remains unclear how widely spread this phenomenon is among the fishes. Many recent studies have shown some level of TSD in fishes (eg. D'Cotta et al., 2000; Goto et al., 2000; Pavlidis et al., 2000; Kitano et al., 2001) and future studies may well increase the number of fish species in which TSD is known to occur. Of the teleost species for which GSD has been demonstrated, various genetic systems of sex determination exist, including heterogametic males (XX females, XY males), heterogametic females (ZZ males, WZ females) and polygenic and clonal systems (Devlin and Nagahama, 2002). Fishes also display an extraordinary variety of reproductive strategies, with systems of gonochorism, progyny, protandry and hermphroditism recognised, and within those species that change sex, developmental, social and environmental cues have all been implicated as having roles in the onset of the changes (Devlin and Nagahama, 2002).

1.3 Production of monosex female populations

Concomitant with increased development of, and reliance on, aquaculture of finfish, has been an increase in the need to control and manipulate fish reproduction (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; 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) (Pandian and Sheela, 1995). However, in some species, notably the commercially valuable European sea bass, *Dicentrarchus labrax* and, as noted earlier, salmonids, females are preferred for their reduced propensity to early maturation, with a concomitant growth benefit (Bye and Lincoln, 1981; Blazquez et al., 1995).

Piferrer (2001) described the alternative methods for the production of female monosex populations, as 'direct' or 'indirect'. In the direct method, all female stocks are produced by the application of exogenous estrogens during the labile period (Piferrer, 2001). In contrast, the indirect method, appropriate for species having homogametic females, is a 2-generational process. Some females are sex inverted, typically by the use of exogenous androgens, to develop and mature as males. In a species having females homogameity, all sperm produced by these "neomales" will carry an X chromosome, which, when used to fertilise eggs from a normal female , will result in the production of all female offspring (Figure 1.1). The process of indirect monosex female production is therefore reliant on the production of neomales by sex inversion.

Since the demonstration by Yamamoto (1969) that androgens and estrogens are the inducers of male and female sex differentiation respectively, numerous workers have investigated steroidal control of teleost sex differentiation much of is detailed in the comprehensive reviews by Donaldson and Hunter (1982), Hunter and Donaldson (1983), Pandian and Sheela (1995), Donaldson *et al.* (1996), Baroiller *et al.* (1999) and Devlin and Nagahama (2002).

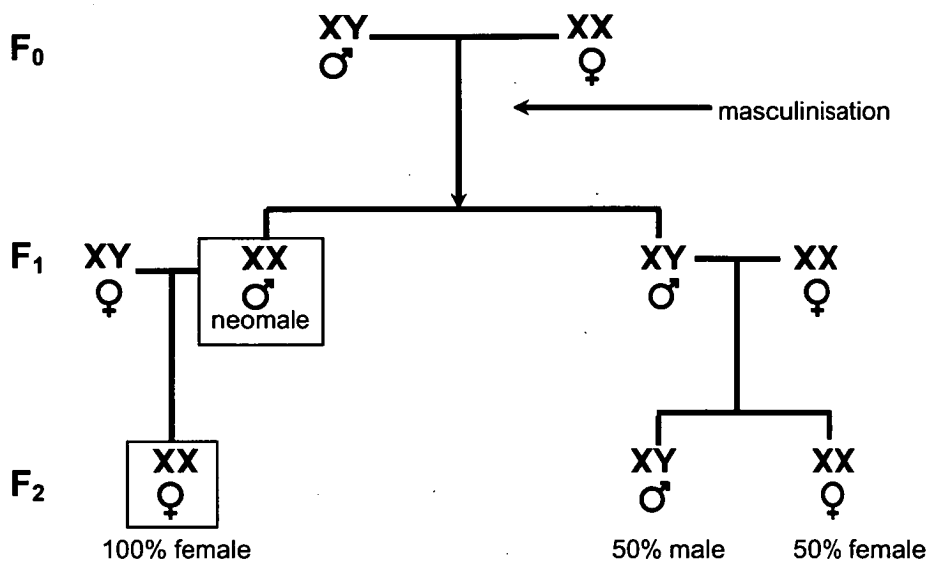

Figure 1.1 Diagram of the procedure for indirect feminisation of species having heterogametic males. (After Piferrer (2001))

The development of a successful protocol for sex control using exogenous steroids involves the consideration of several factors, including the choice of steroid, mode of delivery, dose and timing (Piferrer 2001). At least 16 naturally occurring and synthetic androgens have been used to masculinise fish (Pandian and Sheela, 1995). The most commonly used compound is the synthetic androgen 17α-methyltestosterone (MT), preferred for reasons of efficacy, cost and slower clearance than natural androgens (Pandian and Sheela, 1995). Methods for treating fish with steroid hormones include: immersion, by either the addition of steroids to the culture water, or the transfer of fish to a bath containing steroids; the addition of steroids to the diet; the use of implants containing steroids and by direct injection (Pandian and Sheela, 1995; Piferrer, 2001). Immersion and dietary inclusion are the methods most easily applied and are suitable for the treatment of large numbers of animals in a commercial setting (Pandian and Sheela, 1995). The other methods have been found to be the most appropriate delivery methods for some species, such as silver carp, where immersion and dietary treatments were ineffective (Mizra and Shelton, 1988). Immersion treatment may be applied to fish at all stages of development, whereas dietary treatment is only appropriate to feeding stages, thus the choice of application method must be made in consideration of the timing of treatment (Pandian and Sheela, 1995; Piferrer, 2001). Furthermore, the accuracy of delivery is greater for immersion than dietary

treatment (Pandian and Sheela, 1995) and typically immersion treatments are of shorter duration than dietary treatments (Pandian and Sheela, 1995; Piferrer, 2001). The timing of steroid treatments for sex control is critical to successful treatment. For steroid treatment to be effective for sex control, it must be applied in the labile period, which may occur prior to hatching or the onset of feeding (Piferrer, 2001). The dose of steroid required to effect sex change is species specific and is a combination of steroid concentration and the duration and frequency of treatment (Piferrer, 2001). Excessive doses of estrogens have been associated with developmental abnormalities and increased mortality, and excessive doses of androgens have been associated with gonad malformation and sterility (Piferrer, 2001). Insufficient doses of steroids may result in the development of intersex gonads (Yamamoto, 1969; Piferrer, 2001; Devlin and Nagahama, 2002).

1.4 Estrogen and aromatase (cytochrome $P450_{arom}$)

Aromatase (cytochrome $P450_{arom}$) is the enzyme responsible for the conversion of androgens to estrogens, and is found in all vertebrates, being highly conserved across vertebrate taxa (Callard et al., 1978). Due to its key role in steroidogenesis, and subsequent effects on development, reproduction and behaviour, a substantial body of work has been completed on aromatase activity and regulation across a range of vertebrate taxa (see reviews by Callard et al., 1978; Lance, 1997; Baroiller et al., 1999).

Aromatase converts androstenedione and testosterone to estrone and estradiol (E_2) respectively, by a 3 step process involving the synthesis of 3 intermediate compounds, and utilisation of 3 moles of oxygen and 3 moles of nicotinamide adenine dinucleotide phosphate (NADPH) for each mole of estrogen produced (Cole and Robinson, 1990; Brueggemeier, 2001) (Figure 1.2). The mechanism of the final step is unclear, but terminates with the aromatisation of the A ring of the steroid, by the oxidative cleavage of the bond between C-10 and C-19 and loss of the C-19 carbon as formic acid (Figure 1.2) (Cole and Robinson, 1990; Brueggemeier, 2001).

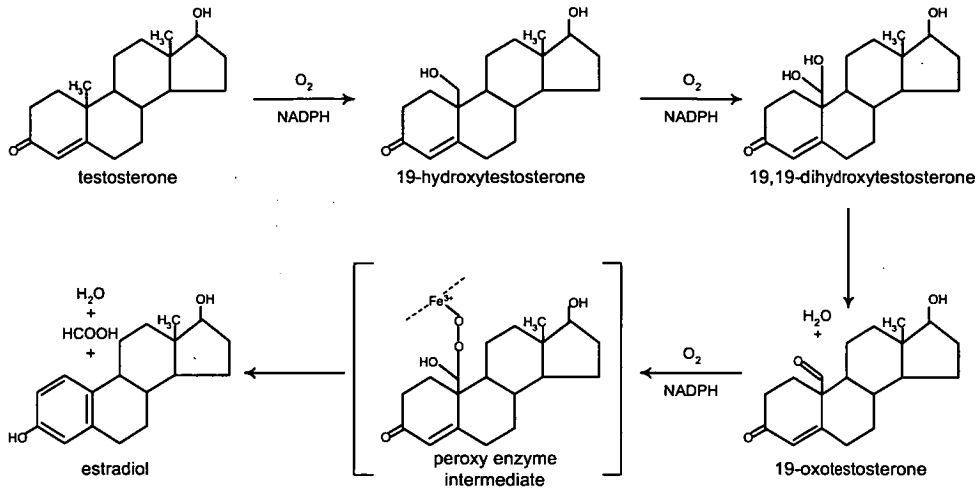

Figure 1.2 Mechanism of the aromatisation of testosterone. (After Brueggemeier (2001))

Evidence from cloning studies suggests that a single protein molecule is responsible for all steps (Cole and Robinson, 1990). Humans carry a single aromatase gene, designated *cyp19* , spanning 123 kilobases and with a coding region of 9 exons (Meinhardt and Mullis, 2002). In contrast, some teleosts, including goldfish, *Carassius auratus* (Tchoudakova and Callard, 1998; Callard et al., 2001) and zebrafish (Callard et al., 2001; Kishida and Callard, 2001; Tong et al., 2001; Trant et al., 2001) carry multiple copies of the *cyp19* gene. These genes, termed *cyp19a* and *cyp19b* for the forms expressed predominantly in the gonad and brain respectively, exhibit greater homology between either the a or b genes between species, than between the 2 genes within a species (Tong et al., 2001; Dalla Valle et al., 2002).

Aromatase activity is greatest in gonadal and brain tissue; however, other tissues may also have some level of activity (Callard et al., 1978; Belvedere et al., 1998; Silverin et al., 2000; Dalla Valle et al., 2001; Zhao et al., 2001). Within the brain, aromatase activity is highest in the forebrain and telencephalon (Callard et al., 1978, 1981; Pasmanik and Callard, 1985; Andersson et al., 1988; Foidart et al., 1995b; Gelinas et al., 1998; Melo and Edmunds, 1999; Silverin et al., 2000). In the ovary, aromatase activity is primarily in the granulosa layer (Devlin and Nagahama, 2002) and shows seasonal variation (Callard et al., 1981; Devlin and Nagahama, 2002).

In mammals, carrying a single copy of *cyp19*, tissue specific gene expression is achieved through multiple promoters, which are differentially expressed in the brain and gonad (Meinhardt and Mullis, 2002). In contrast, there is little overlap in the expression of *cyp19a* and *cyp19b* (Tchoudakova and Callard, 1998). Studies on goldfish indicate that there is temporal variation in the expression of *cyp19a* and *cyp19b*, during development (Callard et al., 2001; Tchoudakova et al., 2001) and furthermore, there are distinct levels of expression of *cyp19b* in developing zebrafish (Trant et al., 2001).

Estrogens fulfill roles as both hormones and neurotransmitters (Callard et al., 1990) and the regulation of estrogens in both roles may be achieved through aromatase regulation. In Japanese quail, *Coturnix japonicus*, male behaviour is moderated by E_2 synthesised in the forebrain, and Balthazart et al. (2001) demonstrated that aromatase activity could be regulated sufficiently rapidly to achieve this, by ion flux. The key role of E_2 in female sex differentiation of non-mammalian vertebrates is supported by a number of studies in which the disruption of E_2 synthesis results in the development of male gonads (eg. Petrini and Zaccanti, 1998) or a male phenotype (eg. Piferrer et al., 1994; Wennstrom and Crews, 1995; Chardard and Dournon, 1999; Guiguen et al., 2000). In species with TSD, brain (Willingham et al., 2000) or gonadal (Jeyasuria and Place, 1997) aromatase activity is higher at temperatures known to cause female development than at male determining temperatures and a mechanism of sex determination based on temperature-dependent aromatase activity has been proposed (Richard-Mercier et al., 1995; Jeyasuria and Place, 1998).

1.5 Aromatase inhibitors

Modulation of E_2 levels by reducing E_2 synthesis is a strategy employed in human medicine for the treatment of estrogen dependent tumors (Brodie et al., 1999; Brueggemeier, 2001). The first compound recognised as having aromatase inhibitory activity was aminoglutethimide (3-[p-aminophenyl]-3-ethyl-piperidine-2,6-dione) (Figure 1.3a), a compound originally developed as an anti convulsant (Brueggemeier, 2001). Similarly, miconazole (1-[2,4-dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole) (Figure 1.3b), an anti-mycotic

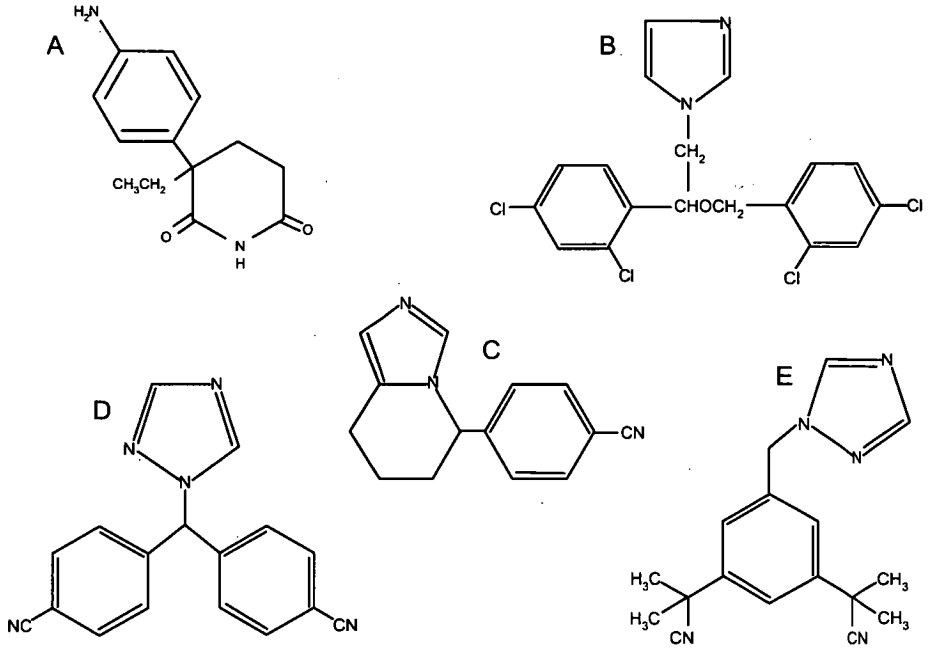

Figure 1.3 Representative non-steroidal aromatase inhibitors a) aminoglutethimide, b) miconazole, c) fadrozole, d) letrozole, e) anastrozole

imidazole was found to inhibit aromatase activity (Mason et al., 1985). Since the discovery of these 'first generation' compounds in the 1970s, an extensive research effort has led to the development more specific and effective aromatase inhibitors (AI). As a result, there is now a better understanding of the structure and function of aromatase, which has, in turn aided the development of more effective AI (Séralini and Moslemi, 2001). Imidazole and triazole derivatives have now been developed that show a greater level of specificity than the first generation compounds, and these include letrozole (CGS 20267) (Figure 1.3d) and anastrozole (ZD 1033) (Figure 1.3e), both of which are currently used in clinical treatment (Demers et al., 1999; Buzdar, 2000; Séralini and Moslemi, 2001), and fadrozole (4-[5,6,7,8-tetra-hydroimidazo-[1,5-a]-pyridin-5-yl benzonitrile HCl), (Figure 1.3c) used in human treatment and experimental studies of aromatase in a wide range of vertebrate taxa (eg. Elbrecht and Smith, 1992; Demers et al., 1993; Wade et al., 1994; Wennstrom and Crews, 1995; Chardard and Dournon, 1999; Kitano et al., 2000).

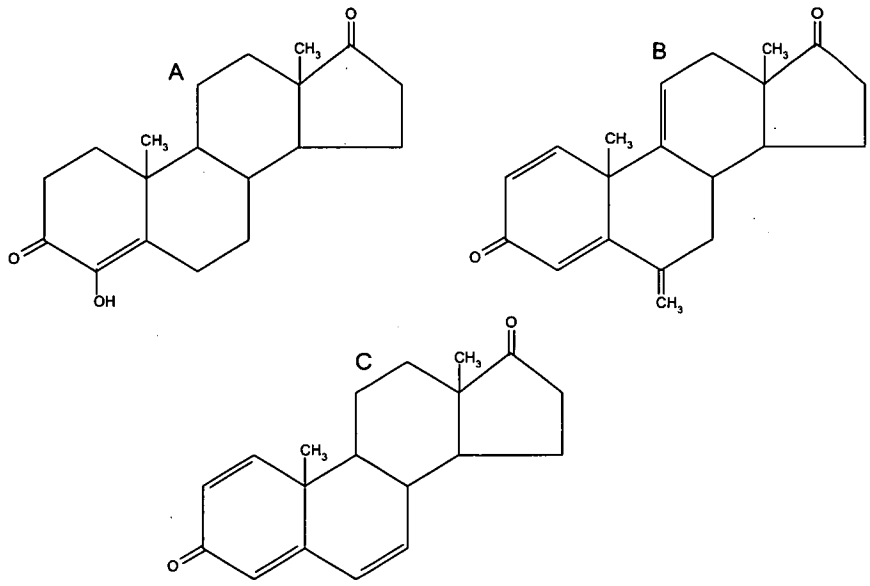

Figure 1.4 Representative steroidal aromatase inhibitors a) OHA b) exemestane c) ATD

In addition, a broad range of steroidal compounds, based structurally on androstenedione, have been investigated and synthesised for use as AI. These compounds include OHA (formestane, 4 androstene-4-ol-3,17-dione) (Figure 1.4a) and exemestane (6-methylenandrosta-1,4-diene-3,17-dione) (Figure 1.4b) both in use clinically (Brueggemeier, 2001; Séralini and Moslemi, 2001) and ATD (1,4,6-androstatriene-3,17-dione) (Figure 1.4c) used in a number of animal studies of aromatase activity and regulation (eg. Alexandre and Balthazart, 1987; Foidart et al., 1995a; Antonolopou et al., 1999).

Inhibition of enzyme function by AI may occur in a number of ways. The majority of non-steroidal AI are competitive inhibitors, interfering with steroid hydroxylation by the formation of a covalent bond between a nitrogen atom and the aromatase heme iron; however, this mode of action may affect other cytochromeP450 enzymes also possessing a heme iron (Brueggemeier, 2001; Séralini and Moslemi, 2001). This lack of specificity accounts for some of the side effects of some of the early non-steroidal AI, although subsequent generations of non-steroidal AI are more specific for aromatase. (Brueggemeier, 2001). Steroidal AI may act competitively or as mechanism-based inhibitors, binding irreversibly to the enzyme. (Numazawa and Tachibana, 1997; Brodie et al., 1999; Séralini and Moslemi, 2001). Mechanism-based AI are more specific in

their action, but side effects of steroidal AI due to their androgenic properties have been reported (Séralini and Moslemi, 2001). Due to the low K_m of aromatase, competitive AI are required at high concentrations to be effective (Cole and Robinson, 1990). In contrast, mechanism-based AI are effective at lower concentrations and furthermore, due to the low turnover rate of aromatase, the effect of inhibitors that irreversibly bind to the enzyme extends beyond the treatment period (Cole and Robinson, 1990).

1.6 Sex control using AI

The development of AI has enabled the development of new approaches to endocrinological research. AI have been used to investigate the role of E_2 as a neurotransmitter and modulator of bird reproductive behaviour (Alexandre and Balthazart, 1987; Kaplan and McGuinnis, 1989; Melo and Edmunds, 1999; Soma et al., 1999). Other studies using AI have dealt with the role of aromatase in the regulation of reproductive hormones (Pasmanik et al., 1988; Afonso et al., 1999a, b; Antonolopou et al., 1999; Sampath-Kumar et al., 2000). The use of AI for sex control has also been the subject of a number of studies, dealing with a range of vertebrate taxa. The female parthenogenetic whiptail lizard, *Cnemidophorous uniparens* was masculinised by the application of letrozole and anastrozole to developing eggs (Wennstrom and Crews, 1995) and masculinisation of the newt, *Pleurodeles waltl* was achieved by immersion treatment with fadrozole (Chardard and Dournon, 1999). Within the teleosts, the Japanese flounder, *Paralichthys olivaceus* and Nile tilapia, *Oreochromis niloticus* have been masculinised by dietary treatment using fadrozole (Kitano et al., 2000; Kwon et al., 2002) and the salmonids, rainbow trout, *Oncorhynchus mykiss* and coho salmon, *O. kisutch* have been masculinised by dietary treatment with ATD (Guiguen et al., 1999) and immersion treatment with fadrozole (Piferrer et al., 1994) respectively.

The use of chemotherapeutants in aquaculture is becoming increasingly more regulated and the development of alternative strategies to masculinisation than through the use of steroid hormones will be of strategic benefit to the aquaculture industry. Furthermore, consumers and regulators are becoming more concerned with the use of steroids in animal production, evidenced by a European Union move in 2002 to ban the use of growth promoter steroids in animal production.

The use of AI for the sex control of fish species in aquaculture therefore offers significant benefits to the aquaculture industry.

1.7 The present study

The high likelihood that the production of farmed salmonids will require a substantial proportion of monosex female stocks both in Tasmania and other salmon producing regions will necessitate the continued production of neomales. The development of alternate methods to steroid hormones for sex inversion of farmed fish is therefore a strategic goal, aimed at alleviating potential future problems associated with the use of steroid hormones. However, in order to optimise the production of Atlantic salmon neomales, it is necessary to develop a greater understanding of the processes of sex differentiation, in particular, the role of E_2 in sex differentiation. AI provide a means to address both of these issues. Accordingly, the present study set out to use AI to investigate the processes of sex differentiation in Atlantic salmon and to develop protocols for their use in the production of Atlantic salmon neomales in a commercial setting through a series of experiments undertaken to investigate:

The efficacy of androgens for masculinisation of Atlantic salmon and optimise androgen-based masculinisation protocols for Tasmanian Atlantic salmon, providing baseline against which to assess the efficacy of AI for masculinisation. The synthetic androgens MT and 17α-methyldihydrotestosterone were applied at a range of doses using dietary and immersion treatments, over various treatment times. Previously published protocols for masculinisation using androgens has been largely developed for *Oncorhynchus* species (eg. Goetz et al., 1979; Baker et al., 1988; Cousin-Gerber et al., 1989; Piferrer et al., 1993; Feist et al., 1995) although some early studies did consider Atlantic salmon with limited success (Johnstone et al., 1978; Johnstone et al., 1979; Johnstone and Youngson, 1984).

The *in vitro* efficacy of AI on both Atlantic salmon gonadal and brain aromatase by comparing E_2 production by isolated follicles or brain homogenate, incubated with testosterone alone or with testosterone and AI. The roles of the 2 forms of aromatase in sex differentiation are presently unclear and recent research has indicated AI efficacy varies for brain and gonadal aromatase (Zhao et al., 2001)

The efficacy of AI for masculinisation of Atlantic salmon and the effects of the timing and dose of AI on sex differentiation. Atlantic salmon alevins were treated with AI by immersion, using a range of doses treatment times, and phenotypic sex and gonadal structure were examined.

The characteristics of uptake and retention of AI, by determining tissue levels of AI at various times following immersion treatment. Although *in vivo* efficacy of AI may be inferred form the *in vitro* studies, it will also be influenced by the efficiency of its uptake and subsequent metabolism.

Detailed below is the planned publication schedule for Chapters 2-5. As a result of this, there are elements of repetition in introductions and methods sections of these chapters.

Chapter 2: Lee, P.S., King, H.R. and Pankhurst, N.W. Preliminary assessment of sex inversion of farmed Atlantic salmon by dietary and immersion androgen treatments. Nth. Am. J. Aquaculture (in press)

Chapter 3 Lee, Pankhurst, N.W. and King, H.R. In prep. Effects of aromatase inhibitors on *in vitro* steroidogenesis in Atlantic salmon (*Salmo salar*) gonadal and brain tissue.

Chapter 4 Lee, P.S., King, H.R. and Pankhurst, N.W. A comparison of aromatase inhibitors for the sex reversal of female Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem (in press)

King, H., Lee, P.S. and Pankhurst, N.W. Photoperiod-induced precocious male sexual maturation in Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem (in press)

Lee, P.S., King, H.R. and Pankhurst, N.W. In prep. Efficacy of aromatase inhibitors for sex inversion, and effects of aromatase inhibitor immersion, on production parameters of farmed Atlantic salmon.

Chapter 5 Lee, P.S., Pankhurst, N.W. and King, H.R. In prep. Uptake and retention of aromatase inhibitors by Atlantic salmon, *Salmo salar* (L.) alevins.

Ethical clearance for animal experimentation work undertaken for this study was conducted under permit No. A0007172 issued by the University of Tasmania, Tasmanian Animal Experimentation Ethics Committee.

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Chapter 2

Preliminary assessment of sex reversal of Atlantic salmon by dietary and immersion androgen treatments.

Sweet Loretta Martin thought she was a woman

But she was another man

John Lennon & Paul McCartney

Get Back

2. Preliminary assessment of sex reversal of farmed Tasmanian Atlantic salmon (*Salmo salar*) by dietary and immersion androgen treatments.

2.1 Introduction

Since early demonstrations of the potency of exogenous steroid hormones to influence sexual development in fish, numerous studies have investigated both the necessary protocols for manipulating development, and some of the mechanisms involved (reviewed by Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001; Devlin and Nagahama, 2002). The studies of Yamamoto (1969) showed that exogenous androgens masculinise differentiating individuals, whereas estrogens are feminising agents, and Yamamoto proposed that these compounds are the natural masculinising and feminising agents respectively in fish. The use of these compounds to produce mono-sex fish populations for aquaculture is now well established and is in routine use where mono-sex populations fulfil production needs or environmental constraints (Hunter and Donaldson, 1983; Piferrer, 2001).

Both natural and synthetic steroids are effective for sex inversion, however they vary considerably in their efficacy (Piferrer and Donaldson, 1991, 1992; Piferrer et al., 1993; Piferrer, 2001). The synthetic androgens 17α-methyltestosterone (MT), and 17α-methyldihydrotestosterone (MDHT) have been shown to have greater potency than either testosterone or 11-ketotestosterone (Piferrer et al., 1993; Pandian and Sheela, 1995), and the synthetic compounds, in particular MT, have been widely used both in experimental studies (e.g. Baker et al., 1988; Piferrer et al., 1993; Blazquez et al., 2001), and commercial production (Johnstone and Maclachlan, 1994) of masculinised fish. Similarly, ethynylestradiol-17α (EE_2), a synthetic estrogen is known to be significantly more potent than the natural 17β-estradiol (E_2) (Yamamoto, 1969; Piferrer and Donaldson, 1992). Devlin and Nagahama (2002) proposed that differences in potency reflect differences in receptor affinity, activities of steroid-receptor complexes and steroid metabolism.

The administration of steroid hormones to manipulate sexual development has been achieved by a number of means, reviewed by Pandian and Sheela (1995). Dietary administration involves the application of a solution of the steroid in a volatile solvent to manufactured, pelletised feed and fish are fed the treated feed over a period of several weeks. Typically, dietary treatments commence at or near the time of first feeding (Pandian and Sheela, 1995). An alternative is immersion treatment, where individuals are placed in an aqueous solution of the steroid for several hours. Unlike dietary treatments, this technique may be applied to individuals before hatching or feeding and the exposure each individual receives may be accurately determined. Immersion treatments are also easier to complete, and may have fewer issues of worker safety (Pandian and Sheela, 1995; Piferrer, 2001). Regardless of the method used to apply steroids, treatment is optimal during the labile period, during which sex differentiation may be influenced by exogenous factors (Pandian and Sheela, 1995; Baroiller et al., 1999; Piferrer, 2001; Devlin and Nagahama, 2002).

Effective steroid doses for sex inversion vary between and within species. In dietary studies, Kitano et al. (2000) achieved 100% masculinisation of the Japanese flounder, *Paralichthys olivaceus*, using MT at a rate of 10 mg kg^{-1} food, for 1260 °C days (DD), while a similar result was obtained for rainbow trout, *Oncorhynchus mykiss*, treated at 0.5 mg kg^{-1} food for 548 DD (Cousin-Gerber et al., 1989). Complete masculinisation of all female stocks of chinook salmon, *O. tshawytscha,* was achieved by immersion for 120 minutes in MT at a concentration of 400 μg l^{-1} (Piferrer et al., 1993) or 200 μg l^{-1} MT (Baker et al., 1988). Repeated immersion treatments were more effective for masculinisation of chinook salmon (Baker et al., 1988); however, masculinisation rates declined with repeated immersion treatments of rainbow trout (Feist et al., 1995). Within limits, the effects of steroid treatments may be dose dependent (Baker et al., 1988; Piferrer and Donaldson, 1991; Piferrer et al., 1993), but high doses have resulted in the production of a significant proportion of sterile individuals (Johnstone et al., 1978; Goetz et al., 1979; Johnstone et al., 1979; Solar et al., 1984). Paradoxical feminisation, the phenomenon of an increased proportion of females produced following high doses of androgens, has also been reported in some species (Goudie et al., 1983; Solar et al., 1984; Piferrer and Donaldson, 1991; Piferrer et

al., 1993). This is attributed to the aromatisation of androgens to biologically active estrogens (Piferrer and Donaldson, 1991; Piferrer et al., 1993).

The production of all female stocks of Atlantic salmon, *Salmo salar* (L.), is preferred in Tasmania, for the production of non-maturing triploids (Jungalwalla, 1991) and because all female stocks have a reduced propensity to early maturation following transfer to seawater (Bye and Lincoln, 1981; Donaldson et al., 1996). Current production methods follow the indirect method described by Piferrer (2001). This method, suitable for species having homogametic females, involves the sex inversion of a small proportion of females, which subsequently develop as phenotypic males, and are used as broodstock to provide milt for crosses with normal females, resulting in the production of unmanipulated, all female, offspring. While early attempts at masculinisation of Atlantic salmon by dietary and immersion treatments were largely unsuccessful (Johnstone et al., 1978), later studies considerably improved treatment efficacy (Johnstone and Maclachlan, 1994), however, no treatment to date has had the level of success or ease of application as those achieved for *Oncorhynchus* spp. This study aimed to develop more effective dietary and immersion treatment regimes for the masculinisation of Atlantic salmon in Tasmania, and to apply such treatments for the production of sex reversed males under commercial conditions. Accordingly, in the first experiment, a series of immersion and dietary protocols were conducted to better define the labile period for immersion treatment of this species, and to ascertain effective dietary treatment levels. Subsequently, the most successful immersion and dietary treatments were repeated on larger groups of fish, which were then ongrown to maturity for assessment of fertility. While MT was initially used as the established dietary treatment, non-aromatisable MHDT was preferred for immersion and latter dietary treatments on the basis that issues of paradoxical feminisation could be avoided.

2.2 Materials and Methods

2.2.i Stock and Husbandry

Experiments were conducted over two years (1991 and 1992) at Saltas Freshwater Operations, Wayatinah, Tasmania, using the progeny of 2+ year old Atlantic

salmon broodstock. Prior to transfer freshwater for final maturation, broodstock had been maintained at Saltas Marine Operations, Dover, Tasmania. Broodstock were reared in a 65m polar circle-style cage, and fed to satiation on a steam pelletised commercial diet (Gibsons Ltd., Cambridge, Tasmania). Sex inverted female fish were used to provide milt in both experiments. These stocks had been masculinised by dietary treatment with MT at 3 mg kg^{-1} feed, following methods similar to Solar *et al.* (1984). Fertilised ova were incubated in commercial upwelling incubators at 8 °C. Once eyed, eggs were transferred to Heath vertical incubator trays (Maricource Inc., Tacoma, USA) and maintained at 7-10 °C until swim up stage. Fry were transferred to 1 m^3 tanks for first feeding and subsequent rearing. Immersion treatments were timed in relation to the day of median hatch (approximately 450 DD post fertilisation), and dietary treatments commenced at first feeding. Populations of parr were maintained in indoor 1 m^3 tanks receiving water 10-12°C, under a natural photoperiod, until sampled for gonadal examination, approximately eight months post-hatch.

2.2.ii Experimental protocols

Experiment 1

Stock solutions (10 mg ml^{-1}) of MT and MDHT were prepared in 100% ethanol, and refrigerated until required. Feed for dietary treatments was prepared by diluting MT stock solution in 100% ethanol, mixing with feed at 200 ml kg^{-1}, and leaving overnight to allow evaporation of the solvent. Feed was prepared with either 1 mg MT kg^{-1} or 3 mg MT kg^{-1} and fed to a separate groups of 6000 fry for a period of approximately 800 DD, commencing at the onset of feeding, at approximately 850 DD post-fertilisation . Bathing commenced at median hatch (Day 0, approximately 450 DD post-fertilisation), with baths undertaken weekly up to 28 days post median hatch (DPMH) or approximately 690 DD post-fertilisation. Groups of fry received either a single bath, or two baths, 7 to 14 days apart as shown in Table 2.1. Bathing was undertaken in Heath trays, with the MDHT stock solution added to water to give a final concentration of 400 μg MDHT L^{-1}, which was recirculated through the Heath trays for 120 min at 0.3 L s^{-1}. Recirculation ceased at the completion of the immersion period and flow through resumed at 0.3 L s^{-1} to ensure dispersal of any remaining steroid.

Duplicate trays, each containing 3000 fry were used for each immersion treatment group. Owing to restrictions imposed by commercial considerations, only a single population of untreated, all female fish, derived from the same parental stocks as the experimental fish, was maintained as a control for both the dietary and immersion treatments. Such populations are known to contain a small proportion of genetic males (H.R. King, unpublished data), resulting from the inclusion of genetic male broodstock in all female production.

Table 2.1 Immersion (17α-methyldihydrotestosterone (MDHT)) and dietary treatment (17α-methyltestosterone (MT)) groups used in Experiment 1.

	Hormonal Treatment			
		MDHT Immersion (DPMH[a])		
Group	Dietary MT	First	Second	Treatment Duration
A[b]	-	na[c]	na	na
B	1 mg kg^{-1}	na	na	800 °C Days
C	3 mg kg^{-1}	na	na	800 °C Days
D	na	0	-	120 min per immersion
E	na	0	7	120 min per immersion
F	na	0	14	120 min per immersion
G	na	7	-	120 min per immersion
H	na	7	14	120 min per immersion
I	na	7	21	120 min per immersion
J	na	14	-	120 min per immersion
K	na	14	21	120 min per immersion
L	na	14	28	120 min per immersion

[a] days post-median hatch

[b] untreated all female population

[c] not applicable

Experiment 2

To exclude the likelihood of paradoxical feminisation, MDHT was used for both dietary and immersion treatments in Experiment 2. A diet with 1 mg MDHT kg^{-1} was prepared and fed to a single group of 6000 fry as in Experiment 1. Duplicate Heath trays, each containing 3000 fry, were immersed for 120 minutes in a tank containing MDHT at 400 $\mu g\ L^{-1}$. Baths commenced on 14 DPMH (approximately 570 DD post-fertilization), with groups of fry receiving second bath, on either 21 DPMH (approximately 630 DD post-fertilization) or 28 DPMH (approximately 690 DD post-fertilization) (Table 2.2). As in Experiment 1, a single population of untreated, all female fish, derived from the same parental stocks as the experimental fish, was maintained as a control for both the dietary and immersion treatments.

Fish that had been ongrown at Saltas Marine Operations were returned to freshwater as 2+ year fish and phenotypic males were examined for sperm duct morphology.

Table 2.2 Immersion (17α-methyldihydrotestosterone (MDHT)) and dietary (MDHT) treatment groups used in Experiment 2.

	MDHT Treatment			
		Immersion (DPMH)[a]		
Group	**Dietary**	**First**	**Second**	Treatment Duration
A[b]	na[c]	na	na	na
B	1 mg kg^{-1}	na	na	800°C Days
C	na	14	21	120 min per immersion
D	na	14	28	120 min per immersion

[a] days post-median hatch

[b] untreated all female population

[c] not applicable

2.2.iii Gonadal examination

In both experiments 1 and 2, a sample of approximately 100 fish was collected from each group nine months post-hatch. Fish were euthenased by a blow to the head, and dissected for gonadal examination under a dissecting microscope. Individuals having oocytes present within gonad were scored as female, whereas all others were scored as male, thus intersex or sterile individuals may have been included with either male or female fish. In Experiment 2, fish remaining after sampling for gonadal examination were retained for a further period of approximately two months, until reaching a weight of approximately 45g. Treatment groups were marked by freeze branding, maintained in 1 m^3 tanks for a further four months, until transfer outdoors to 4m^3 tanks, where they remained until smoltification and subsequent transfer to Saltas Marine Operations. Fish were maintained as part of a population of approximately 5000 fish in a 65 m polar circle style cage and reared under standard Tasmanian commercial conditions from arrival in September 1993, until being returned to Wayatinah in February 1995 for assessment of maturity, fertility and sperm duct patency.

2.2.iv Statistical Analysis

Alterations in sex ratio relative to untreated all female (control) groups were tested using a contingency Chi-squared test for each treatment group. In Experiment 2, differences in sperm duct patency frequency between treatment groups B, C and D were assessed using a Chi-squared contingency test.

2.3 Results

2.3.i Experiment 1

With the exception of a single immersion treatment on median hatch day (Treatment D), all dietary and immersion treatments significantly increased the proportion of phenotypic males (Figure 2.1). Both dietary treatments (Treatments B and C) resulted in complete sex reversal of the population. Single immersion treatments on either 0, 7 or 14 DPMH (Treatments D, G and J) resulted in masculinisation levels of 11, 58 and 61% respectively. Conducting a second immersion treatment, either 7 or 14 days after a bath at 0 DPMH (Treatments E

and F), significantly increased the proportion of masculinisation (61 and 54% males respectively) in comparison to the control population (8% males). Although all other treatments resulted in significant levels of masculinisation compared to control populations, some qualitative differences between treatments were apparent. A second immersion following a bath at 7 DPMH (Treatments H and I) resulted in increased levels of masculinisation (77 and 79% respectively) and second immersion following a bath at 14 DPMH (Treatments K and L) resulted in increased levels of masculinisation (90 and 95% respectively) (Figure 2.1).

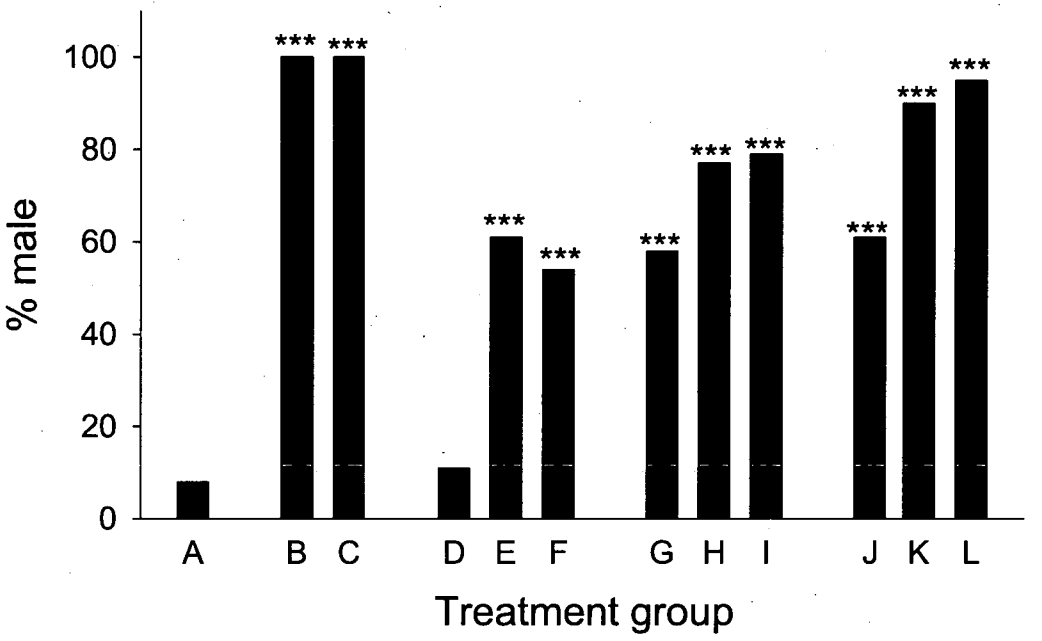

Figure 2.1 Percentage masculinisation in immersion and dietary treatment groups used in Experiment 1. Refer to Table 1 for treatment details. Asterisks indicate a significant difference (***$p<0.001$) in proportion from the untreated, all female group (Group A).

2.3.ii Experiment 2

All MDHT treatments resulted in complete masculinisation of fish sampled at nine months post-hatch, which was significantly different from the untreated all female population (Figure 2.2). The incidence of sperm duct patency, was highest in the immersion treated fish (Treatments C and D, 92 and 84% respectively) (Figure 2.2) and differed significantly between treatment groups B (59%), C and D ($\chi^2 = 14.4$, 2 d.f., $p<0.01$).

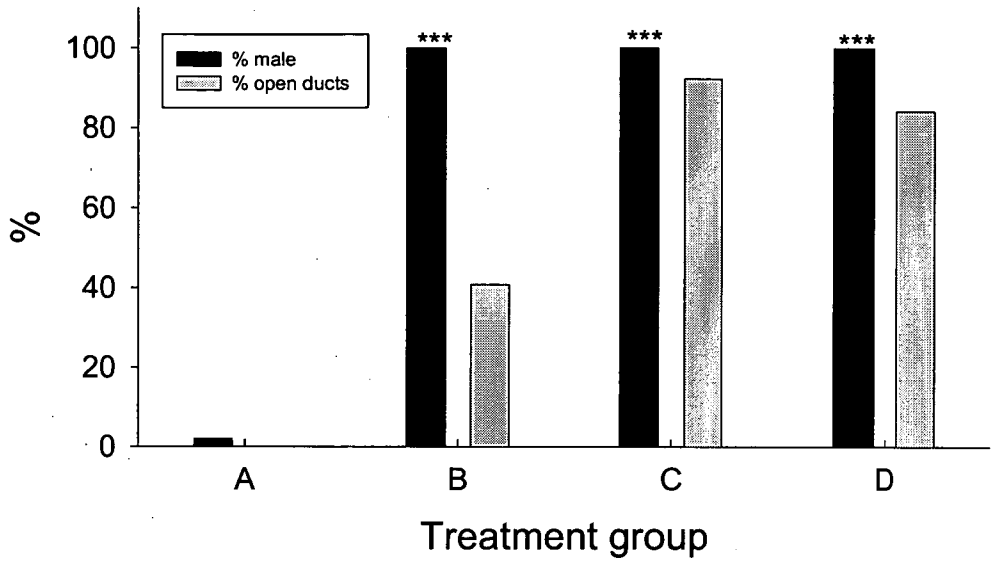

Figure 2.2 Percentage masculinisation and open sperm ducts in immersion and dietary treatment groups used in Experiment 2. Refer to Table 2 for treatment details. Other details as for Figure 2.1

2.4 Discussion

The high levels masculinisation by dietary treatments of both MT and MDHT achieved in this study are consistent with, or improvements upon, the levels achieved previously using dietary androgens for Atlantic salmon. Johnstone et al. (1978), achieved 100% masculinisation of Atlantic salmon following dietary treatment with MT, but 17% of these fish proved to be sterile, with gonads comprised primarily of connective tissue. Johnstone and Maclachlan (1994) reported the same phenomenon at a high level in Scottish commercial hatcheries using a dietary treatment of 3mg MT kg^{-1} feed. Similar effects have been noted in other species treated with high doses of androgens (Goetz et al., 1979; Solar et al., 1984; Piferrer et al., 1994a; Galbreath and Stocks, 1999; Blazquez et al., 2001). As neither fertility nor gonad histology were assessed in Experiment 1, it is not known whether a similar effect occurred in similarly treated animals here (treatment group B), and it is possible that animals with gonads primarily containing connective tissue were present, but having apparently filiform testis, were recorded as males.

The effectiveness of low dose androgens (1 mg kg^{-1} feed) found in this study (Experiment 1 treatment group C; Experiment 2, treatment group B) is substantially higher than that found by Johnstone and Maclachlan (1994) who reported a masculinisation level of 46-56% in mature fish treated with 0.25-1.0 mg MT kg^{-1} feed. However, this figure may have underestimated the level of masculinisation as the sex of immature fish was not determined. Alternatively, the observed differences between the present study and that of Johnstone and Maclachlan (1994) may reflect the differences in treatment duration (500 DD compared to 800 DD) between the studies. Duration of steroid exposure has been previously shown to affect the degree of sex reversal in salmonids (Chevassus and Kreig, 1992; Piferrer and Donaldson, 1992). Increased duration of both dietary (Chevassus and Kreig, 1992) and immersion (Piferrer and Donaldson, 1992) steroid treatments resulted in increased levels of sex inversion.

There was no evidence of paradoxical feminisation in this study, with dietary doses of the aromatisable androgen MT ranging from 1 to 3 mg kg^{-1} feed for 800 DD. Substantially higher doses than those used in this study (>25 mg kg^{-1} feed for 330 – 1320 DD) caused some degree of paradoxical feminisation in rainbow trout (Solar et al., 1984), and high dose (6.4 mg L^{-1}) immersion treatments with MT was associated with the production of an increased proportion of females in coho salmon (Piferrer and Donaldson, 1991). This effect was not apparent with an equivalent dose of non-aromatisable MDHT (Piferrer and Donaldson, 1991) and on this basis, it has been proposed that the mechanism of paradoxical feminisation is by aromatisation of androgens to estrogens (Piferrer et al., 1993). While the doses of MT and MDHT used in this study were sufficient to induce complete sex reversal, there was no evidence of other effects, such as the production of intersex individuals, sterility and paradoxical feminisation, reported by other workers (Johnstone et al., 1978; Johnstone and Maclachlan, 1994; Piferrer et al., 1994a) and respectively proposed to be the result of insufficient or excessive doses (Galbreath and Stocks, 1999).

Results of the present study suggest that treatment by immersion is most effective in the period 14 to 28 DPMH, suggesting that the labile period for masculinisation of Atlantic salmon commences during this time. This result is consistent with other data for Atlantic salmon where immersion was most effective in the period

650-900 DD post-fertilisation (Johnstone and Maclachlan, 1994), equivalent to 16-45 DPMH for conditions used in this study. However, these findings contrast with those of Baker et al. (1988) Piferrer and Donaldson (1989), Piferrer et al. (1993) and Feist et al. (1995) who showed in chinook salmon, coho salmon and rainbow trout respectively, the labile period for androgen immersion treatment commenced at or before hatching, but may extend to 13 DPMH. Variations in the labile period among different taxa are well recognised (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001), probably reflecting interspecific differences in developmental rates (Devlin and Nagahama, 2002). In Atlantic salmon, gonadal primordia appear at 60 DD pre-hatch, while gonadal differentiation in Atlantic salmon continues to 600 DD post-hatch (Laird et al., 1978). In contrast, coho salmon gonadal primordia were detected at 10 DD pre-hatch, differentiation commenced at 270 DD post-hatch (Piferrer and Donaldson, 1989) and differentiated gonads were discernible at 490 DD post-hatch (Goetz et al., 1979). This suggests that observed differences in the labile period between Atlantic and Pacific salmon arise from differences in the timing and duration of gonadal differentiation.

The qualitative increase in masculinisation following repeated immersion suggests that under the rearing conditions used, double treatments may be more effective than single. Repeated immersion in MT has been used to produce a high masculinisation rate for Atlantic salmon (Johnstone and Maclachlan, 1994); however, this study did not compare the treatment with a single immersion. Other studies investigating the effects of multiple immersion treatments on sex reversal in salmonids have had varying results. Baker et al. (1988) found increased levels of masculinisation in chinook salmon following a second immersion treatment; however, a decrease in masculinisation was seen with additional immersion treatments of rainbow trout (Feist et al., 1995). Single immersion treatments of coho and chinook salmon have resulted in masculinisation rates of 70-100% (Piferrer and Donaldson, 1989, 1991; Piferrer et al., 1993; Piferrer et al., 1994b). It is likely that the increased effectiveness of a second immersion treatment may be due to maintenance of an increased tissue loading of androgen, allowing increased exposure of susceptible individuals over time. The results of this study are therefore consistent with a requirement by Atlantic salmon, in comparison to

Pacific salmon, of increased levels of androgens, or elevated levels of androgens for a longer period, for masculinisation to occur.

The results of Experiment 2 showed that both immersion and dietary treatment of Atlantic salmon with MDHT are successful means of sex reversal. This is the first time MDHT has been reported to masculinise Atlantic salmon, and the first successful use of MDHT by dietary treatment. MDHT has been successfully used in immersion treatments of coho and chinook salmon (Piferrer and Donaldson, 1991; Piferrer et al., 1993) and rainbow trout (Krisfalusi and Cloud, 1999) however, dietary treatment of channel catfish with MHDT was unsuccessful (Davis et al., 1990).

The increased incidence of patent sperm ducts in immersion-treated fish, compared to dietary treatment is consistent with other studies. The association between sex reversal by dietary administration of androgens and malformations of the gonad has been noted by several authors (Johnstone et al., 1979; Johnstone and Youngson, 1984; Cousin-Gerber et al., 1989; Feist et al., 1995), while studies involving immersion treatments only, report few malformations. In a study directly comparing the effects of immersion and dietary androgen treatments in rainbow trout, Feist et al. (1995) found a higher incidence of patent ducts in immersion treated fish. While the dose and duration of dietary androgen treatment can affect the incidence of sperm duct patency (Cousin-Gerber et al., 1989), the phenomenon of impaired development of the testes in dietary treated animals may be a function of the developmental timing of dietary treatment, with gonads at a later stage of development less plastic in their growth response.

The current reliance of hatcheries on gonad morphology to distinguish sex inverted male Atlantic salmon from genetic males, means that malformations of the sperm duct and other traits typical of dietary treated animals are necessary for the recognition of sex inverted broodstock, although their presence restricts the use of such animals to a single season (Johnstone and Maclachlan, 1994). Furthermore, the recognition of sex inverted males by morphological features may still be subject to error, as the presence of a small proportion of males in the all female lines used in this study indicates. The absence of a determinant of genotypic sex therefore not only generates a restriction on the commercial use of

immersion treatments for the sex inversion of Atlantic salmon, but on the quality of stock produced from sex inverted broodstock produced using dietary treatments.

The present study has demonstrated the effectiveness of both dietary and immersion androgen treatments for masculinisation of Atlantic salmon, and that masculinisation by immersion results in the production of a high proportion of sex inverted males with patent sperm ducts. Furthermore, the study has demonstrated the successful application of these treatments on a commercial production scale. This has significant implications for the Atlantic salmon farming industry in Tasmania, which is becoming increasingly reliant on all female stocks for year round production.

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Chapter 3

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

If the brain were so simple we could understand it,
we would be so simple we couldn't.

Lyall Watson

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

3.1 Introduction

The benefit to aquaculture productivity generated by using monosex populations has resulted in research which has elucidated much about the processes of sex determination and differentiation in fish reproduction (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001; Devlin and Nagahama, 2002). The seminal studies of Yamamoto, (summarised in Yamamoto, 1969) showed that androgens and estrogens are respectively the masculinising and feminising agents in fish. It is through the delivery of these steroid hormones to some or all of a population, that monosex populations are commercially produced (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001).

An alternative to the use of steroid hormones to control sex determination or differentiation is the use of compounds that affect steroid synthesis or release, particularly that of estrogens. Aromatase (cytochrome $P450_{arom}$) is the enzyme responsible for the conversion of androgens to estrogens. In fish, aromatase activity is greatest in the gonad and brain tissue; however, other tissues may also have some level of activity (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 aromatase gene. Molecular studies have demonstrated differential expression of these genes in different tissues (Callard et al., 2001; Dalla Valle et al., 2001; Tchoudakova et al., 2001). Notably, different genes are predominantly expressed in the brain and 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).

Regulating aromatase activity has become an important strategy in human medicine in the treatment of estrogen dependent tumors, and a large number of

compounds with aromatase inhibitory activity are now recognized (Brodie et al., 1999; Séralini and Moslemi, 2001). Aromatase inhibitors have been shown to be effective in studies on all major classes of non-mammalian vertebrates (e.g. Piferrer et al., 1994; Foidart et al., 1995; Richard-Mercier et al., 1995; Chardard and Dournon, 1999) with a number of studies dealing with teleosts. Antonolopou et al. (1995; 1999) and Afonso et al. (1999; 2000) showed aromatase inhibitors to affect circulating steroids, aromatase activity and response to gonadotropin in salmonids. 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 two isozymes (gonad and brain) responded differently to a range of aromatase inhibitors, and generally, the ovarian isozyme was more sensitive to the aromatase inhibitors tested, than was the brain isozyme.

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). Researchers of mammalian systems may use lower cost species, such as teleosts, in *in vivo* studies (e.g. Nigrelli, 1953; Shilling et al., 1999) to allow sufficiently robust studies to be carried out. An alternative approach is *in vitro* incubation of isolated tissue or homogenates, which has proven to be an informative tool for investigating steroidogenesis and its relationship to reproductive physiology and behaviour across a range of taxa. This method has been successfully applied to various tissue types in fishes, including gonadal (e.g. Kagawa et al., 1982; Pankhurst and Riple, 2000), neural (e.g. Callard et al., 1981; González and Piferrer, 2002), adrenocortical (Bisson and Hontela, 2002) and mesenteric (Moore et al., 2002) tissue. Most studies on teleosts have focussed on gonadal physiology, in particular the role of, or response by, gonadal tissues to hormonal changes during maturation (e.g. Ueda et al., 1983; Ijiri et al., 1995; Pankhurst, 1997) or the process of gonadal steroidogenesis (e.g. Kagawa et al., 1982; Haddy and Pankhurst, 1998; Amiri et al., 1999). Neurological studies have primarily been concerned with the level of steroidogenic activity in relation to neuroanatomy, with consideration given to the role of the specific areas of the brain in hormonally mediated behaviour (e.g. Pasmanik and Callard, 1985) or involvement

of brain tissue in steroid conversion for feedback loops regulating hypothalamic and pituitary endocrine activity (e.g. Antonolopou et al., 1995)

Incubation conditions, including temperature, duration and medium components can all influence *in vitro* tissue steroidogenesis (Tyler et al., 1990; Rahman et al., 2002). The production of compounds such as steroids may increase with increasing incubation time (Zhao and Wright, 1985; González and Piferrer, 2002); however, extended incubation can 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 (Ijiri et al., 1995; Lokman and Young, 1995; Pankhurst, 1997; Rahman et al., 2002) or hormones that promote the activity of steroidogenic enzymes (Zhao and Wright, 1985), or 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. Optimisation of *in vitro* conditions ensures high levels of enzyme activity (Shilling et al., 1999; González and Piferrer, 2002), and thus representativity of responses.

In vitro incubation of gonadal or brain tissue provides an appropriate model for testing the efficacy of aromatase inhibitors (Afonso et al., 1997; Bhatnagar et al., 2001). Measurement of aromatase activity may be achieved either indirectly, by measurement of estradiol (E_2) synthesis (Afonso et al., 1997) or directly, by the measuring stereospecific loss of 1β-^{3}H from ^{3}H-androstenedione (González and Piferrer, 2002). While the direct method is rapid, accurate and allows for kinetic studies of aromatase activity (González and Piferrer, 2002), it requires the use of facilities equipped to deal with radioactive materials. In contrast, incubations for indirect determination may be conducted in locations such a hatcheries, with only the assay procedure requiring specialized facilities.

Accordingly, the aims of the present study were to optimise protocols for *in vitro* incubation of Atlantic salmon, *Salmo salar* (L.), gonadal and brain tissue from

animals sampled directly at a hatchery facility, for detection of basal and steroid precursor-stimulated E_2 synthesis, and then to use the protocol to assess the efficacy of several aromatase inhibitors on steroidogenesis.

3.2 Materials and Methods

3.2.i 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 2-5) facilities. Fish used in Experiment 1, 3, 4 and 5 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 pelletised diet (Nutreco) to satiation.

3.2.ii 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. Whole fish and ovary weights were determined and gonosomatic index (GSI) calculated as:

$$\text{GSI (\%)} = \frac{\text{gonad weight}}{\text{whole body weight}} \quad \text{X 100}$$

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 one hour in 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. Oocyte diameter was determined to 0.01mm from 30 follicles using digital vernier calipers. Each experiment was performed twice using follicles from a different fish.

At the end of the incubation period, the culture medium was aspirated by Pasteur pipette, to 1.5 mL Eppendorf tubes and frozen (-18 °C) until assay.

3.2.iii Brain tissue preparation and incubation

Brain tissue was dissected out, following terminal anesthesia in AQUI-S™ and spinal transection and whole body and brain tissue weights determined. All brain tissue was maintained in chilled CCS during processing. Tissue was collected from 2-5 fish, dissected into pieces, homogenised in CCS and pooled before addition to culture wells. Tissue was homogenized using either a hand held teflon grinder or a Polytron PT 1200C homogenizer (Kinematica AG, Lucerne, Switzerland), added to culture plates as described below and plates gently agitated on either an Amersham N4201 fixed speed orbital shaker (Amersham Biosciences Corp., Piscataway, USA) or on an Amersham ZMD 201 variable speed orbital shaker during incubation at 10 °C.

3.2.iv Experimental protocols

Experiment 1 – Gonadal tissue incubation

Follicles were incubated at 5, 10 or 15 °C for 6, 18 or 42 h. Incubations were undertaken in either CCS or CCS with the steroids 17 α-hydroxyprogesterone (17P) or testosterone (T) as 10 µL of 1 or 10 µg mL^{-1} in ethanol to give final concentrations of 10 or 100 ng mL^{-1}. Steroid doses were selected on the basis of effective concentrations for similar studies on rainbow trout (Haddy and Pankhurst, 1998). Incubations at 10 °C and 15 °C were carried out by immersing the culture plates in water baths of the appropriate temperature, whereas incubations at 5 °C were conducted in a Contherm 1150CP incubator (Contherm Scientific Ltd., Petone, New Zealand). All wells without steroids received an equivalent volume of ethanol. There were six replicate wells for each treatment and 10 follicles per well, except for a treatment of 5 follicles per well, conducted at 10 °C for 18 h.

Experiment 2 – Efficacy of AI on ovarian E_2 production

Five AI were evaluated for efficacy in reducing *in vitro* E_2 production. The compounds tested were: 1,4,6-androstatriene-3,17-dione (ATD, Steraloids Inc.); 4-androstene-4-ol-3,17-dione (OHA, Sigma); 4-[5,6,7,8-tetra-hydroimidazo-[1,5-a]-pyridin-5-yl benzonitrile HCl] (fadrozole, Novartis); 1-[2,4-dichloro-β-([2,4-

dichlorobenzyl]oxy)-phenethyl]imidazole (miconazole, Sigma) and 3-[p-aminophenyl]-3-ethyl-piperidine-2,6-dione (aminogluthetimide, Aldrich).

Follicles were incubated in the Contherm incubator at 10°C for 18 h, in either CCS; CCS with the AI ATD, OHA, fadrozole, aminoglutethimide and miconazole added as 10 μL of 0.01, 0.1 or 1mg mL^{-1} in ethanol to give final concentrations of 0.1, 1 or 10 μg mL^{-1}; CCS with T added as 10 μL of 10 μg mL^{-1} in ethanol to give final concentrations of 100 ng mL^{-1}; or CCS with AI and T. AI doses were selected to include the range of IC_{50} reported in studies of the effects of ATD, OHA, fadrozole and aminoglutethimide on teleost follicles (Pelissero et al., 1996; Afonso et al., 1997) and teleost (Antonolopou et al., 1995) and avian (Wozinak et al., 1992) brain homogenates *in vitro*. All wells without both T and AI received an equivalent volume of ethanol. There were 6 replicate wells per treatment and 5 follicles per well.

Experiment 3- Brain tissue incubation

Brain tissue homogenate prepared as above was pipetted into wells to provide 20, 50 , 100, or 200 mg tissue per well. CCS was added to wells to give a total volume of 990 μL and the steroids 17P or T added as before. There were six replicate wells for each treatment. Following 18 h incubation, well contents were transferred to 1.5 mL Eppendorf tubes and centrifuged at 1200*g* for 10 min. The supernatant was transferred to a new 1.5 mL Eppendorf tube, and frozen (-18 °C) until analysis of E_2 and T in 100 μL aliquots.

Experiment 4- Solvent extraction of steroids

Brain tissue prepared as above was added to wells as for Experiment 3. Complete Cortlands solution (990 μL) was then added to all wells and T or 17P added as 10 μL of 1 or 10 μg mL^{-1} to give final concentrations of 10 ng mL^{-1} and 100 ng mL^{-1} respectively. There were four replicate wells for each treatment. Tissue was incubated for 18 h, well contents were transferred to 1.5 mL Eppendorf tubes, and frozen (-18 °C) until analysis. On thawing, tube contents were transferred to clean 20 mL glass vials, 5 mL diethyl ether added and the vial vortexed. Vials were allowed to stand for 5 minutes, then the aqueous phase was frozen by immersion

in a bath of methanol-dry ice. The organic phase was poured off into a clean glass vial, the solvent evaporated off under N_2 and allowed to stand overnight. The precipitate was resuspended in 1 mL phosgel buffer by vortexing, and 200 µL aliquots removed for measurement of E_2 and T as described in section 3.2.v.

Experiment 5 – Efficacy of AI on E_2 production by brain tissue

Brain homogenate, at 50 mg tissue per well was added to 980 µL CCS, and T was added as before at 10 ng mL^{-1}. The AI OHA, miconazole, aminogluthetimide and fadrozole were added as before to give final concentrations of 0.1, 1.0 or 10.0 µg mL^{-1} respectively. Due to significant levels of cross reactivity with the E_2 RIA (Table 3.1), ATD was added as above, to provide final concentrations of 0.1 and 1.0 µg mL^{-1} only. Six replicate wells were used for each treatment and homogenates were incubated for 42 h. To measure differences in basal and T-mediated E_2 synthesis between homogenates incubated for 18 and 42 h, additional homogenates were incubated for 18 h with and without T at 10 ng mL^{-1}. Following incubation, well contents were stored and extracted as for Experiment 4.

Table 3.1 Effective E_2 equivalence due to cross reactivity of AI with E_2 antibody. Values are mean ±SE (n=6).

	AI concentration (µg mL^{-1})		
AI[a]	0.1	1.0	10.0
	pg $follicle^{-1}$ [b]		
ATD	10 ± 1	27 ± 1	79 ± 3
OHA	<3[c]	10 ± 1	29 ± 0
miconazole	<3	<3	<3
aminoglutethimide	<3	<3	<3
fadrozole	<3	<3	<3
	pg mL^{-1}		
ATD	50 ± 1	136 ± 2	394 ± 6
OHA	22 ± 1	51 ± 1	144 ± 1
miconazole	<15	<15	<15
aminoglutethimide	<15	<15	<15
fadrozole	<15	<15	<15

[a] see text for abbreviations

[b] for incubations of 5 follicles and 1 mL medium per well

[c] at or below assay detection limit of 3 pg $follicle^{-1}$ or 15 pg mL^{-1} medium

3.2.v Steroid measurement

T and E_2 were measured by RIA, based on the methods of Pankhurst (1997), using reagents detailed in Pankhurst and Conroy (1987). Aliquots of unextracted incubation medium (100 μL) (Experiments 1, 2, and 3) or resuspended medium and tissue extract (200 μL) (Experiments 4 and 5) were added to duplicate polypropylene tubes. ^{3}H-labelled steroid (200 μL) and 200 μL antibody were added, tubes covered and incubated overnight. Unbound label removed by the addition of 200 μL ice cold dextran coated charcoal, and centrifugation 4 °C for 10 min at 3000 rpm on a Beckman CS-6R centrifuge (Beckman Coulter Inc. Fullerton, USA). The supernatant was decanted to 5 mL plastic Mini Ploy-Q scintillation vials, 4 mL Ecolite scintillation medium (ICN Biomedical, Costa Mesa, USA) added, and activity measured in a Beckman LS 6500 scintillation counter. Assay detection limits were 30 pg mL^{-1} incubation medium (3 or 6 pg $follicle^{-1}$) in Experiments 1, 2 and 3, and 15 pg mL^{-1} incubation medium in Experiments 4 and 5. Interassay variability was measured using aliquots of a pooled internal standard and was (%CV[n]) 14.8 [8] and 13.9 [12] for T and E_2 respectively. Cross reactivity of AI with the E_2 RIA, was determined by incubating CCS spiked with 0,1, 1.0 or 10.0 μg mL^{-1} AI at 10 °C for 18 h, and assaying for E_2 as above (Table 3.1).

3.2.vi Statistical Analyses

Factorial data were analysed by one-way ANOVA. Mean comparisons were made using a Tukeys honestly significant difference test. Comparisons of the effects of follicle number on *in vitro* steroidogenesis (Experiment 1) and the effects of incubation time on brain E_2 production (Experiment 5) were tested by t-tests. All analyses were performed using SPSS 10.1 for Windows. Heteroscedastic data were log transformed prior to analysis.

3.3 Results

ATD cross-reacted with the E_2 antibody at all levels used in Experiment 2, whereas OHA showed significant cross reaction at 1.0 and 10.0 μg mL^{-1}. No cross reactivity was detected with any of the nonsteroidal AI (Table 3.1). These values

represent the maximum possible contribution of the AI to the measured values, and do not account for losses due to metabolism of the AI or absorption by tissue. Due to high levels of cross reaction, data from incubations with ATD at 1.0 and 10.0 μg mL^{-1} and OHA at 10.0 μg mL^{-1} were excluded from analyses.

The fish used in Experiment 2 both had greater GSI and oocyte diameter than those used in Experiment 1 (Table 3.2). These values reflect more advanced vitellogenesis in the fish used in Experiment 2, conducted in April, compared to those used in Experiment 1, conducted in January.

Table 3.2 Fish whole weight, ovary weight, GSI and oocyte diameter for animals used in Experiments 1 and 2.

	Fish #	Whole weight (kg)	Ovary weight (g)	GSI (%)[a]	Oocyte diameter (mm)[b]
Experiment 1	Fish 1	6.95	123	1.8	1.90 ±0 .05
	Fish 2	8.00	652	8.1	3.34 ± 0.11
Experiment 2	Fish 1	7.18	1121	15.6	4.29 ± 0.07
	Fish 2	9.48	1580	16.7	4.89 ± 0.06

[a] $GSI\ (\%) = \frac{\text{gonad weight}}{\text{whole body weight}} \times 100$

[b] mean ± S.E., n=30

3.3.ii Experiment 1 – gonadal tissue incubation

Treatment of the follicles from Fish 1 with T at 10 and 100 ng mL^{-1} elevated E_2 levels above controls at all temperatures and incubation times, except 10 °C for 42 h, where only T at 100 ng mL^{-1} was effective (Figure 3.1). Similarly, T at 100 ng mL^{-1} stimulated higher E_2 production than T at 10 ng mL^{-1} at all times and temperatures except in the 10 °C, 42 h combination. 17P at 10 ng mL^{-1} was not effective at stimulating E_2 production any time or temperature, whereas 17P at 100 ng mL^{-1} resulted in increased E_2 at all times and temperatures except 10 °C for 6 h. Production of E_2 in response to 17P at 100 ng mL^{-1} was less than that following treatment with T at 100 ng mL^{-1} except at 5 °C for 42 h. E_2 production by follicles from Fish 2 was lower than for Fish 1, although in most instances, above the assay detection limit of 6 pg $follicle^{-1}$, and followed most trends

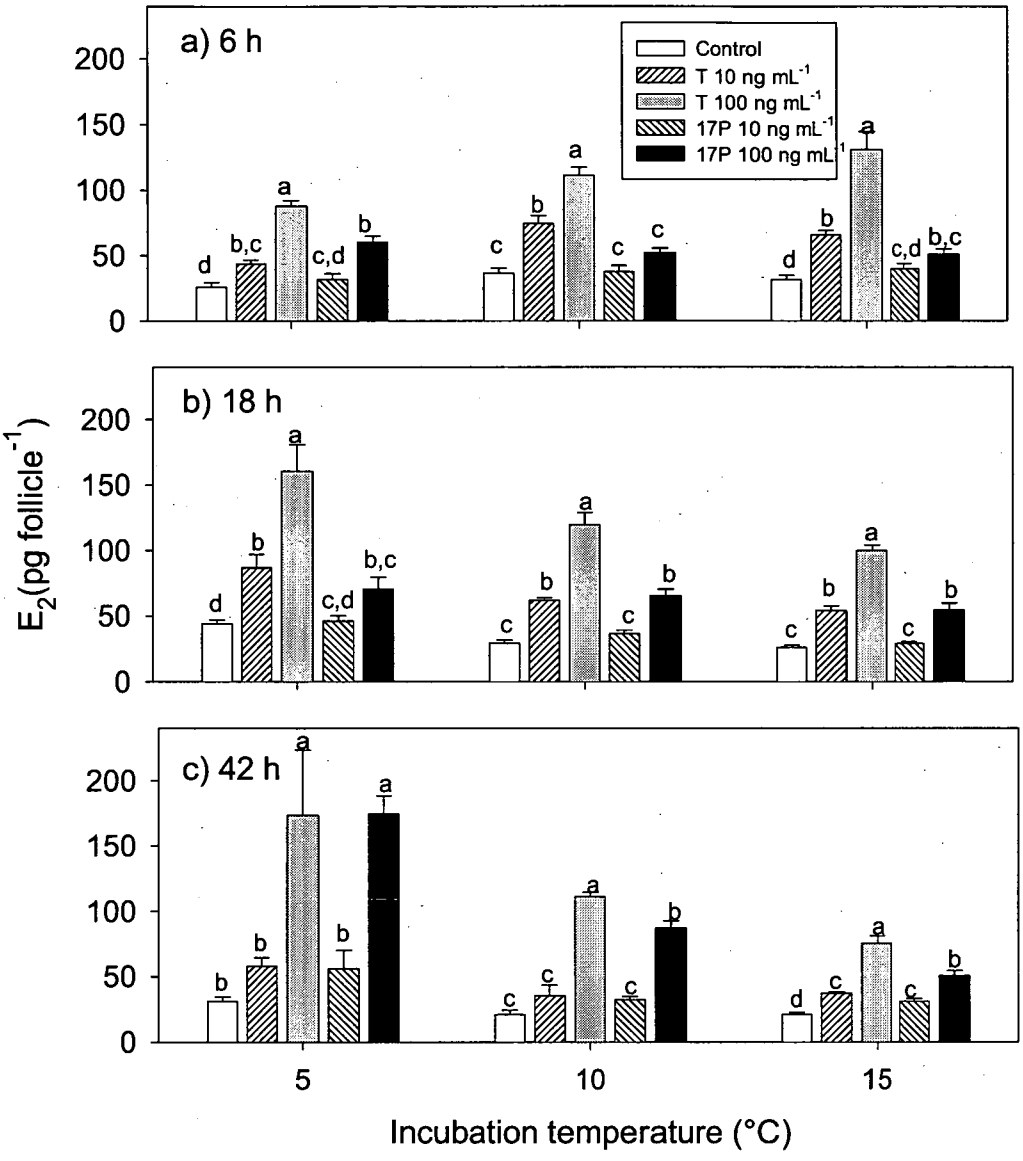


Figure 3.1 E_2 production by follicles from Fish 1, after a) 6 hours b) 18 hours or c) 42 hours incubation, at 5, 10 or 15 °C, with steroid precursors added as shown. Values are mean + SE (n=6) Within each time/temperature combination, values with the same superscript are not significantly different ($p > 0.05$).

observed in incubations of Fish 1 follicles (Figure 3.2). The addition of T at100 ng mL^{-1} increased E_2 production over control levels at all temperatures and incubation times, and above levels attained with the inclusion of T at 10 ng mL^{-1} in all temperature and times except 10 °C for 42 h. The addition of 17P at 100 ng mL^{-1} increased E_2 production over control levels for incubations at 10 °C for 6 or 18 h; at 5 °C for 18 h and at 15 °C for 18 or 42 h. There was no significant effect of 17P added at 10 ng mL^{-1}.

Addition of 17P at 100 ng mL^{-1} increased T production over controls for all times and temperatures for incubations of follicles from both Fish 1 and Fish 2 (Figures 3.3 and 3.4). The addition of 17P at 10 ng mL^{-1} increased T production over controls at 10 °C and 15 °C for 18 h, and at 5 °C and 15 °C for 42 h for Fish 1 (Figure 3.3), but only at 5 °C for 18 h for Fish 2 (Figure 3.4).

Follicular production of E_2 was higher in all treatments for incubations of 5 follicles per well than for 10 follicles per well (Figure 3.5) in both Fish 1 and 2. For incubations of 5 follicles per well, the addition of T at 10 or 100 ng mL^{-1} or 17P at 10 or 100ng mL^{-1} increased E_2 production over controls, for both Fish 1 and Fish 2. In contrast, only the addition of T at 10 or 100 ng mL^{-1} or 17P at 100ng mL^{-1} increased E_2 levels over controls for incubations of 10 follicles per well, for both Fish 1 and Fish 2 (Figure 3.5). T production was also higher for incubations of 5 follicles than 10 follicles when 17P was added at 10 or 100 ng mL^{-1} for Fish 1 and Fish 2 (Figure 3.6). Production of T was increased over controls with 17P at 10 or 100 ng mL^{-1} for both 5 and 10 follicles for Fish 1, but only with 17P at 100 ng mL^{-1} for Fish 2. To compare the degree of variability in E_2 production in incubations of five or ten follicles, the coefficient of variation (CV) was calculated, as a measure of variability, independent of mean values. This value was similar to, or greater, in incubations of five follicles, compared to incubations of ten follicles for Fish 1, but the converse applied for Fish 2 (Table 3.3).

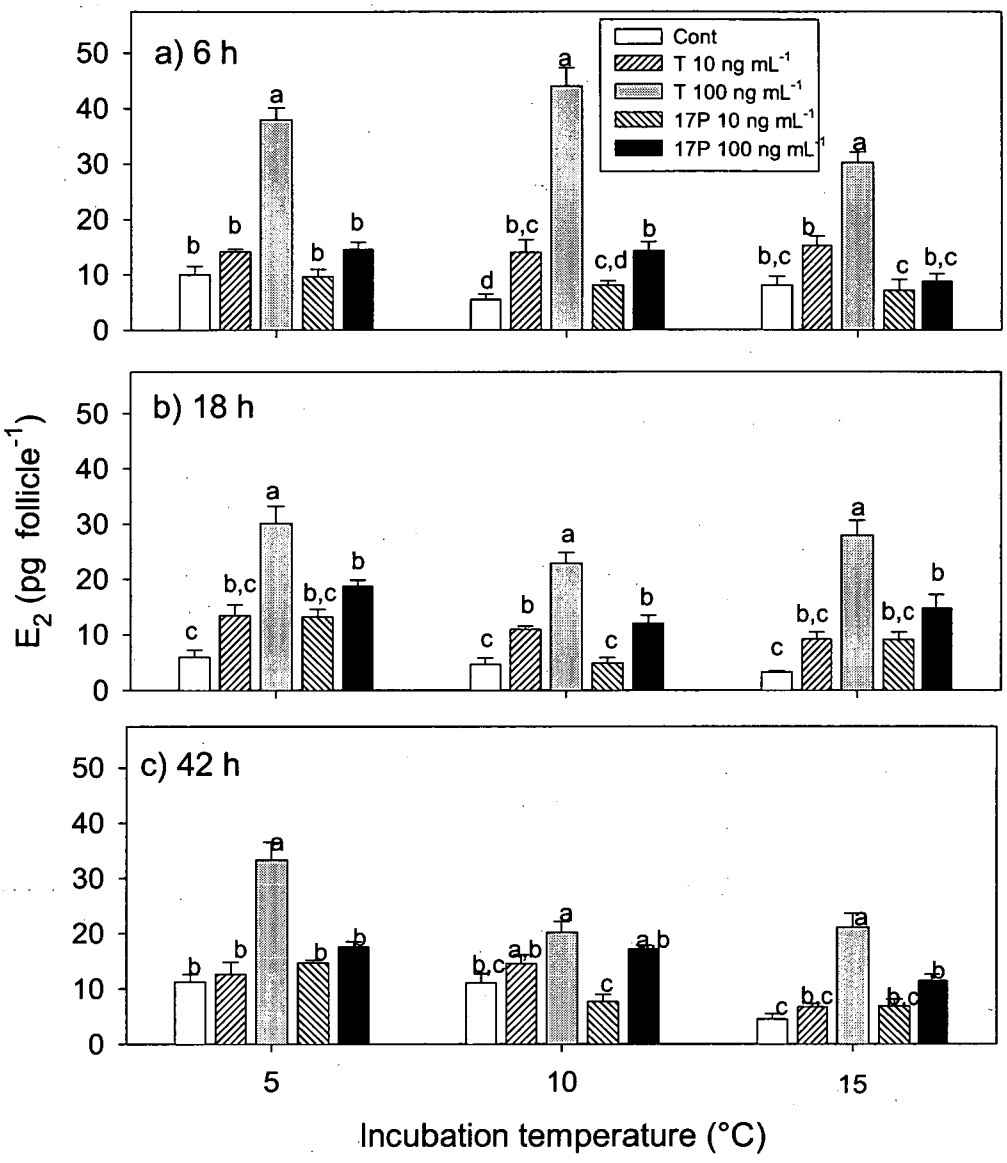


Figure 3.2 E_2 production by follicles from Fish 2, after a) 6 hours b) 18 hours or c) 42 hours incubation, at 5 °C, 10 °C or 15 °C. Other details as for Figure 3.1.

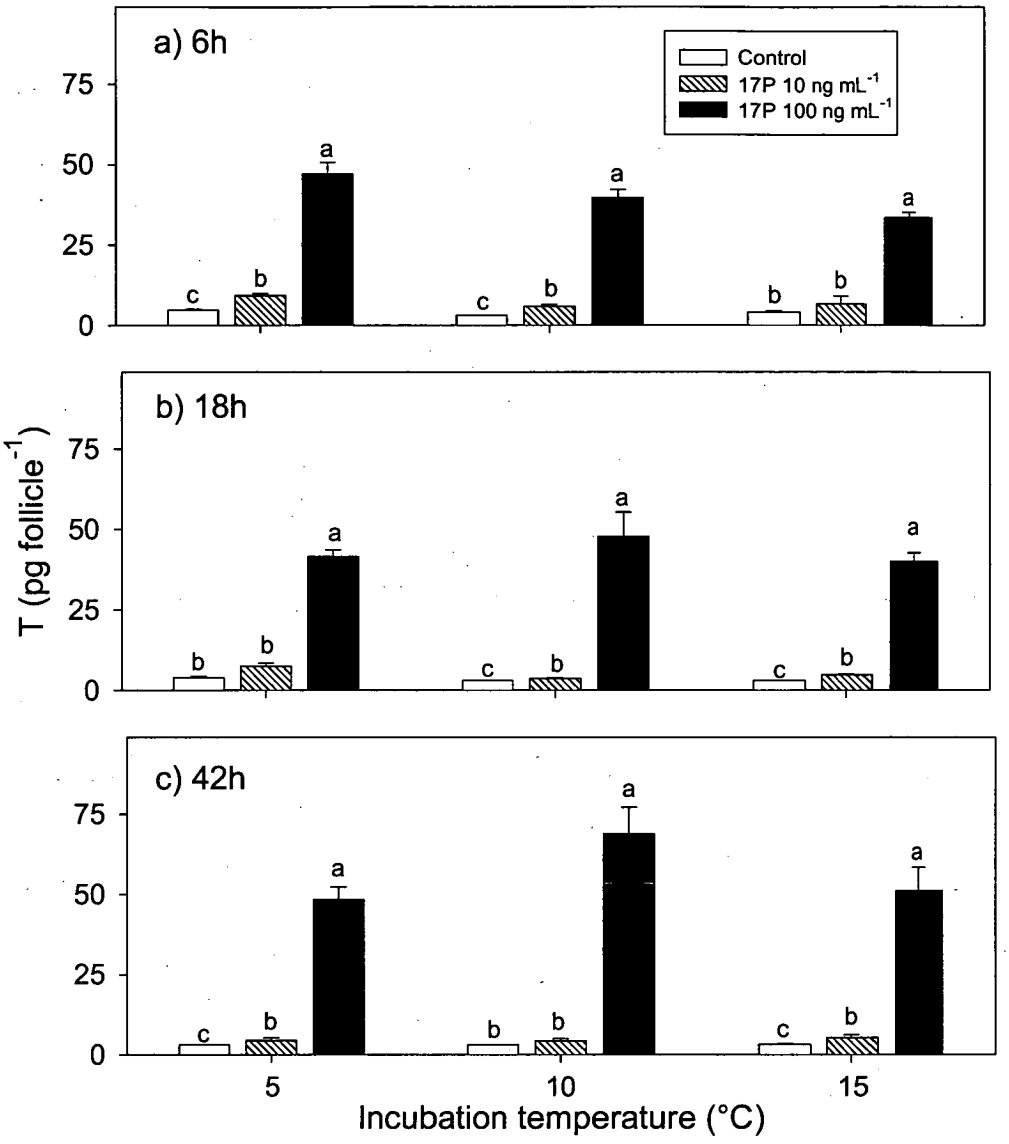


Figure 3.3 T production by follicles from Fish 1, after a) 6 hours b) 18 hours or c) 42 hours incubation, at 5 °C, 10 °C or 15 °C. Other details as for Figure 3.1.

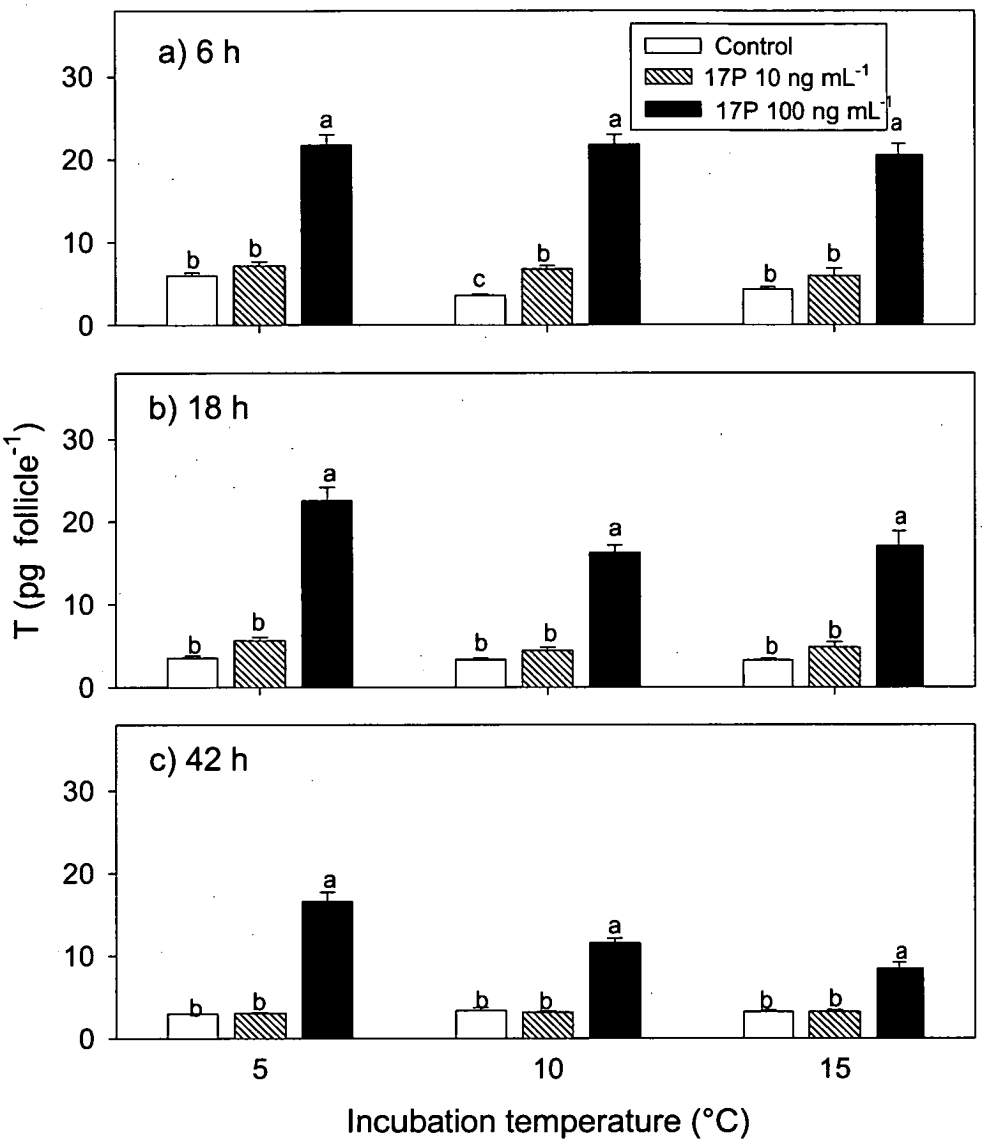


Figure 3.4 T production by follicles from Fish 2, after a) 6 hours b) 18 hours or c) 42 hours incubation, at 5 °C, 10 °C or 15 °C. Other details as for Figure 3.1.

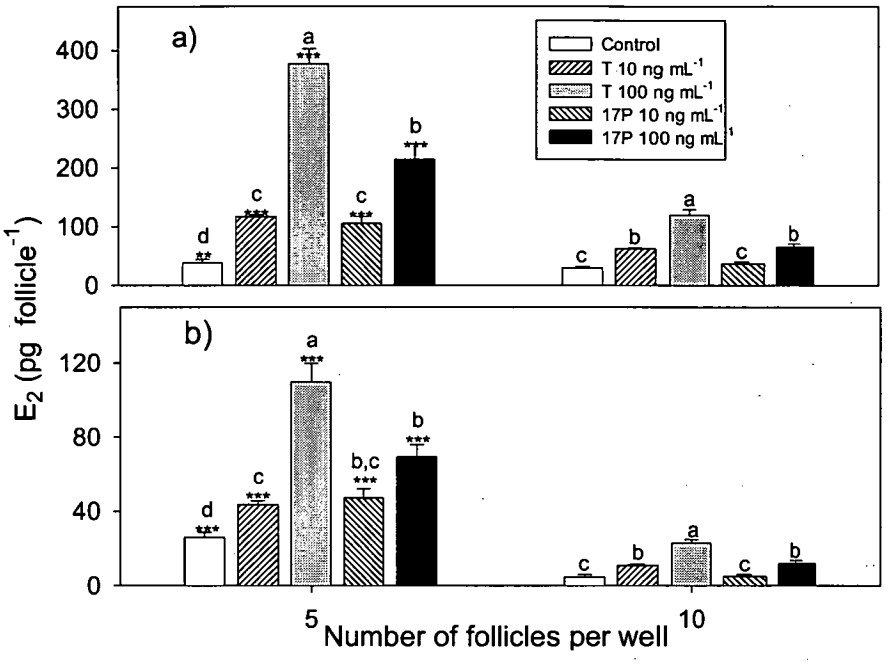
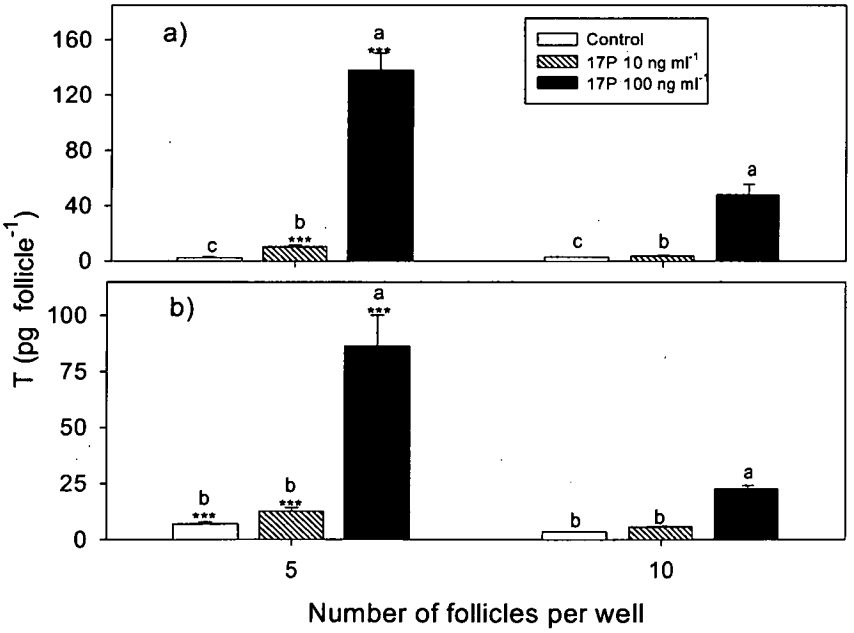


Figure 3.5 E_2 production by follicles from a) Fish 1 and b) Fish 2, after 18 hours incubation at 10 °C. Other details as for Figure 3.1. Within data for each fish, for each follicle number, values with the same superscript are not significantly different. Asterisks indicate a significant difference between values for 5 and 10 follicles, tested



separately for each steroid treatment (*** $p<0.001$; ** $p<0.01$)

Figure 3.6 T production by follicles from a) Fish 1 and b) Fish 2, after 18 hours incubation at 10 °C. Other details as for Figures 3.1 and 3.5.

Table 3.3 Coefficient of variation (CV)[a] of E_2 production following incubation at 10 °C for 18 h, of either five or ten follicles per well.

		follicles per well	
	Steroid treatment	**5**	**10**
Fish 1	Control	37.9	17.8
	T 10 ng mL^{-1}	7.3	7.6
	T 100 ng mL^{-1}	16.9	19.2
	17P 10 ng mL^{-1}	27.7	18.0
	17P 100 ng mL^{-1}	30.4	18.8
Fish 2	Control	25.9	60.5
	T 10 ng mL^{-1}	11.3	12.8
	T 100 ng mL^{-1}	22.7	20.4
	17P 10 ng mL^{-1}	25.6	49.9
	17P 100 ng mL^{-1}	23.3	28.6

[a] $CV\ (\%) = \frac{S.D.}{mean} \times 100$

Incubation at 10 °C for 18 h, allowed best discrimination between the different levels of T and 17P and on this basis, these assay conditions were considered as optimum. Increased E_2 production by incubations of five follicles over ten follicles, with no clear concomitant increase in variability resulted in the use of five follicles for subsequent AI efficacy testing.

3.3.iii Experiment 2 – Efficacy of AI on ovarian E_2 production

Incubations of follicles with fadrozole at 0.1 or 10.0 μg mL^{-1} or miconazole at 1.0 or 10.0 μg mL^{-1} significantly reduced basal E_2 production with respect to the control for Fish 1, and incubations with fadrozole at 0.1 or 10 μg mL^{-1} or ATD at 0.1 μg mL^{-1} decreased E_2 production for Fish 2 (Figure 3.7). In Fish 1, there was a significant increase in apparent E_2 levels when OHA was added at 1.0 μg mL^{-1}, however, this is likely to be an artifact of the cross reactivity between OHA and the E_2 RIA (Table 3.1).

For Fish 1, the addition of T at 100 ng mL^{-1} to the incubation medium, significantly increased E_2 production over control levels for Fish 1 (Figure 3.8), and the addition of fadrozole at 0.1, 1.0 and 10.0 μg mL^{-1}, OHA at 0.1 or 1.0 μg mL^{-1}or ATD at 0.1 μg mL^{-1} all significantly decreased E_2 production compared with incubation with T only. In contrast, for Fish 2, E_2 production was significantly increased over controls

in incubations with T added at 100 ng mL^{-1} aminoglutethimide added at 0.1 or 1.0 ng mL^{-1} with T at 100 ng mL^{-1}, but no other significant differences were apparent.

3.3.iv Experiment 3 - Brain tissue incubation

E_2 in the incubation medium was at or below detection levels of 30 pg mL^{-1} in all incubations without added T (Figure 3.9). Increased levels of E_2 were detected in treatments which received T at 100 ng mL^{-1}; however, this is likely to be a result of cross reaction of T with the E_2 RIA. Pankhurst and Riple (2000) determined a maximum E_2 equivalent of 70 pg mL^{-1} for an addition of 100 ng T mL^{-1} using the same RIA protocol followed in this study. In incubations of 20 mg tissue mL^{-1}, the addition of 17P significantly increased T production over the control; however, this effect was not observed for incubations of greater amounts of tissue (Figure 3.10). There was also a decline in T production, in response to 17P, with increasing amounts of tissue incubated (Figure 3.10). A similar trend was evident in free T levels in the medium where 100 ng T mL^{-1} was added (Table 3.4).

Table 3.4 T levels (mean ± SE [n=4]) in incubation media following incubation for 18 hours at 10 °C of 20, 50, 100 or 200 mg mL^{-1} brain tissue with 100 ng T mL^{-1} added.

Brain tissue (mg mL^{-1})	T (ng mL^{-1})
20	2.78 ± 0.08
50	2.00 ± 0.26
100	1.19 ± 0.07
200	0.34 ± 0.06

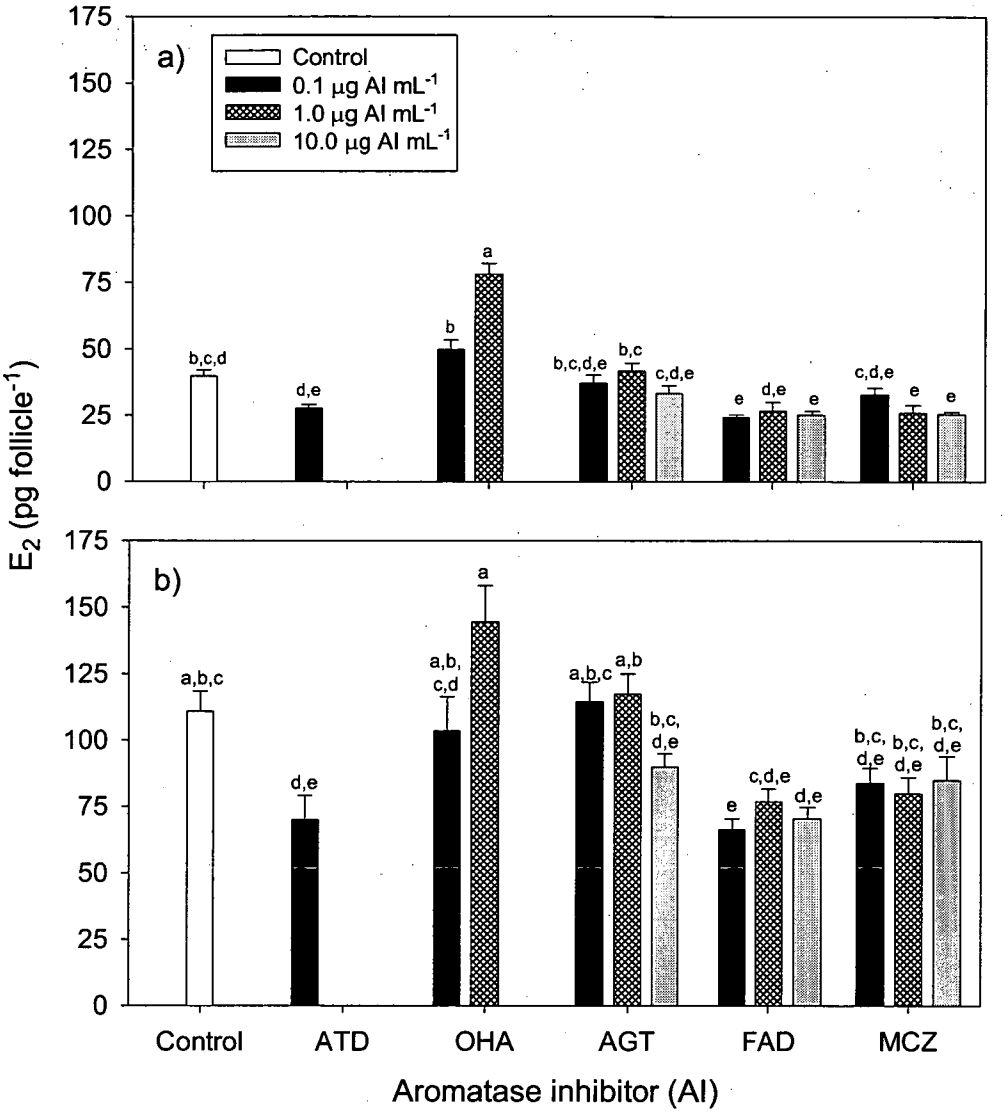


Figure 3.7 Basal E_2 production by follicles from a) Fish 1 and b) Fish 2, after 18 hours incubation at 10 °C with AI added as shown. Other details as for Figure 3.1.

* AGT – aminoglutethimide; FAD – fadrozole; MCZ – miconazole; ATD – 1,4,6–androstatriene-3,17-dione; OHA – 4-androstene-4-ol-3,17 -dione

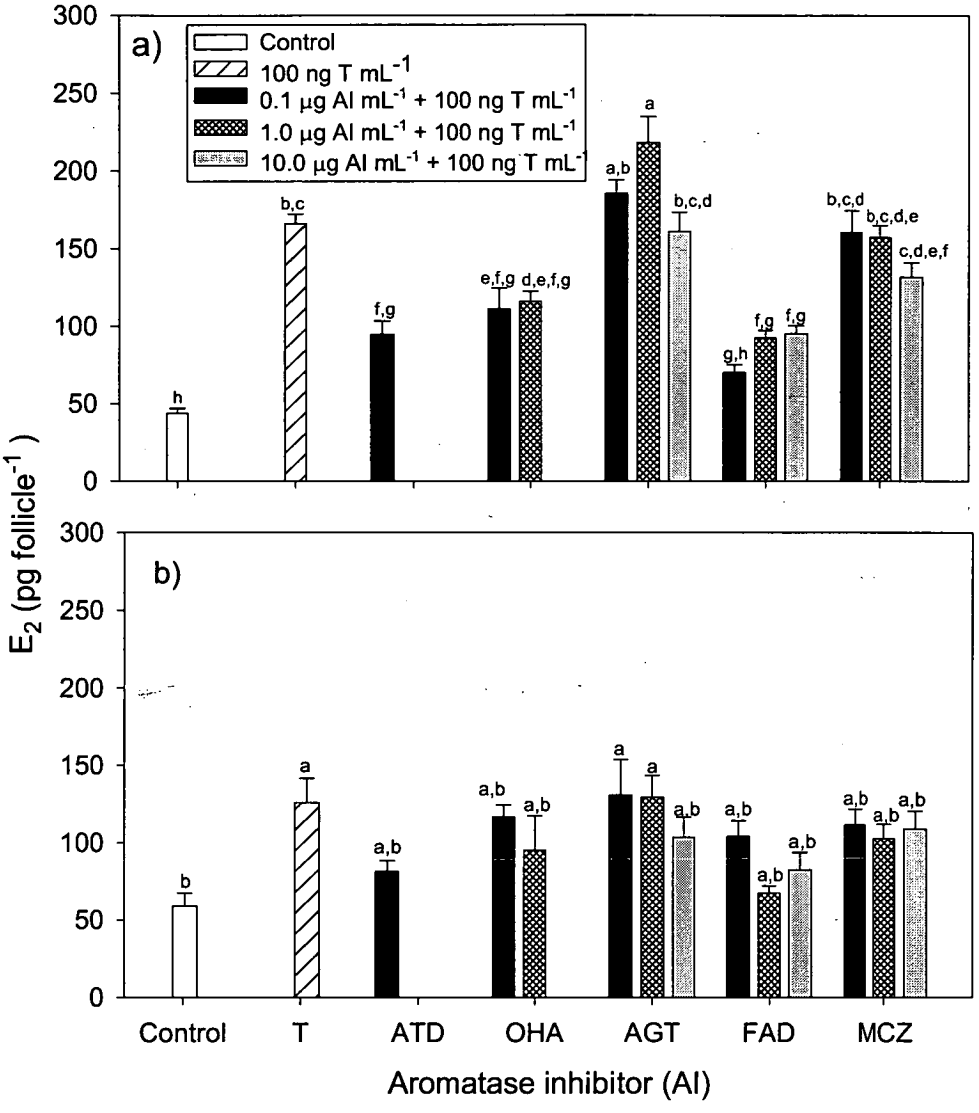


Figure 3.8 E_2 production by follicles from a) Fish 1 and b) Fish 2, after 18 hours incubation at 10 °C with T and AI added as shown. Other details as for Figures 3.1 and 3.7.

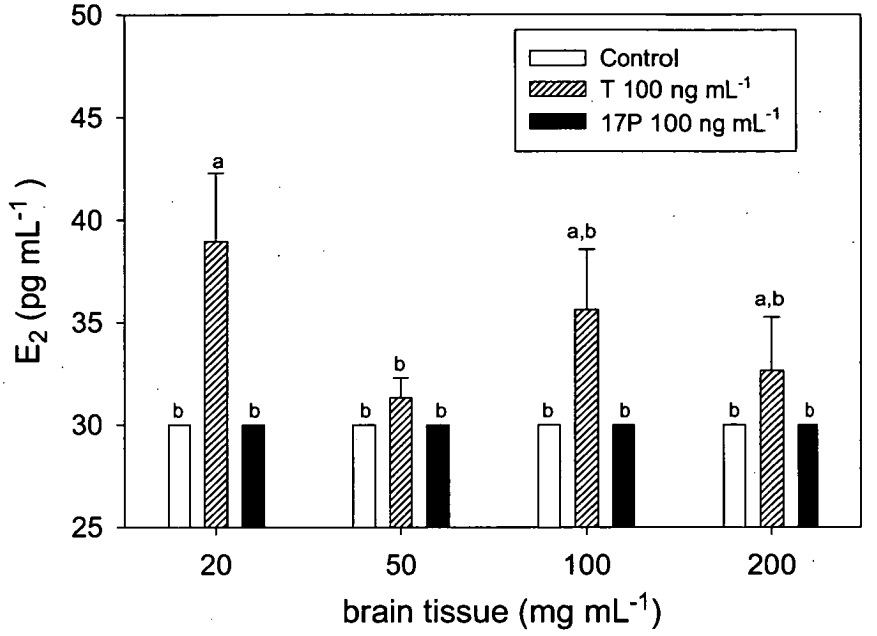


Figure 3.9 E_2 levels in incubation medium from 20, 50, 100 or 200 mg mL^{-1} brain tissue incubated for 18 hours at 10 °C. Other details as for Figure 3.1.

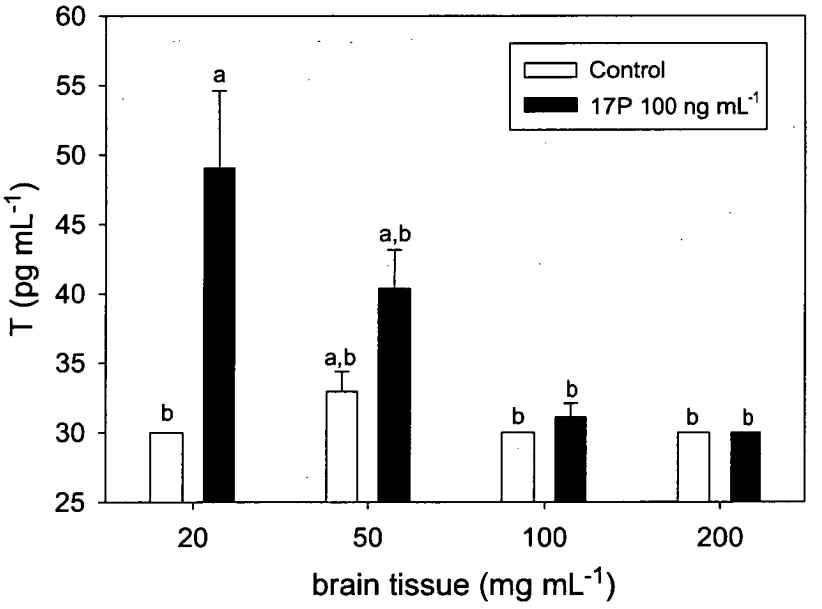


Figure 3.10 T levels in incubation medium from 20, 50, 100 or 200 mg mL^{-1} brain tissue incubated for 18 hours at 10 °C. Other details as for Figure 3.1.

3.3.v Experiment 4 - Solvent extraction of steroids

In incubations of 50 mg tissue, production of E_2 was significantly higher with the addition of 10 ng T mL^{-1} than for both control and 17P incubations (Figure 3.11). No significant differences were detected among any other treatments, and 50 mg was selected as a standard tissue inclusion rate for the subsequent experiment. Levels of T were higher than for E_2, but similarly variable (Figure 3.12). No significant effects of tissue level or presence of 17P were detected. High variability of results was due to extremely high (up to 10-fold increase) levels of the measured steroid determined for a single well within some treatment groups.

3.3.vi Experiment 5 – Efficacy of AI on E_2 production by brain tissue

Incubation of brain tissue homogenate with 10 ng T mL^{-1} increased E_2 production significantly over incubations with no added T, after both 18 and 42 hours incubation (Figure 3.13). With added T, E_2 production after 42 hours was significantly higher than at 18 hours.

With the exception of aminoglutethimide at 1.0 μg mL^{-1}, the addition of AI at all doses significantly decreased T-stimulated E_2 production (Figure 3.14). Fadrozole was most effective in reducing E_2 production, with addition at 1.0 μg mL^{-1} being more effective than 0.1 μg mL^{-1} . Miconazole was more effective at 10 μg mL^{-1} than at 1.0 μg mL^{-1}. There is no clear trend in differences in the efficacy of different doses of OHA and ATD, which may be an artifact of the cross-reaction between the steroidal aromatase inhibitors and the E_2 RIA (Table 3.1).

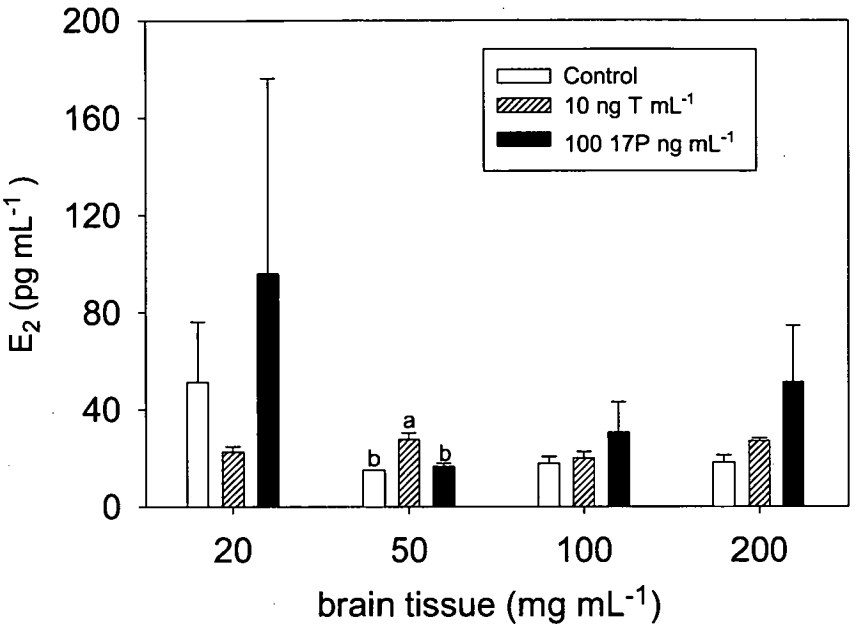


Figure 3.11 E_2 levels in ether-extracted homogenate of 20, 50, 100 or 200 mg mL^{-1} brain tissue incubated for 18 hours at 10 °C. Values are mean+SE (n=4) Other details as for Figure 3.1.

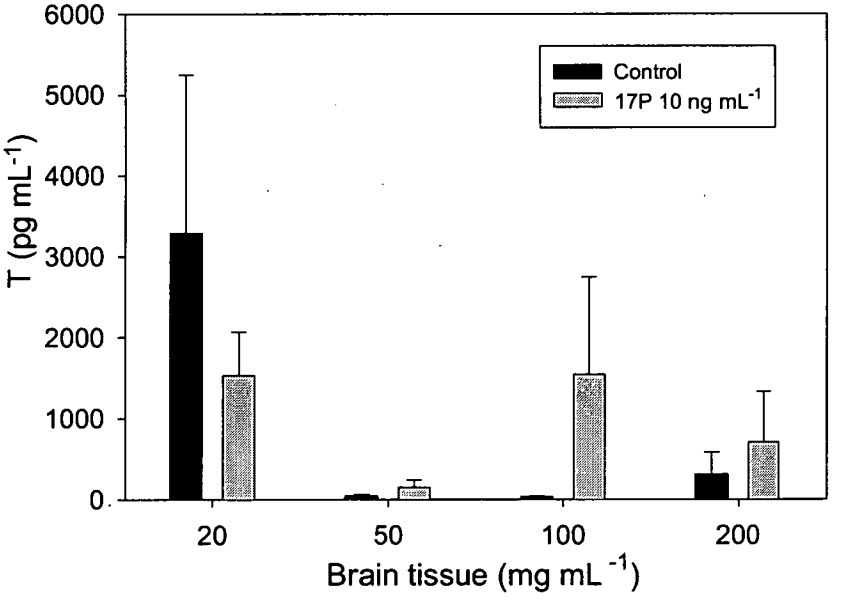


Figure 3.12 T levels in ether-extracted homogenate of 20, 50, 100 or 200 mg mL^{-1} brain tissue incubated for 18 hours at 10 °C. Values are mean+SE (n=4) Other details as for Figure 3.1.

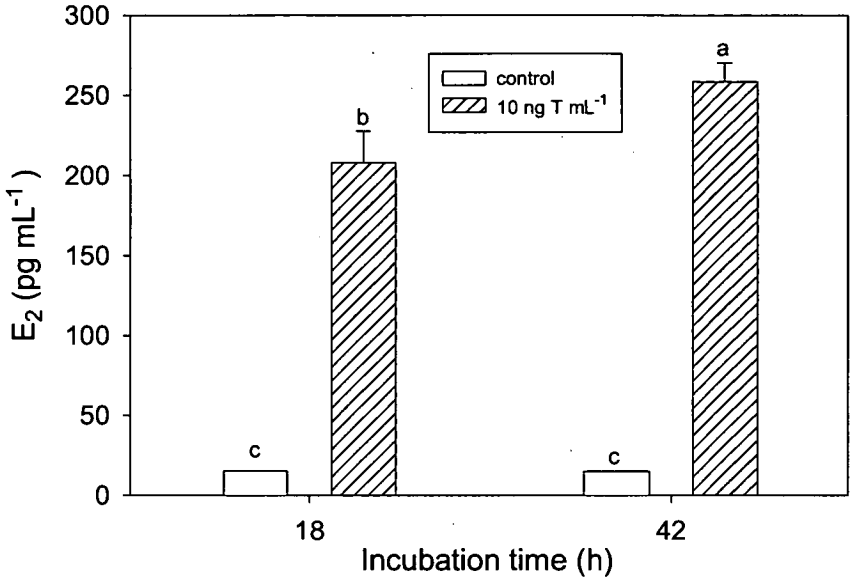


Figure 3.13 E_2 production by brain tissue homogenate following 18 or 42 h incubation with or without added T. Other details as for Figure 3.1.

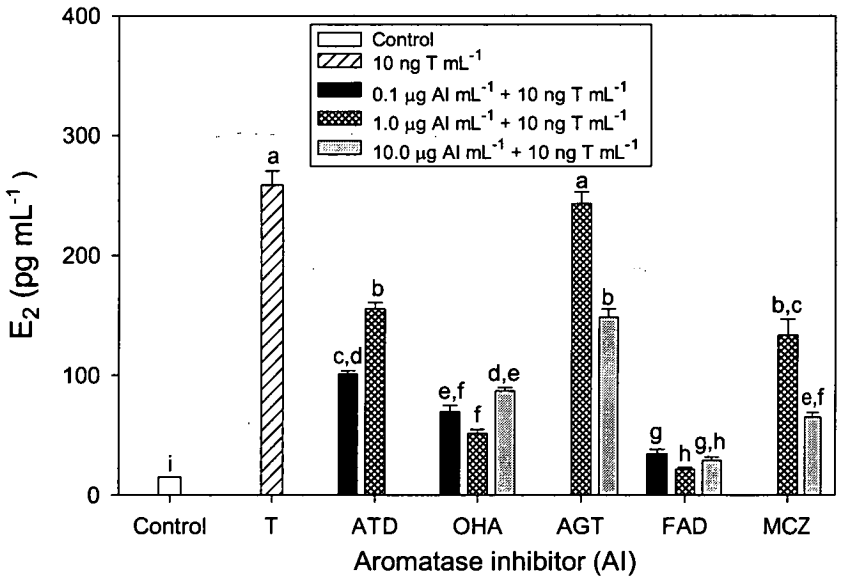


Figure 3.14 Effects of AI on E_2 production by brain tissue homogenate following 42 h incubation with added T. Other details as for Figure 3.1. and 3.7.

3.4 Discussion

Although trends in response to treatment were similar, there were up to 4-fold differences in basal steroid production between individuals in both Experiments 1 and 2. Pankhurst et al. (1996) reported similar variation in E_2 production in incubations of rainbow trout follicles, while individual variation in studies of the follicles of marine fish was considerably lower (Pankhurst, 1997; Pankhurst and Riple, 2000). The high level of variability amongst salmonids may be a function of individual variation in gonadal steroidogenic activity associated with the extended period of E_2 production in these species (Fitzpatrick et al., 1986; King and Pankhurst, 2003). Individual variability in circulating E_2 levels of female fish in the study population increases throughout vitellogenesis, reaching a maximum in March (King and Pankhurst, 2003). Follicles from individuals sampled in January (Experiment 1) and March (Experiment 2) may thus have had highly variable basal levels of steroidogenesis, reflected in the observed results. In conjunction with increased levels of variation between individuals is the seasonal trend of increasing follicle size and mean circulating E_2 levels from October to March (King and Pankhurst, 2003) The greater GSI and follicle diameter for samples used in Experiment 2 is consistent with more advanced stage of vitellogenesis of fish, compared to those in Experiment 1, and basal E_2 levels were generally higher in Experiment 2 than for comparable incubation conditions in Experiment 1. This agrees with *in vitro* studies of the follicles of Japanese eel, *Anguilla japonica* (Ijiri et al., 1995), rainbow trout, *Oncorhynchus mykiss* (Carragher and Sumpter, 1990), amago salmon, *Oncorhynchus rhodurus* (Kagawa et al., 1983), where increased vitellogenic state and follicle size were associated with increased levels of E_2 production. However, the comparison of basal E_2 and follicle size between individuals in Experiment 1 contrasts with these findings, for reasons that remain unclear. It should be noted however that maximum oocyte size does not always coincide with maximum aromatase activity or circulating E_2 levels (Sampath-Kumar et al., 2000) and that steroidogenic activity *in vitro* may not always represent the situation *in vivo,* where circulating steroids levels are the net result of secretion and metabolism and not steroidogenic activity of tissues *per se*.

Stimulation of E_2 and T production by follicles by the addition of T or 17P respectively was apparent after 6 h of incubation and this effect continued for up to 42 h. This is similar to the findings of Pankhurst and Riple (2000) and Haddy and Pankhurst (1998) in incubations of follicles of greenback flounder, *Rhombosolea tapirina* and rainbow trout respectively, and confirms that follicular enzyme systems were functional under the range of incubation periods tested. Conversion of 17P by follicle preparations confirmed activity of 17-hydroxysteroid-dehydrogenase and 17α-hydroxylase (Lemmen et al., 2002), but follicular E_2 production in response to T generally exceeded the response to equivalent doses of 17P. The capacity of follicles to more efficiently utilize T as a substrate for E_2 production than 17P has been ascribed to the greater number of reaction steps necessary for the conversion of 17P to E_2 and the greater possible range of products from 17P conversion (Haddy and Pankhurst, 1998). There was also a clear dose response to the added steroid substrates in the present study, consistent with the findings by Afonso et al. (1997) for coho salmon, *Oncorhynchus. kisutch*, and suggesting that substrate may be a limiting factor for *in vitro* steroid production by follicles.

Discrimination of the effects of treatment on E_2 levels was generally poorer after 42 h incubation, due to increased variability between replicates and reduced absolute E_2 levels in the incubation medium. These results are consistent with the findings of Haddy and Pankhurst (1998) and Pankhurst and Riple (2000). Haddy and Pankhurst (1998) showed that decreased levels of free steroids after 18 h static incubation of rainbow trout follicles was concomitant with increased production of glucuronides and uptake of steroids from the medium by follicles. Discrimination of treatment effects in the present study was less clear after 18 h incubation at 5 °C than at either 10 or 15 °C, with only high doses of steroids consistently eliciting a significant response. Increased incubation temperature, within limits, increases aromatase activity (Zhao et al., 2001; González and Piferrer, 2002) and the differences observed in the present study may reflect this. After 6 h incubation, E_2 production consistently increased with the addition of T at 100 ng mL^{-1} whereas the effects of low doses of T and 17P were equivocal. Doses of 100 ng mL^{-1} T or 17P were effective in increasing E_2 production during incubation of greenback flounder follicles for 6 h (Pankhurst and Riple, 2000) and

rainbow trout follicles for 3 h (Haddy and Pankhurst, 1998). Discrimination of differences in T production by follicles in the present study was less dependent on incubation conditions, as the effect of 10 ng 17P mL^{-1} was marginal, whereas 100 ng 17P mL^{-1} was always effective in increasing T. A similar trend in the effects of dose of 17P was evident in the incubation of greenback flounder follicles (Pankhurst and Riple, 2000) and reflects the capacity of follicular enzyme systems to deal with high levels of 17P.

Despite a higher degree of variability between replicates, there were significantly higher levels of both E_2 and T production by incubations of five follicles than incubation of ten follicles in the present study. This effect was consistent for both individuals tested, despite differences in follicle size. Factors in the medium or access to the medium may be limiting to follicle steroidogenesis for larger numbers of follicles. In a seasonal study, Kagawa et al. (1983) removed yolk from large follicles prior to incubation, to accommodate the required number of follicles in the assay, with no evidence of diminished steroidogenic activity. As steroid synthesis by follicles occurs in the thecal and granulosa layers (Kagawa et al., 1982), it is likely to be a function of surface area, unlike metabolic requirements that vary with volume, thus metabolic requirements may limit steroidogenesis over extended incubation for incubations of ten follicles. In addition, Haddy and Pankhurst (1998) showed significant uptake of steroids by follicles *in vitro* and a similar result was obtained by Piferrer and Donaldson (1994) for eggs and newly hatched coho salmon alevins. Thus follicles represent an effective steroid sink, and the reduction of steroids in the medium in incubations of 10 follicles may be due to this process.

The use of pooled brain tissue in the present study prevented comparison of brain steroidogenic activity between individuals; however, considerable variability between replicate wells was apparent in Experiment 4. Several studies have shown aromatase activity to be anatomically localized to the preoptic regions and the telencephalon in the teleost brain (Callard et al., 1981; Pasmanik and Callard, 1985; Andersson et al., 1988; Gelinas et al., 1998; Callard et al., 2001; Melo and Ramsdell, 2001). Thus incomplete removal, or poor homogenisation of the forebrain in the present study, could explain the heterogeneity of enzyme activity in the homogenate. E_2 production by brain tissue responded to T in the incubation

medium, with the effect continuing for up to 42 h of incubation. However, no comparison can be drawn with other studies as the ^{3}H-androstenedione determination method used by other workers (Andersson et al., 1988; Antonolopou et al., 1995; Zhao et al., 2001) excludes the capacity to measure aromatization levels in the absence of additional substrate.

The results of increasing E_2 synthesis by brain homogenate after 42 h incubation contrasts with the decline in E_2 synthesis with extended incubation of follicles; however, such differences are likely to be a result of the extraction of steroids from tissue in addition to the incubation medium. There was no evidence of increased levels of E_2 production with incubation of increasing amounts of brain tissue, and yields of T declined with increasing mass of tissue incubated, after 18 h incubation, most likely as a result of the uptake of steroids from the medium by tissue. In contrast, González and Piferrer (2002) showed an increase in weight-corrected aromatase activity with increasing levels of homogenised tissue; however, this occurred over short (30 minute) incubation, and was less apparent beyond this. Under similar incubation conditions to the present study, Haddy and Pankhurst (1998), showed uptake of steroids by follicles after 18 h incubation, although this occurred to a lesser extent than in the present study. Such differences may reflect differential capacity of the brain and follicular tissue to act as steroid sinks. Other studies of aromatase activity of brain tissue using ^{3}H-androstenedione (e.g. Callard et al., 1978; Pasmanik and Callard, 1985; Andersson et al., 1988; Zhao et al., 2001; González and Piferrer, 2002) routinely extract steroids from the homogenate.

Levels of E_2 production achieved in Experiment 2 and 5 with gonadal and brain tissue respectively incubated with T, equate to approximately 0.5-0.8 fmol mg^{-1} hr^{-1}. These estimates are orders of magnitude lower than levels recorded for other teleost species, determined using the ^{3}H-androstenedione method (Pasmanik and Callard, 1985; Andersson et al., 1988; Melo and Ramsdell, 2001). This may reflect reduced sensitivity of the methods used in the current study, due to the reliance on measuring the synthesis end product, which may be influenced by factors such as reduced rates of steroid synthesis or increased steroid uptake by follicles, during extended static incubation (Haddy and Pankhurst, 1998); and the use of T as a substrate, which has been shown to be a poorer substrate than

androstenedione for teleost aromatase (Zhao et al., 2001). There was no evidence of greater aromatase activity in brain than gonadal tissue in the present study in contrast to results reported in other studies (Tchoudakova and Callard, 1998; Zhao et al., 2001; González and Piferrer, 2002), although the extended incubation times used in the present study are likely to confound any differences in enzyme kinetics. In addition, the use of large follicles will reduce the synthesis rate, per unit weight, in comparison to that of homogenates of immature ovaries due to the relatively lower amount of steroidogenically active follicular tissue.

AI reduced E_2 biosynthesis by both follicular and brain tissue in the present study although there were some differences in efficacy between tissue types. This may be due to the expression of different isoforms of aromatase in brain and gonad of Atlantic salmon, as occurs in goldfish (Tchoudakova and Callard, 1998) and zebrafish (Kishida and Callard, 2001) which may be differentially sensitive to AI (Zhao et al., 2001). Aminoglutethimide was ineffective in follicles, but significantly reduced E_2 production by brain homogenates. This contrasts to the findings of Zhao et al. (2001) who found no difference in aminoglutethimide efficacy on brain or ovarian homogenates, although efficacy in both was low. The results of the present study concur with those of Chardard and Dournon (1999) who found no effect of aminoglutethimide on aromatase activity of newt, *Pleurodeles waltl*, gonad, but contrast with Pelissero et al. (1996) who showed that aminoglutethimide reduced the aromatase activity of microsomes from rainbow trout ovarian homogenates. These differences may reflect the different systems used, with microsomal preparations reported to have higher aromatase activity than homogenates (González and Piferrer, 2002), and higher sensitivity to AI, than cellular preparations (Bhatnagar et al., 2001). Miconazole was effective at high doses ($\geq$ 1mg L^{-1}) on brain and follicular tissue, consistent with the findings of Chardard and Dournon (1999), who showed that immersion of developing newts in miconazole at 1 mg L^{-1} resulted in a significant decline in gonadal aromatase activity. However, in the present study, miconazole was ineffective in follicle preparations in the presence of added T. Miconazole is a competitive inhibitor (Séralini and Moslemi, 2001), and reduced efficacy in the presence of added T may reflect relatively low affinity for the aromatase enzyme. Fadrozole displayed the highest efficacy of all AI, over the concentrations tested,

in both follicular and brain tissue. This supports previous findings that fadrozole was an effective *in vitro* inhibitor of aromatase activity in coho salmon follicles (Afonso et al., 1997) when used at concentrations of 2.2 and 22 mg L^{-1}. Furthermore, treatment of developing newts with fadrozole by immersion (Chardard and Dournon, 1999) and of Atlantic salmon parr by implantation, (Antonolopou et al., 1995) resulted in reduced aromatase activity in gonadal and brain tissues respectively. Wozinak et al. (1992) also showed fadrozole to be a more potent inhibitor of brain aromatase than either ATD or OHA. Notwithstanding the potential for cross reaction of ATD and OHA with the E_2 assay to underestimate the extent of aromatase inhibition, both ATD and OHA were effective on gonadal and brain tissue. This supports previous work on these compounds on Atlantic salmon *in vivo* and *in vitro* by Antonolopou et al. (1995) who found brain aromatase activity was reduced by both ATD and OHA. Similarly, Zhao et al. (2001) and Moore et al. (2002) showed OHA to be a potent inhibitor of goldfish brain gonadal and mesenteric aromatase respectively. The relatively high efficacy of fadrozole, ATD and OHA is to be expected from these compounds as "second generation" aromatase inhibitors (Brodie et al., 1999; Séralini and Moslemi, 2001). Such compounds have been developed specifically for increased potency and specificity (Brodie et al., 1999; Séralini and Moslemi, 2001).

The results of the present study show that effective methods for the *in vitro* assessment of aromatase activity in both gonadal and brain tissues of Atlantic salmon are feasible. The methods provide an approach to enable relatively simple assessment of the efficacy of compounds affecting steroidogenesis in these tissues, and are suitable for use in standard laboratory or hatchery facilities. Use of these methods to screen several aromatase inhibitors has shown that fadrozole, ATD and OHA are potent inhibitors of *S. salar* gonadal and brain aromatase activity, while miconazole is less potent but still effective, and aminoglutethimide is effective on brain aromatase only. These data do not discount the possibility that Atlantic salmon may possess more than one functional form of aromatase.

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Chapter 4

Efficacy of aromatase inhibitors for sex inversion of farmed Atlantic salmon.

But love is blind and lovers cannot see
The pretty follies that themselves commit;
For if they could, Cupid himself would blush
To see me thus transformed to a boy.

William Shakespeare
The Merchant of Venice, Act II Scene 6

4. Efficacy of aromatase inhibitors for sex inversion of farmed Atlantic salmon.

4.1 Introduction

The commercial production of sex inverted females ("neomales") for the indirect production of all female stocks (Piferrer, 2001) has traditionally been achieved through the use of steroid hormones, and a substantial research effort has been directed to the optimisation of their use (Chapter 2 see also reviews by Pandian and Sheela, 1995; Baroiller et al., 1999; Piferrer, 2001; Devlin and Nagahama, 2002). Masculinisation of salmonids has been achieved following both chronic and acute (Piferrer, 2001) application of androgens, typically by dietary (Johnstone et al., 1978; Goetz et al., 1979; Solar et al., 1984) or immersion (Johnstone et al., 1978; Goetz et al., 1979; Baker et al., 1988; Piferrer and Donaldson, 1991; Piferrer et al., 1993) treatments respectively, and these methods are currently in routine use in commercial production systems. However, a shift in public attitudes and policy against the use of hormones for animal production, exemplified by a ban by the European Union in 2002 on the use of growth promoter hormones in animal production, may necessitate the development of alternative methods for sex inversion of farmed fish. One such avenue is the use of compounds that inhibit or otherwise modulate normal endocrine processes.

Compounds that interfere with steroid synthesis or steroid receptors have been used in a number of studies of teleost reproductive and developmental endocrinology, mainly as tools to elucidate the role of steroid hormones in sex differentiation (eg. Guiguen et al., 1999; Kitano et al., 2001; Kwon et al., 2002) and maturation (eg. Afonso et al., 1999; Antonolopou et al., 1999). Specifically, aromatase (cytochrome $P450_{arom}$) inhibitors (AI); compounds which inhibit the conversion of androgens to estrogens, have been used to investigate the key role of estrogens in teleost sex differentiation (Piferrer et al., 1994c; Nakamura et al., 1998; Guiguen et al., 1999; Kitano et al., 1999; Kwon et al., 2002; Lee et al., 2002) and consequently as a non-hormonal alternative for sex manipulation of a number of teleost species, including coho salmon, *Oncorhynchus kisutch*, (Piferrer et al., 1994c) rainbow trout, *O. mykiss* (Guiguen et al., 1999), Japanese

flounder, *Paralichthys olivaceus* (Kitano et al., 2000) and tilapia, *Oreochromis niloticus* (Guiguen et al., 1999). As is the case for steroid hormones, the administration of AI for sex inversion of fishes has been successfully achieved via the diet (Guiguen et al., 1999; Kitano et al., 2000) and by brief immersion (Piferrer et al., 1994c).

The development of an effective treatment regime using exogenous compounds is dependent on optimising the treatment variables of timing, dose and frequency (Piferrer, 2001). Non-steroidal, competitive AI are required in relatively high concentrations due to the low K_m of aromatase (Cole and Robinson, 1990) and their successful application via immersion for sex inversion, has been at concentrations of 0.3-10 mg L^{-1}(Piferrer et al., 1994c; Chardard and Dournon, 1999). In contrast, there are no data on effective doses for immersion treatment with mechanism-based steroidal aromatase inhibitors, which bind irreversibly to the enzyme; however, these compounds are known to be effective at concentrations several orders of magnitude lower than competitive inhibitors (Cole and Robinson, 1990; Séralini and Moslemi, 2001). Steroid hormones themselves are effective for the sex inversion of salmonids when applied by immersion at doses as low as 0.08 mg L^{-1} (Piferrer et al., 1993) and steroidal AI may be effective at similar concentrations. "First generation" (Séralini and Moslemi, 2001) AI, such as aminoglutethimide (3-[p-aminophenyl]-3-ethyl-piperidine-2,6-dione) have relatively low efficacy (Buzdar, 2000; Séralini and Moslemi, 2001) whereas the effectiveness of "second generation" compounds, such as fadrozole (4-[5,6,7,8-tetra-hydroimidazo-[1,5-a]-pyridin-5-yl benzonitrile HCl]) may be 500 times higher (Cole and Robinson, 1990; Brodie et al., 1999; Brueggemeier, 2001).

In numerous studies of the masculinisation of fishes using androgens, various abnormalities in gonadal development have been observed, which could compromise fertility and/or the use of masculinised individuals in commercial production. In some gonochorists, steroid hormone treatments have resulted in individuals with "intersex" gonads (Yamamoto, 1969), comprising significant proportions of both testicular and ovarian tissue, (eg. Piferrer et al., 1993; Blazquez et al., 1995). This is thought to arise from insufficient dosage or an inability of the gonad to respond to the exogenous hormones (Devlin and

Nagahama, 2002). The development of gonads with a predominance of connective tissue (eg. Johnstone et al., 1978; Goetz et al., 1979; van den Hurk and Slof, 1981; Solar et al., 1984; Piferrer et al., 1994b; Blazquez et al., 2001) is seen as a result of excessive steroid treatment and has been proposed to be due either to incompatibility between exogenous steroids and internal processes, or pathological effects of steroids on the developing gonad (Devlin and Nagahama, 2002). The production of neomales with blocked or otherwise malformed sperm ducts is most frequently observed following dietary androgen treatment (Chapter 2 Feist et al., 1995). The fertility of neomales may thus be compromised on a number of levels, although few studies have attempted to quantify this, possibly due, in part, to the time needed for individuals to reach maturity. Some studies have reported comparable fertility of sex inverted and normal males (Johnstone et al., 1979; Piferrer et al., 1994c), whereas others demonstrated reduced neomale fertility due to the need to manually extract milt (Geffen and Evans, 2000), or poor sperm production and quality (Kirankumar and Pandian, 2002).

Some non-steroidal AI, including aminoglutethimide and fadrozole, are also known to cause side effects in humans, due to their effects on the function of cytochrome P450 enzymes other than aromatase (Buzdar, 2000; Brueggemeier, 2001; Séralini and Moslemi, 2001) and some steroidal AI, including ATD (1,4,6-androstatriene-3,17-dione) and OHA (4-androstene-4-ol-3,17-dione), may cause side effects due to their androgenic properties (Séralini and Moslemi, 2001). In animal studies, miconazole (1-[2,4-dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole) a first generation non-steroidal AI, was toxic to larval newts when used at a concentration of 0.5 mg L^{-1} for several weeks (Chardard and Dournon, 1999) but no toxic side effects have been reported for the second generation AIs fadrozole, ATD and OHA, across a range of vertebrate taxa (Elbrecht and Smith, 1992; Piferrer et al., 1994c; Richard-Mercier et al., 1995; Wennstrom and Crews, 1995; Kitano et al., 2000). The development of a suitable administration protocol for AI should therefore not only consider efficacy, but toxic or other side effects, and adjust the dose and frequency of application accordingly.

Although several AI have been demonstrated to be effective on Atlantic salmon aromatase *in vitro* (Chapter 3), their effectiveness on Atlantic salmon alevins *in vivo* is not known. The present study was conducted to assess the *in vivo* efficacy of AI for Atlantic salmon, with the strategic aim of developing a commercially appropriate protocol for their use in the production of Atlantic salmon neomales. The study was conducted in 2 phases over consecutive years, with the initial phase designed to screen several AI for efficacy and toxicity, and the latter phase aimed at investigating the effects of dose and treatment timing on the efficacy of the most promising AI. All five AI previously shown to be effective on either brain or gonadal aromatase *in vitro* (Chapter 3) were investigated, although fadrozole was not available for the first phase of the study. Atlantic salmon alevins were treated with aromatase inhibitors by immersion, for reasons of both controlled dosage (Pandian and Sheela, 1995) and the increased likelihood of generating individuals with patent sperm ducts (Chapter 2 Feist et al., 1995). In the screening phase, non-steroidal AI were tested at a maximum dose of 12.5 mg L^{-1}, a dose based on the methods of Piferrer et al. (1994c), and the steroidal AI were tested at a maximum dose of 500 μg L^{-1}, based on the demonstrated effective dose for androgens (Chapter 2). Initially, single or repeated immersion treatments were conducted, at times shown to be effective for androgen treatment (Chapter 2). Based on the studies of Duston and Saunders (1992), an artificial photoperiod was applied to animals throughout the 10 months of each phase of the study, to induce elevated levels of precocious maturation, enabling the assessment of sperm quality and fertility of neomales. In order to better evaluate the commercial effectiveness of AI, production parameters, including growth and mortality, were also followed over each phase of the study.

4.2 Materials and Methods

4.2.i Stock and Husbandry

Experiments were conducted at Saltas' two hatcheries, at Wayatinah, Tasmania on the Derwent and Florentine Rivers (respectively "Wayatinah" and "Florentine"). Both hatcheries, utilise intensive recirculation systems for the production of fry (<0.5 g) and natural river waters for the production of parr and smolts. Experiments were conducted over two years (2000/01 and 2001/02) using

the progeny of 2+ year old Atlantic salmon broodstock. Neomales were used to provide milt in both experiments. These stocks had been masculinised by dietary treatment with 17α-methyldihydrotestosterone (MDHT) at 1 mg kg^{-1} feed, for 800 °C days (DD), based on daily water temperature and following methods similar to Solar et al. (1984).

Milt was collected from neomales by removal and maceration of the testes, dilution of the homogenate with milt extender solution (Appendix Ia), and refrigeration until use. Fertilisation was achieved by combining the eggs of 6 female fish, dividing this into 4 containers, and adding the milt from a single neomale, together with milt activator solution (Appendix Ib) to each container. Fertilised eggs from a total of approximately 24 female fish fertilised by up to 8 neomales were maintained in a single upwelling incubator, at 8°C. Eyed eggs were transferred to mesh sided boxes (110mm x 150mm x 30mm), with 500 individuals per box. In a first experiment, transfer occurred at the onset of hatching and in a second experiment, transfer was undertaken several days before the expected commencement of hatching. Boxes were maintained at 8.0 ± 0.1 °C in Heath vertical incubator trays (Maricource Inc., Tacoma, USA), in two separate recirculation systems, with a maximum of 6 boxes per tray, until the swim up stage. Immersion treatments were timed in relation to the day of median hatch, at approximately 450 DD post-fertilisation.

Fry were subsequently transferred to indoor 1 m^3 fibreglass tanks (Figure 4.1) and reared at 10-12 °C under a photoperiod of 8 hr L:16 hr D, from August until December. Tanks were supplied with water from the Florentine facility recirculation system. In September 2001, all tanks were treated with 200 ppm formalin to control an infection of the freshwater protozoan parasite *Costia* (=*Ichthyobodo*) sp. in the hatchery. All tanks experienced elevated mortality levels at the time of the infection and immediately post-treatment. In December, fish were transferred to 4 m^3 fibreglass tanks, located outdoors at Saltas' Wayatinah facility (Figure 4.2). Tanks received river water at natural temperatures, which ranged from 6 to 17 °C (Figure 4.3) and fish were subject to natural photoperiod until final sampling in June. The photoperiod regime used for experiments (Figure 4.4) was based on a model which had previously been shown

Figure 4.1 Arrangement of six $1m^3$ tanks used for first feeding of juvenile Atlantic salmon, indoors at Saltas' Florentine facility.

Figure 4.2 Four m^3 tanks used for ongrowing of Atlantic salmon parr, outdoors at Saltas' Wayatinah facility.

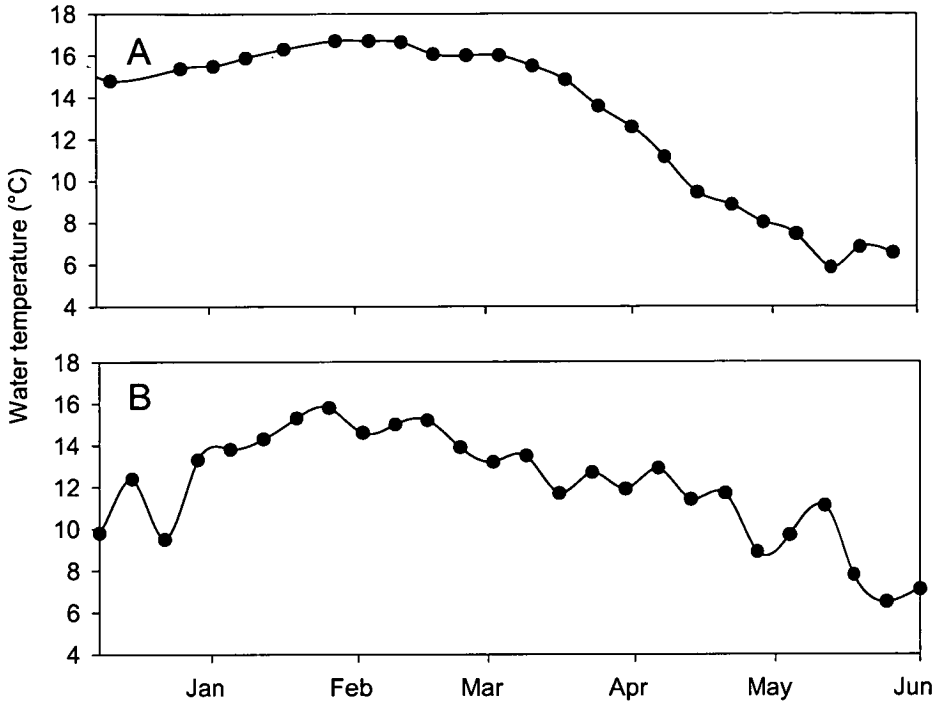

Figure 4.3 Mean weekly water temperature for a) December 2000 to June 2001 and b) December 2001 to June 2002 in outdoor tanks at Wayatinah.

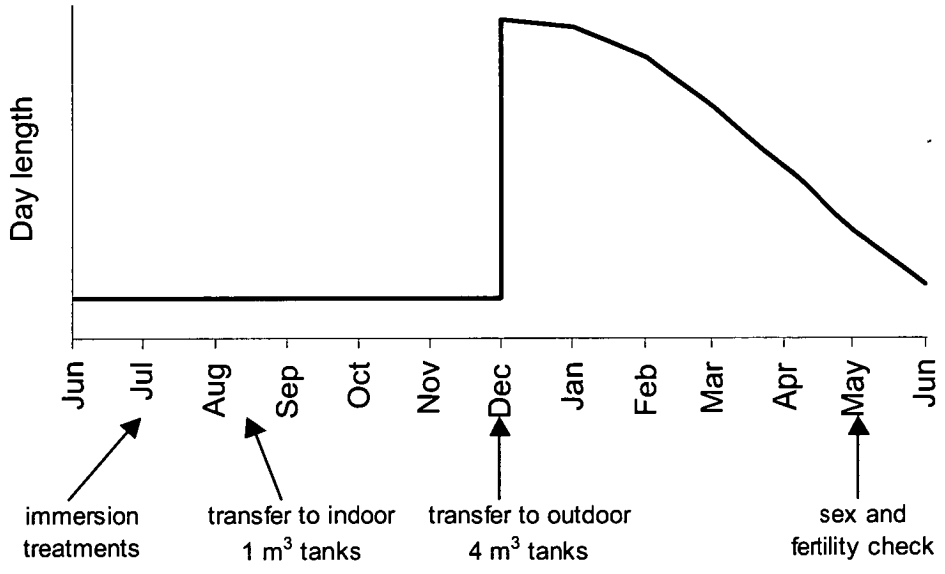

Figure 4.4 Manipulated photoperiod used in Experiments 1 and 2.

to relate photoperiod and growth to the incidence of male maturation in Atlantic salmon parr (Duston and Saunders, 1992)

4.2.ii Immersion protocol

Immersion treatments were undertaken in stainless steel trays (35 cm x 15 cm x 10 cm), containing 750 ml water, with treatments added as detailed below. Stock solutions of the AI and MDHT were prepared in 100% ethanol, and added to the immersion trays in a volume of 2 ml per tray, with control trays receiving only ethanol. Two mesh boxes, each with 500 alevins, were placed in each tray (Figure 4.5), left for 2 hours then returned to the Heath trays.

Figure 4.5 Atlantic salmon alevins undergoing immersion treatment in mesh boxes, in stainless steel containers.

4.2.iii Experiment 1: 2000-01

Four AI were used: ATD (Steraloids Inc.) ; OHA (Sigma); miconazole (Aldrich) and aminogluthetimide (Sigma). Maximum doses of 12.5 mg L^{-1} and 500 μg L^{-1} were respectively used for non-steroidal AI miconazole and aminoglutethimide and steroidal AI ATD and OHD. A 5-fold dilution series from the maximum dose was used in order to assess the extent of any toxic effects of the AI. Thus, ATD and OHA were used at concentrations of 4, 20, 100 and 500 μg L^{-1} , while

miconazole and aminoglutethimide OHA were used at concentrations of 0.1, 0.5, 2.5, 12.5 mg L^{-1} . In addition, MDHT (500 µg L^{-1}) was used as a positive control. For each level of AI or MDHT, groups of 1000 alevins were treated either once at 14 DMPH, or twice, at 14 and 28 DMPH.

4.2.iv Experiment 2: 2001-02

Two AI were used: ATD and fadrozole (Novartis), each at two doses. ATD and fadrozole were tested at doses that had previously been shown to be partially effective in sex reversal, in Experiment 1 and Piferrer et al. (1994c) respectively. Higher doses were also used to assess whether efficacy could be increased with increased dose. Thus ATD was used at doses of 0.5 and 5 mg L^{-1}, and fadrozole was used at doses of 10 and 50 mg L^{-1}. In addition, MDHT (500 µg L^{-1}) was used as a positive control. For each dose of AI or MDHT, groups of 1000 alevins were treated twice, either at 7 and 14 DMPH ("Early" immersion treatment), or 14 and 21 DMPH ("Late" immersion treatment). Immersion treatments were repeated at shorter intervals than in Experiment 1, as reduced interval time has been shown to increase steroid uptake in alevins (Piferrer and Donaldson, 1994). The timing of immersion treatments was made earlier than in Experiment 1 to further assess the effects of developmental stage on AI efficacy.

4.2.v Population assessment

Assessment of sex ratios in all experimental groups was conducted in June, at the completion of the following spawning season, 10 months after the treatment of alevins with AI. One hundred fish were sampled from each treatment group. Fish were euthenased in Aqui-S™ and examined externally for signs of maturity. Sperm duct patency was assessed by applying gentle abdominal pressure and checking for signs of expressed milt. A sample of 25 individuals (Experiment 1) or all individuals (Experiment 2) was weighed and standard length of each fish determined. Gonads from all individuals were dissected out and phenotypic sex was determined by visual observation of the gonad. Representative samples of testes and ovaries were retained from 5 individuals from each treatment group, fixed in Bouins fixative and processed for histological examination. Where phenotypic sex could not clearly be determined from visual examination of the

gonads, gonads were fixed in Bouins fixative and sex determined histologically. In the absence of any recognisable gonadal tissue, animals were recorded as sterile. Gonads from 25 individuals were weighed for determination of gonosomatic index (GSI), calculated as:

$$GSI\ (\%) = \frac{\text{gonad weight}}{\text{whole body weight}} X\ 100$$

4.2.vi Sperm quality

Where present, five mature males with patent sperm ducts were collected from each treatment group and milt was obtained from these by insertion of a polypropylene pipette tip into the vent and application of gentle abdominal pressure. Expressed milt was refrigerated in the tips until required. Sperm quality was assessed by applying a small amount of milt to a microscope slide, adding 10 µl of sperm activator and recording sperm activity (Table 4.1) every 15 seconds, until no activity was observed. Parameters used for comparisons of sperm activity between treatment groups were the proportion of motile sperm observed 30 sec after the addition of activator and the duration of sperm activity. Male fertility was estimated using groups of 100 eggs, collected from a single female that had ovulated 3-7 days previously. Eggs were placed in 120 ml plastic vials, 250 µl of milt and 50 ml sperm activator were added, the vials gently swirled, and allowed to stand for 2 minutes. After standing, eggs were rinsed twice with fresh water, incubated at 8 °C for 15 hours and embryos fixed in clearing solution (1:1:1 v/v methanol:acetic acid:water). Embryos were examined at 10 X magnification,

Table 4.1 Sperm motility criteria used in sperm activity assessment.

Score	Criteria
0	No motile sperm
1	Less than 25% sperm motile
2	25-50% sperm motile
3	50-75% sperm motile
4	Greater than 75% motile

fertility determined as the proportion of eggs that had undergone first cell division. Sperm quality and fertility of AI treated neomales were compared with that of precociously mature males of the same age from a mixed sex population.

4.2.vii Histological procedures

Tissue was fixed in Bouins fixative for 24-72 hours, then transferred to 70% ethanol. Standard histological techniques, similar to those outlined by Hinton (1990) were employed. Samples were dehydrated in an alcohol series and toluene, and embedded in paraffin in a Tissue-Tek VIP 4617, using the infiltration regime given in Appendix IIa. Seven micron sections were cut on a Leitz Wetzlar 1512 microtome fitted with disposable blades (Feather S35), and stained with Mayer's haematoxylin and Young's eosin, with Scott's tapwater substitute used as a blueing agent (Appendix IIb). Sections from gonads of some representative males (n=7) and females (n=5) from Experiment 2 were used for the determination of spermatocyte and ooctye size. The largest diameter of 20 spermatocytes or 10 ooctyes was measured for each individual using images captured with a Leica DC 300F videocamera at 100 and 400X magnification respectively, and measurements were made using Leica IM 50 image analysis software.

4.2.viii Statistical Analyses

Morphometric, mortality and fertility data were analysed by one-way ANOVA, and mean comparisons made using a Tukeys honestly significant difference (HSD) test. Comparisons of masculinisation rates were made by contingency Chi-squared tests between each treatment group and the appropriate control, but the differences in the frequency of intersex and sterile animals among groups was not tested as some of these individuals were only able to be detected in histological samples which were considered to be unrepresentative of the population. Sperm activity data were analysed by Mann-Whitney U-test for comparisons of activity 30 sec after activator addition, and by ANOVA for duration of activity. All analyses were performed using SPSS 10.1 for Windows. Percentage data were arcsine transformed prior to analysis.

4.3 Results

4.3.i Experiment 1: 2000-01

Although there were differences in mortality rates between groups of alevins during the period from treatment to first feeding (Table 4.2), elevated mortality showed no relationship with AI type or dose, and coincided with events of reduced water flow affecting one of the rearing systems. On this basis, only animals treated at the highest dose were retained for subsequent study. The differences in mortality following first feeding (Table 4.3) arose either because of difficulties in establishing feeding (control, double immersion, and aminoglutethimide, single immersion tanks), or reduced flow for extended periods (MDHT, single immersion and aminoglutethimide, double immersion tanks), both of which elevated mortality rates. Mortality data for each treatment were not collected following transfer to 4 m^3 tanks; however, total mortality in these tanks from transfer in December 2000 to final sampling in May 2001 was less than 1%.

Table 4.2 Percent mortality (mean ± range[a]) of Atlantic salmon alevins immersion treated in different doses of the aromatase inhibitors ATD, OHA, miconazole and aminoglutethimide.

Aromatase inhibitor	Immersion regime (DPMH)[a]	Dose (µg L^{-1})				
		0	**4**	**20**	**100**	**500**
Control	14	21.7±0.6	-	-	-	-
	14, 28	24.4±10.4	-	-	-	-
ATD	14	-	16.5±7.4	23.6±4.0	26.4±7.6	23.3±7.4
	14, 28	-	17.4±3.6	19.0±1.6	24.5±3.0	19.4±5.6
OHA	14	-	20.0±2.8	22.5±15.0	16.6±6.0	16.3±1.0
	14, 28	-	25.9±2.2	21.0±10.4	16.6±6.4	18.2±2.8
		Dose (mg L^{-1})				
		0	**0.1**	**0.5**	**2.5**	**12.5**
miconazole	14	-	15.4±3.2	18.9±3.4	19.3±10.6	17.6±6.4
	14, 28	-	22.8±0.4	60.2±79.6	17.6±2.0	21.3±1.4
aminoglutethimide	14	-	21.3±7.0	23.4±6.4	20.3±2.6	22.6±17.2
	14, 28	-	16.7±3.4	62.5±75.0	23.7±12.6	14.5±3.8

[a] data from duplicate rearing trays

Table 4.3 Production parameters for Atlantic salmon, immersion treated in either MDHT or the aromatase inhibitors ATD, OHA, miconazole or aminoglutethimide.

Treatment	Immersion regime (DPMH)[a]	First feeding mortality (%)[b,c]	Weight (g)[d,e] (mean±SE)	Length (mm)[d,e] (mean±SE)	CF[d,e,f] (mean±SE)	GSI (%)[d,e,g] (mean±SE)
Control	14	17.2	110.03 ± 7.36 [w,x]	198.47 ± 4.27 [x,y]	1.35 ± 0.01	0.11 ± 0.01
	14, 28	39.4	98.69 ± 6.74 [w,x]	194.23 ± 4.33 [x,y]	1.29 ± 0.01	0.31 ± 0.17
MDHT	14	98.6***	ND	ND	ND	ND
500µg L^{-1}	14, 28	14.8***	95.53 ± 11.09 [w,x,y]	186.04 ± 6.81 [x,y,z]	1.37 ± 0.02	3.19 ± 0.58
ATD	14	20.1	92.36 ± 6.48 [w,x,y]	187.46 ± 4.33 [x,y,z]	1.35 ± 0.02	0.14 ± 0.01
500µg L^{-1}	14, 28	28.7***	109.35 ± 6.93 [w,x]	200.23 ± 4.24 [x,y]	1.31 ± 0.02	0.22 ± 0.11
OHA	14	16.8	86.32 ± 6.02 [w,x,y,z]	187.13 ± 4.35 [x,y,z]	1.26 ± 0.01	0.14 ± 0.01
500µg L^{-1}	14, 28	16.3***	60.96 ± 3.18 [z]	168.87 ± 2.83 [z]	1.24 ± 0.01	0.16 ± 0.00
miconazole	14	14.1	67.71 ± 4.15 [y,z]	171.90 ± 3.53 [z]	1.29 ± 0.02	0.15 ± 0.01
10 mg L^{-1}	14, 28	21.7***	79.39 ± 6.17 [x,y,z]	183.31 ± 4.40 [y,z]	1.22 ± 0.01	0.15 ± 0.01
aminoglutethimide	14	32.3***	106.82 ± 7.08 [w,x]	201.13 ± 4.58 [x,y]	1.26 ± 0.01	0.12 ± 0.01
10 mg L^{-1}	14, 28	83.7***	112.80 ± 7.76 [w]	204.00 ± 4.59 [x]	1.28 ± 0.01	0.13 ± 0.01

[a] DPMH - days post-median hatch
[b] cumulative mortality from first feeding (September 2000) to December 2001
[c] Asterisks indicate difference in mortality rate from appropriate controls (* $0.01<p<0.05$; ** $0.001<p<0.01$; *** $0.001<p$)
[d] data collected in May 2001
[e] values sharing the same superscript are not significantly different ($p>0.05$)
[f] CF = 100 x weight (g) / (fork length (mm))^3 x 1000
[g] GSI (%) = gonad weight/ total weight x 100

Weight and fork length of OHA, double immersion treated and miconazole, single immersion treated fish were significantly lower than controls, and but not different to other fish treated with the same AI (Table 4.3). There were no significant differences in condition factor. GSI was significantly higher in the MHDT treated group, reflecting the increased frequency of mature males in that group (Figure 4.6).

There was a significantly higher proportion of males in fish treated with MDHT and ATD single and double immersion, than in control groups (Figure 4.6). Maturation levels among males were 75, 46 and 46% for MHDT and ATD single and double immersion treated fish respectively, and patent sperm ducts were observed in 86 , 83 and 67% of the mature fish in those same groups (Figure 4.7).

Females, mature and immature males displayed similar distributions of body weight, fork length and condition factor (Figure 4.8 a-c), whereas differences in GSI were apparent between mature and immature males (Figure 4.8 d). Due to the low number of male fish present in samples at 9 months post-hatch, statistical analyses were not performed on these data.

Histological examination of representative male and female gonads (n = 50) from all treatment groups agreed with the assignation of sex based on visual examination in 94% of cases. Most testes were comprised of spermatogonia arranged in clusters (Figure 4.9), however some specimens also contained secondary spermatocytes (Figure 4.10 a,c inset). Ovaries consisted of perinucleolar oocytes with surrounding somatic cells (Figure 4.11) and were structurally similar in control and AI treated populations. In those individuals where sex was unable to be determined visually (n=18), gonads from two individuals were ovotestes, with both gamete types mixed throughout the gonad (Figure 4.10 a,b) In another two cases the gonads were comprised primarily of spermatogonia, with clusters of small numbers of oocytes also present in addition to areas of sterile tissue ("mostly male", Figure 4.12a). The presence of small numbers of oocytes in a predominantly male gonad has been frequently observed in 2+ year old mature sex inverted male Atlantic salmon (P.S. Lee and H. R. King, unpublished observations). On this basis, and giving consideration to the likely

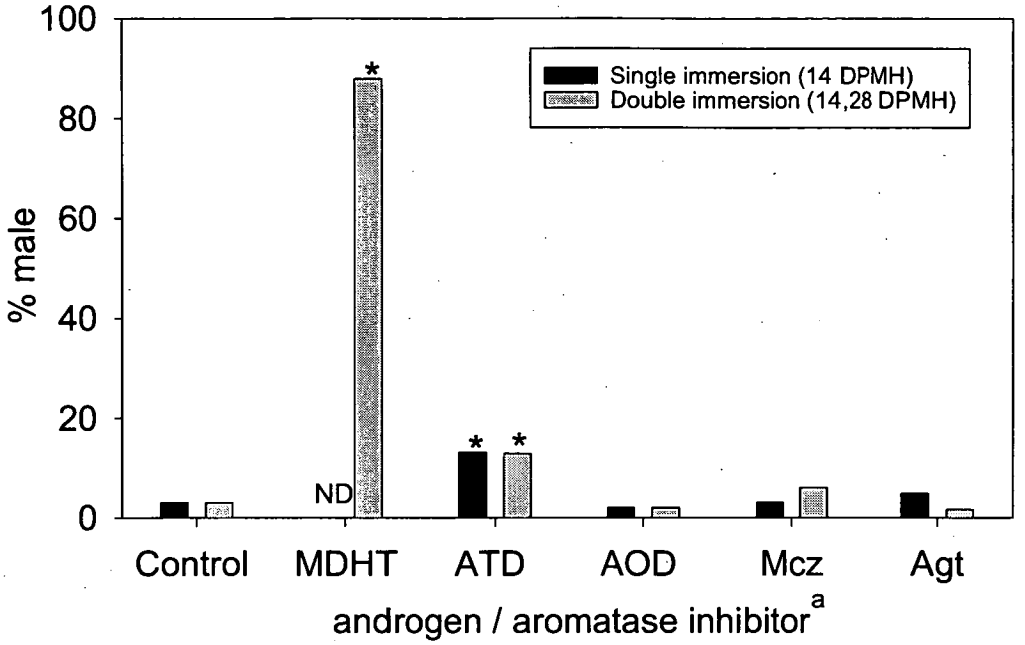

Figure 4.6 Percentage masculinisation following single or double immersion with MDHT or aromatase inhibitors. See text for abbreviations. Asterisks indicate significant difference in male frequency from control population. ND – no data available. [a] Mcz – miconazole; Agt – aminoglutethimide

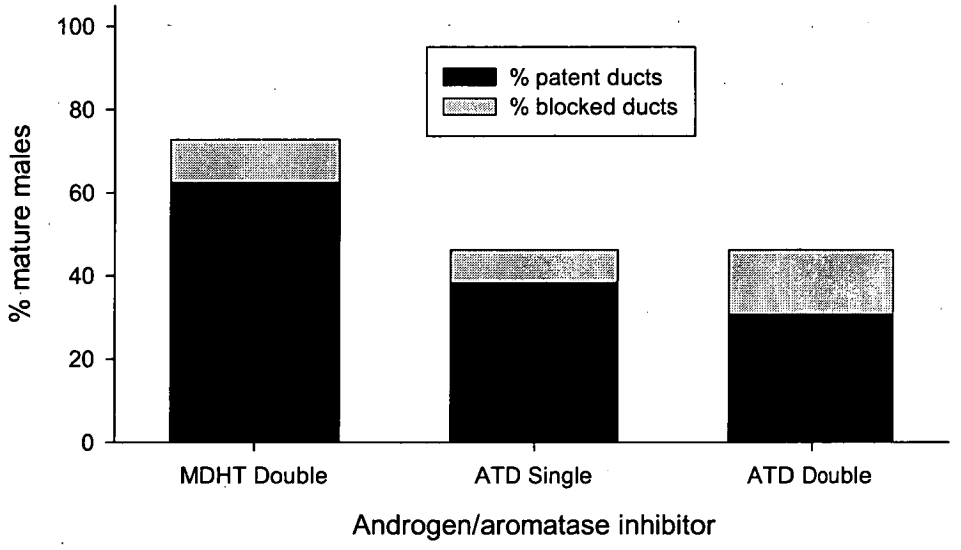

Figure 4.7 Proportions of sperm duct status of mature fish, expressed as a percentage of mature sex inverted male fish produced by immersion in either MDHT or ATD in Experiment 1. See text for abbreviations.

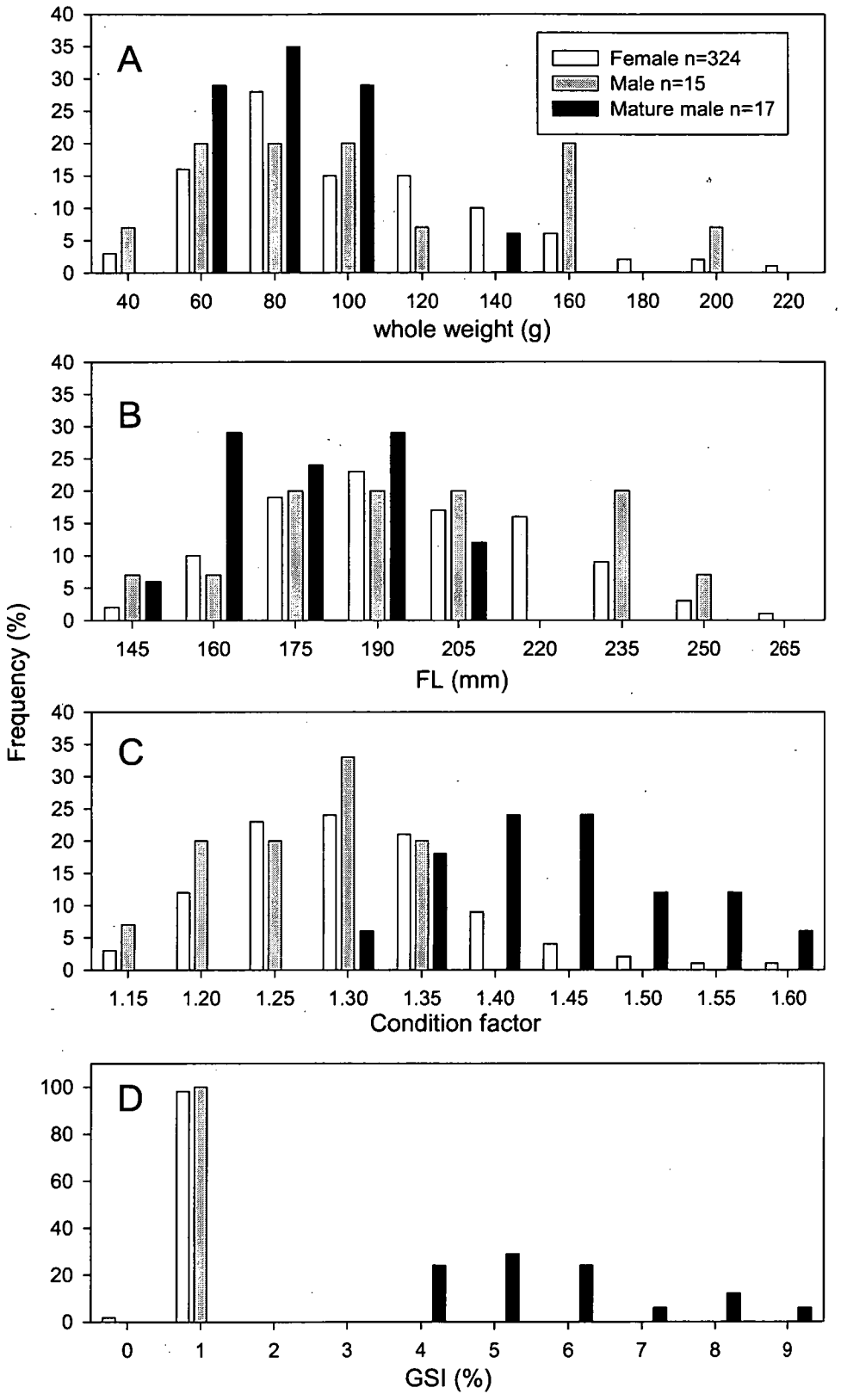

Figure 4.8 Percent frequency of pooled data (N = 356) of: a) whole weight; b) fork length (FL); c) condition factor and d) GSI, for fish from Experiment 1.

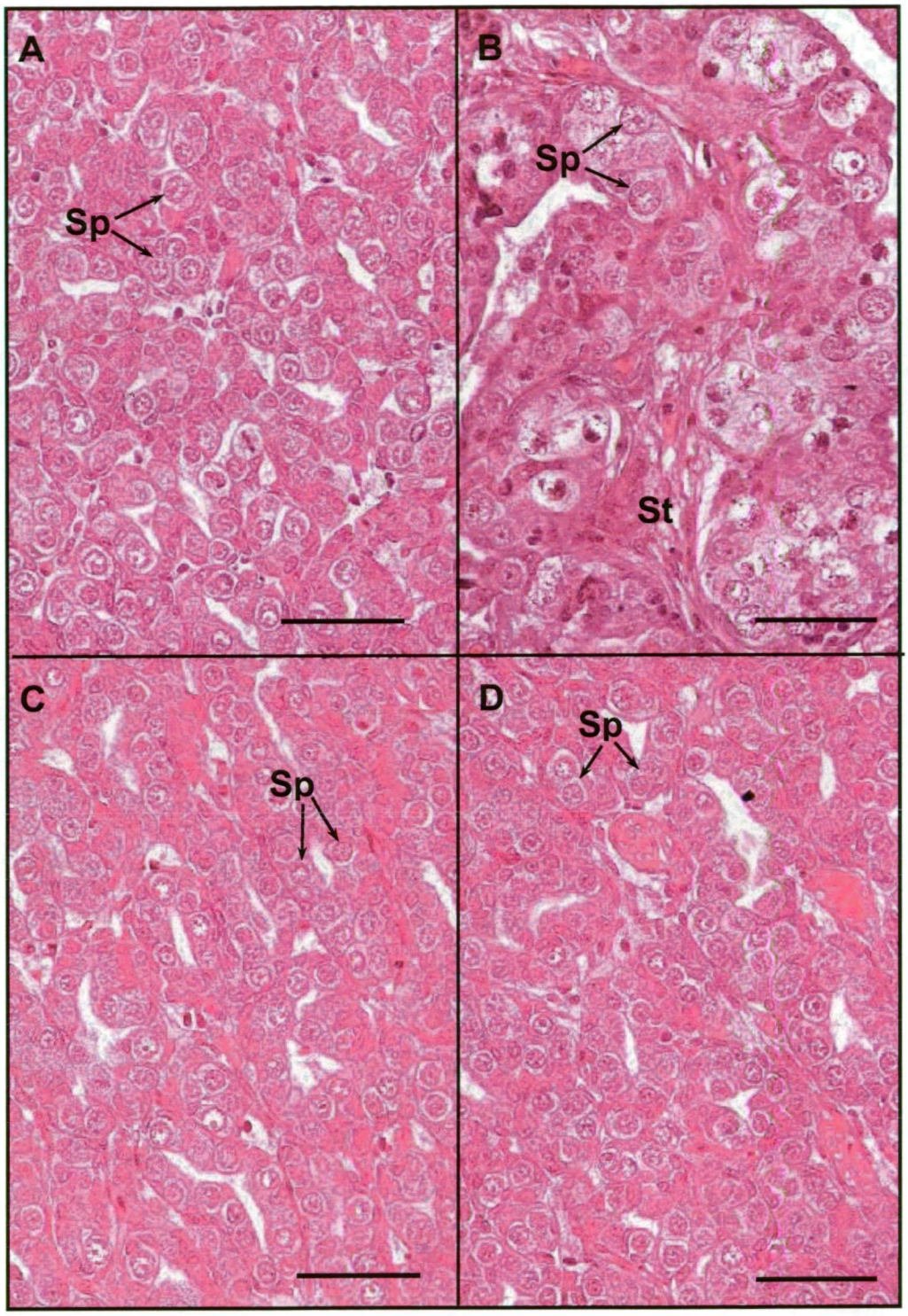

Figure 4.9 Sections of testis showing spermatocytes (Sp), from individuals treated with a) MDHT b)ATD c) aminoglutethimide and d) miconazole. Note sterile connective tissue (St) in b). Scale bar =50 μm.

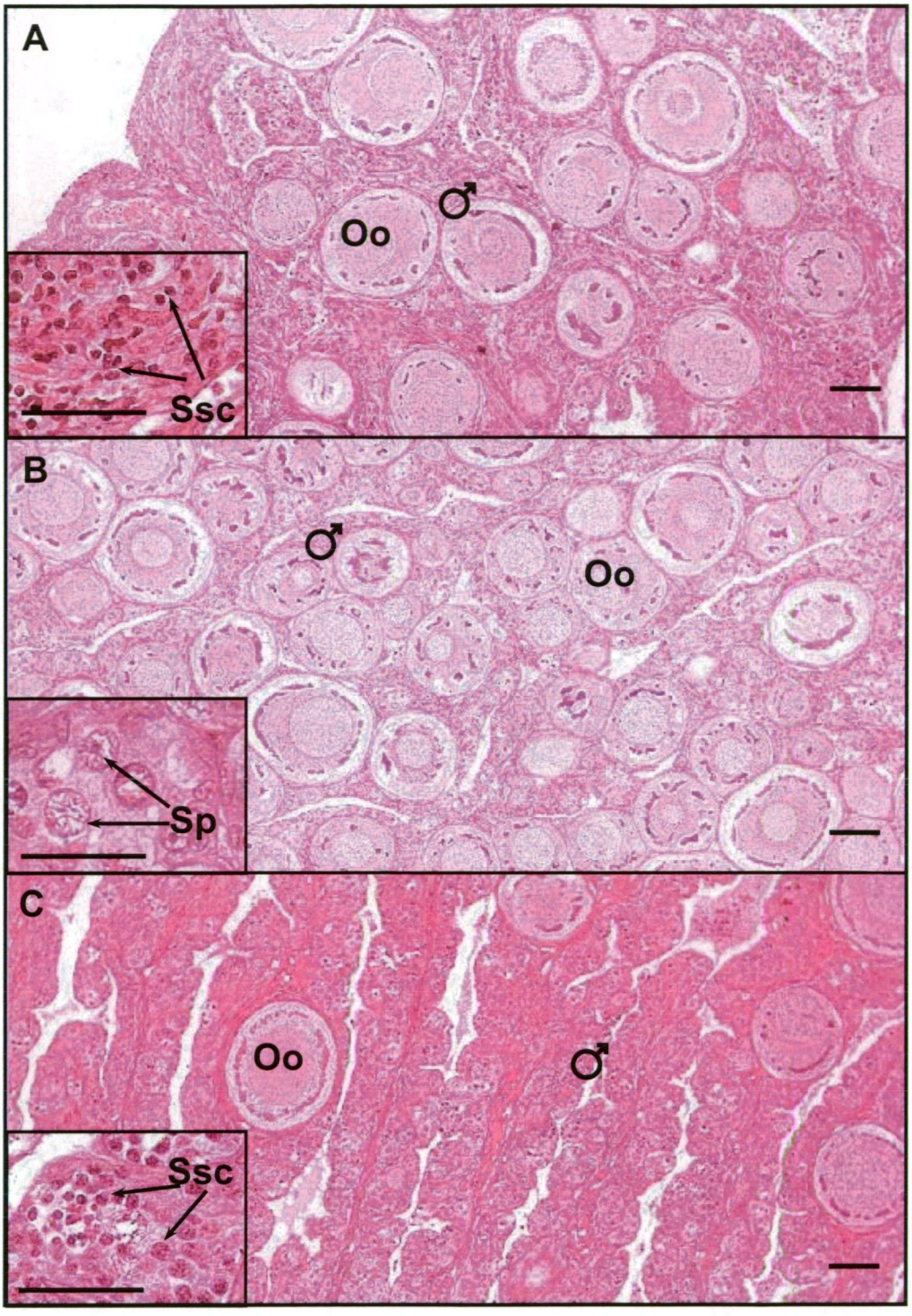

Figure 4.10 Sections of ovotestis, from individuals treated with a) and b) miconazole c) aminoglutethimide, showing oocytes (Oo), male gonadal tissue (♂), spermatogonia (Sp) and secondary spermatocytes (Ssc). Detail of male gonadal tissue in inset. Other details as for Figure 4.9.

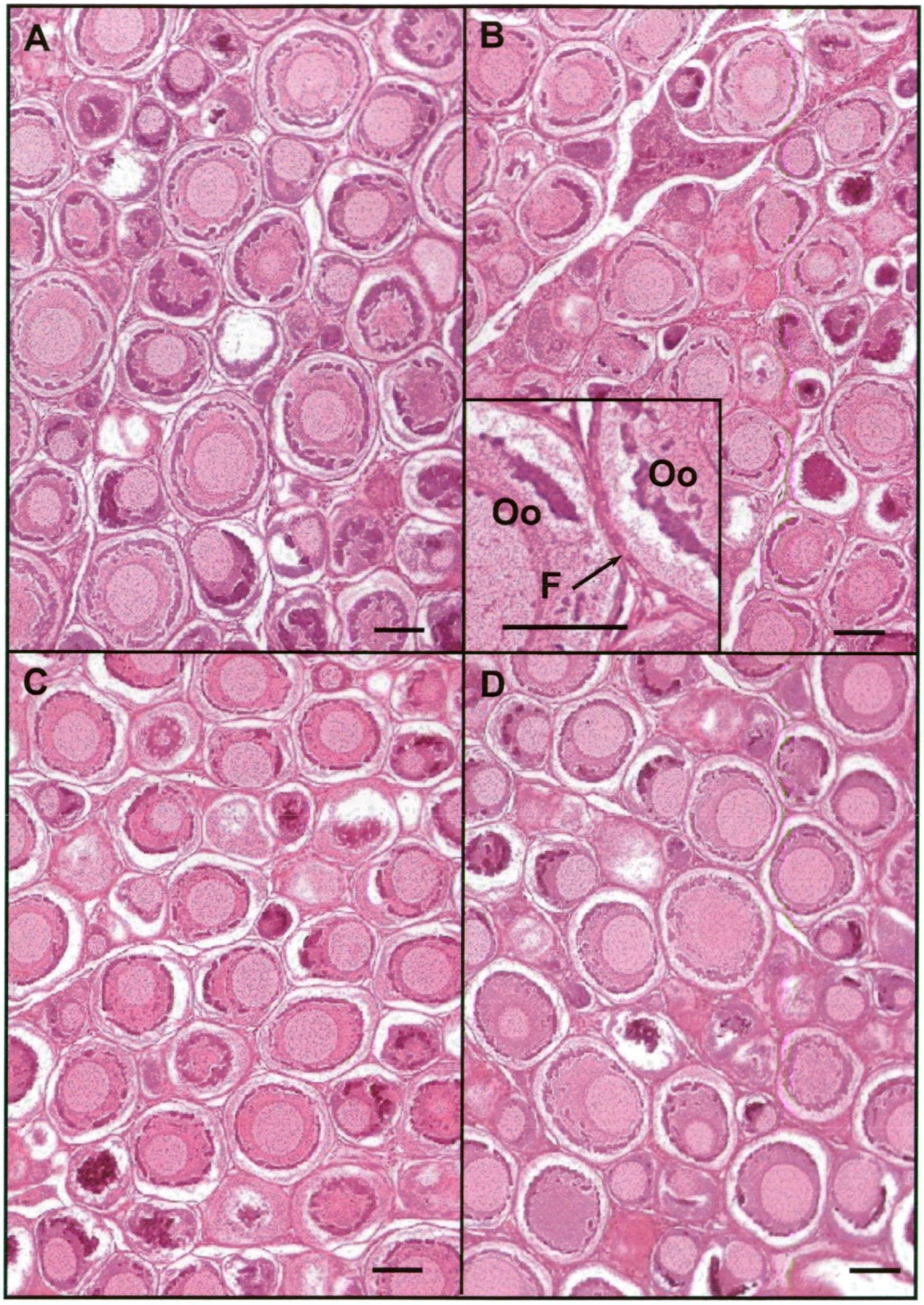

Figure 4.11 Sections of ovaries, from a) control b) aminoglutethimide c) miconazole and d) OHA treated individuals. Inset shows detail of oocyte (Oo) and surrounding follicular cells (F). Other details as for Figure 4.9.

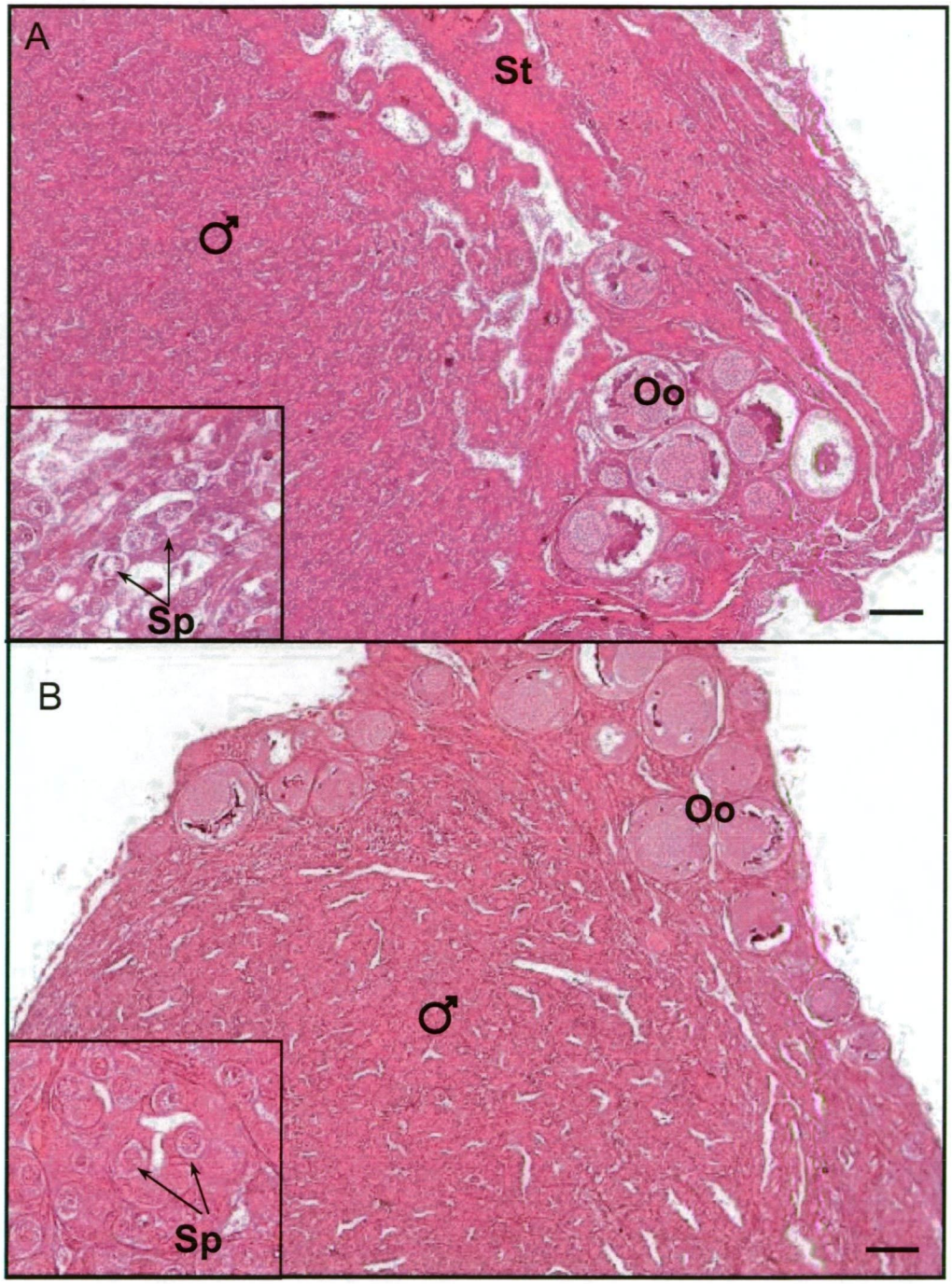

Figure 4.12 Sections of gonads containing a small cluster of oogonia (Oo) amongst testis (♂) and sterile connective tissue (St) from a) MDHT treated and b) Control individuals. Inset shows spermatogonia in detail. Other details as for Figure 4.9.

assignation of similar fish as males based solely on visual examination, this and subsequent mostly male individuals were included as males in the data set. In distinguishing the gonads of intersex individuals from the mostly male gonads described above, the key criterion was the presence of oocytes located throughout testis tissue (Figure 4.10), rather than in an isolated cluster (Figure 4.12a). The sex of the remainder of individuals determined by histological examination was: male (10); female (3); and sterile (1). Of the three representative gonad samples where histological determination of sex differed from visual assessment, two that had been visually assigned as male were intersex, and one visually assigned as female was mostly male.

Sex inverted males, obtained by immersion in either ATD or MDHT displayed no difference in either sperm activity 30 sec after activator addition (χ^2=3.98, 3 d.f., $p>0.05$), activity duration ($F_{3,16}$ =1.49, $p>0.05$) or fertility ($F_{3,16}$ =1.68, $p>0.05$), from normal precocious males (Table 4.4).

Table 4.4 Sperm activity parameters and fertility of sex inverted and normal male fish in Experiment 1. See text for abbreviations

Treatment	Sperm activity[a] at 30 sec (mean[range])	Duration of sperm activity (sec)	Fertility (%)
Control	2.8[3]	54.0 ± 11.2	69.3 ± 7.8
MDHT, double immersion	2.0[2]	54.0 ± 11.2	74.8 ± 4.9
ATD, single immersion	2.4[3]	72.0 ± 22.4	68.3 ± 4.8
ATD, double immersion	1.4[3]	63.0 ± 25.3	66.7 ± 6.9

[a] Defined in Table 4.1

4.3.ii Experiment 2: 2001-02

There was no effect of either AI dose or timing of immersion treatment on survival to 10 months post-hatch over the course of the experiment (Table 4.5). Increased mortality prior to first feeding in fry treated with ATD at 5 mg L^{-1} and fadrozole at 10 mg L^{-1} on 14 and 21 DPMH (Table 4.5) was the result of fungal infection which affected trays in one system. Increased mortality following first feeding was the result of the *Costia* sp. infection and treatment discussed

Table 4.5 Production parameters for Atlantic salmon, immersion treated in either MDHT or the aromatase inhibitors ATD or fadrozole in Experiment 2. Details as for Table 4.3.

AI/ androgen dose	Immersion regime (DPMH)	Post-hatch mortality (%)	First feeding mortality (%)	Weight (g) (mean±SE)	Length (mm) (mean±SE)	CF (mean±SE)	GSI (%) (mean±SE)
Control	7,14	17.9	23.4	65.85 ± 2.32	178.3 ± 2.1 ^{y,z}	1.13 ± 0.02	0.25±0.08 ^{y,z}
	14,21	13.4	22.3	65.97 ± 2.11	179.5 ± 1.7 ^{y,z}	1.11 ± 0.01	0.78±0.35 ^{y,z}
MDHT	7,14	21.4	22.3	67.64 ± 2.62	177.9 ± 2.1 ^{y,z}	1.15 ± 0.01	1.02±0.40 ^{y,z}
0.5 mg L^{-1}	14,21	15.2	33.0***	65.23 ± 2.72	176.0 ± 2.3 ^{y,z}	1.14 ± 0.01	1.57±0.43 ^{y,z}
ATD	7,14	21.3	18.9*	70.41 ± 3.17	177.5 ± 2.5 ^{y,z}	1.19 ± 0.01	1.48±0.47 ^{y,z}
0.5 mg L^{-1}	14,21	15.2	23.8	69.78 ± 2.77	179.2 ± 2.2 ^{y,z}	1.16 ± 0.01	0.86±0.31 ^{y,z}
ATD	7,14	16.9	19.3*	60.90 ± 2.54	170.8 ± 2.2 ^{y}	1.16 ± 0.01	1.26±0.41 ^{y,z}
5 mg L^{-1}	14,21	20.1***	24.3	65.38 ± 3.53	172.6 ± 2.8 ^{y,z}	1.19 ± 0.02	1.60±0.42 ^{z}
fadrozole	7,14	20.3	19.9	67.17 ± 2.43	176.6 ± 2.1 ^{y,z}	1.25 ± 0.10	0.14±0.01 ^{y}
10 mg L^{-1}	14,21	17.6*	18.9	68.74 ± 2.04	180.8 ± 1.7 ^{z}	1.13 ± 0.01	0.16±0.02 ^{y}
fadrozole	7,14	17.0	32.3***	63.30 ± 2.20	175.4 ± 1.9 ^{y,z}	1.13 ± 0.01	0.17±0.01 ^{y,z}
50 mg L^{-1}	14,21	16.2	28.4***	65.15 ± 2.38	177.8 ± 1.8 ^{y,z}	1.12 ± 0.01	0.16±0.01 ^{y}

previously. As in Experiment 1, total mortality in the 4 m^3 tanks from transfer in December 2001 to final sampling in May 2002 was less than 1%.

Mean fork length of fry treated with ATD at 5 mg L^{-1} on 7 and 14 DPMH was significantly lower than that of fry treated with fadrozole at 10 mg L^{-1} on 14 and 21 DPMH (Table 4.5). GSI was significantly higher in the group treated with ATD at 5 mg L^{-1} on 14 and 21 DPMH than in animals treated with Fadrozole at 10 mg L^{-1} on 7 and 14 DPMH or 14 and 21 DPMH, or at 50 mg L^{-1} on 14 and 21 DPMH, reflecting the low frequency of mature males in the fadrozole treated animals (Figure 4.13). There were no significant differences in either weight or condition factor between the treatment groups..

There was a significantly higher proportion of males in fish treated with MDHT and ATD, regardless of dose and timing of immersion, than in control groups (Figure 4.13). Furthermore, the proportion of males in fish treated with MDHT at 7 and 14 DPMH was lower than those treated at 14 and 21 DPMH, whereas the proportion for fish treated with ATD at 500 μg L^{-1} at 7 and 14 DPMH was greater than at 14 and 21 DPMH. Maturation levels were 39 and 47 % for MHDT , 67 and 62 % for ATD at 500 μg L^{-1} and 59 and 72% for ATD at 5 mg L^{-1} , for immersion at 7 and 14 or 14 and 21 DPMH respectively (Figure 4.14). Patent sperm ducts were observed in 87, 96, 88, 80, 87, 90% of the mature fish in those same groups (Figure 4.14). Analysis of pooled data for female, mature and immature male fish (Figure 4.15) showed that mature males had significantly lower weight (45.7 g) and FL (155.5 mm) and significantly higher CF (1.18) than either immature males (weight = 69.9 g, FL = 180.2 mm, CF = 1.14) or females (weight = 70.1 g, FL = 181.0 mm, CF = 1.14) (weight $F_{2,1156} = 78.0$, $p<0.001$; FL $F_{2,1156} = 133.8$, $p<0.001$; CF $F_{2,1156} = 9.8$, $p<0.001$). GSI of mature males (4.24 %) was higher than that of females (0.13 %), which was higher than immature males (0.07 %) ($F_{2,678} = 4397$, $p<0.001$). Histological determination of phenotypic sex of representative samples agreed with visual assessment in all cases (n = 50). There were 47 individuals in which sex could not be determined visually, with histological examination determining them to be: male (22 including mostly male (4)); female (14) and intersex (7). The four gonad types described above: testis

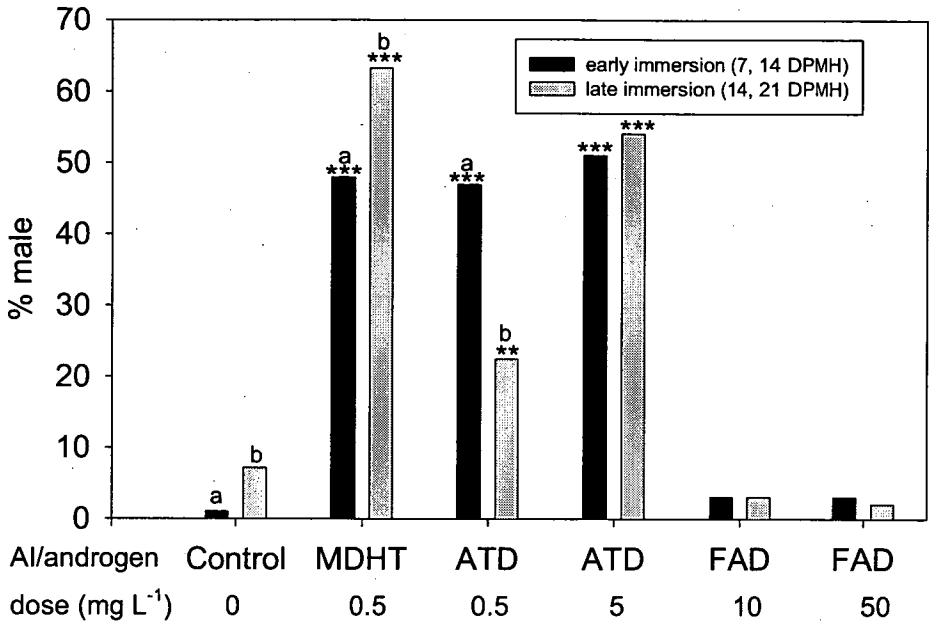

Figure 4.13 Percentage masculinisation following immersion with MDHT or AI. See text for abbreviations. For each level of androgen or aromatase inhibitor treatment, different superscripts indicate significant difference ($p<0.05$) between early and late immersion treatments. Asterisks indicate significant difference in male frequency from control populations (** $0.001<p<0.01$; ***$p<0.001$).

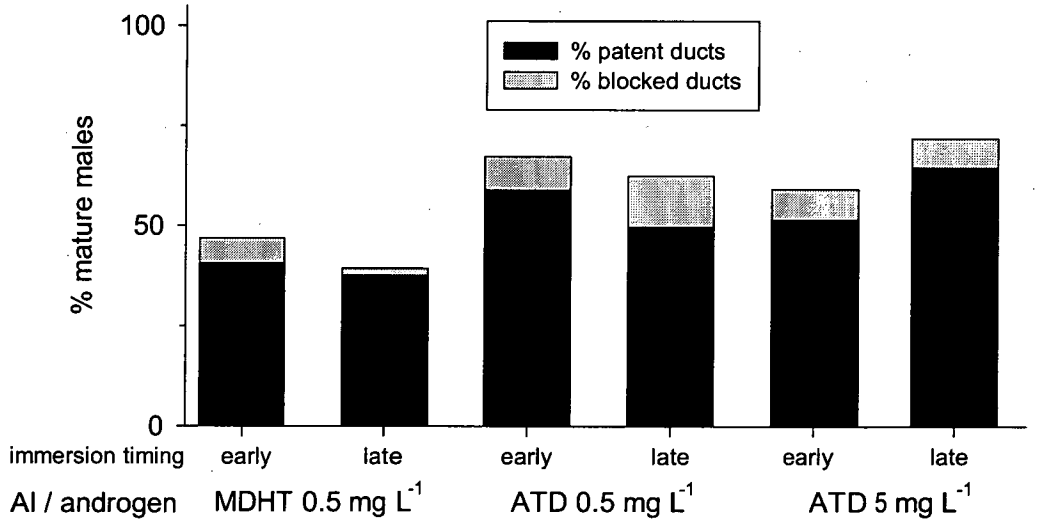

Figure 4.14 Proportions of sperm duct status of mature fish, expressed as a percentage of mature sex inverted male fish produced by immersion in either MDHT or ATD in Experiment 2.

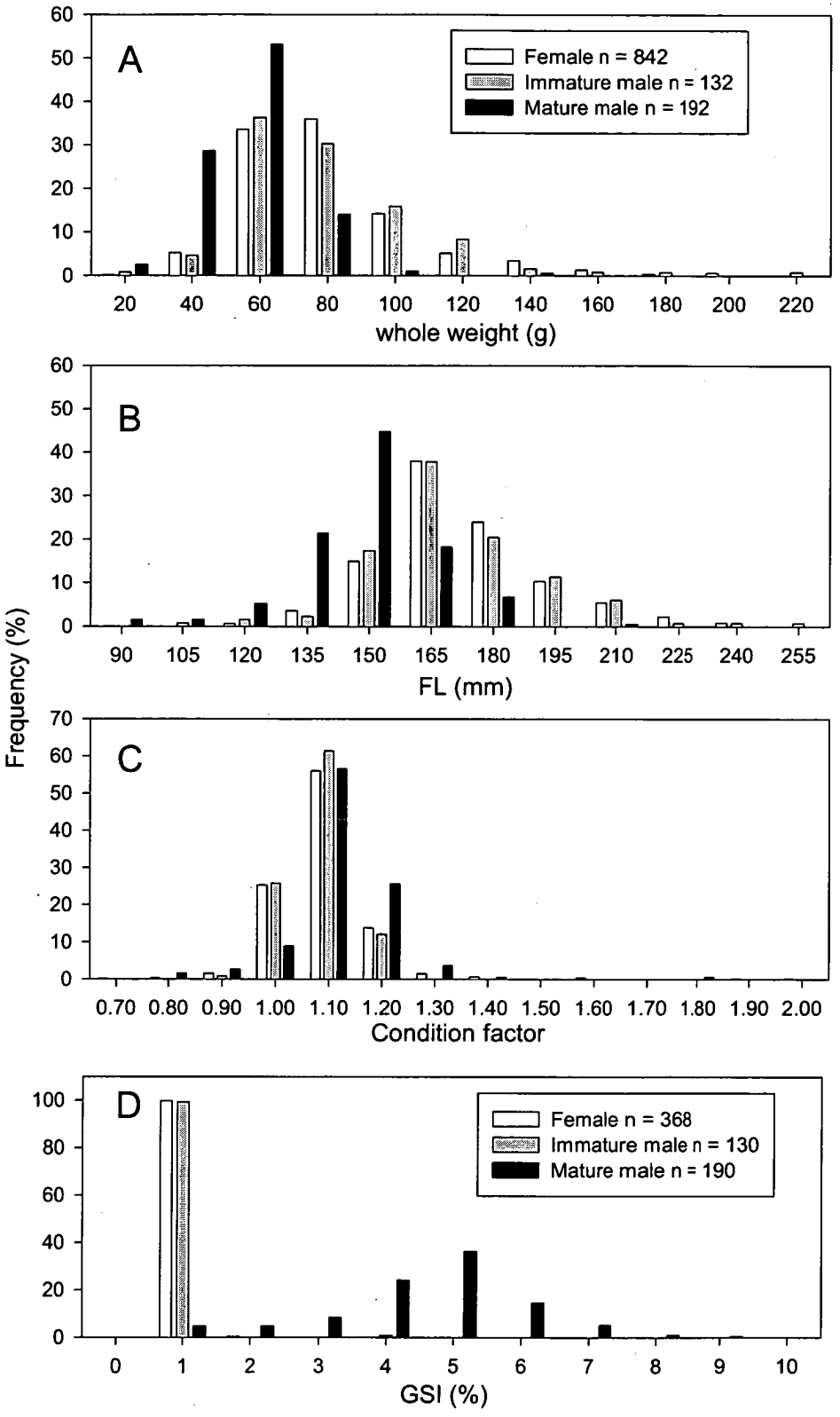

Figure 4.15 Percent frequency of pooled data of: a) whole weight; b) fork length (FL); c) condition factor and d) GSI, for fish from Experiment 2.

(Figure 4.16) ; ovotestis (Figure 4.17); ovaries (Figure 4.18); and mostly males (Figure 4.19) were structurally similar between treatment groups, and to those described for Experiment 1. Within the groups treated with AI, large areas of sterile tissue were present in some ovotestis with (Figure 4.18) . Oocyte size (mean diameter ±S.E.) of individuals was in the range of 134.7±7.8 to 160.0±7.8 μm, with spermatocyte size in the range of 7.4±0.1 to 10.6±0.4 μm. Sperm activity 30 sec after activator addition (χ^2=3.98, 3 d.f., p>0.05), activity duration ($F_{3,16}$ =1.49, p>0.05) or fertility ($F_{3,16}$ =1.68, p>0.05) of sex inverted males, obtained by immersion in either ATD or MDHT did not differ to that of normal precocious males (Table 4.6).

Table 4.6 Sperm activity parameters and fertility of sex inverted and normal male fish from Experiment 2. See text for treatment details and abbreviations.

AI/androgen dose	Immersion regime (DPMH)	Sperm activity[a] at 30 sec (mean[range])	Duration of sperm activity (sec) (mean ± SE)	Fertility (%) (mean ± SE)
Control	na	1.6 [2.0]	36.0 ± 6.0	66.5 ± 3.6
MDHT	early	1.6 [3.0]	33.0 ± 5.6	63.8 ± 2.3
0.5 mg L^{-1}	late	2.2 [3.0]	39.0 ± 7.6	72.8 ± 2.9
ATD	early	2.8 [2.0]	48.0 ± 5.6	74.0 ± 3.6
0.5 mg L^{-1}	late	3.8 [1.0]	54.0 ± 3.7	75.6 ± 2.4
ATD	early	2.4 [3.0]	42.0 ± 5.6	71.9 ± 2.5
5 mg L^{-1}	late	2.0 [2.0]	33.0 ± 3.0	69.6 ± 2.8

[a] Defined in Table 4.1

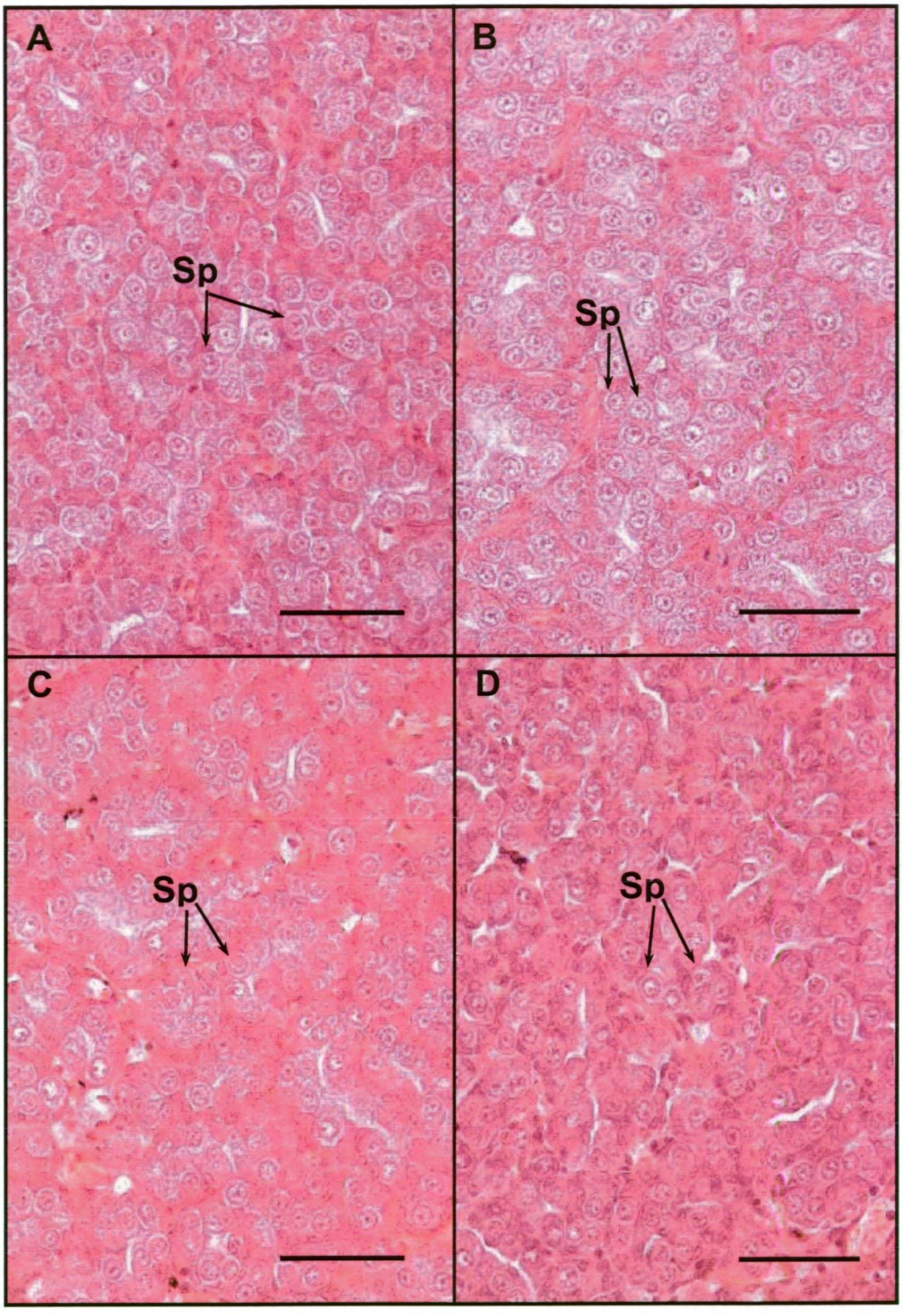

Figure 4.16 Sections of testis from a) control or individuals treated with, b) ATD, c) fadrozole and d) MDHT. Other details as for Figure 4.9.

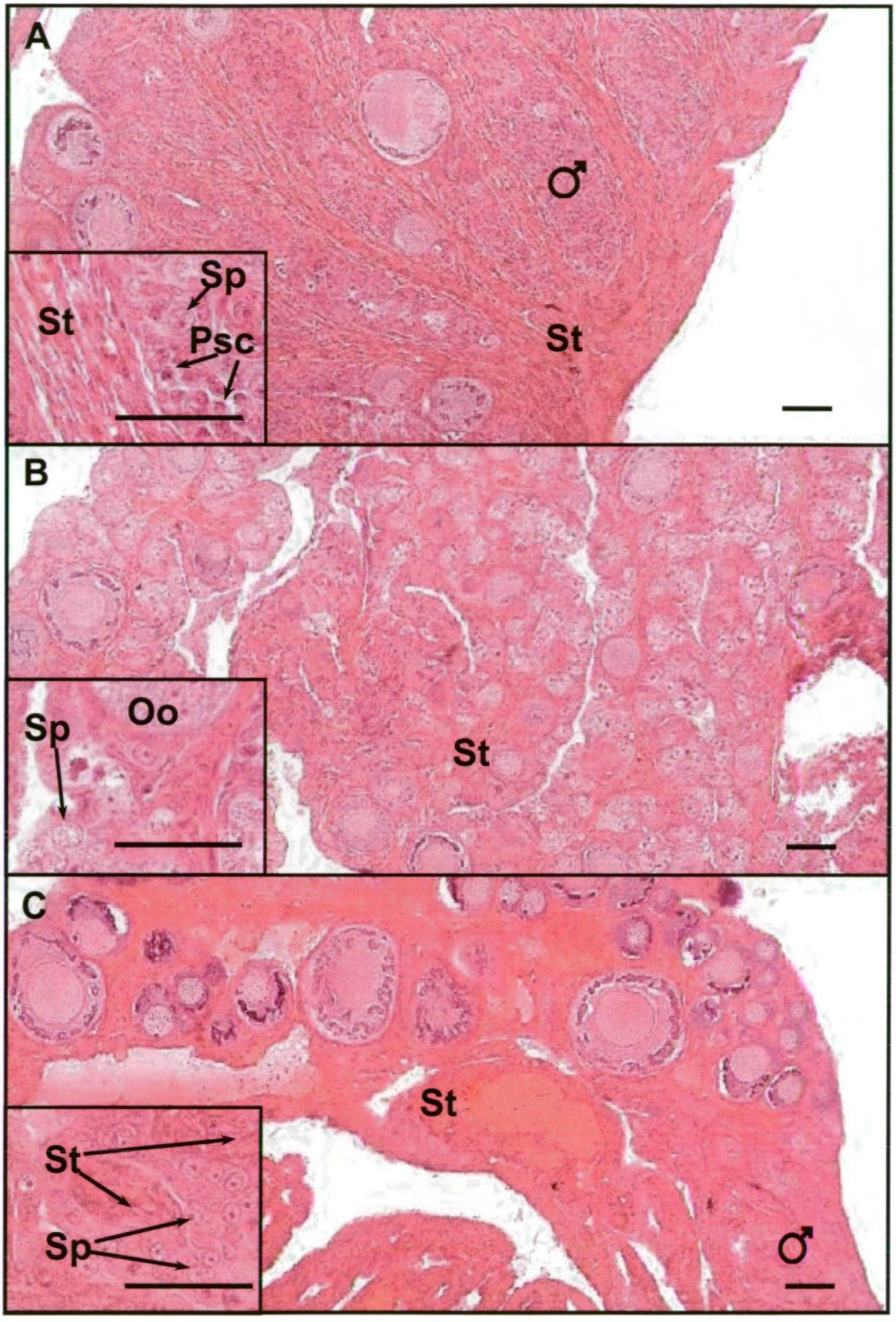

Figure 4.17 Ovotestis from individuals treated with a) and b) fadrozole at 10 mg L^{-1} c) ATD at 5 mg L^{-1}. Note extensive areas of sterile connective tissue (St) in addition to male gonadal tissue ♂. Detail of male gonadal tissue with primary spermatocytes (Psc) and spermatogonia (Sp) shown in insets. Other details as for Figures 4.9 and 4.12.

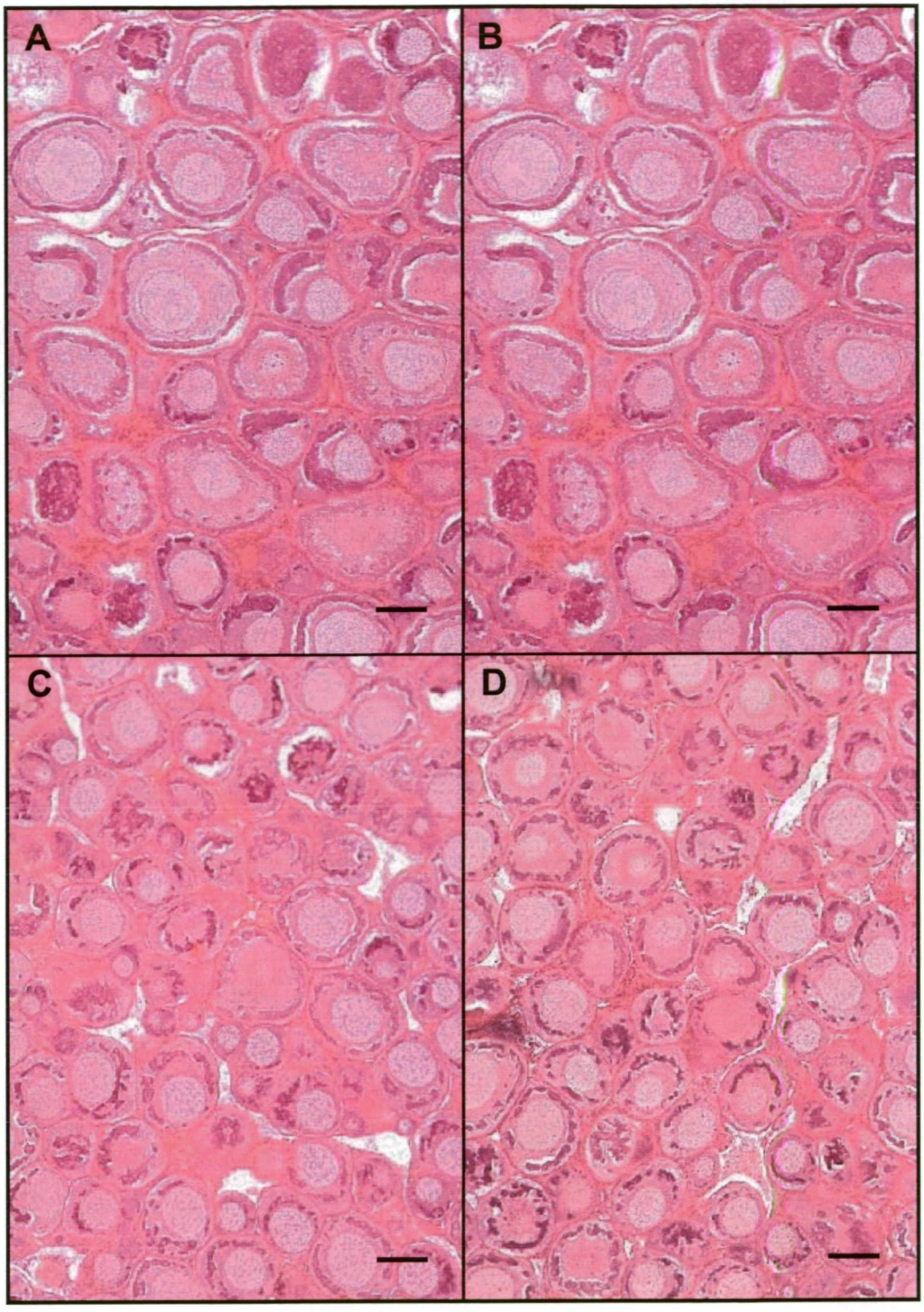

Figure 4.18 Sections of ovaries from individuals treated with a) ATD at 5mg L^{-1} b) ATD at 0.5mg L^{-1} c) and d) MDHT. Other details as for Figure 4.9

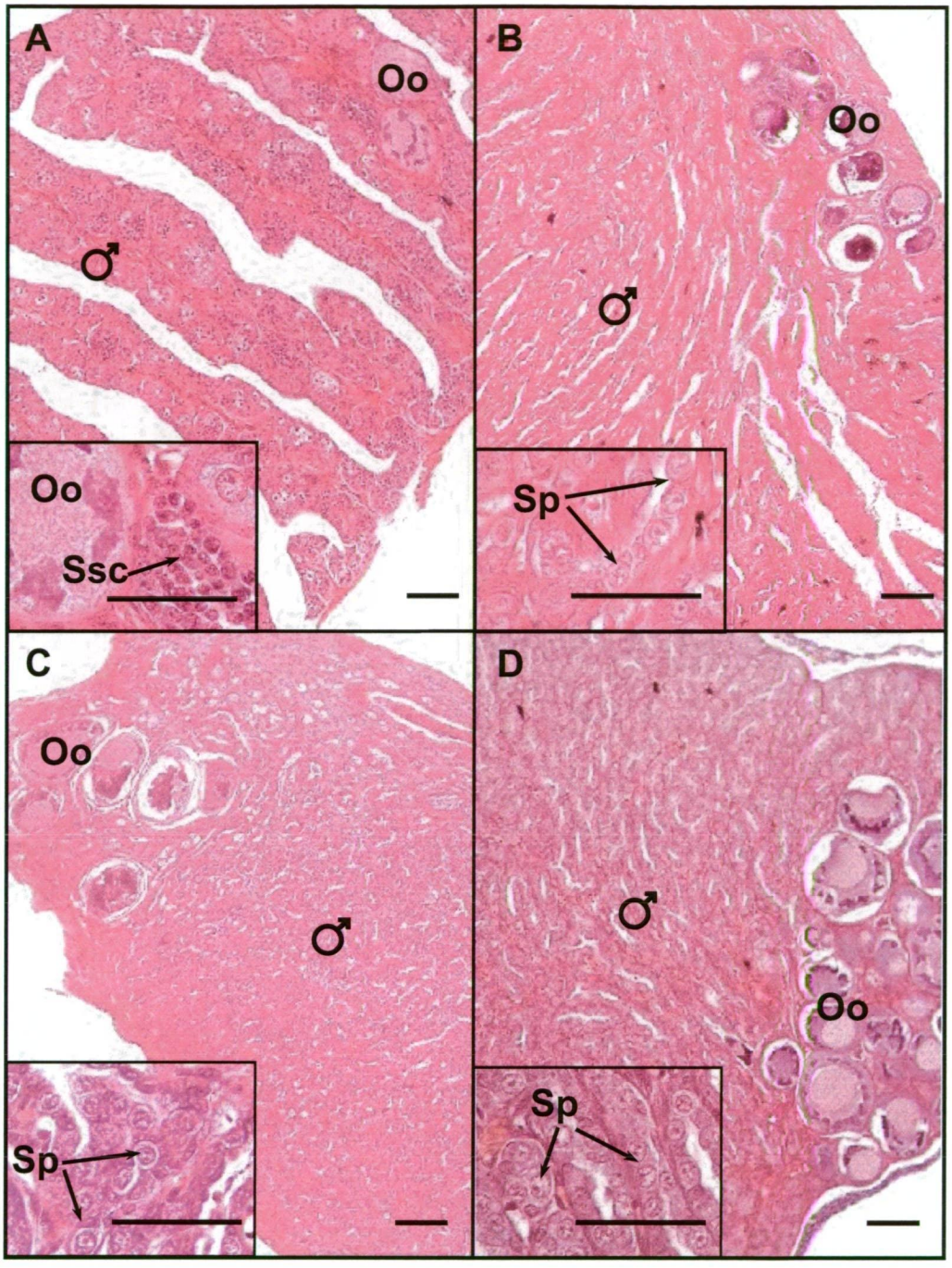

Figure 4.19 Sections of gonads containing predominantly testis tissue (♂), with a cluster of oocytes (Oo). Animals treated with a) and d) ATD, b) and c) MDHT. Other details for Figures 4.9 and 4.11. Insets shoe detail of male gonadal tissue, including secondary spermatocytes (Ssc) in a), and spermatogonia (Sp) in a)-d).

The persistence of a small proportion of males in the control all female population may arise from the use of genetic males during spawning, alternatively there may have been movement of fish between the 1 m^3 tanks. At the conclusion of both experiments there were a small number of males with blocked sperm ducts present in the control populations. While the spontaneous development of these cannot be ruled out, it is possible that they came from an adjacent tank. Jump screens were used during Experiment 2 to prevent transfer between tanks; however, the relatively high incidence of females in the Control (Late) tank, again including some males with blocked sperm ducts, indicated that some mixing may have occurred.

4.4 Discussion

The present study represents the first achievement of masculinisation of salmonids following immersion treatment with ATD. Furthermore, masculinisation rates of greater than 50% following immersion treatment with ATD were comparable to levels achieved with MDHT in the same study, and demonstrate the commercial viability of immersion treatment with ATD for salmonid masculinisation. ATD had previously been shown to be an effective masculinising agent for rainbow trout, *Oncorhynchus mykiss*, when it was administered chronically, via the diet (Guiguen et al., 1999). In their study of the efficacy of ATD applied acutely for the masculinisation of developing chickens, Elbrecht and Smith (1992) induced a male pattern of gonadal development with a single, 2 mg dose. In contrast, similar acute treatment with ATD was ineffective in masculinising turtles with temperature-dependent sex determination (Wibbels and Crews, 1992; Pieau et al., 1994).

The poor efficacy of fadrozole in the present study is in contrast to the results of previous *in vitro* efficacy experiments (Chapter 3) and *in vivo* studies on a range of taxa (Elbrecht and Smith, 1992; Piferrer et al., 1994c; Wennstrom and Crews, 1995; Chardard and Dournon, 1999; Kitano et al., 2000; Kroon and Liley, 2000; Kitano et al., 2001; Kwon et al., 2002). Using similar techniques to those in the present study, Piferrer et al. (1994c) achieved 22% masculinisation of chinook salmon, *O. tshawytscha,* and although the difference in masculinisation rates

between the present study and that of Piferrer et al. (1994c), may be a result of interspecific differences, the failure of fadrozole to induce masculinisation in the present study, even when applied at times that were effective for another AI, suggests that other factors may have contributed the observed results. Fadrozole has increased aqueous solubility under acidic conditions (Ackermann and Kaiser, 1989), and aqueous solubility negatively correlates with drug uptake rate (Hunn and Allen, 1974). This may mean that bath conditions in immersion studies may influence uptake. Ammonia and carbon dioxide excretion by alevins during bathing would tend to reduce bath pH, and although bath pH was not measured in the present study, the density of alevins in immersion treatments was more than 10-fold higher than in the study by Piferrer et al.(1994c), raising the possibility that different immersion conditions between studies might affect results.

Notwithstanding the results of the present study, previous successful studies have achieved only moderate levels of masculinisation of chinook salmon (Piferrer et al., 1994c) and newts, *Pleurodeles waltl* (Chardard and Dournon, 1999), following immersion treatment with fadrozole. In contrast, higher levels (>95%) of masculinisation have been achieved using fadrozole applied directly to the developing eggs of the parthenogenetic whiptail lizard *Cnemidophorous uniparens* (Wennstrom and Crews, 1995), and the chicken (Elbrecht and Smith, 1992). Furthermore, complete masculinisation following dietary administration of fadrozole has been reported in the Japanese flounder, *Paralichthys olivaceus,* (Kitano et al., 2000, 2001) and Nile tilapia, *Oreochromis niloticus* (Kwon et al., 2002). Such differences in efficacy may be a consequence poor uptake from an aqueous solution, possibly as a result of the effects of pH on aqueous solubility as discussed above. The characteristics of fadrozole uptake and their relevance to its efficacy in the present study are discussed further in Chapter 5.

The frequency of application may have also affected the efficacy of fadrozole in the present study, as there is a quantifiable relationship between treatment frequency, drug half life and tissue levels (Notari, 1980; Grahame-Smith and Aronson, 1984). Due to a low K_m and turnover rate of aromatase, competitive inhibitors, such as fadrozole, are required in higher concentrations, and have a shorter effective half-life, than mechanism-based inhibitors which bind

irreversibly to aromatase, such as ATD (Cole and Robinson, 1990; Séralini and Moslemi, 2001). Taking these observations into account, the efficacy of fadrozole may be improved by reducing stocking density during immersion treatment, reducing the inter-bath interval to less than 7 days and applying more than 2 treatments. However, should the efficacy of fadrozole be improved by this approach, the need to repeatedly handle alevins over a relatively short time period may still limit its attractiveness in a commercial setting

OHA is an effective inhibitor of salmonid aromatase *in vitro* (Chapter 3, Antonolopou et al., 1995; Shilling et al., 1999) and masculinises developing amphibian gonads (Yu et al., 1993; Petrini and Zaccanti, 1998); however, it was neither an effective masculinising agent of Atlantic salmon in the present study nor effective in reducing aromatase activity when administered *in vivo* to juvenile Atlantic salmon (Antonolopou et al., 1995). This may be due to a compensatory increase in aromatase production as proposed by Antonolopou et al. (1995). Alternatively, it may result from rapid glucuronation of OHA, as occurs in humans (Cole and Robinson, 1990). Additionally, poor efficacy of OHA in the present study may be related to poor uptake, discussed in detail in Chapter 5. The low efficacy of the miconazole and aminoglutethimide is consistent with the relatively poor efficacy in previous *in vitro* studies (Chapter 3) and with the findings of Chardard and Dournon (1999) in their studies on the newt.

Masculinisation by MDHT in Experiment 1 is at a comparable levels to previous studies, as is the reduction in efficacy of androgens applied prior to 14 DPMH (Chapter 2). The relatively low levels of masculinisation of animals treated at 14 and 21 DPMH in Experiment 2 is in contrast with previous studies (Chapter 2). The reasons for this are unclear, and it may be a reflection of annual variability of the stocks. Importantly, notwithstanding the relatively low incidence of sex inversion achieved with MDHT in Experiment 2, commercially acceptable levels of masculinisation of were achieved with ATD.

Previous work (Chapter 2) showed no difference in the efficacy of androgens applied in dual immersion treatments separated by either 7 or 14 days; however, in the present study there was an increase in masculinisation from 13 to 24%

when ATD was applied at a 7 (Experiment 2) rather than a 14 (Experiment 1) day interval. Such differences may reflect differences in the mode of action of these compounds, or differences in uptake and clearance rates. The turnover of steroid hormones in salmonids is rapid, with an estimated half life of 48 hours in alevins (Piferrer and Donaldson, 1994), and shorter periods in fry and juveniles (Johnstone et al., 1978; Johnstone et al., 1983; Piferrer and Donaldson, 1994), thus by 7 or 14 days, only 1-10% of the initial steroid uptake would remain. (Grahame-Smith and Aronson, 1984). Provided clearance is not complete, repeated doses of a drug increase tissue levels and hence efficacy; however, to be optimally effective, the interval between doses should be equal to or less than the drug half life (Notari, 1980; Grahame-Smith and Aronson, 1984). This was borne out in the study of Piferrer and Donaldson (1994) who showed E_2 efficacy was improved when the inter-bath interval of coho salmon alevins was reduced from 7 to 2 days. In contrast, the efficacy of AI such as ATD that irreversibly bind to aromatase, is extended beyond the period of clearance, as aromatase has a slow turnover rate (Cole and Robinson, 1990), and the optimum inter-treatment interval is therefore likely to be substantially longer than for steroids. The influence of the timing of immersion treatments with ATD on uptake and retention of ATD is discussed further in Chapter 5.

The masculinisation of Atlantic salmon achieved with ATD in the present study supports the hypothesis that the onset of ovarian differentiation in fishes is dependent on the presence of estrogens (Yamamoto, 1969; Piferrer et al., 1994c; Nagahama, 2000; Piferrer, 2001), or a sufficiently high estrogen:androgen ratio (Bogart, 1987), and that the differentiation of gonadal tissue to testis occurs in the absence of estrogens (Kwon et al., 2002). The improved efficacy of ATD when used at 7 compared to 14 DPMH contrasts with results obtained with MDHT in the present and previous (Chapter 2) studies, and suggests differences in their roles in masculinisation. These data suggest that the presence of E_2 at a time soon after hatch is a key factor in the onset of female sex differentiation, whereas androgens exerts a masculinising effect at a later time, which extends beyond the period of first feeding. Similar evidence of asynchrony between the onset of feminisation and masculinisation in fishes was shown by Piferrer and Donaldson (1989) who determined that the sensitivity of coho salmon to estrogen preceded

that for androgens by several days, and by Nagahama (2000) who showed that in tilapia the levels of several steroidogenic enzymes increased prior to ovarian differentiation, but not until after differentiation of the testis. Petrini and Zaccanti (1998) also showed an earlier response to exogenous estrogens than to androgens in the toad *Bufo bufo*. Although the study of Piferrer et al. (1994c) involved the addition of fadrozole or androgens at a time of sensitivity to both estrogens and androgens, the relatively poor masculinisation rate attained with fadrozole alone compared to levels obtained with fadrozole and androgens in combination, raises the possibility that the earlier addition of fadrozole may have improved its efficacy. In the present study, the increase in efficacy of ATD when used in late immersion treatments at a dose of 5 mg L^{-1}, compared to 0.5 mg L^{-1} suggests an additional role for ATD. ATD has androgenic properties (Wozinak et al., 1992; Séralini and Moslemi, 2001) and this increased efficacy is possibly due to its androgenic effects. The potential for ATD to induce masculinisation through action as an androgen was also noted by Guiguen et al. (1999), who achieved complete masculinisation of rainbow trout using a dose of 50 mg kg^{-1} ATD in the diet, (an order of magnitude higher than typical dietary androgen doses for salmonids). Notwithstanding a demonstrated reduction in aromatase activity following ATD treatment, Guiguen et al. (1999) noted that "We cannot exclude a superimposed androgenic effect concomitantly to the decrease in estrogen production, since ATD has a steroidal structure". It remains possible that the labile period for feminisation (and hence maximum sensitivity to AI) extends earlier than 7 DPMH in Atlantic salmon, and the efficacy of both ATD and fadrozole may be improved with earlier application. Masculinisation by non-steroidal AI administered in the diet has been demonstrated for species in which the labile period for E_2 sensitivity occurs after the onset of feeding, including tilapia (Kwon et al., 2002) and flounder (Kitano et al., 2000), but it has not been reported for salmonids. Similarly, dietary administration of tamoxifen, an E_2 receptor antagonist, caused masculinisation of Japanese flounder (Kitano et al., 2001) but not rainbow trout (Guiguen et al., 1999). These examples support the premise that on the model of sex determination discussed previously, the period of maximum efficacy of non-steroidal AI would be expected to coincide with the time of sensitivity to exogenous E_2, while the efficacy of androgenic steroids and steroidal AI may extend beyond this period. The comparison of an effective non-

steroidal AI applied by immersion during the labile period, and by the diet would provide a useful test of this hypothesis.

The artificial photoperiod regime used in both Experiments 1 and 2 in the present studies was successful at inducing up to 70% precocious male maturation reducing the time requirement to achieve gonadal function from 3 years to 9 months. The high level of sperm quality and fertility of neomales obtained by treatment with all doses of ATD concurs with the findings of Piferrer et al. (1994c). Regrettably, few studies of AI have continued to the point of male maturation to allow more detailed comparisons. The effects of masculinisation by MT on the fertility of neomales have varied, with studies on rainbow trout reporting either comparable fertility to normal males despite the need to macerate gonads to extract milt (Johnstone et al., 1979) or a significant decline in fertility (Geffen and Evans, 2000). The fertility of Siamese fighting fish declined with increasing of MT, due in part to reduced sperm motility and sperm count (Kirankumar and Pandian, 2002), whereas Geffen and Evans (2000) found no specific traits of the sperm from sex-reversed rainbow trout could account for declining fertility. The fertility of sex-reversed males is also compromised with high doses of androgens, affecting gonadal development (Johnstone et al., 1978; Goetz et al., 1979; Krisfalusi and Cloud, 1999). The need to minimise any deleterious effects of steroid hormones has been a consideration in the development of a strategy of minimum intervention for sex control (Piferrer, 2001), but the use of aromatase inhibitors for masculinisation may alleviate some of the fertility problems associated with androgens.

Treatment with AI and MDHT did not affect the production parameters of growth, condition factor or GSI. Similarly, the growth and gonadal development of chinook salmon was unaffected following immersion treatment of alevins with fadrozole (Piferrer et al., 1994c). In contrast, varying effects on gonad growth and maturation were found in juvenile Atlantic salmon treated with AI (Antonolopou et al., 1995) and gonad size was reduced in chinook salmon following repeated immersion treatment with MT (Goetz et al., 1979). Other workers have reported both positive and negative effects of steroid hormones on the growth of fishes (Pandian and Sheela, 1995; Blazquez et al., 2001; Piferrer, 2001). Piferrer (2001)

described 2 response types in growth whereby a Type A response is characterised by a dose dependent increase in growth rate up to a threshold dose and a declining growth rate at higher doses. The data for androgens clearly fit this model, as the administration of androgens to teleosts has both positively (Goetz et al., 1979; Baker et al., 1988; Riley et al., 2002) and negatively (Blazquez et al., 1995; Kirankumar and Pandian, 2002) affected growth. The basis for the increase in growth is proposed to be a result of stimulation of GH/IGF axis by androgens (Riley et al., 2002). In contrast, the Type B response is characterised by the lack of, or a negative effect on growth with increasing dose, typified by the growth response following treatment with E_2 (Johnstone et al., 1978; Goetz et al., 1979; Piferrer, 2001). The absence of an effect on growth by all AI used in the present study suggests a Type B response, despite the reported weak androgenic properties of the steroidal AI, ATD and OHA (Séralini and Moslemi, 2001).

The patterns of mortality in the present study showed no relationship to AI treatment, and aside from those due to equipment failures, mortality rates of AI treated fish were generally similar to those of controls. No toxic effects of AI have been reported for fishes in either dietary (Guiguen et al., 1999; Kwon et al., 2002) or immersion (Piferrer et al., 1994c) treatments, despite the use of high doses of these compounds in some studies (Guiguen et al., 1999). In contrast, fadrozole and miconazole have been reported to be toxic to developing European pond turtles (Pieau et al., 1994) and newts (Chardard and Dournon, 1999). Although this may solely reflect interspecific variation, differences in dose or duration of exposure, AI action may also contribute to these observations. The doses of miconazole of 0.25-0.5 mg L^{-1} used by Chardard and Dournon (1999) were considerably lower than those used in the present study; however, treatment duration was extended, and mortalities occurred after 3 days of exposure. Similarly, mortalities in European pond turtles treated *in ovo* occurred following a fourth injection of fadrozole (Pieau et al., 1994). Miconazole, an antimycotic imidazole, may inhibit a number of P450 enzymes (Séralini and Moslemi, 2001), and although fadrozole is known to have fewer side effects, it may also inhibit other pathways (Demers et al., 1993). The results of the present study suggest that toxic side effects will not be an issue at the doses and treatment frequency used here.

The incidence of 67-90% sperm duct patency in sex inverted males obtained through immersion treatment with AI is comparable to that of 86-96% in MDHT-treated fish in the present study, and previous results of 84-92% for fish treated with MDHT by immersion (Chapter 2), and higher than that of 59% in fish treated with dietary MT or MDHT (Chapter 2). Other studies of salmonids have found that the masculinisation of salmonids by androgens via the diet resulted in a high proportion of neomales with blocked or malformed ducts (Johnstone et al., 1978; Johnstone and Youngson, 1984; Feist et al., 1995) whereas immersion treated males had proportionally greater open ducts (Feist et al., 1995). Johnstone and Maclachlan (1994) found low incidence of patent ducts in males, regardless of treatment mode; however, in that study, immersion treatment was continued at weekly intervals, from hatch to 1 month after first feeding. Regrettably, in the only study of sex inversion of salmonids by dietary administration of AI, only data on juvenile fish was published (Guiguen et al., 1999). Collectively, these data indicate that, despite working through apparently different mechanisms, both AI and androgens produce neomales with a high incidence of patent sperm ducts, when applied by immersion.

Treatment of Atlantic salmon alevins in the present study with either ATD or MDHT resulted in the production of individuals with fully developed testes and high fertility. Histologically the testes of neomales were, in most instances, identical to those of normal males and to those of sex inverted female salmonids described previously (Krisfalusi and Cloud, 1999). Testis tissue was packed with spermatogonia arranged in lobules (Piferrer et al., 1994a; Krisfalusi and Cloud, 1999) and spermatogonial size was similar to that of diploid, masculinised, coho salmon gynogens (Piferrer et al., 1994a). The onset of meiosis within the testis, indicated by the presence of secondary spermatocytes in some individuals, is consistent with the onset of maturation (Patiño, 1995; Nakamura et al., 1998), reflected in spermiation in a high proportion of sex inverted males. The testis of some individuals contained significant areas of connective tissue, similar to that described for sex reversed males treated with androgens (Johnstone et al., 1978; Goetz et al., 1979; van den Hurk and Slof, 1981; Blazquez et al., 1995; Krisfalusi and Cloud, 1999), although few individuals in the present study had gonads comprised solely of connective tissue, of the type described by Goetz et al. (1979)

Blazquez et al. (1995), Krisfalusi and Cloud (1999) and van den Hurk and Slof (1981). This is typically associated with high doses of, or extended treatment with, androgens.

Gonads having both male and female gametic tissue, as observed following AI treatment in the present study, have also been previously reported as developing in a number of gonochoristic fish species following steroid treatment (Johnstone et al., 1978; Blazquez et al., 1995). These intersex individuals (Yamamoto, 1969) are most frequently associated with intermediate doses or timing of androgens (Johnstone et al., 1978; van den Hurk and Slof, 1981; Baker et al., 1988; Blazquez et al., 2001). The persistence of ovarian tissue in the testis of males, also observed in AI treated fish in the present study, is a frequent occurrence for sex reversed Atlantic salmon males produced by dietary androgen treatment (H.R. King, unpublished data). Ovarian structure was similar to that which had been previously described for salmonids (Piferrer et al., 1994a), with synchronous oocytes in the perinucleolar stage, and a lamellar structure of ovaries evident in a number of specimens.

Based on the results of the present study, an effective commercial regime, utilising a minimum intervention strategy recommended by Piferrer (2001) has been developed for the masculinisation of Atlantic salmon. Dual immersion of alevins in ATD at a concentration of 0.5 mg L^{-1} commencing no later than 7 DPMH will provide a commercially effective rate of masculinisation. Efficacy may be further improved by reducing the inter-bath interval. If the hypothesis that it is the reduction of estrogen causing masculinisation following AI treatments is correct, then treatment with AI could be improved by application during the period of maximum sensitivity to E_2. This remains to be determined for Atlantic salmon, but is probably earlier than 7 DPMH.

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Chapter 5

Uptake and retention of aromatase inhibitors by Atlantic salmon alevins following immersion treatments.

Nothing endures but change.

Heraclitus

5. Uptake and retention of aromatase inhibitors by Atlantic salmon alevins following immersion treatments.

5.1 Introduction

The responsible and cost-effective use of drugs in aquaculture relies on the implementation of treatment regimes that optimise efficacy and dose. A number of readily controlled variables relating specifically to the administration of the drug may be considered in the development of such a regime, including drug selection, dose, frequency and mode of treatment (Allen and Hunn, 1986; Piferrer, 2001). Additionally, operational considerations such as worker safety, ease of implementation, environmental issues and marketing consequences may also be taken into consideration. Many of these issues are addressed in the "minimum intervention approach" proposed by Piferrer (2001) specifically for the development of protocols for steroid hormone treatment of new finfish species in aquaculture. However, the principles of this approach including the determination of the appropriate treatment period and progressive reduction in dosage and treatment duration over repeated experiments, could readily be applied to any drug treatment in aquaculture, to yield the desired outcomes of high effectiveness, while minimising treatment duration, dosage, cost, environmental impact, operator risk and side effects(Piferrer, 2001).

Drug treatment of fishes is primarily undertaken via either the feed, by immersion treatment or by injection. Although the latter method is widely used for some vaccine administration, particularly for high value species such as salmonids, mass treatment by the former two methods is preferred for reasons of cost, ease of delivery and worker safety (Pandian and Sheela, 1995). Dietary treatment may be undertaken over weeks or months, but due to differential feed intake between members of a population, may result in variable doses between individuals (Pandian and Sheela, 1995). In contrast, immersion treatment is usually relatively brief (< 1day) (Pandian and Sheela, 1995; Piferrer, 2001) and offers significant advantages over dietary delivery in relation to precision of dose and surety of delivery of similar doses across a treatment group (Pandian and Sheela, 1995).

Treatment by immersion relies on the uptake of drugs by fishes via gills and skin and, as most drugs are not taken up by active membrane transport, their uptake is a direct function of lipid solubility (Hunn and Allen, 1974; Allen and Hunn, 1986). Larval fish may also accumulate high levels of lipid soluble compounds in yolk or oil reserves, effectively extending exposure to the treatment agent beyond the immersion period (Piferrer and Donaldson, 1994).

Data on drug uptake and retention are essential for the development of an optimum treatment protocol and can contribute to the implementation of a minimum intervention strategy for treatment. Steroid hormones, widely studied for the control of sex differentiation of fishes, are taken up by the diet (Hishida, 1965; Johnstone et al., 1983) or via the gills in immersion treatment (Piferrer and Donaldson, 1994; Specker and Chandlee, 2003). Although immersion treatment may be applied to all developmental stages of fish, including pre-hatching, the efficiency of uptake does change with developmental stage treatment (Piferrer and Donaldson, 1994; Specker and Chandlee, 2003), and this should be taken into consideration in the development of a treatment protocol. Knowledge of drug pharmacokinetics is necessary for the development of a multi-dosage regime, as the optimum timing of subsequent doses will be a function of drug half-life (Notari, 1980). Additionally, clearance data are necessary for cultured animals intended for human consumption.

The application of treatments for the manipulation of sex differentiation is one area of fish endocrinological research and production where drugs are routinely used. Historically, steroid hormones were used for studies of sex manipulation, having been identified by Yamamoto (1969) as the agents responsible for fish sex differentiation. More recently, an alternative approach to the control of sex inversion in fishes has been developed using drugs that interfere with either steroid synthesis or cellular reception of steroids. To date, sex inversion has most successfully been achieved using aromatase inhibitors (Piferrer et al., 1994; Guiguen et al., 1999; Kitano et al., 2000; Kwon et al., 2002).

Aromatase inhibitors (AI) block the synthesis of estrogens from androgens, through action on the aromatase (cytochrome $P450_{arom}$) enzyme, and are used extensively in human medicine for the treatment of estrogen–dependent tumors

(Brodie et al., 1999; Brueggemeier, 2001). Many aromatase inhibitors have been developed, and these may be categorised by mode of action, into two main groups. Mechanism-based or suicide AI are compounds structurally based on androstenedione, and which bind irreversibly to the aromatase enzyme complex (Cole and Robinson, 1990; Numazawa and Tachibana, 1997; Brodie et al., 1999; Brueggemeier, 2001; Séralini and Moslemi, 2001). A second class of compounds, competitive AI, bind reversibly with aromatase in dynamic competition with natural substrates (Cole and Robinson, 1990; Brueggemeier, 2001; Séralini and Moslemi, 2001). Competitive AI may be steroidal or non-steroidal in nature, (Brueggemeier, 2001) with non-steroidal imidazole and triazole derivatives showing the greatest clinical efficacy (Cole and Robinson, 1990; Santen et al., 1990; Buzdar, 2000; Brueggemeier, 2001; Séralini and Moslemi, 2001). In addition to the many human studies, competitive and mechanism-based AI have been shown to be effective in reptiles (Richard-Mercier et al., 1995; Wennstrom and Crews, 1995), amphibians (Petrini and Zaccanti, 1998; Chardard and Dournon, 1999), birds (Elbrecht and Smith, 1992; Foidart et al., 1994) and fishes (Piferrer et al., 1994; Guiguen et al., 1999).

Based on their molecular structure, different AI would have a predicted range in lipid solubility and hence potential uptake capacity by alevins. In particular, the uptake of compounds carrying a charge would be expected to be poor, while in general, that of steroidal compounds should be relatively high. Both fadrozole and OHA displayed poor efficacy for sex inversion of Atlantic salmon (Chapter 4), which contrasted markedly with high *in vitro* efficacy (Chapter 3) and this anomaly may be due to poor uptake and/or retention of these compounds by alevins. The high efficacy of ATD *in vitro* was supported by moderate *in vivo* efficacy, although optimisation of the treatment protocol could further increase efficacy and potentially enable dosage to be reduced. Determining the levels of uptake, and retention profiles for ATD, OHA and fadrozole, following their administration to Atlantic salmon alevins by immersion would provide data to address both of these issues.

The technique of direct measurement by gas or liquid chromatography (GC and LC respectively) in combination with mass spectrometry (MS) may be applied to the measurement of a wide range of compounds in biological materials (Throck,

1985), although detection levels may limit the application of this method in some instances. Ackermann and Kaiser (1989) developed a method for the determination of fadrozole in plasma and urine using GC/MS which enabled detection of fadrozole to a lower limit of 0.2 $\mu g\ mL^{-1}$ in extracted samples. However, the authors also noted problems in the chromatography, in particular the occurrence of 'ghost' peaks, where a proportion of the sample was retained in the column and eluted with subsequent samples. On this basis, LC was preferred for the present study. The technique of tandem mass spectrometry (MS/MS) was developed to enable the detection of compounds against a complex chromatographic background (Throck, 1985) and therefore applicable to less purified samples. It relies on the use of two mass spectrometers operating in series, with only selected ions from the first spectrometer passed on to the second. The selection and subsequent analysis of only those ions arising from the target compound, results in a final spectrum of limited molecular species, and consequently higher sensitivity than standard MS (Throck, 1985). Radioimmunoassay (RIA) is a highly sensitive means for the determination of a range of compounds, in pg quantities, from animal tissues or fluids (eg. Crain et al., 1995; Navarro et al., 1995; Pankhurst and Riple, 2000; Lokman et al., 2002) and its use is well established in endocrinological research. Heterologous RIA relies on displacement of labeled tracer by compounds that have enough structural similarity to the homologous ligand to cross-react with the antibody. This method has been applied to measure compounds where specific antibodies or labeled tracers are not available and a level of sensitivity in excess to that achievable with chromatography is required (e.g. Crain et al., 1995; Navarro et al., 1995). The steroidal structure of both ATD and OHA and low dosage regime used for these AI (Chapter 4) made analysis by RIA appropriate for these compounds.

Accordingly, alevins from Experiments 1 and 2 in the study described previously (Chapter 4) were sampled at the completion of immersion treatment and for up to 7 days post-immersion. Tissue levels of ATD or OHA were determined by heterologous RIA based on a testosterone assay and tissue levels of fadrozole were determined by LC MS/MS.

5.2 Materials and Methods

5.2.i Stock and Husbandry

Experiments were conducted at Saltas' two hatcheries, at Wayatinah, Tasmania on the Derwent and Florentine Rivers (respectively "Wayatinah" and "Florentine"). Both hatcheries, utilise intensive recirculation systems for the production of fry (<0.5 g) and natural river waters for the production of parr and smolts. Experiments were conducted over two years (2000/01 and 2001/02) using the progeny of 2+ year old Atlantic salmon broodstock. Sex inverted female fish ("neomales") were used to provide milt in both experiments. These stocks had been masculinised by dietary treatment with 17α-methyldihydrotestosterone (MDHT) at 1 mg kg^{-1} feed, for 800 °C days (DD), following methods similar to Solar *et al.* (1984). Fertilisation and incubation was conducted as described previously (Chapter 4).

5.2.ii Immersion protocol

Immersion treatments were undertaken as described previously (Chapter 4).

5.2.iii Aromatase inhibitor uptake and retention

Alevin samples were collected for studies of AI uptake and retention, commencing at the completion of the immersion period, and at 4.5, 9, 24, 72, 168 and 240 hr post-immersion. Six individuals were collected with forceps, blotted dry on a paper towel, and placed either as a group (Experiment 1) or singly (Experiment 2) in Eppendorf tubes and frozen (-18 °C) until analysis. Samples were thawed, separated where necessary, and individuals were homogenised separately in Eppendorf tubes using a hand-held teflon tissue grinder. Individuals from Experiment 1 that could not be separated intact were discarded.

5.2.iv Experiment 1: 2000-01

Of the four AI used, as described in Chapter 4, tissue levels were determined only for : 1,4,6-androstatriene-3,17-dione (ATD) and 4-androsten-4-ol-3,17-dione (OHA). For each dose of AI, groups of 1000 alevins were treated either once, at 14 DMPH, or twice, at 14 and 28 DMPH.

5.2.v Experiment 2: 2001-02

Two AI were used: ATD and 4-[5,6,7,8-tetra-hydroimidazo-(1,5-α)-pyridin-5-yl] benzonitrile HCl (Fadrozole), at 500 μg L^{-1} and 5 mg L^{-1} , and at 10 and 50 mg L^{-1} respectively. For each dose of AI, groups of 1000 alevins were treated twice, either at 7 and 14 DMPH ("Early" immersion treatment), or 14 and 21 DMPH ("Late" immersion treatment).

5.2.vi ATD and OHA assay

ATD and AOD levels in tissue were determined by heterologous RIA based on the displacement of T by the AI in a T RIA system. Fish in 1.5mL Eppendorf tubes were homogenised with a hand held teflon grinder in 500 μL ethyl acetate, the tubes were then centrifuged at 1200 *g*, for 10 minutes, 150 μL aliquots of the supernatant added to duplicate polypropylene tubes and left overnight to allow evaporation of the solvent. Extracts were assayed for T using the methods of Pankhurst (1997), detailed in Chapter 3. Cross reactivity of ATD and OHA with the T antiserum were 0.64 and 0.13% respectively and minimum detection levels were 0.03 ng $fish^{-1}$ and the minimum detection level of T in controls was 0.03 pg $fish^{-1}$. Interassay variability was measured using aliquots of a pooled internal standard and was (%CV[n]) 14.5[12] and 12.4[9] for assays for Experiments 1 and 2 respectively. Assay performance characteristics are described in the results.

5.2.vii Fadrozole assay

Fadrozole levels were determined by LC MS/MS, following extraction based on the methods of Ackermann and Kaiser (1989). Fish were homogenised using a hand-held teflon grinder, in 800μL 0.01M HCl, and 100 μL of 500 ng mL^{-1} miconazole, used as an internal standard. Tube contents were then transferred to a 20 mL glass scintillation vial with 4 mL diethyl ether, 2mL pH 10 buffer (Ackermann and Kaiser, 1989), vortexed and allowed to stand for 5 minutes. The homogenate was then frozen in a dry ice/methanol bath, the organic phase transferred to a clean 20 mL glass scintillation vial, 1mL 0.1M HCl added, vortexed and allowed to stand for 5 minutes. The homogenate was again frozen in

a dry ice/methanol bath, the organic phase discarded and the aqueous phase transferred to a 2 mL glass vial and frozen (-18 °C) before assay by LC MS/MS.

Fadrozole levels were determined using a Waters Xterra MS C18 5 micron column (2.1mm x 150mm.) on a Waters Alliance HPLC, coupled to a Finnigan LCQ mass spectrometer fitted with an Atmospheric Pressure Chemical Ionization (APCI) source. The HPLC was operated isocratically with a mobile phase of methanol and 2% acetic acid (9:1 v/v) at a flow rate of 0.4 mL min^{-1}. The APCI conditions were: nitrogen sheath gas 35 psi; source current 5 μA; capillary temperature 150 °C; vaporiser temperature 470 °C. Sample volume was 100 μL and trap fill time was 150 msec. Samples were analysed by selection reaction monitoring of product ions from the protonated molecular ions. For fadrozole, the fragmentations *m/z* 224→81, *m/z* 224→82 and *m/z* 224→156 were monitored using an isolation width of 4 daltons and collision energy of 20%. For miconazole the product ions at *m/z* 159 &161 from the molecular ion cluster starting at *m/z* 415 were monitored; the isolation window was centred at 417 with a width of 8 daltons, and the collision energy was 23%.

Standard curves were constructed for each run, using untreated fish spiked with 100 μL fadrozole in 0.01M HCl, to produce concentrations of 500, 250, 125 and 25 ng $fish^{-1}$ and the minimum detection level was 25 ng $fish^{-1}$.Three replicate fish were used for each concentration of fadrozole. A separate standard curve was constructed for the determination of residual levels of fadrozole in bath water after immersion.

5.2.viii Statistical analyses

Aromatase inhibitor uptake and retention data failing Cochrans test for homoscedasticity were $\log_{10} + 1$ transformed and analysed by one way ANOVA for each sampling period. Mean comparisons were made by a Tukeys honestly significant difference test using harmonic mean sample size for analyses where sample sizes were uneven (Day and Quinn, 1989). Regression curve slopes of AI standard curves and extract serial dilution were compared using t tests (Zar, 1984). All analyses were performed using SPSS 10.1 for Windows.

5.3 Results

5.3.i RIA validation

Standard curves in the range 100 – 3200 pg mL^{-1} and 200 – 12800 pg mL^{-1} were constructed for ATD and OHA respectively. The slopes of standard curves were not different to serially diluted extract for either ATD (t_4=0.170, p>0.05, Figure 5.1a) or OHA (t_5=0.027, p>0.05, Figure 5.1b).

5.3.ii Fadrozole assay validation

Chromatographs produced following the LC MS/MS analysis (Figure 5.2) had a single, well formed peak, with no evidence of sample retention in the column as reported previously for fadrozole determination using gas chromatography (Ackermann and Kaiser, 1989). Two measures of the amount of fadrozole could be obtained from the data, total area under the fadrozole curve, and the ratio of areas of the fadrozole and internal standard (miconazole) curves. Standard curves constructed using alevins spiked with fadrozole showed less variability when the ratio data were used (Figure 5.3) and this was subsequently used in all calculations.

5.3.iii Experiment 1: 2000-01

Tissue levels of ATD increased significantly with increasing dose (Figure 5.4a); however, the proportion of ATD taken up from the bath declined with increasing dose from 100% uptake for treatment at 4, 20 and 100 μg L^{-1} to 62% for treatment at 500 μg L^{-1} (Table 5.1). Within 4.5 hr of the completion of the first immersion treatment, tissue levels in alevins treated at a dose of 4 μg L^{-1} had declined to those of controls, whereas all other doses were significantly different to each other and controls (Figure 5.4a). After 9 hr, alevins treated at 500 μg L^{-1} had higher tissue levels than all other treatments, whereas there was no difference between tissue levels in alevins treated at 20 or 100 μg L^{-1}, and treatment at 4 μg L^{-1} did not elevate tissue levels above control levels (Figure 5.4a). As only a single control sample was available at 24 hr post-treatment, this was excluded

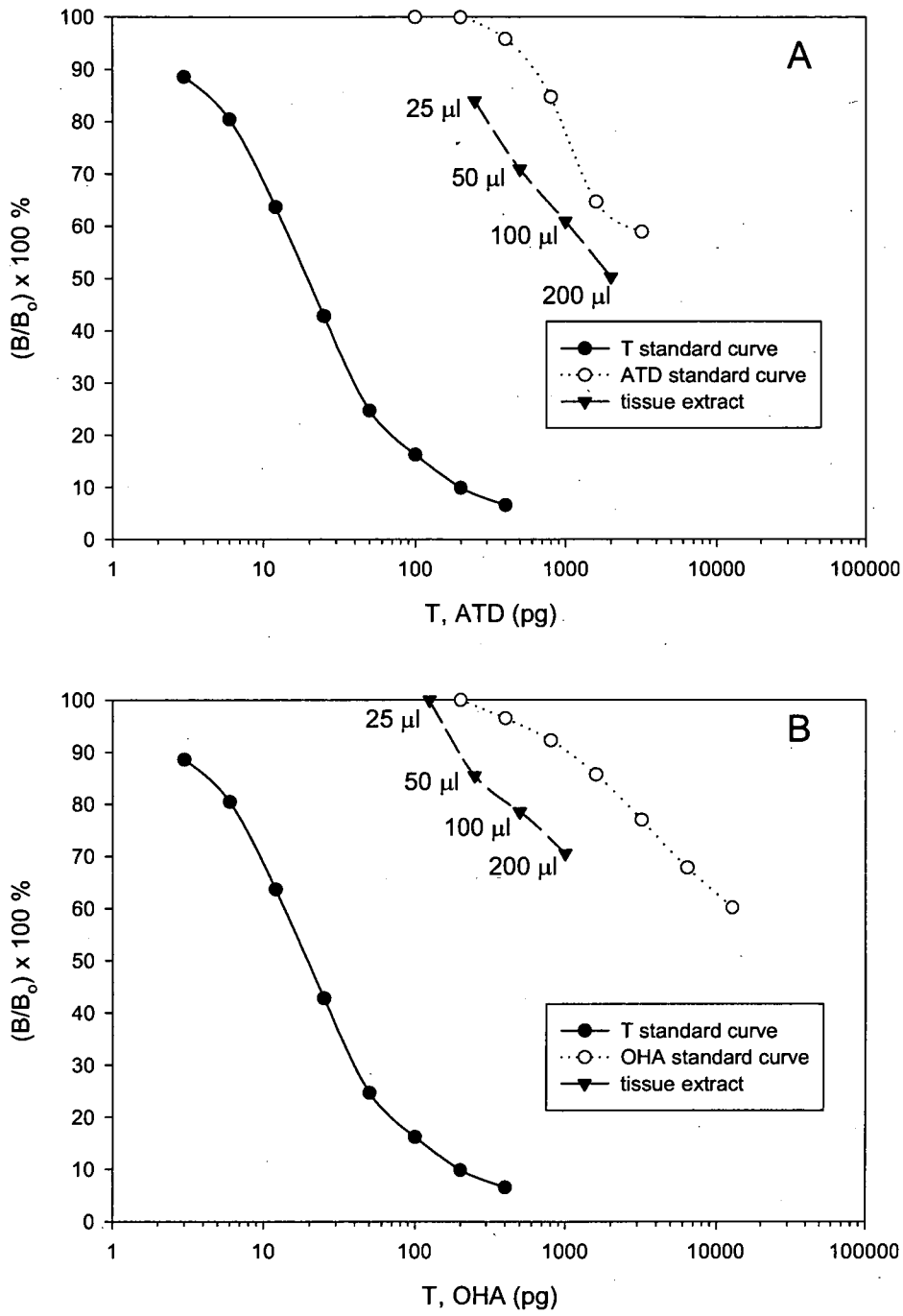

Figure 5.1 RIA binding curves for a) ATD and b) OHA showing displacement curves for T, ATD and OHA standards, and serial dilutions of tissue extract, in assays using ^{3}H-T tracer and a T antibody.

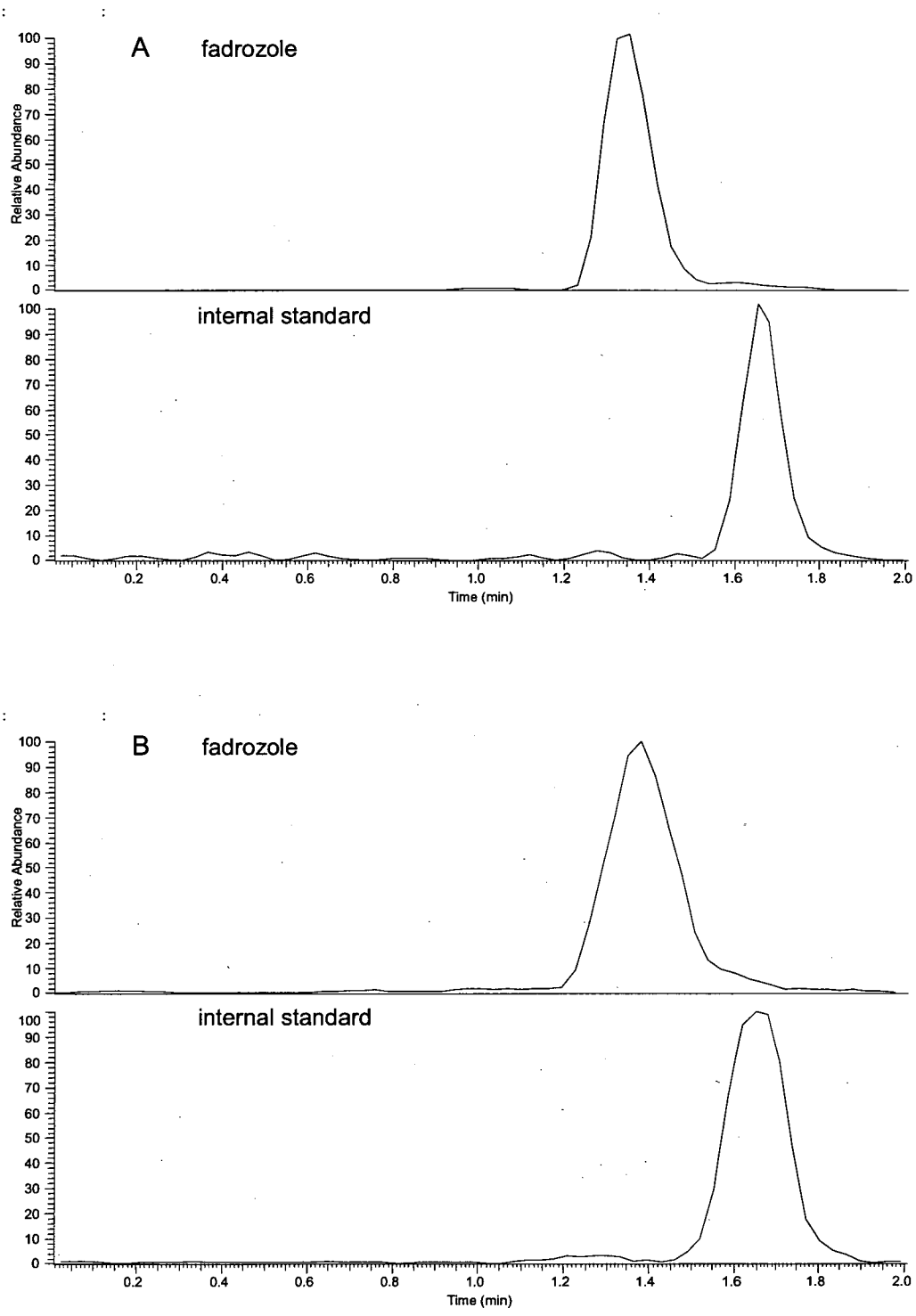

Figure 5.2 Chromatograms of fadrozole and internal standard (miconazole) from a representative a) standard (alevin spiked with 500ng fadrozole) and b) sample (treated with fadrozole at a dose of 50 mg L^{-1}).

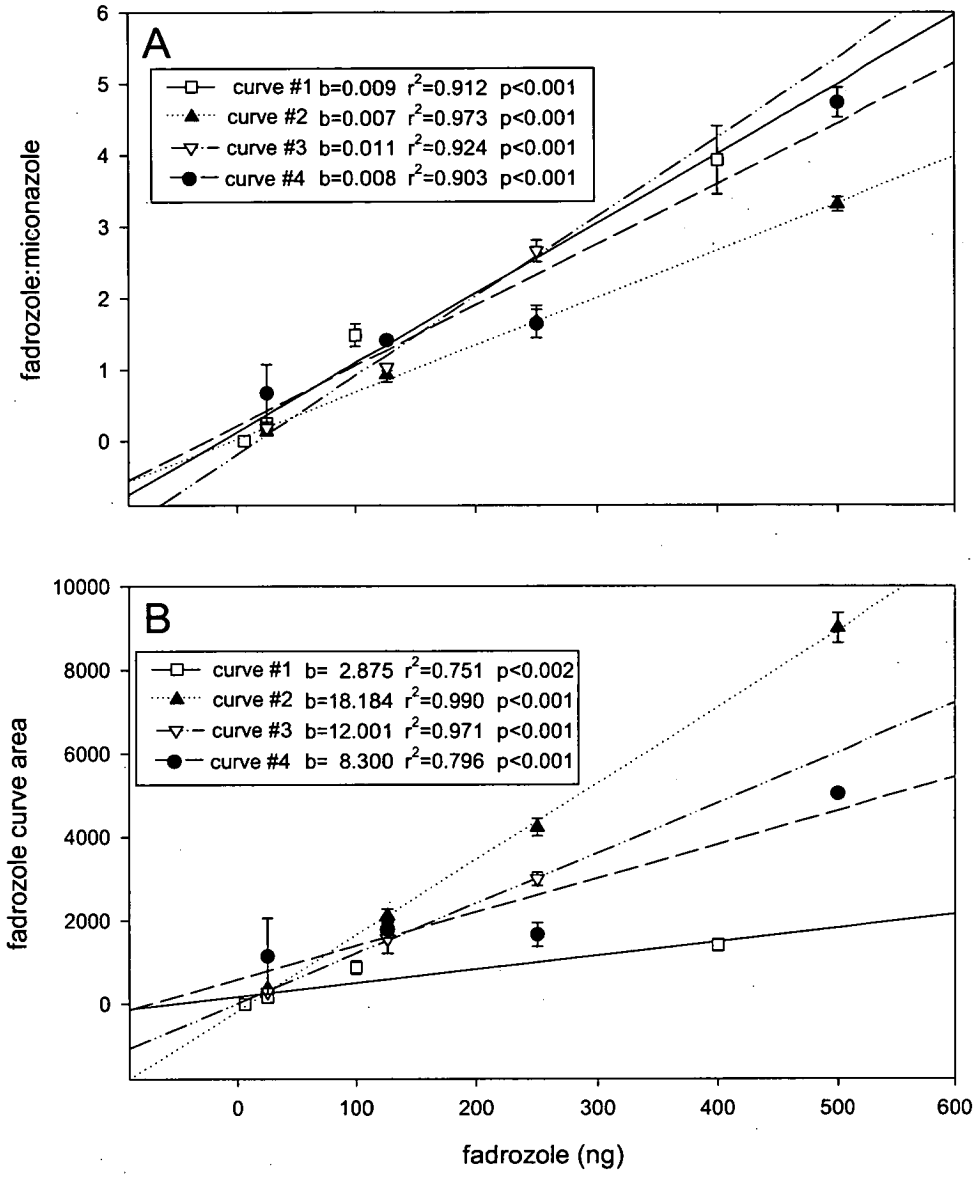

Figure 5.3 Standard curves constructed using alevins spiked with fadrozole, with miconazole as an internal standard. Values are a) fadrozole curve area:miconazole curve area and b) fadrozole curve area. Legends indicate slope (b), correlation coefficient (r^2) and regression significance value (p), determined by ANOVA for each curve. Values are mean ± SE (n=3).

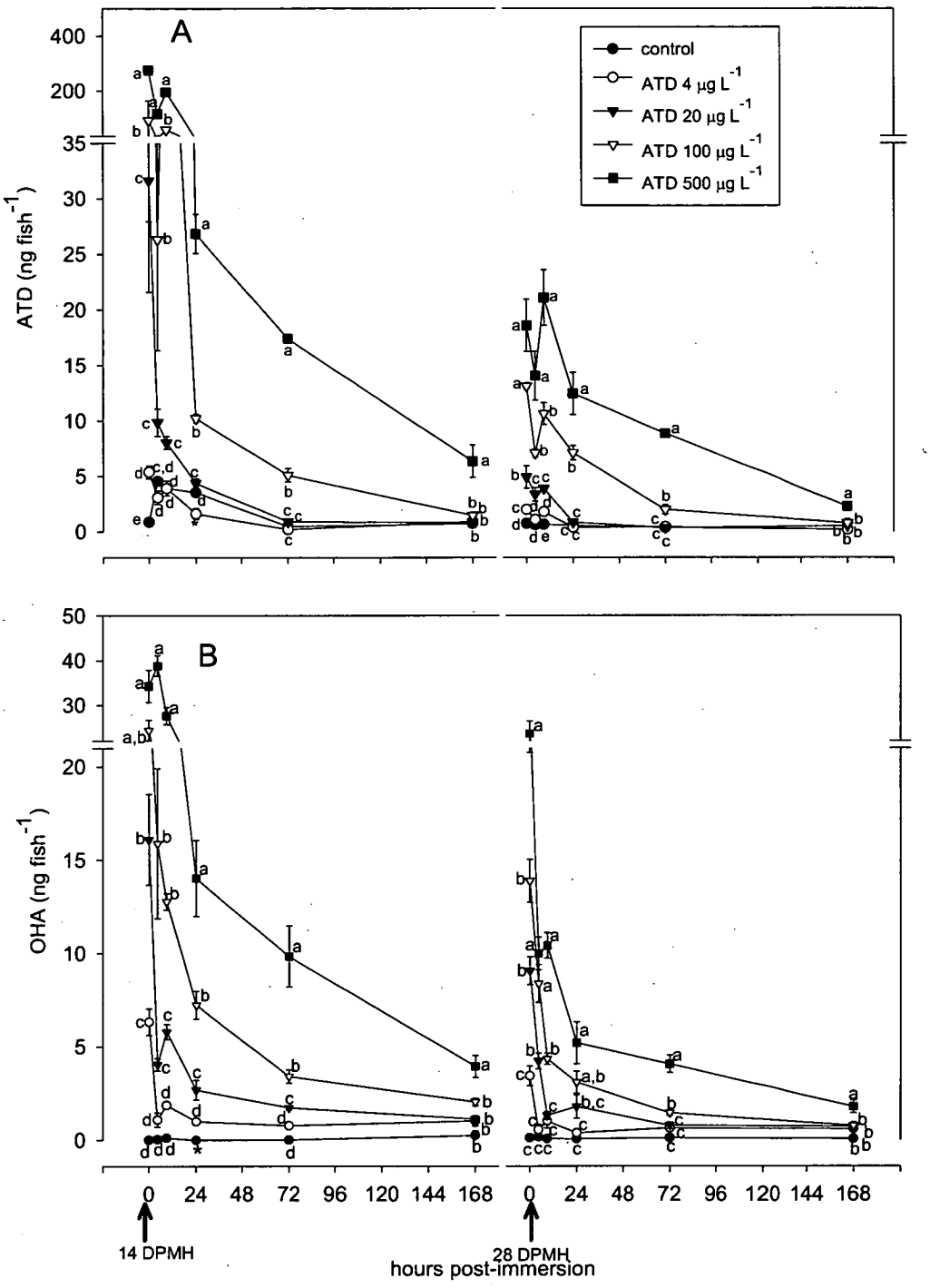

Figure 5.4 Tissue levels of a) ATD and b) OHA in Atlantic salmon alevins following 120 minutes immersion in solutions of AI of 4, 20, 100 or 500 µg L^{-1}, on 14 and 28 DPMH (arrows). Values are mean ± S.E. (n=3-5). For each sample time, comparisons are made between doses, and values sharing superscripts are not significantly different ($p>0.05$).

* n=1

Table 5.1 Uptake and tissue levels (mean ± S.E.) of the AI ATD and OHA by Atlantic salmon alevins, following 120 min immersion in solutions of AI at 4, 20, 100 or 500 μg L^{-1}.

	1st bath – 14 DPMH		2nd bath – 28 DPMH	
AI dose	Tissue level (ng $fish^{-1}$)[a]	Uptake from bath (%)	Tissue level (ng $fish^{-1}$)[a]	Uptake from bath (%)
Control	1.2 ± 0.3	-	0.7 ± 0.1	-
ATD 4 μg L^{-1}	5.4 ± 0.6	100[b]	2.0 ± 0.1	36
ATD 20 μg L^{-1}	31.6 ± 3.7	100[b]	4.9 ± 1.0	24
ATD 100 μg L^{-1}	92.5 ± 7.1	100[b]	13.1 ± 0.2	14
ATD 500 μg L^{-1}	275.1 ± 10.7	62	18.6 ± 2.3	4
Control	0.1 ± 0.0	-	0.6 ± 0.1	-
OHA 4 μg L^{-1}	6.3 ± 0.7	100[b]	3.4 ± 0.5	81
OHA 20 μg L^{-1}	16.1 ± 2.4	91	9.1 ± 0.7	48
OHA 100 μg L^{-1}	24.4 ± 2.3	28	13.9 ± 1.2	15
OHA 500 μg L^{-1}	34.3 ± 3.6	8	23.6 ± 2.8	5

[a] mean ± S.E, n=3-6
[b] calculated value >100%

from analysis. Tissue levels at this time were different among all AI doses. At 3 days post-treatment, there were no differences between treatment at 4 or 20 μg L^{-1} and controls, whereas tissue levels were still significantly elevated in alevins treated at 500 and 100 μg L^{-1} (Figure 5.4a). After 7 days, only alevins treated at 500 μg L^{-1} had tissue levels above controls. Regardless of dose, tissue levels declined by approximately 50%, within 9 hours and 90% within 24 hours of the immersion treatment (Figure 5.4a).

Tissue levels of ATD immediately following the second immersion at 28 DPMH were significantly higher than controls for all doses; however, levels were an order of magnitude lower than those achieved following the first immersion treatment (Figure 5.4a). This was also reflected in reduced uptake rates, ranging from 36% for 4 $\mu g\ L^{-1}$ to 4% for a dose of 500 $\mu g\ L^{-1}$ (Table 5.1) .Tissue levels in alevins treated at 500 $\mu g\ L^{-1}$ were no different from those treated at 100 $\mu g\ L^{-1}$ (Figure 5.4a). After 4.5 hours, tissue levels in alevins treated at 4 $\mu g\ L^{-1}$ were not different to controls; however, tissue levels were different among all other doses. There were differences in tissue levels between all doses at 9 hours post-treatment, whereas after both 24 hours and 3 days, only those alevins treated at 100 or 500 $\mu g\ L^{-1}$ had tissue levels higher than controls (Figure 5.4a). As with the first treatment, only alevins treated at 500 $\mu g\ L^{-1}$ had elevated tissue levels 7 days after treatment (Figure 5.4a).

Trends in the uptake and retention of OHA were similar to those of ATD; however, uptake rates and tissue levels were substantially lower than those for ATD, particularly following the first immersion treatment (Figure 5.4b). Uptake rates declined with increasing concentrations of OHA from 100% for 4 $\mu g\ L^{-1}$ to 8% for 500 $\mu g\ L^{-1}$. Immediately following immersion, tissue levels of OHA in all alevins exposed to OHA were higher than controls, with levels in alevins treated at 500 $\mu g\ L^{-1}$ higher than those treated at 20 or 4 $\mu g\ L^{-1}$, but not 100 $\mu g\ L^{-1}$, and there was no difference in tissue levels between alevins treated at 100 or 20 $\mu g\ L^{-1}$. Similarly , after 4.5 and 9 hours, and 3 days , there were differences between all treatment groups, except 4 $\mu g\ L^{-1}$ and controls (Figure 5.4b). Tissue levels in all treatment groups differed 24 hr post-treatment; however, as for ATD, no comparisons were possible with controls due to low sample size (n=1) (Figure 5.4b). After 7 days, only those animals treated with OHA at 500 $\mu g\ L^{-1}$ had tissue levels higher than controls (Figure 5.4b).

Tissue levels following the second immersion treatment with OHA were lower than for the first treatment, but not to the same degree as occurred for ATD (Figure 5.4b) with uptake rates of 81% for a dose of 4 $\mu g\ L^{-1}$ to 4% for 500 $\mu g\ L^{-1}$. Alevins treated at 500 $\mu g\ L^{-1}$ has tissue levels higher than all other doses, whereas there was no difference in tissue levels between alevins treated at a dose of 20 or

100 μg L^{-1} and treatment at 4 μg L^{-1} did not elevate tissue levels above controls (Figure 5.4b). After 4.5 hr, there was no difference in tissue levels between alevins treated at 100 or 500 μg L-1, although these levels were higher than for all other treatments. At 9 hours post treatment, tissue levels in alevins treated at 20 μg L^{-1} had declined to those of controls and alevins treated at 4 μg L^{-1} , whereas tissue levels were significantly higher in alevins treated at 100 and 500 μg L^{-1}. After 24 hr and 3 days, only alevins treated at 100 or 500 μg L^{-1} had tissue levels greater than those of controls, and by 7 days post-treatment tissue levels were only elevated in the 500 μg L^{-1} treatment (Figure 5.4b).

5.3.iv Experiment 2: 2001-02

Uptake of ATD immediately following immersion did not differ with dose, but exceeded control levels for all immersion treatments. Consequently the uptake rate following treatment with ATD at 5 mg L^{-1} was substantially lower than that for treatment at 0.5 mg L^{-1} (Table 5.2). For the early immersion regime, treatment with ATD at 0.5 or 5 mg L^{-1} at 7 DPMH elevated tissue levels of ATD above control values immediately after immersion and at 4.5, 9 and 24 hr post-treatment, and differences due to dose only occurred at 4.5 hr (Figure 5.5a). At 3 days post-treatment, a dose of 5mg L^{-1} increased tissue levels above controls, but a dose of 0.5 mg L^{-1} did not and after 7 days there were significant differences between both doses of ATD and controls (Figure 5.5a). Immediately following a second immersion treatment at 14 DPMH tissue levels of alevins treated with a dose of 0.5 or 5 mg L^{-1} were greater than controls; however, after 4.5hr, there was no difference between alevins treated at 0.5 mg L^{-1} and controls, but treatment at 5 mg L^{-1} maintained elevated tissue levels. At 9 hr post-treatment, tissue ATD levels differed between both doses of ATD and controls whereas after 24 hr, ATD treated alevins had increased tissue levels of ATD, but this did not differ between doses (Figure 5.5a). After 7 days, tissue levels began to increase in alevins treated at 5 mg L^{-1}, and these remained higher than both alevins treated at a dose of 0.5 mg L^{-1} and controls, up to 10 days post-treatment, where animals treated at 0.5 mg L^{-1} had tissue ATD levels elevated above controls at 7 and 10 days, but not 3 days post-treatment (Figure 5.5a). Tissue levels in alevins treated at a dose of 0.5 mg L^{-1} declined to approximately 50% of initial levels after 7 days for both the first and

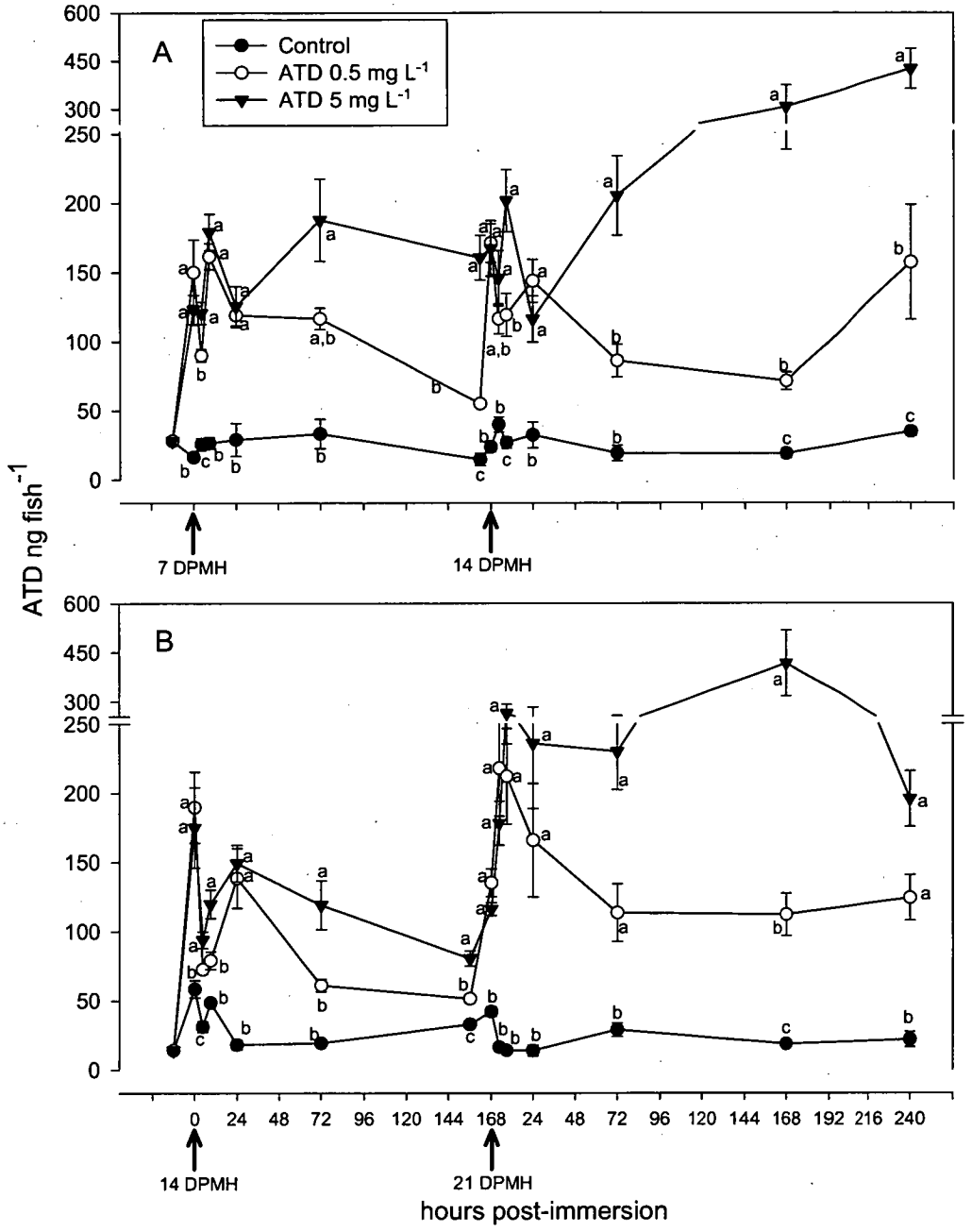

Figure 5.5 Tissue levels of ATD in Atlantic salmon alevins, following 120 minutes immersion in solutions of ATD at 0.5 or 5 mg L^{-1} at a) 7 and 14 DPMH (arrows) or b) 14 and 21 DPMH (arrows) Values are mean ± S.E.[1] Other details as for Figure 5.4.

[1]Control n=3; ATD treatments n=6

second immersion treatments, whereas alevins treated at a dose of 5 mg L^{-1} showed no decline, and appeared to be increasing up to 10 days after the second treatment.

Table 5.2 Percentage uptake and tissue (mean ± S.E.) levels of the AI ATD by Atlantic salmon alevins, following 120 min immersion in solutions of ATD at 0.5 or 5.0 mg L^{-1}, at either 7 and 14 or 14 and 21 DPMH.

	1st bath – 7 DPMH		**2nd bath – 14 DPMH**	
AI dose	**Tissue level (ng $fish^{-1}$) [a]**	**Uptake from bath (%)**	**Tissue level (ng $fish^{-1}$) [a]**	**Uptake from bath (%)**
Control	16.3±2.4	-	23.±3.3	-
ATD 0.5 mg L^{-1}	150.1±23.6	35%	171.0±14.1	38%
ATD 5.0 mg L^{-1}	123.0±10.7	3%	167.2±20.1	4%
	1st bath – 14 DPMH		**2nd bath – 21 DPMH**	
Control	58.5±6.3	-	42.1±3.5	-
ATD 0.5 mg L^{-1}	189.7±25.6	32%	135.1±10.0	17%
ATD 5.0 mg L^{-1}	175.1±29.0	3%	116.1±4.7	1%

[a] mean ± S.E., control n=3; ATD 0.5 and 5 mg L^{-1} n=6

Immersion treatment utilising the "late" treatment regime resulted in similar tissue levels as the second treatment of the early regime, also conducted at 14 DPMH; however, subsequent trends in retention were different (Figure 5.5b). Tissue levels of ATD in alevins treated with ATD at 0.5 or 5 mg L^{-1} declined to approximately 50% of initial levels 7 days post-treatment. Tissue levels were greater than controls, but not different between ATD treated groups immediately after treatment; however, after 4.5 hr, alevins treated at 0.5 mg L^{-1} had lower levels than those treated at 5 mg L^{-1}, but higher than controls (Figure 5.5b). At 9 hr post-treatment, treatment at 5mg L^{-1} resulted in higher tissue levels than either treatment at 0.5 mg L^{-1} or controls, whereas after 24 hr both ATD treated groups were higher than the controls (Figure 5.5b). After 3 days, alevins treated at 5 mg L^{-1} had higher tissue levels than alevins treated at 0.5 mg L^{-1} and controls and by

7 days post-treatment, there were differences between all treatment groups (Figure 5.5b).

Following the second immersion treatment of the late treatment regime, the trend in retention was similar to that of the early regime, in that tissue levels of ATD treated alevins stayed at or above uptake levels (Figure 5.5b). Both ATD treated groups maintained similarly higher tissue levels than controls immediately after treatment and for 4.5, 9, 24 hr and 3 days post-treatment (Figure 5.5b). At 7 days post-treatment tissue levels in alevins treated at 5 mg L^{-1} were higher than those treated at 0.5 mg L^{-1} which were higher than controls, whereas after 10 days, there was do differences between the ATD treated groups, but both had levels greater than controls (Figure 5.5b).

Uptake of fadrozole was less than 5% for all immersion periods and doses (Table 5.3). In contrast to the data for ATD, the trends of retention of fadrozole by alevins were similar for both first and second immersion treatments, for both early and late regimes (Figure 5.6). In all cases, tissue levels declined after immersion, reducing to approximately 50% of uptake levels by 3 days post-treatment. Tissue levels in alevins treated with fadrozole remained greater than control levels up to 3 days- post treatment, with levels in animals treated at 50 mg L^{-1} greater than those in alevins treated at 10 mg L^{-1} (Figure 5.6). In the early treatment groups, 7 days after both the first and second immersion treatments, only animals treated at 50 mg L^{-1} had elevated tissue levels, whereas in the late group, this was only the case following the second bath (Figure 5.6).

Table 5.3 Percentage uptake and tissue (mean ± S.E.) levels of the AI fadrozole by Atlantic salmon alevins, following 120 min immersion in solutions of fadrozole at 10 or 50 mg L^{-1} at either 7 and 14 or 14 and 21 DPMH.

	1st bath – 7 DPMH		2nd bath – 14 DPMH	
AI dose	**Tissue level (ng $fish^{-1}$) [a]**	**Uptake from bath (%)**	**Tissue level (ng $fish^{-1}$) [a]**	**Uptake from bath (%)**
control	ND[b]	-	ND	-
fadrozole 10 mg L^{-1}	210.0 ± 12.6	2%	629.2 ± 42.4	7%
fadrozole 50 mg L^{-1}	945.4 ± 40.2	3%	1974.3 ±4 43.8	5%
	1st bath – 14 DPMH		**2nd bath – 21 DPMH**	
control	ND	-	ND	-
fadrozole 10 mg L^{-1}	330.3 ± 54.9	4%	172.2 ± 10.7	2%
fadrozole 50 mg L^{-1}	996.3 ± 96.1	2%	861.6 ± 49.3	2%

[a] mean ± S.E. n=3

[b] ND not detected

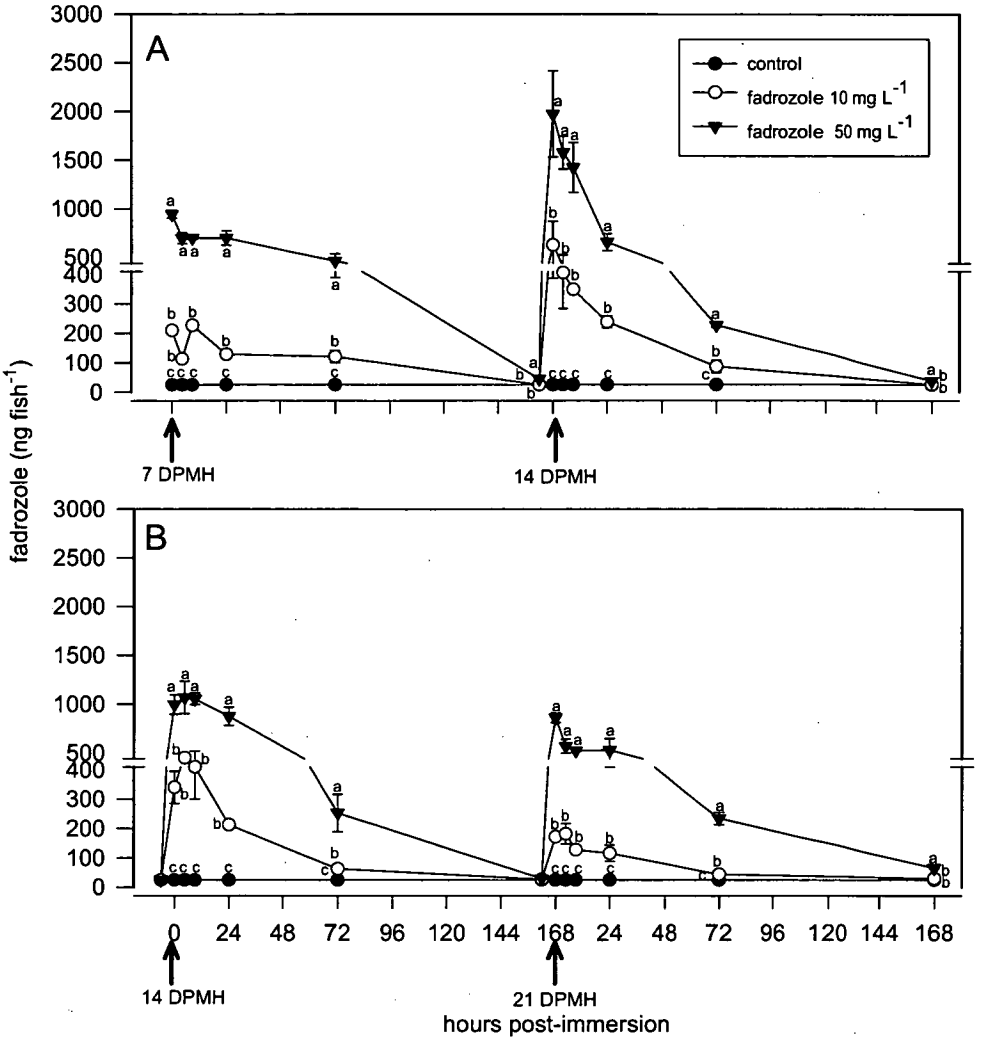

Figure 5.6 Tissue levels of fadrozole in Atlantic salmon alevins, following 120 minutes immersion in solutions of fadrozole at 10 or 50 mg L^{-1} at a) 7 and 14 DPMH (arrows) or b) 14 and 21 DPMH (arrows) Values are mean ± S.E.[1] Other details as for Figure 5.4.

[1]Control n=3; fadrozole treatments n=6

5.4 Discussion

The assay methods developed as part of the present project provide methods for determining tissue levels of ATD, OHA and fadrozole at biologically significant levels. The use of a heterologous RIA provides an effective means of determining biologically significant levels of both ATD and OHA in the tissues of alevins; however the inability of the assay to distinguish the AI from native androgens, may limit the capacity to better elucidate steroidogenic processes post-immersion. Levels of native androgens in control fish in the present study of ca. 8 pg T equivalents $fish^{-1}$ (data not shown) were similar to those reported in salmonid alevins of a similar age (Feist et al., 1990; Feist and Schreck, 1996) and androgen levels of rainbow trout and coho salmon alevins declined for up to 40 days post-hatch (Feist et al., 1990; Feist and Schreck, 1996), indicating that the potential for interference with the assay by native androgens is low. Nonetheless, the addition a separation step following the extraction of steroids in ethyl acetate may provide a means of determining the presence of androgens and AI, separately, using the T RIA.

The use of HPLC for fadrozole separation is a significant change from previous studies, where the use of gas chromatography was associated with some problems, including 'ghosting' (Ackermann and Kaiser, 1989; Schneider et al., 1996). Similar problems were not apparent with HPLC separation in the present study. Although the level of sensitivity of the fadrozole assay used in the present study was less than that of Ackermann and Kaiser (1989), significant levels of fadrozole were detectable in the tissues of single alevins up to 7 days after immersion indicating suitability of the assay for such studies.

The uptake and retention data for Atlantic salmon alevins treated with AI by immersion are similar to those obtained by Piferrer and Donaldson (1994) for coho salmon, *Oncorhynchus kisutch* immersed in solutions of T and E_2. These data confirm the capacity of alevins to incorporate substantial levels of steroidal compounds and retain these in the tissues well beyond the immersion period, as proposed by Hunter and Donaldson (1983), and demonstrated for T and E_2 by

(Piferrer and Donaldson, 1994). The retention of steroids for a significant period following immersion treatment was proposed as a major factor in the efficacy of short duration immersion treatments (2 h) for sex control of salmonids (Piferrer and Donaldson, 1994).

Clearance rates of steroidal AI were similar to those reported previously for steroids in larval fish (Hishida, 1965; Piferrer and Donaldson, 1994; Specker and Chandlee, 2003), with a half life of approximately 24-50 h for steroidal AI. Catabolic byproducts of the AI were not quantified; however, glucuronation of OHA in the liver is known to be a major route of its catabolism in mammals (Cole and Robinson, 1990; Brueggemeier, 2001) and fish are known to be capable of both glucuronation and conjugation of steroids (Fostier et al., 1983; Yeoh et al., 1996). The glucuronated form of the steroidal AI would not have been available to the RIA (Haddy and Pankhurst, 1998) and so this cannot be excluded, although E_2 excretion by larval medaka, *Oryzias latipes*, was predominantly in the free form (Hishida, 1965). Both OHA and ATD show similar rates of clearance, suggesting a similar process to be responsible for their elimination. Regardless of the nature of their clearance, the efficacy of both ATD and OHA as mechanism-based aromatase inhibitors would continue beyond their clearance, due to the slow turnover of aromatase (Séralini and Moslemi, 2001).

At concentrations of 4 - 500 $\mu g\ L^{-1}$, the uptake of steroidal AI in the present study was dependent on the concentration in the medium. This is also the case for the uptake of steroids by coho salmon (Piferrer and Donaldson, 1994) and summer flounder, *Paralithys dentatus*, (Specker and Chandlee, 2003). However, there was no difference in the in the uptake of ATD following the first immersion at a dose of either 5 or 0.5 $mg\ L^{-1}$, suggesting that the levels attained represented maximum concentrations for a 2 h immersion. This suggests that any further increases in uptake would require extending immersion time rather than increasing the AI concentration. The tissue level of ATD of 275 $ng\ fish^{-1}$ achieved in Experiment 1 equates, on the basis of estimated alevin weight of 150 mg (H.R. King, unpublished data), to approximately 6.5 $nmol\ g^{-1}$, comparable to levels of E_2 achieved in coho salmon by Piferrer and Donaldson (1994) of 25 $nmol\ g^{-1}$. Notably, although similar immersion protocols were used in both studies, tissue levels of steroids determined by Piferrer and Donaldson (1994) were based on

retained radiolabel following immersion with labeled steroid, and were recognised by those authors as being an upper estimate, as they potentially included metabolites. In their study of the uptake of E_2 by summer flounder juveniles after 48 h incubation, Specker and Chandlee (2003) recorded E_2 levels of 1.1 nmol g^{-1}, 600% higher than in the immersion solution of 0.2 nM (approximately 50 $\mu g\ L^{-1}$) , indicating a concentrating of E_2 in the tissues. Similar analysis of the data for salmonids from the present study and that of Piferrer and Donaldson (1994) indicates that tissue concentrations of ATD and E_2 reached 400 and 1700% of bath concentrations of 500 and 400 $\mu g\ L^{-1}$ respectively. The capacity to concentrate steroidal compounds to such high levels may be a feature of salmonids, made possible by the large yolk size of alevins. The uptake of aqueous drugs by alevins occurs primarily across the gills (Allen and Hunn, 1986), at a rate determined by physical processes (Hunn and Allen, 1974), and steroids appear to accumulate primarily in the yolk (unpublished observations, cited in Piferrer and Donaldson, 1994). Thus in salmonids the yolk acts as both a steroid 'sink' during immersion, and a reservoir, providing extended delivery of steroid-like compounds, post-immersion.

The reduced level of uptake of ATD between immersion treatments on 14 and 28 DPMH in Experiment 1 in the present study is consistent with the role of yolk as a reservoir for AI, as yolk loss occurred over that time. The absence of similar differences in Experiment 2 may reflect the earlier developmental state of alevins at the time of treatment and the smaller inter-treatment interval, with a smaller loss of yolk over the period between treatments. Although no studies have quantified the uptake of steroids by alevins at different stages of development, the studies of Piferrer and Donaldson (1994) and Specker and Chandlee (2003) compared alevins or larvae respectively with subsequent developmental stages. In contrast to the results of the present study, uptake rates by coho salmon fry after 4 h immersion, were greater than for alevins, whereas maximum concentrations (C_{max}) of T and E_2 after extended immersion were greatest in alevins (Piferrer and Donaldson, 1994). However, as discussed previously, the data of Piferrer and Donaldson (1994) may reflect differences in steroid metabolism between alevins and fry rather than differences in uptake over time. Summer flounder larvae retained higher concentrations of E_2 than juveniles after 4 h immersion; however,

there was a significant increase in tissue levels in juveniles after 24 h immersion, and the authors postulated unidentified processes not related to body lipid levels to be regulating E_2 uptake in juveniles (Specker and Chandlee, 2003). Thus developmental stage of animals has a clear influence on the uptake of steroids, not only in relation to physical uptake capacity but in metabolic processing of steroids, and this should be considered in the development of an immersion protocol.

Reducing the inter-bath interval from 14 to 7 days resulted in changes to the patterns of uptake and retention of ATD in the present study. Tissue levels following the second immersion treatment were greater than those after the first; however, this resulted from elevated tissue levels before treatment, as net uptake was similar (approximately 200 ng $fish^{-1}$) for each immersion treatment. This contrasts with an increase in uptake of E_2 by coho salmon alevins following a second treatment 48 h after the first which was ascribed to either the induction of hormone receptors or saturation of hormone metabolising enzymes (Piferrer and Donaldson, 1994). To achieve similar effects with ATD, the inter-bath interval would probably need to be reduced to near the estimated half life (approximately 24 h), as recommenced by Piferrer and Donaldson (1994).

The substantial difference in uptake between ATD and OHA in Experiment 1 may explain in part the relative efficacy of these compounds *in vivo*. Piferrer and Donaldson (1994) estimated the immersion time to achieve C_{max} of T and E_2 by coho salmon alevins to be in approximately 20 and 50 h respectively. On this basis it is unlikely that maximum uptake of AI would have been reached in 2 h, so the observed differences in alevin content in the present study are likely to reflect differences in the uptake rate. Lipid solubility is a major determinant of drug uptake across fish gills (Hunn and Allen, 1974). Therefore, differences in the lipid solubility between ATD and OHA may result in differential uptake rates. OHA has a highly electronegative hydroxyl group on C-4, making the molecule more polar, and less lipid soluble (Stryer, 1981) than ATD. Although there are no data available on effective tissue levels in fish for the AI tested, *in vitro* data indicate increased efficacy of AI with increasing dose (Chapter 3), and dose dependence of steroids for sex inversion has also been demonstrated (Devlin and Nagahama, 2002). On this basis, the efficacy of OHA could possibly be improved by

increasing the effective dose, either through increased concentration in the bath or by extending the duration of immersion. Alternatively, administration via the feed may be a more effective means of delivery.

Although tissue levels of fadrozole exceeded those of ATD, efficacy was poor, suggesting levels may have been insufficient for aromatase inhibition. Competitive inhibitors, such as fadrozole, are required at high concentrations, due to the low K_m of aromatase (Cole and Robinson, 1990). The relatively low tissue levels achieved in the present study may have been due to poor uptake efficiency of fadrozole which was an order of magnitude lower than that of ATD. Under conditions of low pH, fadrozole monohydrochloride is more water soluble (Ackermann and Kaiser, 1989), and therefore less available for uptake by alevins (Allen and Hunn, 1986). Carbon dioxide is a major waste metabolite produced by fishes and although neither carbon dioxide concentrations nor pH were determined during immersion treatments, carbon dioxide excretion during immersion treatment would be expected to reduce bath pH. The capacity for alevins to affect bath pH was potentially greater in the present study than in that of Piferrer and Donaldson (1994) since alevins were held at a substantially higher density of 1300 fish L^{-1} compared to 20 fish L^{-1}, and this in turn may account for the differences in sex inversion frequency between the two studies. Modifying the immersion methods by extending immersion time, increasing immersion treatment frequency, reducing fish density and adding a pH buffer to the immersion solution may all contribute to enhanced uptake of fadrozole and hence increased tissue levels. However, whether such measures will increase the efficacy of fadrozole for sex inversion of Atlantic salmon is uncertain.

The maintenance of elevated tissue levels of ATD in the present study following a second treatment at a dose of 5 mg L^{-1} compared to the decline after the first treatment, may reflect saturation of hormone metabolising enzymes by ATD. Piferrer and Donaldson (1994) proposed a similar mechanism to account for enhanced E_2 uptake by coho salmon alevins following repeated immersion treatment. It is also possible that native androgens, including T, may be elevated, although no data on potential feedback mechanisms to account for this were collected in the present study. Sustained high tissue levels of ATD may be the underlying cause of the increased efficacy of high doses of ATD in comparison to

low doses, administered at 7 and 14 DPMH. Steroidal AI are known to exert androgenic effects when present in high concentrations (Séralini and Moslemi, 2001) and androgen efficacy increases after 14 DPMH (Chapter 2), thus the increase in the efficacy of ATD could have resulted from both the aromatase inhibitory, and androgenic properties of ATD. These data add further evidence to the premise that there is asynchrony between estrogen and androgen sensitivity with androgen sensitivity later in development (Chapter 4, Piferrer and Donaldson, 1989; Petrini and Zaccanti, 1998).

Treatment of Atlantic salmon alevins by immersion in solutions of AI represents a viable and commercially appropriate method of delivering some of these compounds; however, consideration needs to be given to factors influencing the lipid solubility of each AI and methods adjusted accordingly. The uptake of molecules having lower lipid solubility, such as OHA may be enhanced by extending the immersion period or increasing dose. For compounds such as fadrozole, where solubility is influenced by pH, buffering the immersion solution may overcome poor uptake. Although adequate levels of sex inversion were attained in the present study using an inter-bath interval of 7 days, reducing this to 3 days may further improve AI uptake and efficacy.

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Chapter 6

General Discussion

Science unfolds and controls nature

Toki-o Yamamoto

6. General Discussion

6.1 The present study

The routine use of androgens for sex reversal is well established in the commercial production of a number of cultured fish species (Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Piferrer, 2001; Devlin and Nagahama, 2002); however, the literature regarding the masculinisation of salmonids is, with a few notable exceptions (Johnstone et al., 1978; Johnstone and Youngson, 1984; Johnstone and Maclachlan, 1994), dominated by studies on *Oncorhynchus* spp. The present studies were conducted to develop a more complete understanding of the process of sex differentiation in Atlantic salmon, *Salmo salar* and to evaluate aromatase inhibitors (AI) as a means of masculinisation.

6.2 Sex differentiation in Atlantic salmon

The successful use of AI for sex inversion (Chapter 4) supports the hypothesis that the process of female sex differentiation in lower vertebrates is dependent on the presence of estrogens (Nagahama, 2000; Devlin and Nagahama, 2002). By implication, male sex differentiation is a default state, initiated in the absence of estrogens but able to be induced by androgens (Chapters 2 and 4, Nagahama, 2000; Devlin and Nagahama, 2002) and this has some implications for potential mechanisms of genetic sex determination in fishes. In mammals, the expression of a male phenotype depends on the presence of the *Sry* gene (Devlin and Nagahama, 2002), the product of which is known to bind to the promoter regions of aromatase and other genes (Haqq et al., 1993). No functional *Sry* gene has been found in fish (Nagahama, 2000; Devlin and Nagahama, 2002); however, the gene *Ftz-F1* is believed to have a role in regulating aromatase transcription in fish (Watanabe et al., 1999) although there is no evidence of male-specific expression in fish (Nagahama, 2000).

The demonstration in the present study that the labile period for the induction of sex inversion by the inhibition of estrogen synthesis (Chapter 4) precedes that for masculinisation by androgens (Chapter 2) supports the results of a

previous study on coho salmon *Oncorhynchus kisutch* (Piferrer and Donaldson, 1989). A similar asynchrony was found for the production of steroidogenic enzymes during the development of tilapia, with the presence of enzymes being detected in females several days before males (Nakamura et al., 2000) and this has been proposed to reflect the relative timing of the onset of differentiation of ovaries and testis (Piferrer, 2001). It should be noted that evidence from the present study for the existence of an estrogen sensitive period of development in Atlantic salmon is inferred from data of estrogen synthesis inhibition, and needs to be confirmed by E_2 immersion treatment during development. In general, the timing of the labile period in Atlantic salmon determined from the present study is later than that of coho salmon (Piferrer and Donaldson, 1989) and this is consistent with studies of gonadal ontogenesis suggesting that gonadal differentiation in Atlantic salmon commences later than in *Oncorhynchus* spp. (Laird et al., 1978; Goetz et al., 1979; Piferrer and Donaldson, 1989).

6.3 Sex inversion with AI

All AI investigated in the present study demonstrated a level of aromatase inhibitory activity (Chapter 3) and *in vitro* efficacy which largely agreed with the findings of mammalian studies (Cole and Robinson, 1990; Buzdar, 2000; Brueggemeier, 2001; Séralini and Moslemi, 2001), confirming the high degree of conservation of steroidogenic enzyme structure across vertebrate taxa (Callard et al., 1978). In consequence, third and fourth generation AI, such as letrozole and anastrozole, which have very high efficacy against mammalian aromatase (Brueggemeier, 2001; Séralini and Moslemi, 2001), are likely to be similarly effective against salmon aromatase and may have applications for masculinisation of fishes in the future.

Although the AI used in the present study were effective against both brain and gonadal aromatase (Chapter 3), there were indications of a differential response, suggesting that in Atlantic salmon, as in other fish species (Callard et al., 2001; Tchoudakova et al., 2001; Tong et al., 2001; Trant et al., 2001), more than one aromatase isozyme may be present. Furthermore, the differential efficacy of AI in preparations of brain and gonadal tissue is

consistent with the findings of Zhao et al. (2001) who demonstrated differences in efficacy of AI against goldfish, *Carassius auratus*, brain and gonadal aromatase. As a result of the effectiveness of all AI against both brain and gonadal tissue, the present study did not permit distinction between the roles of brain and gonadal aromatase in female sex differentiation. Future studies may be able to utilise differential response of the aromatase isozymes to AI, to differentiate their roles in this process.

The induction of commercially significant levels of masculinisation using AI by immersion (Chapter 4) is the first such demonstration for fish, and, along with the study of Piferrer et al. (1994) demonstrates the feasibility of the production of functional, fertile salmonid neomales without the use of steroid hormones. Although masculinisation was achieved in the present study using a steroidal AI with androgenic properties at high concentrations (Séralini and Moslemi, 2001), the data in relation to treatment timing (Chapter 4) are consistent with the hypothesis the effectiveness of ATD is due primarily to E_2 synthesis inhibition rather than androgenic effects. However, the efficacy of AI other than ATD for sex inversion was not consistent with their efficacy against Atlantic salmon aromatase *in vitro* (Chapter 3) or their efficacy for sex inversion in other species (Piferrer et al., 1994; Chardard and Dournon, 1999; Guiguen et al., 1999; Kwon et al., 2002). Fadrozole and OHA were highly effective *in vitro* in the present study and effective in masculinising the developing gonads of birds (Elbrecht and Smith, 1992), amphibians (Petrini and Zaccanti, 1998; Chardard and Dournon, 1999), reptiles (Wennstrom and Crews, 1995) and fishes (Piferrer et al., 1994; Kitano et al., 2000) but neither was effective in the present study. These results may be explained by the low rates of uptake of both of these compounds by alevins during the standardised immersion treatment protocol used in this study (Chapter 5). Immersion for 2 h was shown to be an effective, commercially appropriate method of administering steroid hormones for sex control in salmonids (Goetz et al., 1979; Baker et al., 1988; Piferrer and Donaldson, 1989, Chapter 2) but its effectiveness is clearly contingent upon the high lipid solubility of the compounds used and hence their rapid uptake across the gills of alevins during the brief treatment period (Hunn and Allen, 1974; Allen and Hunn,

1986). Although potential differences in lipid solubility between ATD, OHA and fadrozole could only be inferred from differences in their structure in the present study, the results presented in Chapter 4 are consistent with the proposal by Allen and Hunn (1986) that uptake rates depend on lipid solubility. As discussed in Chapter 5, modification to the immersion protocol in terms of dose, duration or water pH may provide a means of addressing issues of poor uptake, although the effectiveness of these measures would need to be demonstrated.

As proposed by Piferrer (2001), factors for consideration in the application of steroids for sex inversion include: the choice of compound, treatment method and dose, and these may equally be applied to the choice of AI for sex inversion, provided relevant data are available to predict their effects. The results of the *in vitro* component of the present study (Chapter 3) indicate that there is considerable variation in the efficacy of AI for Atlantic salmon, consistent with differences in efficacy in mammalian systems (Cole and Robinson, 1990; Brueggemeier, 2001). However, it was also demonstrated that *in vitro* efficacy did not necessarily translate to efficacy for sex inversion (Chapter 4), necessitating the application of additional data on AI uptake to be considered when interpreting the results of studies of AI for sex inversion (Chapter 5). Additionally, data on the lipid solubility of AI may be used to predict uptake rates (Hunn and Allen, 1974; Allen and Hunn, 1986)

6.4 Commercial implications

Utilising the methods developed for the masculinisation of *Oncorhynchus* spp. (Goetz et al., 1979; Baker et al., 1988; Piferrer and Donaldson, 1989; Piferrer et al., 1994), the present study has established commercially appropriate protocols for the production of Atlantic salmon neomales using androgens and AI. The success of immersion for the administration of both categories of masculinising agents has enabled the production of neomales with patent sperm ducts, providing further evidence that sperm duct patency is a function of the timing of treatment. Blocked sperm ducts significantly impair production efficiency, and the high incidence of patent sperm ducts and high

fertility in neomales produced using AI in the present study is a significant factor for consideration in the potential commercial application of AI.

Based on the results of the present study, non-steroidal AI do not appear to be suitable for sex inversion of Atlantic salmon. In general, the structure of non-steroidal AI would be expected to be associated with lower lipid solubility than steroidal compounds, and therefore result in lower uptake rates by alevins (Allen and Hunn, 1986), making non-steroidal AI unsuited to use in immersion treatments. Furthermore, since AI were most effective when applied during the estrogen sensitive period, non-steroidal AI would not be suited for application via the diet, as this would commence well after the optimum window for estrogen treatment.

The recommended use of any new compound in commercial aquaculture must take into consideration several factors. Compounds used in animal production require approval from national or international regulatory authorities. These authorities may take into consideration issues such as the current use in humans of a particular drug, and determine its use accordingly. Currently, a number of AI are the preferred treatment for breast cancer (Séralini and Moslemi, 2001) and this may affect the approval for their use in aquaculture. However, commercial usage of a drug also necessitates its ready availability. Of the AI used in the present study, ATD is available, but its longevity in the marketplace is unclear as it has no application in human medicine. Fadrozole and OHA are no longer used in human medicine, however, OHA is available commercially.

Provided specific guidelines are met, steroid hormones are readily available and approved for use in aquaculture in Australia and these presently represent a cost effective means of masculinisation in salmonids. Future potential regulatory changes aside, the public perception of the use of steroids in animal production may be the greatest impediment to their use in the future. Indirect feminisation provides a robust method for the production of all female stocks that are completely untreated, with masculinisation agents only used for broodstock production (Piferrer, 2001). The commercially significant level of masculinisation of Atlantic salmon was achieved in the present study using a

steroidal AI, ATD and it is therefore possible that potential negative regulatory or marketing issues associated with the use of steroids may not be alleviated by this approach for the production of neomales. Based on uptake data for both steroids and steroidal AI, a reasonable argument may be mounted that the quantities taken up by alevins during immersion treatment are low, (approximately 0.2 μg ATD, Chapter 5; or 0.8 μg T, Piferrer and Donaldson (1994)), and that once taken up, these compounds have a half life of approximately 48 h (Hishida, 1965; Piferrer and Donaldson, 1994; Specker and Chandlee, 2003, Chapter 5). The compounds would therefore not be detectable at spawning, 3 years after treatment. Additionally, AI may offer the perceived benefit to the public of having a specific mode of action, in contrast to steroid hormones, which effect processes other than sex differentiation. The release of steroid hormones or other aquaculture drugs, into the environment is also an issue for consideration. This may be addressed by the use of immersion treatment for AI or androgens for sex inversion. Unlike dietary treatment, immersion treatment involves relatively small quantities of water, which may be appropriately disposed of at the completion of the treatment.

6.5 Future studies

Although the present study addresses some issues of teleost sex differentiation, there is much about the processes of sex differentiation that still remains unclear. Studies of the type undertaken by Kitano et al. (1999; 2001), Guiguen et al. (1999) and Nagahama (2000) which combine molecular techniques with AI or steroid receptor antagonists would complement the present work and address issues such as the nature of control of *cyp19* expression.

The existence of asynchronous labile periods for androgens and estrogens in salmonids has potential implications for the timing of the expression of sex determining genes. If, as occurs in mammals, *cyp19* activity in genetic males is suppressed at the promoter level by the products of a male determining gene (Devlin and Nagahama, 2002), expression of this gene, is likely to commence in the period immediately post-hatch in Atlantic salmon. Molecular studies at

this time may be a means of further investigating this model, and ultimately may elucidate the sex determining gene(s) in Atlantic salmon.

The differential sensitivity of brain and ovarian aromatase isozymes to AI (Zhao et al., 2001) and E_2 receptor antagonists (Kishida and Callard, 2001) may also provide an avenue of further research into sex differentiation. If compounds can be found that significantly differentiate between the 2 isozymes, they may be used evaluate the relative contributions to sex differentiation of estrogens of gonadal and neural origin.

The possibility remains that future regulatory controls or market pressures may prevent the use of steroidal AI for sex inversion, and methods for sex inversion using non-steroidal AI may become necessary at that time. Further studies are needed to improve the efficacy of non-steroidal AI for sex inversion and such studies may address the issues of dose, treatment duration and bath pH discussed previously. Although the development of a steroid-free method for masculinisation of salmonids may be achieved using AI, alternative approaches to sex control, such as the use of environmental manipulation may ultimately prove to be the preferred approach. Numerous recent studies have demonstrated a role for temperature in sex determination of fishes (Kitano et al., 1999; D'Cotta et al., 2000; Goto et al., 2000; Pavlidis et al., 2000; Fujioka, 2001; Felip et al., 2002; Kwon et al., 2002). The potential for thermal regulation of sex determination in Atlantic salmon remains to be explored.

6.6 Concluding remarks

Borrowing from the quote from Yamamoto (1969) cited at the beginning of this chapter, science has gone a long way to unfolding and controlling the processes of sex differentiation in fish in the nearly 50 years since Yamomoto's seminal studies on sex differentiation in medaka commenced. Such advances have undoubtedly provided aquaculturists with a number of highly efficient tools with which to 'control nature'.

The ongoing need to produce monosex or sterile populations of farmed Atlantic salmon in Tasmania, and farmed finfish elsewhere, is likely to remain

high as producers continue to maximise production efficiencies and minimise risks. The challenge is therefore to apply the increasing knowledge of sex determination and differentiation to develop tools for use in aquaculture, that not only satisfy production requirements, but which are sensitive to the needs of an increasingly better informed and proactive public. For its part, the aquaculture industry also needs to address the issues of public perception of the use of steroids and other compounds in aquaculture by making clear the quantities of compounds involved, and explaining the benefits of indirect treatment protocols for the production of untreated fish for consumption.

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Appendices

Appendix I Atlantic salmon milt extender and activator solutions

Appendix Ia Extender solution for Atlantic salmon milt

Distilled water 2 L

KCl 14.40 g

NaCl 3.84 g

$NaH_2PO_4.H_2O$ 0.80 g

$MgSO_4.7H_2O$ 0.48 g

CaCl 0.48 g

$NaHCO_3$ 2.00 g

glucose 2.00 g

Appendix Ib Activator solution for Atlantic salmon milt

Distilled water 2 L

NaCl 10.00 g

tris-methylamine 1.34 g

glycine 0.88 g

Appendix II Histological procedures

Appendix IIa Infiltration regime – Tissue Tek VIP 4617

Step No.	Solvent	Time	Step No.	Solvent	Time
1	70% ethanol	60 min	8	100% ethanol	60 min
2	80% ethanol	60 min	9	toluene	60 min
3	85% ethanol	60 min	10	toluene	60 min
4	90% ethanol	60 min	11	paraffin	30 min
5	95% ethanol	60 min	12	paraffin	30 min
6	100% ethanol	60 min	13	paraffin	30 min
7	100% ethanol	60 min	14	paraffin	30 min

Appendix IIb Haematoxylin and eosin staining procedure

Step No.	Solvent/stain	Time	Step No.	Solvent/stain	Time
1	xylene	2 min	11	tap water	20 dips
2	xylene	2 min	12	Young's eosin	150 sec
3	100% alcohol	20 dips	13	tap water	10 dips
4	100% alcohol	20 dips	14	70% alcohol	20 dips
5	100% alcohol	20 dips	15	100% alcohol	20 dips
6	70% alcohol	20 dips	16	100% alcohol	20 dips
7	tap water	20 dips	17	xylene	20 dips
8	Mayer's haemalum	5 min	18	xylene	20 dips
9	tap water	20 dips	19	xylene	20 dips
10	Scott's tapwater substitute	30 sec	20	Mountant: DPX	