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Effect of Culture Environmental Conditions on Sex Inversion of Asian
seabass (Barramundi), *Lates calcarifer* (Bloch)

Thesis submitted by

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ABSTRACT

Asian seabass, *Lates calcarifer*, is a protandrous catadromous fish species cultured worldwide for commercial aquaculture. Farmed Asian seabass exhibit precocious sex inversion before two years of age and this phenomenon is the major impediment to maintaining broodstock in a hatchery. In the wild, age, seasonal temperatures and movement of the fish to a brackish or saline environment apparently affect spawning and sex inversion of Asian seabass. This series of experiments investigated the role and relationships between age and the hatchery conditions which appear to induce sex inversion in captivity. Asian seabass grown in freshwater under natural conditions were transported to the research facility at James Cook University, Australia to conduct four experiments as follows.

In the first experiment investigating age effects, Asian seabass of four different sizes grown in freshwater were transferred to salinewater (32 g L⁻¹) at 28 °C and 14: 10 L:D photoperiod and fed to satiety twice daily. Eight groups of Asian seabass (n=8/group, two groups per each size) according to their body weight were allocated to 3000 L tanks in a single enclosed room. In the rest of the experiments, 14 month old seabass grown in freshwater under natural temperature in the same farm were transferred to the research facility and held in freshwater at 28 °C until acclimatized to the experimental conditions at different salinities (0 g L⁻¹, 20 g L⁻¹ or 30 g L⁻¹ salinity), different temperatures (22 °C, 25 °C, 28 °C, 31 °C and 34 °C) and both salinity versus temperature (24 °C, 29 °C or 34 °C at each of 0 g L⁻¹ or 30 -32 g L⁻¹) in experiments two, three and four, respectively. Each experiment additional rearing conditions were similar, except the variable environmental parameter tested. Fish were anaesthetized to collect the blood samples for plasma steroids assays at the beginning and at the end of the experimental period. Upon collection of blood, the fish were sacrificed; brain and gonad were removed aseptically, and labeled vials were placed in liquid N₂ at -80 °C for aromatase assay for all experimental fish, while histological analyses were conducted for last two experiments.

Brain aromatase activity appeared to respond to age/size rather than environmental conditions, while gonadal aromatase was detectable only in the 700 – 1000 g fish group, plasma T increased in response to the environmental change in fish groups of 300 – 500 g and 700 – 1000 g while the 50 – 100 g and 2.5 – 4 kg fish had no increases (P>0.05). Plasma E₂ increased significantly in all groups of fish in experiment one, while 11 KT was detected in the 700 – 1000 g and 2.5 – 4 kg fish and was significantly different (P<0.05). Results indicated that the hormonal conditions are pre-requisite for inducing sex change in captive Asian seabass from 435 ± 27 g body weight.

The results of the second experiment indicated that there were no differences ($p > 0.05$) between the aromatase activities in the brains of fish held at 0 g L⁻¹, 20 g L⁻¹ or 30 g L⁻¹ salinity, while no differences ($p > 0.05$) between the gonadal aromatase activities were also observed in any of the treatment groups except for fish held in 10 g L⁻¹ and 30 g L⁻¹ salinities, respectively. The highest gonadal aromatase level was recorded in fish held in 0 g L⁻¹ and 20 g L⁻¹. Plasma T concentration in fish in all treatments at the end were not different ($P > 0.5$), while the highest E₂ level was recorded in fish held at 0 g L⁻¹ followed by fish held at 10 g L⁻¹ and 30 g L⁻¹, respectively. However, no measurable amount of 11-KT was detected in any salinity group of fish in this experiment.

Results of the third experiment which examined the effects of temperature on sex change indicated that there was an increase in plasma E₂ levels with increasing temperature from 25 °C, while no significant difference was observed among all treatment temperatures except at 25 °C. However, fish held at 22 °C expressed higher E₂ level than at either 25 °C and 28 °C. Significantly higher plasma T levels were detected in fish held at 31 °C and 34 °C, while a reducing trend was observed towards lower temperature regimes. Fish held at 22 °C had significantly lower plasma T than all others as well as those sampled at the beginning. The plasma 11-KT was at non-detectable levels in all experimental temperatures as shown in the initial fish sampled. The average aromatase activity in the brain was highest at 28 °C among all temperatures, but no significant differences were observed. The average aromatase activity in the gonad was higher at 31 °C, followed by 34 °C and 28 °C. No or very low levels of gonad aromatase activity was recorded in fish sacrificed prior to treatment. The aromatase activity was greater in brain than in gonad suggesting that the initial responses to changes in environmental temperatures occur in aromatase produced in the brain.

The results of the final experiment indicated that there was an increase in plasma E₂ level with temperature in fish held at 34 °C, whereas no significant difference was observed at 24 °C and 29 °C, although the highest plasma T level was detected in fish at 34 °C which, except for those fish held at 24 °C in freshwater, had significantly lower levels than at the beginning. Plasma 11-KT was significantly greater in fish held at 24 °C compared with 29 °C or 34 °C, which was opposite to that of E₂. Aromatase activity in the brain was higher at 29 °C than at either 24 °C or 34 °C, whereas gonadal aromatase was recorded the highest at 34 °C. It is apparent from the data presented in the final experiment that there is a relationship between culture water temperature, independent of salinity, and induction of sex change as demonstrated by histological staging and measured through changes in the concentrations of aromatase and reproductive hormones.

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Full name</u>
T	Testosterone
E ₂	17 β- estradiol
11-KT	11 Keto Testosterone
GtH ₁	Gonadotropin 1
GtH ₁₁	Gonadotropin 11
17,20β-DP	17,20β-Dihydroxy-4-pregnen-3-one
LHRHa	Lutenizing hormone releasing hormone analogue
SCC	Side-chain cleavage system
GABA	δ-amino-butyric acid
GnRHa	Gonadotropin Releasing Hormone analogue
HCG	Human Chorionic Gonadotropin
LH	Luteinizing hormone
FSH	Follicular stimulating hormone
PG	Prostaglandin
L : D	Light and Dark
ppt	Part par thousand
°C	Celsius
g	gram
mL	millilitre
L	Litre

CHAPTER 1

GENERAL INTRODUCTION

The Asian seabass *Lates calcarifer* (Bloch, 1790), also known as barramundi or Giant perch, is a protandrous hermaphroditic species (Moore, 1979; Davis, 1982) that belongs to the family *Latidae* (Xia *et al.*, 2010; Ayson and Ayson, 2012; Laughnan *et al.*, 2013; Pethiyagoda and Gill, 2013). The genus *Lates* consists of eleven species, seven of which are confined to the freshwaters of Africa and four species, including two recently described cryptic species, euryhaline South-East Asian species (*L. calcarifer*, *L. japonicas*, *L. lakdiva*, *L. uwisara* (Pethiyagoda and Gill, 2013). Asian seabass is an important species for both aquaculture and fisheries throughout its natural distribution within the Indo-West Pacific region between longitude 50° E and 160° E and latitude 24° N and 25° S. In Australia, at least three different stocks (Shaklee and Salini, 1985; Pusey *et al.*, 2004) of Asian seabass are naturally found as far south as the Noosa River (26° 30'S) on the east coast and the Ashburton River (22° 30'S) on the west coast (Schipf, 1996). Production of Asian seabass occurs in both large and small-scale aquaculture enterprises, and has increased in many countries, particularly in South-East Asia after the techniques for captive breeding were first developed in Thailand in the early 1970's. Since then considerable progress in culture techniques for this species have been achieved and the culture of Asian seabass in fresh and saline waters has expanded throughout the Indo-Pacific region, including Australia (Schipf, 1996; Szentes *et al.*, 2012; Ayson and Ayson, 2012). In the Asian Pacific region, Malaysia, Indonesia, Taiwan and Thailand remain the leading countries involved in commercial Asian seabass production, with Hong Kong, Singapore and Australia also playing an important role (Boonyaratpalin and Williams, 2002; Ayson and Ayson, 2012).

In Australia, research into culturing Asian seabass began in 1984 and farming began around 1986, but rapid expansion only occurred after 1992, particularly because of refinement of more extensive larval rearing techniques and cost efficient feed formulations (Tucker *et al.*, 2002). Since then, Australia has recorded a rather rapid and continuing increase in Asian seabass production whereas fish are now being farmed in one form or another (ponds, seacages, tanks) throughout the country, with the exception of Tasmania (O'Sullivan, 1998). However, obtaining seeds for culture operations from wild broodstock is a practice which is expensive, highly seasonal and unreliable (Ayson and Ayson, 2012) and conflicts with wild stock resource management (Barlow *et al.*, 1996). The variability of seed supply and high costs incurred with wild seed supply limited the expansion of the culture industry. Hence, the shortage of Asian seabass fingerlings for grow-out culture facilities led to the development of commercial

hatcheries throughout the country for year-round reproduction of Asian seabass (Ayson and Ayson, 2012). These now retain captive broodstock for seed production since the first large scale Asian seabass farm was established in 1986 (Schipf, 1996). Though year-round hatchery production is possible through the manipulation of environmental cues, the hatchery production is limited by the inability of culturists to maintain functional males in the breeding population due to their early sexual maturity (Szentes *et al.*, 2012; Ravi *et al.*, 2014) and precocious sex inversion to female (Schipf, 1996; Pankhurst, 1998; Allan and Stickney, 2000; Guiguen *et al.*, 2010; Robinson *et al.*, 2010). This often leads to a shortage of suitable male broodstock. Consequent difficulties in hatchery seed production and maintenance of the sex of broodstock also inhibit the development of a genetic improvement program for this species (Pankhurst, 1998; Allan and Stickney, 2000; Robinson *et al.*, 2010).

Water temperature and salinity appear to be the controlling environmental factors of reproduction in Asian seabass (Davis, 1985; Ayson and Ayson, 2012) compared with European seabass, *Dicentrarchus labrax*, where photoperiod appears to be of greater significance (Carrillo *et al.*, 1989). Environmental factors such as salinity, temperature and photoperiod are closely controlled in culture systems. These environmental cues can therefore be manipulated to alter the spawning times of seabass broodstock and hence solve the ever increasing and aseasonal demands for eggs and fry (Carrillo *et al.*, 1995; Ayson and Ayson, 2012). The first evidence of temperature influence on spawning time came from long-term observations of the reproductive cycle under ambient conditions. By keeping European seabass broodstock under natural photoperiod, but heating the water during autumn and winter, spawning is delayed by one month with respect to controls (Zanuy *et al.*, 1986). In general, exposure to one month of long days (L:D 15:9) during the earlier part of the reproductive cycle advances vitellogenesis, maturation and spawning time of European seabass (Carrillo *et al.*, 1995). Therefore, the use of environmental manipulation to change spawning time is a particularly attractive proposition for flatfish, bass, bream and mullet because the very high fecundities of these fish mean that only small numbers of broodstock need to be maintained under artificial conditions (Bromage, 1995). Bishop *et al.* (2001) recorded Asian seabass in waters with a temperature range of 26 °C to 35 °C in the Northern Territory, where some precocious sex change fish populations were also recorded. Temperature effects on sex ratio were confirmed in several fish species with sexual dimorphic growth patterns, e.g., Bluegill sunfish (*Lepomis macrochirus*), Nile tilapia (*Oreochromis niloticus*), Rainbow trout (*Oncorhynchus mykiss*), European seabass (*Dicentrarchus labrax*), Turbot (*Scophthalmus maximus*) and Japanese flounder (*Paralichthys olivaceus*), in which there is complete sex-reversal from males to females, or from females to males by exposure to high or low temperatures (Shen and Wang, 2014).

Different cues, varying from species to species, may induce sex changes, but the underlying physiology directing this process has received little attention (Guiguen *et al.*, 2010). However, steroid production has been shown to mediate both natural and induced sex change and has been examined during gonadal sex change in many hermaphrodite species. Changes in serum E₂ levels are often associated with the sex inversion process of both protandrous and protogynous hermaphrodite species. E₂ levels can induce sex change either from male to female through E₂ supplementation, or female to male through E₂ level decreases or aromatase activity inhibition (Guiguen *et al.*, 2010). Aromatase is part of a key enzymatic complex bound to the endoplasmic reticulum (Diotel *et al.*, 2010). This enzymatic complex is the last of the steroidogenic synthetic pathway, and allows the conversion of aromatizable androgens into estrogens. In most fish studied, two aromatase gene forms are expressed in both the gonad and the brain, which control the production of sexual steroids (Diotel *et al.*, 2011). A recent gene study undertaken by Ravi and colleagues (2014) found that 32 of the 37 genes investigated showed sexually dimorphic expression. As would be expected, these included a number of genes involved in steroidogenesis, but particularly showed high levels of aromatase expression in testes.

According to the reviewed literature, it is apparent that the relationship between aromatase enzyme activity and sex inversion of Asian seabass is far from clear. No detailed studies have yet been conducted on the relationships between changes of serum steroid levels and aromatase activities with manipulated environmental parameters for sex inversion of the hermaphroditic Asian seabass. In a protandrous hermaphroditic fish, the black porgy (*Acanthopagrus schlegelii*), the increase of gonadal aromatase activity was associated with natural and control sex change (Lee *et al.*, 2001) and the inhibition of aromatase activity blocked natural sex change (Lee *et al.*, 2002). In a protogynous Blackeye goby (*Rhinogobiops nicholsii*), the inhibition of aromatase activity by Fadrozole caused complete sex change towards male (Kroon and Liley, 2000). These observations revealed that aromatase is directly involved in E₂ production in teleosts and may play an important role in sex change (Zhang *et al.*, 2004). Anderson and Forrester (2001) described the increased aromatase activity and associated decreased 11-KT: E₂ ratio and suggested that this aromatase activity may be seen as either a cause of sex reversal, or a consequence of the sex change process of Asian seabass (as seen in sex reversal of the black porgy (Chang *et al.*, 1997). Therefore, this study investigates the relationship between levels of serum steroid hormones, aromatase enzyme levels and their abundance in both brain and gonadal tissues under different culture environmental conditions of captive Asian seabass and their relationships with precocious sex inversion from male to female.

The objectives of this study are to provide further detail regarding the hormonal control of the sex inversion process of this hermaphroditic species and attempts to clarify the effect of various environmental factors on sex inversion of Asian seabass.

Objectives:

- 1) Investigate the age/size at which sex inversion takes place under captivity.
- 2) Investigate the effect of the environmental parameters temperature and salinity have on sex inversion of Asian seabass.
- 3) Investigate whether concentrations of the aromatase enzyme and steroid levels change with different environmental parameters.
- 4) Investigate whether the aromatase enzyme plays a pivotal role in sex inversion process of Asian seabass by regulating the endogenous levels of steroids (T, E₂ or 11-KT).

The above key objectives have been incorporated into the overall design of this project in order to have a better scientific understanding of sex inversion of Asian seabass. Chapter Two of this thesis gives an overview of the general reproductive biology of Asian seabass which is important to understand the underlying mechanisms of sex inversion in the species. Chapter Three of this thesis investigated the effect of age/size on plasma sex steroids and aromatase activity of Asian seabass when transferred to ideal hatchery conditions. Eight groups of Asian seabass at different sizes were raised under hatchery conditions and the changes in steroidal hormones and aromatase activity in brain and gonad during the experimental period were investigated. This experiment addressed objectives 1 and 3 and the results indicate that the hormonal conditions are pre-requisite for inducing sex inversion in Asian seabass from a body weight of $435 \pm 27\text{g}$ in captivity. Chapter Four investigated the effect of different salinity conditions from freshwater to saline water on the sex inversion process of Asian seabass. This experiment addressed objectives 2 and 3. Chapter Five investigates the effect of different temperature regimes on the sex inversion process of Asian seabass. Experiments involved observation of temperature related variation in aromatase activity, serum sex steroids and histology of gonads of Asian seabass. The 2nd, 3rd and 4th objectives were addressed by the experiment in Chapter Six which focused on the effects of culture environmental conditions, in particular, temperature and salinity on sex inversion of Asian seabass where the change in gonadal morphology, concentrations of plasma steroids and aromatase activity of gonad and brain were investigated with different temperature and salinity regimes.

The findings of this study further widen our understanding of the mechanisms involved in the precocious sex inversion process in Asian seabass.

CHAPTER 2

A LITERATURE REVIEW ON THE REPRODUCTIVE BIOLOGY OF ASIAN SEABASS

2. INTRODUCTION

Asian seabass, *Lates calcarifer*, also known as barramundi in Australia, is an important coastal, estuarine and freshwater fish in South-East Asia and Australia (Russell and Garrett, 1985; Grey, 1987; Schipp, 1996; Pusay *et al.*, 2004; Szentes *et al.*, 2012). Asian seabass spends most of its growing period in freshwater bodies such as river and lakes, which are connected to the sea (Ruangpanit, 1986). Spawning takes place in brackish to marine conditions (Yue *et al.*, 2012) near the mouths of rivers with an incoming tide, which allows the egg and hatchlings to drift into estuaries. Larvae and juveniles develop in coastal swamps and the young migrates upstream at the end of the wet season where they remain for the rest of their life until they reach sexual maturity as males. In the wild, males mature at about three to four years of age and convert to females at between six and eight years old (Guiguen *et al.*, 1994; Allan and Stickney, 2000; Ayson and Ayson, 2012), although Davis (1984a) described populations of sexually precocious Asian seabass changing their sex at four to five years of age and at a smaller size (30-40 cm) in the Gulf of Carpentaria. This hermaphroditic species does not possess an ovotestis gonad, and sex inversion involves profound morphological and histological changes that result in strongly dimorphic testes and ovaries (Guiguen and Jalabert, 1995; Ravi *et al.*, 2014). Initial breeding programs used eggs and sperm that are hand stripped from wild spawners, but later used captive as well as wild spawners. Australian studies on the reproductive capacity showed that females are very fecund, producing up to 10 million eggs at 100 cm and 30-40 million eggs at 120 cm body length (Davis, 1984b; Ayson and Ayson, 2012; Yue *et al.*, 2012).

Asian seabass has a complex life history, being a protandrous hermaphrodite as well as being catadromous (Moore, 1979; Davis, 1982; Mathew, 2009; Mukai *et al.*, 2014; Budd *et al.*, 2015). Movement to the spawning areas and maturation of gonads is thought to be triggered by an increase in water temperature (Kitano *et al.*, 1999; Ayson and Ayson, 2012), which occurs at the end of the dry season (Grey, 1986; Pusey *et al.*, 2004; Piferrer *et al.*, 2012). The fish spawns according to the lunar cycle during the late evening, which is synchronised with the incoming tide that allows the eggs and hatchlings to drift into estuaries. The strong association between the life cycle of Asian seabass and the monsoon cycle in Australia and Papua New Guinea water is the same as that identified in Thailand (Barlow, 1981 cited in Grey, 1986).

Post-larvae enter coastal swamps on the peak spring tide and until the end of the wet season remain there (Grey, 1986, Ayson and Ayson, 2012; Yue *et al.*, 2012). As the water recedes, the young fish move upstream to the river where they generally remain until they sexually mature as males. Hatchery based studies indicate that larval survival is optimal in the salinity range of 20-25 g L⁻¹ and that hatching success is optimal at salinities of about 30 g L⁻¹ (Pusey *et al.*, 2004., Liu *et al.*, 2012). However, not all fish migrate (Milton and Chenery, 2005), as there seems to be a resident population of all age groups that remain in coastal waters (Opnai and Tenakanai, 1986; Ayson and Ayson, 2012). The species exhibits similar spawning behaviour in Asia, although there are differences in size of maturation between Asian seabass from Australia compared to Papua New Guinea and equatorial Asia (Grey, 1986). These populations also show a distinct genetic difference sufficient to allow traceability of origin (Yue *et al.*, 2012).

Asian seabass undergo a prolonged breeding season that starts just before the summer monsoon (Davis, 1985a; Piferrer *et al.*, 2012). The prolonged spawning season appears to be the result of landlocked fish arriving late on the spawning ground when flooding has occurred late in the wet season. However, the timing and duration of the breeding season vary between regions, river systems and from year to year (Davis, 1985; Mathew, 2009). In Queensland, Asian seabass have a single annual spawning period that extends from October to March. Peak spawning in northern Queensland stocks occurs during November and early December (Davis, 1984b; Russell and Garrett, 1985) and during late December to early January in southern east coast stocks, whereas Central Queensland stocks have two spawning peaks that take place in November and January (Garrett, 1986). James and Marichamy (1986) described different spawning seasons throughout India, with a similar two peaks of spawning, as well as a single spawning based on the observed mature specimens and availability of fingerlings. Water temperature appears a primary factor controlling the commencement of spawning in Asian seabass (Davis, 1985). In Tahiti in French Polynesia (a translocated population), seabass exhibits a single annual reproductive period from October to February beginning with the warm and wet season (Guiguen *et al.*, 1994). However, Asian seabass in Thailand spawn all year (Yue *et al.*, 2012), which is a common phenomenon in most equatorial counties in South-East Asia (Budd *et al.*, 2015), and the peak season at Songkhla Lake occurring during April-September (Ruangpanit, 1986).

2.1 Spawning grounds

The Asian seabass spawns in a saltwater environment as it was found that their gonadal development is retarded in landlocked freshwater (Davis, 1985). Thus, spawning occurs in brackish waters (28-36 g L⁻¹ salinity) near the mouths of rivers (Grey, 1986; Ayson and Ayson, 2012; Yue *et al.*, 2012). The spawning grounds are generally located in coastal water near coastal swamps during the months of November to February in Papua New Guinea. However, spawning could also easily take place upstream, provided salinities are high enough (~30 g L⁻¹), with high salinity appearing to be the main requirement of spawning grounds (Davis, 1986; Mathew, 2009). Significant numbers of Asian seabass larvae in Van Diemen Gulf were caught in plankton trawls when salinities were 30 g L⁻¹ or higher (Davis, 1985). Most of the mature Asian seabass in Northern Australia are resident in tidal waters throughout the year, spawn before the wet season floods lower local salinities as they do not have to make the catadromous migration to spawning grounds (Davis, 1984a). Spawning grounds appear to be located close to larval and juvenile habitats, or within the path of currents that transport the larvae to such habitats (Garrett and Russell, 1982 cited in Garrett, 1986). The positions of spawning grounds probably differ slightly from year to year depending on coastal salinities (Moore, 1982; Mathew, 2009). Suitable salinities have been observed on the mudflats adjacent to the mouths of most rivers during the first months of the spawning season in northern Australia (Davis, 1986). Aggregations of fish with ripe-and-running gonads have been found in the lower estuaries of rivers and their adjacent foreshores. In Australia, spawners are found in central and southern Queensland, just inside and outside of river and creek mouths; northeast Queensland rivers, in shallow side gutters near the mouths (Garrett, 1986), and around coastal mudflats, near the mouths in the southern Gulf of Carpentaria (Davis, 1985). In Thailand, spawners are found in the river mouths, salt-water lakes and along the coastal area where the salinity and depth range between 30 and 32 g L⁻¹ and 10-15 m, respectively (Ruangpanit, 1986). Coastal spawning grounds are usually close to the nursery habitat of larvae and juvenile fish.

2.2 Spawning of Asian seabass

Asian seabass appears to be a surface spawner (Garrett, 1986, Mathew, 2009). They display three strategies that maximise spawning success: male fish outnumber the females in the breeding event, spawn mainly at the beginning of the incoming tide and perform dusk or evening spawning. Spawning begins at the onset of, or just prior to, the monsoon season. Thus, the reproductive strategy of Asian seabass is well suited to the formation of water bodies associated with monsoon rains, which provides an environment for growth and survival of young fish. Water temperature and salinity appear to be controlling factors (Davies *et al.*, 1986; Mathew, 2009; Piferrer *et al.*, 2012). Asian seabass spawned in a Northern Gulf of Carpentaria

estuary when water temperatures ranged from 27 °C to 33 °C and salinities from 28 to 34 g L⁻¹ (Davis, 1985). In Thailand, mature seabass migrate towards the mouth of the river or lake from inland waters into the sea, where the salinity range is 30-32 g L⁻¹, for gonadal maturation and subsequent spawning (Ruangpanit, 1986) which has also been linked to the lunar cycle. In Papua New Guinea, falling water levels induce mature fish to migrate from freshwater habitat to the coastal waters where high salinities are required for egg fertilisation and hatching (Garrett, 1986). Spawning can take place well upstream in rivers as long as salinities are high (Davis, 1985). Local spawning of Asian seabass in rivers rather than on communal spawning grounds is consistent with genetic evidence of stock heterogeneity (Shaklee and Salini, 1985). Male seabass spawn at least once and sometimes for a number of years before changing sex (Davis, 1984a; Ayson and Ayson, 2012; Ravi *et al.*, 2014).

The fecundity of Queensland Asian seabass is one of the highest reported in a teleost: average fecundity of central coast barramundi is 0.6×10^6 eggs/kg of body weight, whilst Gulf of Carpentaria seabass are estimated to produce 2.3×10^6 eggs/kg of body weight (Garrett, 1986) and seasonal average of 4×10^6 eggs/kg of body weight in Australia (Mathew, 2009). Research indicated that the fecundity in Asian seabass was related to the size and weight of the fish (Ruangpanit, 1986; Garrett, 1986; Yue *et al.*, 2012). In Papua New Guinea, sampled fish of weight ranging from 7.7 to 20.8 kg gave fecundity estimates of 2.3×10^6 to 32.2×10^6 eggs per fish (Moore, 1982). In Thailand, female weighing from 5.5 to 11 kg gave 2.1 - 7.1×10^6 eggs (Wongsomnuk and Maneewongsa, 1974 cited in Ruangpanit, 1986).

Spawning behaviour in fish is cued by physical environmental factors, social interactions and, in many species, sexual pheromones (Stacey *et al.*, 1994 cited in Pankhurst, 1998). Environmental factors that stimulate spawning are changes of temperature, photoperiod, salinity and lunar cycle (Kitano *et al.*, 1999; Piferrer *et al.*, 2012; Shen *et al.*, 2014). As standard management practices good quality and a suitable amount (1% of body weight) of food should be given to spawners. Overfeeding can result in failure to spawn. For seabass clean, filtered saltwater should be used, with adequate dissolved oxygen (not less than 6 ppm DO), and pH range of 7.5-8.5. Salinity in the broodstock tank should be maintained between 28 and 32 g L⁻¹. Disturbances during the spawning season in tanks cause stress to the Seabass. The breeder should ensure that spawners of appropriate age are chosen, that the size of males and females is compatible, and that both sexes are evenly represented. On occasion hormone injections of lutenising hormone releasing hormone analogue (LHRHa) are needed to stimulate the fish to spawn naturally in captivity. The dosage for natural spawning is usually lower than for spawning by stripping which is about 50-200 IU/Kg of body weight (Maneewong, 1986).

Since the Asian seabass spawning is synchronised with the monsoon season (Ayson and Ayson, 2012) and it does not spawn normally in confined areas (Pillay, 1990), it is inevitable to find methods for uninterrupted seed supply to cater for the demand with the expansion of the Asian seabass culture. Artificial propagation of Asian seabass was first achieved in Thailand in 1971 by stripping the ripe and running wild spawners, which were collected from the natural fishing grounds. However, Wongsomnuk and Maneewongsa (1973) successfully induced the cultured broodstock to spawn in captivity by injection of HCG opening the possibility of artificial propagation of the species (Ruangpanit, 1986). In 1986, Kungvankij successfully induced Asian seabass to spawn by environmental manipulation.

2.2.1 Artificial fertilization

In early Asian seabass culture, spawners were caught in natural spawning grounds and immediately checked for degree of maturity. If the female had ripe eggs and the male was in the running stage, stripping was carried out in the field. As soon as the ripe female had been stripped, the male spawner was stripped for milt. If only males were available, stripped milt was collected into a dry glass and stored in an icebox or refrigerator (Kungvankij, 1986; Ruangpanit, 1986). The preserved milt available for immediate use or could be viable for about one week in cold storage at 5-15 °C.

Fertilised eggs were washed repeatedly with clean salinewater (28-32 g L⁻¹ salinity) and sent for hatching after mixing the sperm and eggs together. Success using this method of spawning is severely limited by the difficulties in obtaining ripe broodstock and results in very low hatch rates and has been almost entirely replaced by hormonally induced natural spawning.

2.2.2 Induced spawning by hormonal injection

The broodfish are stocked in the pre-spawning tank for two months and the females are inspected twice a month for ovarian maturity. Eggs are examined via a biopsy with a polyethylene cannula and the egg diameter measurement is recorded. When the seabass eggs reach the tertiary yolk globule stage or have a diameter of 0.4-0.5 mm, the female is ready for hormone injection (Kungvankij, 1986; Ruangpanit, 1986). For males, only those with running milt are chosen. Before the injection, the spawner should be weighed and the hormone requirement computed. Spawners should be injected intramuscularly, either below the dorsal

pin or the base of the pectoral fin. After injection, they should be transferred from the pre-spawning tank to the spawning tank. In the last two decades, after the discovery of GnRH (Schally, 1978) and the synthesis of highly active analogue of GnRH (Crim and Bettles, 1997), spawning induction therapies shifted from the use of GtHs. This was partly due to the fact that by acting at a higher level of the brain–pituitary–gonad axis and stimulating the release of the fish's own LH, GnRHs may provide for a more integrated stimulation of reproductive processes and other physiological functions. Today, both GnRHs and GtHs are used extensively in spawning induction therapies (Mylonas and Zohar, 2007).

Asian seabass that are induced to spawn by hormone treatment usually spawn within 12 hours after the second injection and the schedule of injections must be synchronised with the natural spawning time. Fertilised eggs float on the surface as a milky white scums (Kungvankij, 1986) and can be siphoned out for hatching. If the brood fish do not spawn in the tank after the second injection, they are stripped and the eggs are artificially fertilised (Pillay, 1990). The fecundity ranges from 2-17 million eggs, depending on the size of the spawner.

2.2.3 Induced spawning by environmental manipulation

Captive broodstock of Asian seabass were successfully induced to spawn naturally using environmental stimulation and this type of spawning induction is still often used to spawn fish in Asia (Kungvankij, 1984 cited in Kungvankij, 1986; Szentes *et al.*, 2012; Ayson and Ayson, 2012). Environmental manipulation involves either changing the water salinity to stimulate fish migration, decreasing the water temperature to simulate the decreased water temperature after rain, and lowering followed by subsequent addition of fresh seawater to the tank in order to simulate the rising tide during the lunar phases. The salinity in the pre-spawning brood fish tank is prepared at 20-25 g L⁻¹ and increases up to 30-32 g L⁻¹ after the spawners are stocked (Pillay, 1990; Kungvankij, 1986). This will take about two weeks and it simulates the migration of fish from estuarine feeding areas to coastal spawning grounds.

The pre-spawning behaviour of the spawners is carefully monitored. Pre-spawning activities include: turning laterally during swimming, separation of the female fish from the school, pairing of the male and female fish, and increase in play activity (Kungvankij, 1986; Fortes, 1986). The female fish is separated from the school and feeding is stopped one week prior to spawning. Spawning normally takes place during the full moon and new moon period. At this time the temperature of the water in the spawning tank, containing the females and males, is raised to 31-32 °C by lowering the water level to about 30 cm and exposing the water

to the sun for 2-3 hours (Kungvankij, 1986; Pillay, 1990). Filtered seawater is then rapidly added to the tank to simulate the rising tide and this leads to a sudden lowering of water temperature to 27-28 °C. This induces the fish to spawn during the succeeding night. If no spawning occurs, manipulation is repeated for 2-3 more days, until spawning is achieved. The fish usually spawns intermittently for about 3-7 days (Pillay, 1990). Whether the fish are induced by hormone treatment or environmentally manipulated to spawn, they can continue to spawn for 2-5 days after the first spawning (Kungvankij, 1986). The use of the tank spawning method for Asian seabass either naturally, or by hormone injection has a lot of advantages compared to the previous method of stripping the eggs at their natural spawning ground (Ali, 1986).

2.3 Sexual maturity and sex inversion

The transition process from one sex to another sex state is called sex inversion. Sex inversion in Asian seabass is believed to be intimately linked with the spawning season as females are derived from post-spawning males, and the transition is completed shortly after spawning (Davis, 1982; Yue *et al.*, 2012; Ravi *et al.*, 2014). Sex inversion is initiated as the male testes ripen for the last time and the transition to ovary is completed within about a month of spawning (Davis, 1984a). In the early life stage the majority of the Asian seabass appears to be male. Not all females appear to have sex changed from males (Davis, 1986; Yue *et al.*, 2012). Thus, primary females, which are not derived from male fish, also occur in Asian seabass populations (Moore, 1980 cited in Garrett, 1986) and thus possibly a very small percentage of females develop directly from immature fish. Sexually precocious populations from the Northern Gulf region may fully mature at much smaller lengths of 300-400 mm (Davis, 1984a) and among farmed fish in Queensland and Northern Territory (Schipf, 1996; Pankhurst, 1998; Allan and Stickney, 2000; Ayson and Ayson, 2012). Female Asian seabass in Queensland matured approximately at 840 mm in length (Garrett, 1986). Males matured at between 510 and 700 mm and between 550 and 680 mm, respectively in Papua New Guinea and Northern Australia (Davis, 1982). The smallest sexually active male fish in northeast Queensland stock was 535 mm long (Garrett and Russel, 1982 cited in Garrett, 1986). Female Asian seabass mature at a larger size than the males. Asian seabass in Papua New Guinea, mature as males at 3 to 5 years, whilst secondary females become mature at 8 or 9 years (Opnai and Tenakanai, 1986). Typically, Queensland Asian seabass attain sexual maturity (as males) in their third to fifth year of life (Garrett, 1986) while sexually precocious fish mature in their first or second year (Davis, 1984a). Moore (1979) captured a single 2-year old 420-mm TL female, which was smaller than the size at which males normally mature. There are some primary

females, which mature at 730 mm or 6 years. Patnaik and Jena (1976) also encountered the smallest mature female at 700 mm and male at 505 mm in India (James and Marichamy, 1987). As females, Asian seabass may not commence egg production until they are quite old, for example eight year old (Davis, 1984a).

As the gonads of newly formed females develop rapidly and are soon indistinguishable from those of previously spawned females, a complete reorganisation of the gonad structure and function occurs with sex inversion (Moore, 1979), probably under the influence of hormones (Davis, 1982). The only reliable way to detect transitional stages is by routine examination of histological sections stained with haematoxylin and eosin (Davis, 1986; Chang and Yueh, 1990; Wu *et al.*, 2009). The wide occurrence of protandry in Queensland seabass stocks were confirmed for Gulf of Carpentaria, South-East coast, Central and South-Eastern coast fish. In Van Diemen Gulf, transitional stages were detected from October to January, while in the Gulf of Carpentaria transitional fish were found from October to March.

Environmental manipulations such as social factors, temperature and stocking density and their interactions influence on the genotype to alter fish sex. Social interactions are commonly implicated in the onset of adult sex change in hermaphrodite fish (Budd *et al.*, 2015). A shortage of mature male broodstock is a major challenge in maintaining proper breeding programmes for Asian seabass in culture facilities. Manipulation of social factors provides the possibility to retain protandrous Asian seabass males at mature ages for longer periods in captivity (although this has not been confirmed experimentally). In such cases, genotype is thought to be inhibiting complete control of sex by external factors (Budd *et al.*, 2015). Further, several endogenous and exogenous factors may trigger or mediate sexual inversion. Generally androgens induce masculinization and estrogens feminisation. Testosterone (T) and estradiol (E₂) production was inversely correlated with the proportion of testicular tissue. There are close relationships between developing male tissue and 11-KetoTestosterone (11-KT) in blood plasma or its gonadal biosynthesis (Condeca and Canario, 1999). During feminisation there is a reduction in 11-KT and an increase in E₂ (Godwin and Thomas, 1993; Guiguen *et al.*, 2010) and vice versa during masculinization (Sun *et al.*, 2013). The E₂ to androgens ratio also significantly increased during the course of sex reversal in the protandrous anemonefish, *Amphiprion melanopus* (Godwin and Thomas, 1993). Guiguen *et al.* (1993) suggested that E₂ plays an important role in the protandrous sex reversal process in the Asian seabass. Further, estrogen treatment has been shown to induce male to female sex change in protandrous hermaphrodites, including in the Asian seabass (Anderson and Forrester, 2001; Guiguen *et al.*, 2010). Testosterone should act as a precursor for the synthesis of E₂. Conversion of T into E₂ is

the rate limiting step in E₂ biosynthesis and is catalysed by an aromatase enzyme (Guiguen *et al.*, 2010; Piferrer *et al.*, 2012; Ravi *et al.*, 2014).

It is most likely that the aromatase which is present in the gonadal granulosa cells (Kagawa *et al.*, 1984) is the main limiting factor in E₂ production. Follicular aromatase is required for E₂ production leading to feminisation and is probably activated by gonadotropin (Kagawa and Young, 1984; Ravi *et al.*, 2014). A low aromatase activity leading to E₂ synthesis was found in the pre-vitellogenic ovary of a protandrous Asian seabass (Guiguen *et al.*, 1993). Aromatase activity that catalyses the conversion of T to E₂ is low in the functional male (Sun *et al.*, 2013) and bisexual phases (Chang *et al.*, 1991). Anderson and Forrester (2001) described the increased aromatase activity and associated decreased 11-KT: E₂ ratio may be seen as either a cause of sex reversal or a consequence of the sex change process of Asian seabass (Piferrer *et al.*, 2012) as shown in sex reversal of black porgy (Chang *et al.*, 1997; Guiguen *et al.*, 2010).

Previous research (Bogart, 1987; Pieau, 1996; Shimada, 1998; Jeyasuria and Place, 1998; Diotel *et al.*, 2010; Vizziano- Cantonnet *et al.*, 2011) and a review (Guiguen *et al.*, 2010) showed that the aromatase enzyme is now considered to be a key enzyme in gonadal sex differentiation, at least in fish, reptiles and birds. In fish, the enzyme aromatase is probably one of the key enzymes needed for both ovarian differentiation in gonochoristic fish and for sex inversion in protandrous hermaphroditic fish (Baroiller & Guiguen, 1999; Guiguen *et al.*, 2010; Piferrer *et al.*, 2012; Sun *et al.*, 2013; Ravi *et al.*, 2014). Sexually undifferentiated genotypic females that are treated with a specific aromatase inhibitor developed as phenotypic males in several fish species, also demonstrating the importance of aromatase in sex differentiation (Gonzalez and Piferrer, 2002; Guiguen *et al.*, 2010). Aromatase inhibitors work by either irreversibly deactivating the aromatase enzyme, or through competitive exclusion of aromatase to receptors in estrogen producing cells (Budd *et al.*, 2015)

2.4 Hormonal and enzymatic regulations related to reproduction

Steroid hormones are synthesised from cholesterol (Hussain, 2000) within the endoplasmic reticulum of the developing oocyte (Hiort and Holterhus, 2000). E₂ are produced normally in a steroid biosynthetic (steroidogenic) pathway that involves androgens as a substrate (See Figure 2.1). E₂ synthesis requires either prior synthesis of an androgen or an external source of androgen (Norris, 1997). Besides E₂ having feminizing effects, treatment with aromatase inhibitors that blocked E₂ synthesis were found to induce masculinization when applied during the sex differentiation period of fish (Guiguen *et al.*, 2010).

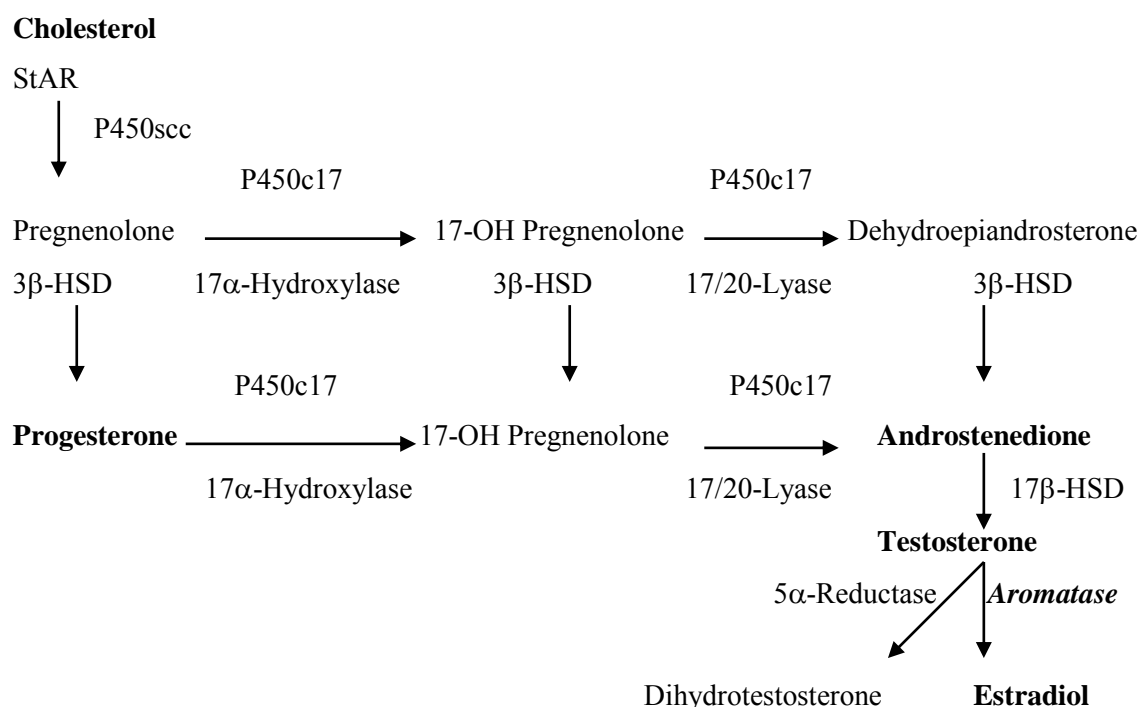


Figure 2.1 Pathways and metabolites of androgen biosynthesis (Adapted from Hiort and Holterhus, 2000)

Aromatase, in particular cytochrome P450, is the critical enzyme for biosynthesis of estradiol-17β from testosterone (Strussmann and Nakamura, 2002; Zhang *et al.*, 2004; Guiguen *et al.*, 2010; Piferrer *et al.*, 2012; Ravi *et al.*, 2014) and is the enzyme that influences the physiological balance between the sex steroid hormone estradiol-17β and testosterone (Conley and Hinshelwood, 2001). The aromatization of androgens to estrogens takes place in the endoplasmic reticulum of the developing oocyte (granulosa cell layer) and is classified as a mixed-function oxidase reaction (Jeyasuria *et al.*, 1996). Most P450 enzymes occur in the smooth endoplasmic reticulum (SER) and often metabolize multiple substrates and the presence of steroidogenic enzymes in SER leading to their recognition as being members of the P450 cytochrome family of proteins which are reflected in the abbreviations used for some of the enzymes (Norris, 1997). In some teleost fishes, such as *Carassius auratus*, *Danio rerio* and *Oreochromis niloticus*, two distinct P450 aromatase isoforms (brain and gonad) have been identified (Zhang *et al.*, 2004; Piferrer *et al.*, 2012; Ravi *et al.*, 2014)). The aromatase activity in the brain and gonad has been well documented and is an evolutionary conserved characteristic of vertebrates (Conley and Hinshelwood, 2001; Zhang *et al.*, 2004).

The aromatase enzyme complex (*CYP19*) is the sole mediator for the conversion, which consists of two membranes bound P450 enzymes namely, a cytochrome P450 aromatase

(P450_{arom}) and NADPH- dependent cytochrome P450 reductase, a product of the *CYP 19 gene* (Simpson *et al.*, 1994; Tchoudakova and Callard, 1998; Zhang *et al.*, 2004; Piferrer *et al.*, 2012; Ravi *et al.*, 2014). The overall reaction involves three steps namely, hydroxylation of the C-19 methyl group, release of formic acid, and a resulting spontaneous aromatization of the A-ring (Jeyasuria *et al.*, 1996). The association of P450_{arom} during vitellogenesis is confirmed by previous studies in rainbow trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*) and tilapia (*Oreochromis niloticus*), which have shown that the mRNA level of P450_{arom} are increased in association with enzyme activity and the involvement of P450_{arom} enzymes in this conversion (Gen and Okuzawa, 2001). The terminal enzyme in this steroidogenic pathway is P450_{arom}, which is responsible for this conversion of androgens into estrogens (Simpson *et al.*, 1994; Trant and Gavasso, 2001; Guiguen *et al.*, 2010; Piferrer *et al.*, 2012).

Gen and Okuzawa (2001) showed that P450_{arom} mRNA levels in ovaries of Red seabream (*Pagrus major*) increased in association with the increase of GSI (Gonado Somatic Index) and serum E₂ levels during gonadal development. This is consistent with a physiological study that E₂ biosynthesis and P450_{arom} mRNA levels raise in parallel during sexual maturation in catfish (Kumar and Ijiri, 2000). Furthermore, Gen and Okuzawa (2001) also observed in the Red seabream that the increase of aromatase activity of ovarian fragments assessed by *in vitro* conversion of T to E₂ was associated with the ascending pattern of P450_{arom} mRNA levels. Therefore, the increase in E₂ production during oocyte development was due primarily to the increased levels of P450_{arom} transcripts (Piferrer *et al.*, 2012; Ravi *et al.*, 2014).

GtH has been reported to induce P450_{arom} activity (conversion of T to E₂) in ovarian follicles of some fish, but it remains unclear whether FSH or LH can stimulate the expression and activation of P450_{arom} in teleosts (Gen and Okuzawa, 2001). Chang *et al.* (1997) also suggests that E₂ acts through positive feedback to stimulate GnRH-GtH expression and secretion. Increase of aromatase activity further stimulates E₂ production and ovarian development, and finally causes sex reversal.

Zanuy and Carrillo (1998) have presented some evidence of an active role of testosterone on sex differentiation of European seabass and have suggested that aromatase has an important key role in sex differentiation regulating endogenous levels of E₂ in fish. However, experiments regarding aromatase enzymes in European seabass found that aromatase activity was significantly higher after morphological gonadal sex differentiation (Guiguen *et al.*, 2010) and prior to the work described in this thesis, a similar experiment regarding aromatase activity in Asian seabass was yet to be published.

Chapter 3: EFFECT OF ONTOGENY ON THE SEX INVERSION OF ASIAN SEABASS

Author contributions – Chapter 3

Details of publications on which chapters is based	
<p>Saman Athauda and Trevor Anderson. 2013. Effect of Age/Size on Plasma Sex Steroids and Aromatase activity of Asian seabass (<i>Lates calcarifer</i>) when transferred to ideal hatchery conditions. European International Journal of Science & Technology. Vol.02, Issue.09: Pp. 29-40.</p>	
Name of candidate and other co-authors	Nature and extent of the intellectual input of each author, including the candidate
<p><u>Candidate:</u> Saman Athauda (SA)</p> <p><u>Co-author:</u> Trevor Anderson (TA)</p>	<p><u>SA</u> and <u>TA</u> co-developed the research question. <u>SA</u> performed the experimental work and laboratory analysis. Data analysis with assistance from TA. <u>SA</u> wrote the first draft which was revised with editorial inputs from <u>TA</u>. <u>SA</u> developed the figure and tables.</p>

CHAPTER 3

EFFECT OF ONTOGENY ON THE SEX INVERSION OF ASIAN SEABASS

3.1 INTRODUCTION

The sex inversion of fish is a complex and labile mechanism under the control of genetic, physiological and/or environmental factors (Baroiller and Guiguen, 1999; Delvin and Nagahama, 2002; Budd *et al.*, 2015). Asian seabass, born as males, reach early maturity and change into females within two years of age in farmed conditions in comparison to six to eight years in the wild (Guiguen *et al.*, 1994; Allan and Stickney, 2000; Ayson and Ayson, 2012; Yue *et al.*, 2012; Szentes *et al.*, 2012; Ravi *et al.*, 2014). Precocious sex inversion has been observed after eighteen months of age in captivity at hatcheries in Queensland and the Northern Territory, Australia (Anderson *et al.*, 1998; Ayson and Ayson, 2012). This precocious sex change may be the result of the captive environment. A previous study by Anderson *et al.* (1998) indicated that precocious development of Asian seabass may be advanced by culture in salinewater conditions compared with freshwater.

In Northern Australia, male Asian seabass in Van Diemen Gulf and South-East Gulf of Carpentaria mature between 3 and 5 years age in the wild. However, sexually precocious maturity was reported in North-East Gulf of Carpentaria fish that were observed to mature in their first or second year (Davis, 1985, Garrett, 1986). Gonad development was retarded in landlocked freshwater Asian seabass from the same region (Davis, 1985). Ayson and Ayson (2012) reported that spermiating males and maturing females of Asian seabass can be found in captivity at the age of two-three years. Furthermore, spermatozoa have been detected at the age of nine months when the Asian seabass are grown in intensive aquaculture systems (Szentes *et al.*, 2012) presumably due to the fish gaining larger body sizes quicker than in the wild when they are in intensive fed farming systems.

It has been suggested (Haddy and Pankhurst, 1998) that salinity may play a role in regulating reproductive activity in fish. There is relatively little published data or information on the effects of salinity on the timing of reproductive development (Bromage *et al.*, 2001) and reproductive physiology in fish (Haddy and Pankhurst, 2000), even though considerable numbers of fish undergo migrations between freshwater and brackish water and vice versa, especially for reproductive activities. Previous studies with other protandrous species; *Amphiprior melanopus* (Godwin and Thomas, 1993) and *Acanthopagrus schlegeli* (Wu *et al.*, 2009) also showed that sex steroids have a significant role in sex change and gonadal

development in teleost fish (Guiguen *et al.*, 2010). Therefore, sex steroids estradiol-17 β (E₂), testosterone (T) and 11-ketotestosterone (11-KT) have received most attention (Fostier and Jalabert, 1983; Borg, 1994).

Protandrous sex inversion in Asian seabass could be associated with a shift in gonadal steroidogenesis from androgens to estrogens (Guiguen and Jalabert, 1995; Ravi *et al.*, 2014). Thus, increases of aromatase activity further stimulate E₂ production and ovarian development, and finally cause sex inversion into females. Results of recent research and a review (Guiguen *et al.*, 2010; Ravi *et al.*, 2014) also showed that the aromatase activity has a stimulatory effect on E₂ production in Asian seabass.

This chapter sought to determine the ontogeny (age/size) at which a change to ideal hatchery conditions (water temperature 28 °C; salinity 32 g L⁻¹ and photoperiod of 14:10 L: D) might induce changes of the serum steroids (E₂, T and 11-KT) concentration and aromatase activity in gonad and brain across different size group of Asian seabass in order to understand the course of sex inversion process of cultured Asian seabass under hatchery conditions.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design and sampling procedures

A group of Asian seabass at different sizes (50 - 100 g, 300 - 500 g, 700 - 1000 g and 2.5 - 4 kg) that range from immature to mature male were obtained from a commercial farm (GFB Fisheries, Kelso) in North Queensland (19 °S, 147 °E) where they were maintained in freshwater (0 g L⁻¹) under natural conditions at a temperature of 28 °C and photoperiod of 12:12 Light: Dark. Fish were randomly taken from the cages and immediately transported in oxygenated tanks (80 L) to the Marine and aquaculture facility (MARFU) at James Cook University, Townsville, Australia, where they were used for the experiment. Fish were fed with commercially formulated barramundi pellets (50% protein, 18 MJ Kg⁻¹, Ridley Aquafeeds, Brisbane, Australia) to satiety twice daily when held on the farm and to satiety once daily in the aquaculture facility at James Cook University. Fish were acclimatized to the aquarium conditions (28 °C and 14:10 L: D).

Eight groups of Asian seabass (n = 8 / group, two groups per size class) according to their body weight were allocated to four 3000 L tanks (each tank was considered as one experimental unit) and were contained in a single enclosed room. A group (n = 8) of fish of

each size class were sampled under the initial freshwater condition prior to commencing the exposure of the remaining fish to experimental conditions of saline water. Gradual changes of water salinity from freshwater (0 g L^{-1}) to saline water (32 g L^{-1}) were undertaken over a one week period for each of the size class fish and subsequently maintained throughout the experimental period of nine weeks at 28°C and at 32 g L^{-1} , respectively. All fish were reared at photoperiod of 14:10 L : D in four 3000 L tanks which were supplied with bio-filtered recirculated water throughout the experimental period. One third of the tank water was changed every day throughout the experimental period in addition to removing uneaten feed, faeces or any foreign material twice a day by siphoning. Both saline and freshwater were supplied from the main supply of the aquarium system to adjust the required salinity levels for each experimental tank and siphoned water was discharged to the waste water system.

Temperature and Salinity were measured twice daily (9.30 am and 2.30 pm) and adjustments were made, if required, to maintain experimental condition. During this period water quality parameters (pH, Ammonia, Nitrate and Nitrite levels) were measured using standard test kits (Aquarium Pharmaceuticals, Inc. USA) once a week throughout the experimental period and were maintained at constant and equal levels in all the units. Dissolved Oxygen was measured everyday by DO meter and maintained above 5 mg L^{-1} . A UV sterilizer (QL 160, Rainbow Aq Products, Elmonte, CA 91734) was used to sterilize the water as a preventive method of disease control in addition to the biological filtration.

Average water quality parameters during the experiment period are shown below (Table 3.1). Ammonia and nitrite levels were measured routinely and were maintained at less than 1.0 mg L^{-1} while nitrate was around 12.0 mg L^{-1} . Mean salinity and temperature were maintained at $30\text{-}32 \text{ g L}^{-1}$ and 28°C , respectively and measured daily, while pH was measured weekly and maintained at 8.0.

Table 3.1 Average water quality parameter of Experiment 1 for all fish tanks. Values are mean \pm S.E.

Salinity (mg L^{-1})	pH	Ammonia (mg L^{-1})	Nitrite (mg L^{-1})	Nitrate (mg L^{-1})	Temperature $^\circ\text{C}$
31 ± 0.24	8.0 ± 0.02	0.64 ± 0.05	0.037 ± 0.004	12.72 ± 0.55	28.3 ± 0.07

Fish were anaesthetized to collect blood samples for steroid hormone assays at the beginning (n = 6 or 8) and at the end (n = 7 or 8) of the nine weeks experimental period. A 2.5 mL blood sample was extracted from the caudal vein using a 5 mL syringe and an 18-gauge hypodermic needle. Blood samples were immediately transferred to 2.5 mL fluoride-oxalate tubes (Sarstedt, Technology Park, SA 5095, Australia), mixed gently and stored on ice until transported to the laboratory. Blood samples were centrifuged at 14000 g for 10 min at 4 °C (Eppendorf centrifuge 5415C, Hamburg, West Germany) and the plasma stored at -80 °C until the assay for E₂, T or 11-KT.

Upon collection of the blood samples, the fish were sacrificed; brain and gonads were removed aseptically, transferred to labeled vials, and placed in liquid N₂ at -80 °C for the aromatase assay.

3.2.2 Sex steroids assay

Plasma levels E₂, T and 11-KT were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Conroy (1987). Briefly, 300 µL of plasma was extracted with 1.5 mL ethyl acetate, 200 µL of the extract was added to each assay tube for evaporation and resuspension in assay buffer, with the reagent and supernatant mixed with ready gel (Ecolite, USA), and ³H-labelled steroid in each sample was measured in duplicate using a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA). Extraction efficiency (recovery level of ³H-labelled steroid from plasma) was determined and assay values were corrected accordingly for E₂, T and 11-KT. Interassay variability was measured using aliquots of a pooled internal standard for E₂, T and 11-KT. The detection limit of E₂, T and 11-KT in plasma was 3 ng/tube, and all samples were measured in a single assay. E₂, T and 11-KT concentrations were determined against a standard curve.

3.2.3 Measurements of aromatase activity

Aromatase activity in the gonad and brain was measured by tritiated water release assay (radiometric method), which had been previously validated for use with Asian Seabass in our laboratory (Anderson and Forrester, 2001). Macerated gonad tissue was thoroughly homogenised at 24000 g in 10 volumes (w/v) of a cold solution containing 100 mM KCl, 10 mM K₂HPO₄, 1 mMethylenediaminetetraacetic acid (EDTA) and 2 mMdithiothreitol (pH 7.4)

using a Heidolphdix 600 homogeniser with 10G tools. The homogenate was then sonicated for 30 seconds (Unisonics FX8) and centrifuged at 1000g for 5 minutes at 4 °C. The non-lipid portion of the supernatant was recovered by piercing the tube immediately above the pellet with a heated 18 gauge needle. Aliquots of extract were stored at -80 °C prior to the assay. Extract (150 µL) was incubated at 30 °C with 450 µL of solution containing 100mM KCl, 10mM K₂HPO₄, 1mM ethylenediaminetetraacetic acid (EDTA) and 2mM dithiothreitol, 5mM glucose-6-phosphate, 1mM β-nicotinamide adenine dinucleotide phosphate (NADP), 2U glucose-6-phosphate dehydrogenase plus 66.67 nM androst-4-ene-3, 17-dione (pH 7.4). At 10 and 30 minutes, a 200 µL aliquot of the reaction mixture was terminated by mixing with 100 µL of 30% trichloroacetic acid containing 60 mg mL⁻¹ charcoal. After standing for 30 minutes, the mixture was then centrifuged at 10,000g for 5 minutes and the supernatant (200 µL) was added to a 1.0 x 3.0 cm column packed with equal volumes of 50-100 and 100-200 mesh AG50W-X4 resin (Bio-Rad, Hercules, CA). Samples were then eluted with 2.3 mL of deionized water, with the final 1.5 mL collected and mixed with 15 mL Ready Gel (Beckman, USA). DPM (radioactivity) was measured over 5 minutes with a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA).

Aromatase activity was expressed using the production of ³H₂O from ³H-androstenedione (³H-A) according to the method of Anderson and Forrester (2001). The Bradford (1976) method was used for determining protein concentrations of the crude supernatant fraction by monitoring the absorbance of protein-dye complex. Aromatase activity was assayed in the gonad and brain tissues of all fish except for those of 50 g of body weight, which had insufficient amount of tissue to run the assay. Aromatase activity was expressed as the reaction velocity for the conversion of androstenedione to tritiated water (formation of ³H₂O during aromatization of ³H-A) using fmoles/mg protein/min as the units.

3.2.4 Data analysis

Each tank was considered as one experimental unit and each fish as one replicate. Two-way Analysis of Variance (ANOVA) was used with Tukey's HSD test to test for the difference between treatments for hormone and aromatase or steroids levels. Least Significant Difference (LSD) was used to compare the significant levels within and in between treatments and their interactions. Significant effects were assumed at p = 0.05. All data were analyzed using SPSS version 12.0 and where appropriate, proportion data were normalized by transformation.

3.2.5 Ethics of the study

All experimental regimes were conducted within the guidelines of “ The Australian Code of Practice for the Care and Use of Animals for Scientific Purpose” Ethical approval was provided by James Cook University Animal Ethics Committee (Approval number – A 754).

3.3 RESULTS

3.3.1 GENERAL

3.3.1.1. Growth

Initial and final average weights of fish are shown in Table 3.1. Average Daily Growth (ADG) decreased as the size of the fish increased, with the highest in the smallest fish (50 - 100 g) and lowest in the largest fish (2.5 - 4 kg). Average size at the end of the experiment was significantly greater than the beginning weight in all fish groups except 2.5 – 4 kg; the largest fish group (Table 3.2).

Table 3.2 Mean fish weight at the beginning and the end of Experiment 1 and average daily growth (ADG) over the experiment for each group of fish. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Group	Initial Wt (g)	Final Wt (g)	ADG (% body weight d ⁻¹)
50 – 100 g	81 \pm 4 ^a	435 \pm 27 ^b	7.05
300 – 500 g	463 \pm 13 ^c	1025 \pm 35 ^d	1.96
700 – 1000 g	636 \pm 18 ^e	1225 \pm 61 ^f	1.49
2.5 – 4.0 kg	4408 \pm 445 ^g	5375 \pm 530 ^g	0.35

ADG = [(Final Average Weight) - (Initial Average Weight)]/Number of experiment days

3.3.1.2. Survival rate

No fish died during the experiment.

3.3.2 AROMATASE

3.3.2.1. Aromatase activity in the brain

The aromatase activity at the beginning of the experiment was significantly greater in the brain of 700 – 1000 g and 2.5 – 4 kg animals than in the brain of 300 - 500 g animals, compared to that at the end of the experiment.

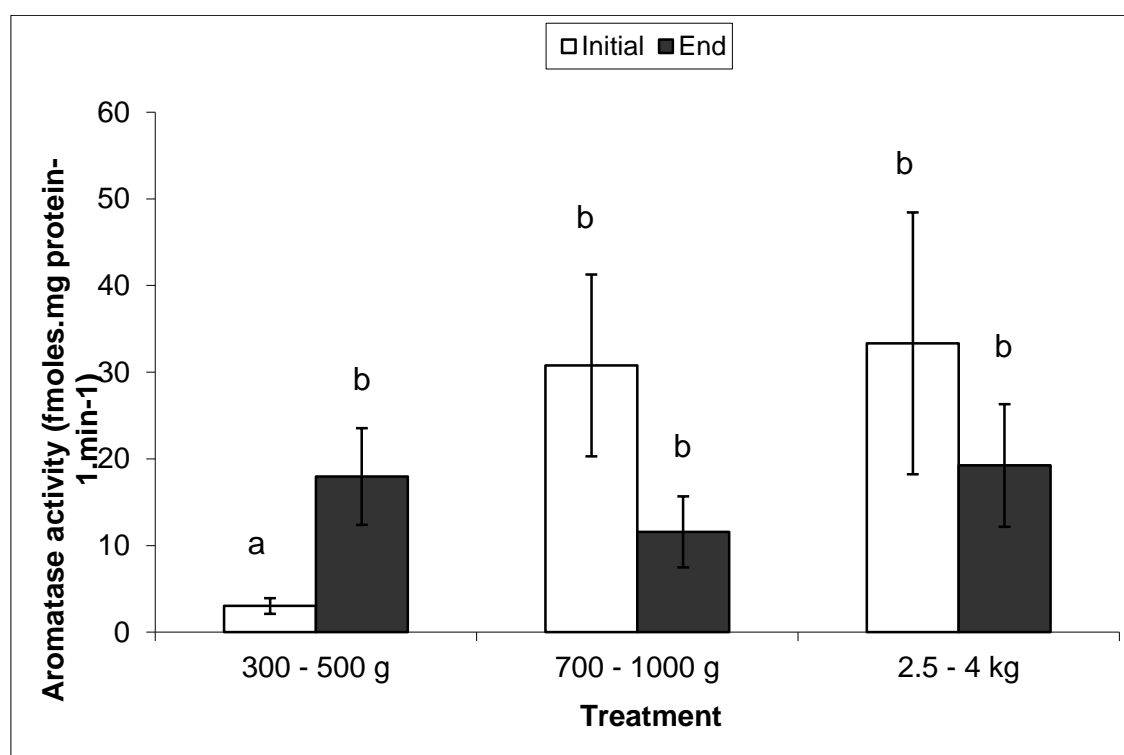


Figure 3.1. Aromatase activity in the brain. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Aromatase activity in the brain of fish group 300 – 500 g was significantly increased ($p < 0.05$) at the end of the experiment and in the remaining two groups (700 - 1000 g and 2.5 – 4 kg) it was lower, but not significantly, compared to their initial aromatase activity. Aromatase was undetectable in the brain of the smallest group (50-100 g) of fish. The aromatase activity in the brain during the experimental period is shown in Figure 3.1. Two-way ANOVA revealed a significant effect of group and the group \times time interaction, but not for time (Table 3.3).

Table 3.3 Result of two-way ANOVA of Group and Time for aromatase activity in the brain of Asian seabass

Main Effects	Sum of squares	DF	Mean Square	F	Significance of F
Group	1.191	2	0.595	4.068	0.026
Time	0.039	1	0.039	0.269	0.607
Group x Time	1.949	2	0.974	6.658	0.003
Residual	5.268	36	0.146		
Total	59.396	43			

3.3.2.2. Aromatase activity in the gonad

The changes in aromatase activity in the gonad are shown in Figure 3.2. Aromatase activity was present in the fish group 700 - 1000 g at the end of the experiment and was significantly greater than in the gonad of the same size fish at the beginning of the experiment. Asian seabass in the 2.5 – 4 kg group had a very small amount of activity that wasn't significantly different to the undetectable values found in all the other sample groups.

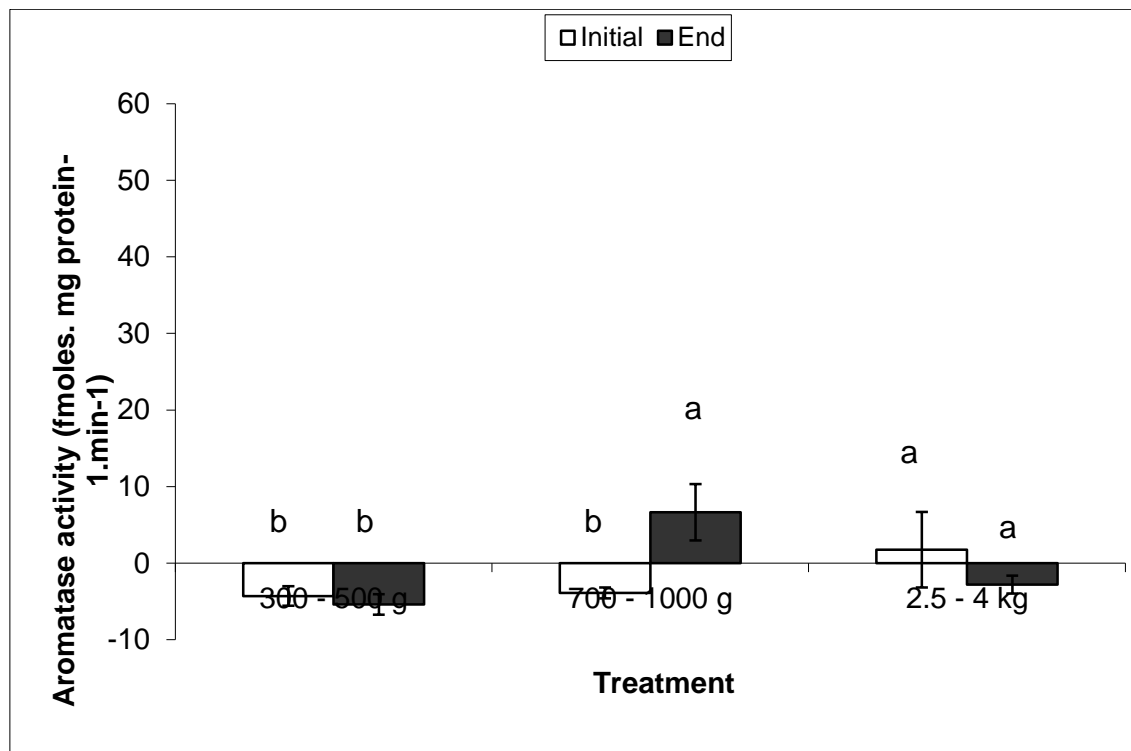


Figure 3.2 Aromatase activity in the gonad. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Table 3.4 Result of two-way ANOVA of Group and Time for aromatase activity in the gonad of Asian seabass.

Main Effects	Sum of squares	DF	Mean Square	F	Significance of F
Group	186.062	2	93.031	3.734	0.034
Time	60.968	1	60.968	2.447	0.127
Group x Time	218.565	2	109.283	4.386	0.020
Residual	897.009	36	24.917		
Total	1548.649	43			

3.3.3 PLASMA STEROIDS

3.3.3.1. Testosterone (T)

Two-way ANOVA of the testosterone data showed a significant effect of group and time, but no interaction of group x time. There was no significant difference in plasma T levels in fish at different weight groups at the beginning of the experiment, although the mean value for the largest group was approximately twice that of the other groups (Figure 3.3). Initial T values were recorded as 0.127, 0.136, 0.163 and 0.295 ng mL⁻¹ in the 50 – 100 g, 300 – 500 g, 700 – 1000 g and 2.5 – 4 kg groups, respectively.

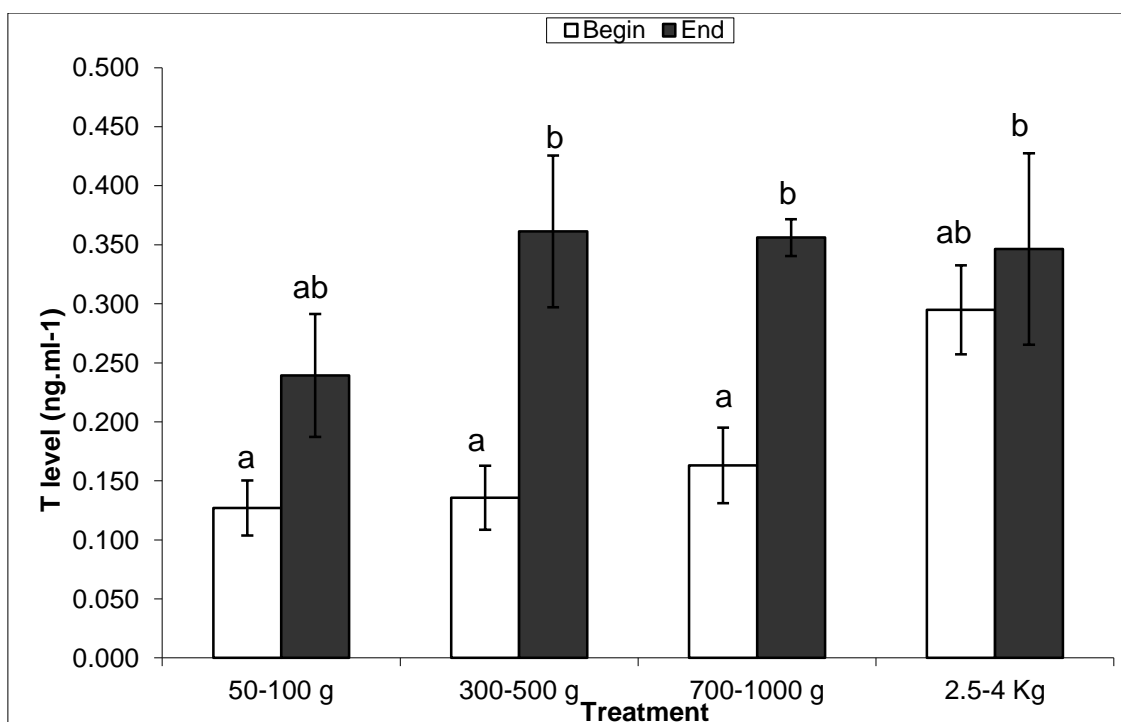


Figure 3.3 Mean Testosterone levels in fish of different weight groups. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Table 3.5 Result of two-way ANOVA of Group and Time for testosterone in the circulation of Asian seabass.

Main Effects	Sum squares	of	DF	Mean Square	F	Significance of F
Group	0.511		3	0.170	3.501	0.022
Time	1.075		1	1.075	22.0730	0. 000
Group x Time	0.387		3	0.129	2.649	0.058
Residual	2.532		52	0.049		
Total	33.054		60			

Within each fish group, plasma T levels increased with the increase appearing to be dependent on fish size. T values were recorded as 0.239, 0.361, 0.356 and 0.346 ng mL⁻¹ in the 50 – 100 g, 300 – 500 g, 700 – 1000 g and 2.5 – 4 kg groups, respectively. The smallest (50 – 100 g) and the largest (2.5 – 4 kg) fish had the smallest relative increases, which were not significantly different ($p < 0.05$).

3.3.3.2. Estradiol 17- β (E_2)

There was no difference in plasma E_2 levels among beginning fish at different weight groups. E_2 concentrations were slightly higher in the larger fish than in the smaller fish. The E_2 levels at the beginning were 0.002 and 0.003 ng mL⁻¹, respectively for the 50 –100 g and 300 – 500 g weight group fish, respectively and 0.034 ng mL⁻¹ was found in both the 700 – 1000 g and 2.5 – 4 kg weight groups. At the end of the experiment period E_2 levels were significantly increased ($p < 0.05$) within each of the fish groups and E_2 increased significantly with fish size (Table 3.6) i.e. 0.056, 0.106, 0.142 and 0.211 ng mL⁻¹ at 50 –100 g, 300 – 500 g, 700 –1000 g and 2.5 – 4 kg, respectively. Plasma E_2 levels of 50 –100 g and 300 – 500 g and 300 – 500 g and 700 – 1000 g were not significantly different to each other. Plasma E_2 in all fish smaller than 700 g were significantly lower ($p < 0.05$) than those of the 2.5 kg – 4 kg plasma E_2 level (Figure 3.4) where the value recorded was 0.211 ng mL⁻¹.

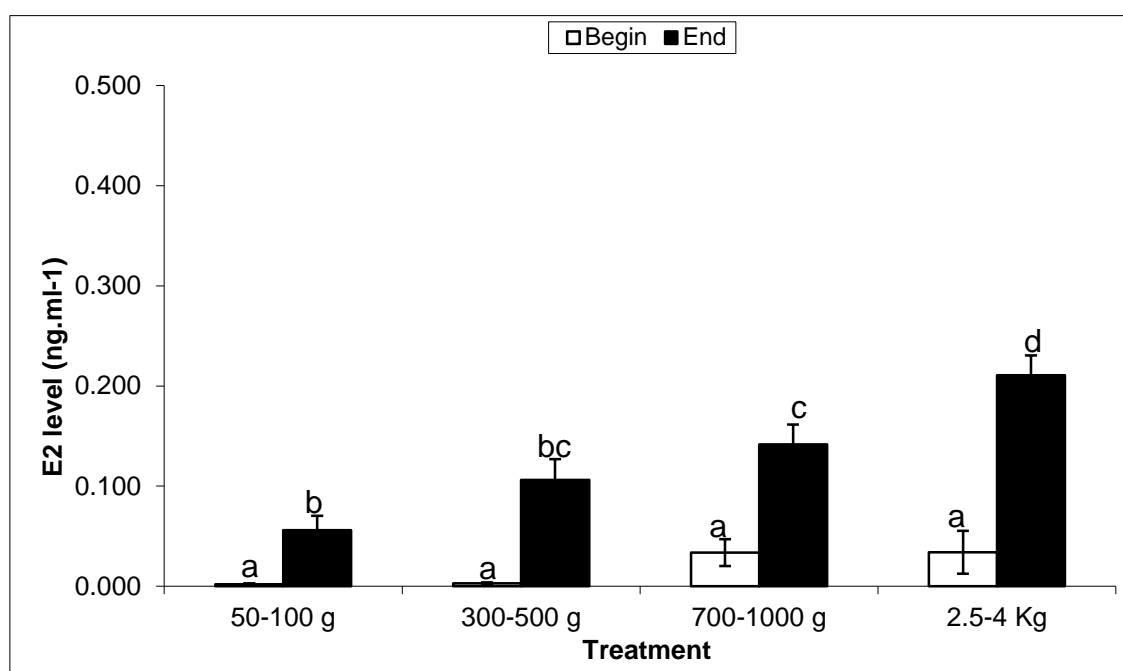


Figure 3.4 Mean Estradiol 17- β levels in fish at different weight groups. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Table 3.6 Result of two-way ANOVA of Group and Time for estradiol 17- β activity in the blood circulation of Asian seabass.

Main Effects	Sum of squares	DF	Mean Square	F	Significance of F
Group	0.104	3	0.035	3.921	0.014
Time	0.460	1	0.460	52.154	0.214

Group	x	0.041	3	0.014	1.544	0.214
Time						
Residual		0.450	51	0.009		
Total		4.378	60			

3.3.3.3. Keto-Testosterone (11- KT)

There were no measurable amounts of 11- KT in the two smallest groups (50 -100 g and 300 - 500 g) of fish. Measurable amount of 11- KT was (0.01 ng mL^{-1} and 0.045 ng mL^{-1}) detected in the plasma from fish in the 700 – 1000 gram and 2.5 – 4 kg groups, respectively. There was no significant difference between the beginning and the end of the experiment in 11- KT concentrations in these fish (Figure 3.5; Table 3.7).

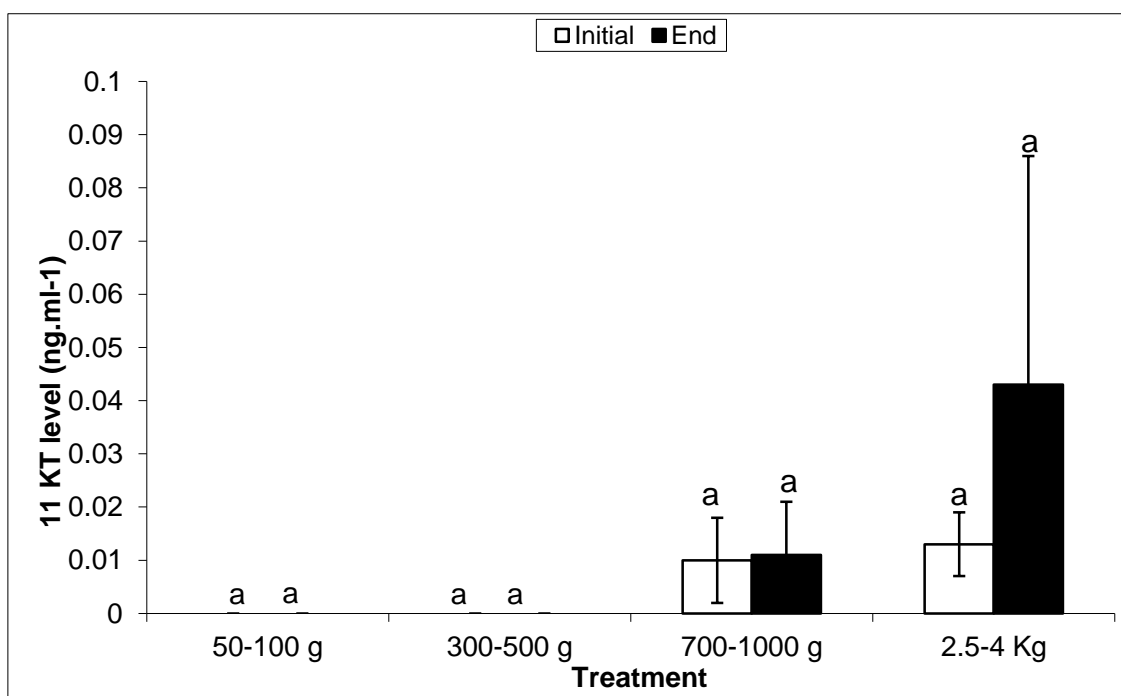


Figure 3.5 Mean 11 Keto Testosterone levels in fish of different weight groups at the beginning and end of the experiment. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Table 3.7 Result of two-way ANOVA of Group and Time for 11 Keto Testosterone activity in the circulation of Asian seabass.

Main Effects	Sum of squares	DF	Mean Square	F	Significance of F
Group	0.162	1	0.162	0.939	0.404

Time	0.328	1	0.328	1.902	0.262
Group x Time	0.371	1	0.317	2.152	0.239
Residual	0.518	3	0.173		
Total	17.450	8			

3.4 DISCUSSION

3.4.1 Growth

The highest Average Daily Growth was recorded in the smallest fish group 50 – 100 g followed by the 300 – 500 g and 700 – 1000 g fish groups, while the lowest was in the largest fish (2.5 – 4 kg). This is commonly observed with fish and shows a standard growth versus size relationship (De Silva and Anderson, 1995). Numerous exogenous factors have been shown to affect the growth of fish. In this experiment, the environmental factors (salinity and temperature and photoperiod) are optimum for Asian seabass growth and dietary factors (protein and energy) are also in sufficient levels. Growth rates observed in this study were similar to previously observed values (Raso and Anderson, 2003) in healthy growing Asian seabass under a healthy environment.

3.4.2 Effect on aromatase activity

Aromatase activity level in the brain increased significantly ($p < 0.05$) after nine weeks in the 300 – 500 g experimental fish group, but other groups (700 – 1000 g and 2.5 – 4 kg) showed no significant change in brain aromatase activity. The significant change that occurred with the change in brain aromatase was the increase in the size of the fish, with no changes occurring in larger animals with the change to a higher salinity environment. The size of the 300- 500 g group increased to be equivalent to the starting size of the 700-1000 g group and the aromatase activity in the brain increased to a level that was not significantly different to that at the beginning of the experiment in the 700-1000 g group. The level of brain aromatase activity was not different between groups in all fish of 636 ± 18 g or above. The brain is a site where environmental effects are largely integrated and temperature and salinity changes might be expected to exert an effect on the brain aromatase activity. However, the data presented here indicate that brain aromatase activity is a function of size/age of the fish rather than being influenced by external environmental parameters.

In protandrous black porgy (*Acanthopagrus schlegeli*), aromatase inhibitor Fadrozole blocked the natural sex change and significantly suppressed the brain aromatase activity (Lee *et al.*, 2001) although it would also have blocked gonadal aromatase activity. Aromatase activity in gonads was not detectable in most groups of Asian seabass in this study with one clear exception of the 700-1000 g fish after nine weeks at 28 °C, in which the gonadal aromatase activity level increased significantly ($p > 0.05$) over its initial level. Previous research (Kitano *et al.*, 1999; Kwon *et al.*, 2001; Piferrer *et al.*, 2012; Ravi *et al.*, 2014) has shown that gonadal aromatase plays a decisive role in sexual differentiation. In the black porgy, the increase of gonadal aromatase activity was associated with both natural and controlled sex changes (Lee *et al.*, 2000, 2001). Recent studies (Ravi *et al.*, 2014) expressed that ovarian aromatase involved in the conversion of testosterone to estrogen and precocious sex change was observed at two to four years of age in Asian seabass.

3.4.3 Effect on sex steroids

The role of T as one of the precursors for E₂ (Devlin and Nagahama, 2002; Piferrer *et al.*, 2012; Ravi *et al.*, 2014) has been well documented. There were significantly ($p < 0.05$) increased T levels in all fish groups (300-500 g and 700-1000 g) while the smallest (50 - 100 g) fish and largest (2.5 – 4 kg) groups of fish were not significantly different to the initial level. Factors affecting levels of T in circulation involve regulation of both synthesis of T and its conversion to other hormones. It is likely that the production of T is not yet fully functionally in 50 – 100 g fish which are immature. In contrast, 2.5 – 4 kg fish contained relatively high concentrations in the circulation at the beginning of the experiment but did not show a significant increase by the end of the experiment, indicating that metabolism of T was approximating its synthesis in these fish.

The end T level of smaller fish (50 – 100 g) is greater, but not significantly than the initial T level circulating in 300 – 500 g fish, although the weight of the two groups (435 ± 27 g in the smaller group *versus* 463 ± 13 g in the larger of the two groups; Table 3.1) were similar and indicated an effect of salinity also on T production in Asian seabass. The concentration of T observed in fish over 1000 g, which was the finishing size of the 300 – 500 g group, had a consistent mean between 350 and 400 pg mL⁻¹ suggesting that this may be the steady state level of T in Asian seabass held in seawater conditions.

All fish in this study showed very low levels of circulating E_2 at the beginning of the experiment. However, by the end of the nine weeks experimental period, there was a significant increase in the circulating E_2 concentration in all the fish groups. The E_2 level showed a clear increase with increasing size, with the lowest value recorded in the 50 –100 g fish, while the highest value was found in the 2.5 – 4 kg fish. A lower level of E_2 is likely to be found in the small fish (50 – 100 g) due to the absence of functional gonadal tissue, while in the larger fish the higher concentration of E_2 will reflect both a greater amount of gonadal tissue and the fact that it is ovarian tissue.

The plasma E_2 level increased in all groups after the fish had been kept for nine weeks under saline water conditions, compared with those of similar size that had been held on the farm in freshwater. These data clearly indicate that salinity has a stimulatory effect on plasma E_2 level in Asian seabass and that effect occurs in all fish for this species over 463 ± 13 g.

E_2 was detected in fish within the 300-500 g size class and T was detected in fish as small as 50 g. Brain and adipose tissue have been shown to produce these steroids (Zhang *et al.*, 2004) with aromatase being involved in the synthesis of E_2 from T (Guiguen *et al.*, 2010; Piferrer *et al.*, 2012; Ravi *et al.*, 2014). The absence of significant aromatase activity in the gonads in the same groups of fish that showed both brain aromatase activity and circulating of E_2 suggests that the brain is an important initial site of E_2 synthesis. The increase in brain aromatase in response to the change of environment occurred at sizes greater than 636 ± 18 g, but the increase in E_2 at smaller sizes in response to increasing salinity suggests that the regulation of aromatase activity is not the sole process by which E_2 is regulated, but it may affect the rate of response to environmental changes (Piferrer *et al.*, 2012; Sun *et al.*, 2013). In this study, 11 KT was detected only in the groups of larger fish (700 –1000 g and 2.5 – 4 kg) at both the initial, freshwater, and after nine weeks of higher salinity conditions (Fig. 3.5). Guiguen *et al.* (1993) showed that 0.1 to 0.2 ng mL⁻¹ 11-KT levels were detected in males during the spawning season; the levels measured in this study were < 0.045 ng mL⁻¹ in 700 – 1000 g and 2.5 – 4 kg group fish and may be due to the fact they were in transitional states from male to female. Further, the fish group 50 – 100g and 300- 500 g did not show any detectable level of 11 – KT as they were yet to be matured. The presence of 11 KT is an indicator of maturity of male in fish (Pankhurst, 1998). Thus, hormonal indicators of mature male were present only in animals that had reached 636 ± 18 g while being held in freshwater conditions and a shift to 28 °C and 32 g L⁻¹ salinity for nine weeks was insufficient to cause 11 KT productions to cease. The presence of high concentrations of E_2 in these animals suggests that remaining for a longer period in these conditions will result in sex change to female which is further supported by previous studies done by Kitano *et al.* (2009); Piferrer *et al.* (2012); Sun

et al. (2013) and Ravi *et al.* (2014) with various species of fish, including Asian seabass. In the case of the smallest group, the absence of 11 KT in the circulation may reflect an absence of functional gonadal material.

E₂ responds to increasing salinity and there is some brain aromatase activity present in fish of 463± 13 g which indicates that the hormonal conditions that are pre-requisite for inducing sex change in Asian seabass (Anderson and Forrester, 2001) are present from this size, much earlier than would be expected from observations if normal (wild) ontogeny of sex change was to occur.

The outcome of this experiment shows that the aromatase enzyme levels affect the production of estradiol which control precocious sex inversion of cultured Asian seabass under domesticated hatcheries. Further study needs to be done at different salinities across temperatures, with histological analysis of the gonads of subjected fish, since this fish is diadromous and presently being cultured as an important aquaculture species in a wide geographical range (Mathew, 2009; Liu *et al.*, 2012; Mukai and Lim, 2014). Hence these findings can be a useful tool for understanding the effect of habitat environment on the reproductive biology of other diadromous fish species used in aquaculture.

3.4.4 Limitation of this study

One aspect of this study, which affected interpretation of the results was the impact of growth and hence body size on the physiology of the fish combined with holding the experimental fish in salinewater (32 g L⁻¹). Table 3.1 shows the size of the experimental fish at the beginning and at the end of the experiment. It is apparent that the fish in the smallest size group (50 – 100 g) at the beginning of the experiment ended at a similar size to the starting size of those fish in the next smallest group (300 – 500 g). The same effect occurred in the body size of the 300 – 500 g group relative to the 700 – 1000 g fish. While this allows for some comparisons to be made between fish held in fresh and saline water and between body sizes, it does remove some independence from some of the comparisons. Since it is not possible to hold fish in a healthy state without them growing, loss of independence of some comparisons was considered to be acceptable.

Some other experimental limitations, which may have affected the results, were experienced in this study. Obtaining 2.5 – 4 kg fish was found to be difficult and resulted in a lower sample size (n = 6) for this group. Although the differences observed are clear, the

reduced statistical power that results from the smaller sample size may have obscured some changes.

Secondly, during acclimatization of the 2.5 – 4 kg fish into aquarium conditions, the fish ceased feeding for two weeks compared to the other size groups that began feeding almost immediately upon being placed in the experimental tanks. While this may have affected the results by impacting on their growth and ultimately perhaps steroids levels, the acclimation period of a month was considered to be sufficient to remove most or all of this effect.

CHAPTER 4

EFFECT OF SALINITY ON THE SEX INVERSION OF ASIAN SEABASS

4.1 INTRODUCTION

Asian seabass is a catadromous hermaphrodite fish species, having a high tolerance to culture in either fresh or salt water farming conditions. Mature Asian seabass in nature migrate from freshwater to salinewater for mass spawning, followed by sex inversion to female. Although the catadromous migratory pattern of mature Asian seabass fish has been reported in previous research (Dunstan, 1959; Garrett and Russell, 1982 and Russell, 1986a quoted in Garrett, 1986; Mathew, 2009; Yue *et al.*, 2012; Ayson and Ayson, 2012). as an euryhaline species, juvenile and adult seabass can thrive within the relatively wide salinity range of 0 - 45 g L⁻¹. Seabass broodstock in aquaculture are generally maintained in seawater and larvae are cultured in brackish or seawater.

The lack of complete control over seasonal maturation, differentiation and reproduction presents challenges for selective breeding of Asian seabass in captivity (Robinson *et al.*, 2010). Early puberty is a major problem in farmed fish, such as Salmonids, *Oncorhynchus tshawytscha* (MacClure *et al.*, 2007), European seabass, *Dicentrarchus labrax* (Felip *et al.*, 2008), Atlantic halibut, *Hippoglossus hippoglossus* (Weltzien *et al.*, 2003), Cod fish, *Gadus morhua* (Karlsen *et al.*, 2006), Tilapia, *Oreochromis niloticus* (Longalong *et al.*, 1999), Sea breams, *Pagrus auratus* (Gines *et al.*, 2003, 2004) and Perches, *Perca flavescens* (Shewmon *et al.*, 2007). Similarly, Asian seabass reach early sexual maturity followed by sex inversion in which they change from male to female within two years of age in farmed conditions (Ayson and Ayson, 2012; Szentes *et al.*, 2012) compared with generally six to eight years of age in the wild (Guiguen *et al.*, 1994). In northern Australia, male Asian seabass in Van Diemen Gulf and southeast Gulf of Carpentaria mature between 3 and 5 years age under wild conditions. In contrast, sexually precocious maturity was reported in North-East Gulf of Carpentaria fish, which mature in their first or second year (Davis, 1984a; Davis, 1986, Garrett, 1986; Ayson and Ayson, 2012). However, gonad development was retarded in landlocked freshwater Asian seabass from the same region (Davis, 1985). Previous studies (Schipf, 1996; Anderson *et al.*, 2000; Szentes *et al.*, 2012; Ravi *et al.*, 2014) also conducted in Australia revealed that cultured Asian seabass mature as males at a much smaller size than in those in the wild, and precocious sex inversion has been observed after eighteen months of age in captivity at hatcheries in Queensland and the Northern Territory. This precocious sex change may be the result of the captive environment used during the spawning season (Schipf, 1996). It has been suggested

that salinity may play a role in regulating reproductive activity in fish (Haddy and Pankhurst, 1998). A previous study (Anderson *et al.*, 1998) indicated that precocious development of Asian seabass may be advanced by culture in saline conditions compared with freshwater.

There is relatively little published data or information on the effects of salinity on the timing of reproductive development (Bromage *et al.*, 2001) and reproductive physiology in fish (Haddy and Pankhurst, 2000), even though considerable numbers of fish undergo migrations between freshwater and salinewater and vice versa. Tamaru *et al.* (1994) reported that striped mullet (*Mugil cephalus*), slowed ovarian development with reduced salinity. Magwood *et al.* (1999) showed that ovulation in the Atlantic salmon (*Salmo salar*) was advanced in fish transferred to freshwater for up to 3-4 months before the time of expected ovulation compared to broodstock maintained in seawater (Bromage *et al.*, 2001). In contrast Zanuy and Carrillo (1984) showed that although European seabass matured in low salinities, spawning did not take place unless the fish were transferred to salinewater and in Striped mullet (*Mugil cephalus*), no fertilized eggs were obtained in freshwater though the species can be induced to spawn over a salinity range of 0-36 g L⁻¹ (Haddy and Pankhurst, 2000). Therefore, it has been suggested that salinity may play a role in regulating reproductive activity (Haddy and Pankhurst, 1998; Ayson and Ayson, 2012) and fertilization, survival and normal development of fish eggs are known to be directly affected by salinity (Haddy and Pankhurst, 2000). Hence, the migratory process of Asian seabass is of particular interest as there are major changes in salinity levels experienced by fish that may contribute to the regulation of physiological processes. Particularly of interest in the present study is the impact of salinity on reproductive processes and sex change. Migration of Asian seabass to a marine environment may trigger some endocrine events resulting in changed hormone levels, which lead towards maturation and the protandrous sex inversion process.

The objectives of the experiment described in this chapter were to determine the effect of different salinities on steroid levels and the process of sex inversion in Asian seabass. Several common steroids; 17 β -estradiol, testosterone and 11-ketotestosterone, are known for their physiological actions in the reproductive processes. Preliminary finding in chapter 3 on sex steroids was that testosterone (T) and estradiol (E₂) production was inversely correlated with the proportion of testicular tissue with size group. There are close relationships between developing male tissue and 11 Ketotestosterone (11-KT) in blood plasma or its gonadal biosynthesis (Condeca and Canario, 1999). During feminisation there is a reduction in 11-KT and an increase in E₂ (Godwin and Thomas, 1993). The E₂ to androgens ratio also significantly increased during the course of sex reversal in the protandrous anemonefish, *Amphiprion melanopus* (Godwin and Thomas, 1993). Guiguen *et al.* (1993) suggested that E₂ plays an

important role in the protandrous sex reversal process in the Asian seabass. Testosterone should act as a precursor for the synthesis of E_2 . Conversion of T into E_2 is the rate limiting steps in E_2 biosynthesis and is catalyzed by an aromatase enzyme (Kitano *et al.*, 1999; Piferrer *et al.*, 2012; Ravi *et al.*, 2014). Furthermore, the aromatase activity that catalyses the conversion of T to E_2 is low in the functional male and bisexual phases (Chang and Yueh, 1991). Anderson and Forrester (2001) described that the increased aromatase activity and associated decreased 11-KT: E_2 ratio may be seen either as a cause of sex reversal or a consequence of the sex change process of Asian seabass (Piferrer *et al.*, 2012) as shown in sex reversal of black porgy (Chang and Lin, 1997). Hence, cytochrome P450 aromatase, which is involved in the synthesis of 17 β -estradiol, was also measured in this experiment.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design and sampling procedures

Fourteen months old adult male Asian seabass were obtained from a commercial farm (GFB Fisheries, Kelso) in north Queensland (19 °S, 147 °E) where they had been maintained in freshwater under natural conditions of temperature and photoperiod (14 h light). Fish were randomly taken from the cages in which they were cultured at the farm and immediately transported in oxygenated tanks (80 L) to the aquarium facility at James Cook University in Townsville, Australia, where they were used for experiments. Fish were fed with commercially formulated barramundi pellet (50% protein, 18 MJ Kg⁻¹, Ridley Aquafeeds, Brisbane, Australia) to satiety once daily and tank cleaning was conducted as required and reared in 3000 L tanks throughout the experimental period. Fish were acclimatized to the aquarium conditions for one month, prior to the experimental period.

At the beginning of the experiment, a group of male fish (n = 8) weighing 853.5 g average weight were allocated to each of the four experimental tanks held in one controlled environment room. Each tank was separately supplied with recirculated seawater treated by both a particulate and biofilter. Salinity of the water in each tank was adjusted daily to 0 g L⁻¹, 10 g L⁻¹, 20 g L⁻¹ or 30 g L⁻¹ respectively, with the aid of a refractometer and by exchanging with salinewater (30 – 32 g L⁻¹) or freshwater as required. Fish were held at summer conditions (28 °C, 14 h light) in the selected salinities for nine weeks. Fish were fed with commercial

barramundi pellet to satiety once daily and tank cleaning was conducted as required. During this period water quality parameters (pH, dissolved oxygen, ammonia, nitrate and nitrite levels) were measured weekly using standard test kits (Aquarium Pharmaceuticals Inc. USA) and maintained at the same level in all the units throughout the experimental period. A UV steriliser (QL 160, Rainbow Aq Products, Elmonte, CA 91734) was used to sterilize some proportion of the water in each tank as a preventive method of disease control in addition to the biological filtration.

Ammonia and nitrite levels were measured routinely and were maintained at less than 1.0 mg L⁻¹ and nitrate was maintained around 12.0 mg L⁻¹. Average temperature was measured daily and maintained at 28 °C, while pH was measured weekly and maintained at 8.0. Salinity was maintained according to the experimental design at 0 g L⁻¹, 10 g L⁻¹, 20 g L⁻¹ and 30 g L⁻¹, respectively. All water exchanges required maintaining favorable conditions for cultured fish and adjustments of water quality parameters occurred at the same time each day.

Prior to sacrifice, each fish was bled. Fish were sacrificed to collect brain and gonads at the beginning (n = 8) and end (n = 7 – 8 fish from each treatment) of the experimental period. A 2.5 mL blood sample was extracted from the caudal vein using a 5 mL syringe and an 18-gauge hypodermic needle. Blood samples were immediately transferred to 2.5 mL fluoride-oxalate tubes (Sarstedt, Technology Park, SA 5095, Australia), mixed gently and stored on ice until transported to the laboratory. Blood samples were centrifuged at 14000 g for 10 min at 4 °C (Eppendorf centrifuge 5415C, Hamburg, West Germany) and the plasma stored at -80 °C until the assay for E₂, T or 11-KT.

Upon collection of the blood samples, the fish were sacrificed; brain and gonads were removed aseptically, transferred to labeled vials, and placed in liquid N₂ at -80 °C for the aromatase assay.

4.2.2 Sex steroids assay

Plasma levels E₂, T and 11-KT were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Conroy (1987). Briefly, 300 µL of plasma was extracted with 1.5 mL ethyl acetate, 200 µL of the extract was added to each assay tube for evaporation and resuspension in assay buffer, with the reagent and supernatant mixed with ready gel (Ecolite, USA), and ³H-labelled steroid in each sample was measured in duplicate using a liquid scintillation counter (Beckman,

QuantaSmart-1.31, USA). Extraction efficiency (recovery level of ^3H -labelled steroid from plasma) was determined and assay values were corrected accordingly for E_2 , T and 11-KT. Interassay variability was measured using aliquots of a pooled internal standard for E_2 , T and 11-KT. The detection limit of E_2 , T and 11-KT in plasma was 3 ng/tube, and all samples were measured in a single assay. E_2 , T and 11-KT concentrations were determined against a standard curve.

4.2.3 Measurements of aromatase activity

Aromatase activity in the gonad and brain were measured by tritiated water release assay (radiometric method) which had been previously validated for use with Asian seabass in our laboratory (Anderson and Forrester, 2001). Macerated gonad tissue was thoroughly homogenised at 24000 g in 10 volumes (w/v) of a cold solution containing 100 mM KCl, 10 mM K_2HPO_4 , 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM dithiothreitol (pH 7.4) using a Heidolph diax 600 homogeniser with 10G tools. The homogenate was then sonicated for 30 seconds (Unisonics FX8) and centrifuged at 1000g for 5 minutes at 4 $^{\circ}\text{C}$. The non-lipid portion of the supernatant was recovered by piercing the tube immediately above the pellet with a heated 18 gauge needle. Aliquots of extract were stored at -80°C prior to assay. Extract (150 μL) was incubated at 30 $^{\circ}\text{C}$ with 450 μL of solution containing 100mM KCl, 10mM K_2HPO_4 , 1mM ethylenediaminetetraacetic acid (EDTA) and 2mM dithiothreitol, 5mM glucose-6-phosphate, 1mM nicotinamide adenine dinucleotide phosphate (NADP), 2U glucose-6-phosphate dehydrogenase plus 66.67 nM androst-4-ene-3, 17-dione (pH 7.4). At 10 and 30 minutes, a 200 μL aliquot of the reaction mixture was terminated by mixing with 100 μL of 30% trichloroacetic acid containing 60mgml $^{-1}$ charcoal. After standing for 30 minutes, the mixture was then centrifuged at 10,000g for 5 minutes and the supernatant (200 μL) was added to a 1.0 x 3.0 cm column packed with equal volumes of 50-100 and 100-200 mesh AG50W-X4 resin (Bio-Rad, Hercules, CA). Samples were then eluted with 2.3 mL of deionized water, with the final 1.5 mL collected and mixed with 15 mL Ready Gel (Beckman, USA). DPM (radioactivity) was measured over 5 minutes with a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA).

Aromatase activity was expressed using the production of $^3\text{H}_2\text{O}$ from ^3H -androstenedione ($^3\text{H-A}$) according to the method previously used in our laboratory (Anderson & Forrester, 2001). The Bradford (1976) method was used for determining protein concentrations of the crude supernatant fraction by monitoring the absorbance of protein-dye complex. Aromatase activity was assayed in the gonad and brain tissues of all fish. Aromatase activity was expressed as the reaction velocity for the conversion of androstenedione to tritiated water (formation of $^3\text{H}_2\text{O}$ during aromatization of $^3\text{H-A}$) using fmoles/mg protein/min as the units.

4.2.4 Data analysis

Analysis of Variance (ANOVA); One-way comparison was used with Tukey's HSD test to identify the differences between treatment for hormone and aromatase levels. Least Significant Difference (LSD) was used to compare the significant levels within and in between treatments and their interactions. Significant effects were assumed at $\alpha < 0.05$. All data were analyzed using SPSS version 12.0 for Windows and where appropriate proportion data were normalized by transformation prior to analysis.

4.3 RESULTS

4.3.1 General

4.3.1.1. Growth

Initial average weight was 853.5 g and final average weight of fish groups 0 g L⁻¹, 10 g L⁻¹, 20 g L⁻¹ and 30 g L⁻¹ were 1114 g, 1144 g, 1126 g and 1354 g respectively (Table 4.1).

Table 4.1 Mean fish weight at the beginning and the end of Experiment 2 and average daily growth (ADG) over the experiment for each group of fish. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Salinity Group	Mean Initial Wt (g)	Final Wt (g) \pm S.E.	ADG(% body weight d ⁻¹)
0 g L ⁻¹	853.5 ^a	1114 \pm 45 ^b	0.4925
10 g L ⁻¹	853.5 ^a	1144 \pm 41 ^b	0.5500

20 g L ⁻¹	853.5 ^a	1126 ± 76 ^b	0.5175
30 g L ⁻¹	853.5 ^a	1354 ± 60 ^c	0.9471

The highest (1354 ± 60 g) Average Daily Growth (ADG) of experimental fish was recorded in fish held in a salinity of 30 g L⁻¹ and was significantly different ($p < 0.05$) to the ADG of the other groups (Table 4.1).

4.3.1.2. Fish Survival Rate

One fish died during the experiment period in the 30 g L⁻¹ treatment group. The cause of this death was not able to be determined.

4.3.2 Aromatase

4.3.2.1. Aromatase activity in the brain

The aromatase activity measured in the brains of Asian seabass before and after the treatment at differing salinities are shown in Figure 4.1. The level of aromatase activity was 11.14 ± 3.25 fmole mg protein⁻¹ min⁻¹ in the brain of fish at the beginning of the experiment and was significantly lower ($p < 0.05$) at the end of the nine week experimental period in the 10 g L⁻¹ treatment group, in which it was significantly reduced to 4.46 ± 1.27 fmole.mg protein⁻¹.min⁻¹ (Figure 4.1). The aromatase activity was significantly greater in the brains of fish held at 0 g L⁻¹, 20 g L⁻¹ or 30 g L⁻¹ than in the brains of the beginning groups of fish and in the brains of the fish in the 10 gL⁻¹ treatment group. Aromatase activity was 56.46 ± 16.81 fmole.mg protein⁻¹.min⁻¹ in the brains of fish held at 0 g L⁻¹, 29.09 ± 11.22 fmole mg protein⁻¹min⁻¹ in the brains of fish held at 20 g L⁻¹ and 35.71 ± 6.45 fmole mg protein⁻¹ min⁻¹ in the brains of fish held at 30 g L⁻¹ (Figure 4.1). There was no significant difference between the aromatase activities in the brains of fish held at 0 g L⁻¹, 20 g L⁻¹ or 30 g L⁻¹ for the nine week experimental period (Figure 4.1).

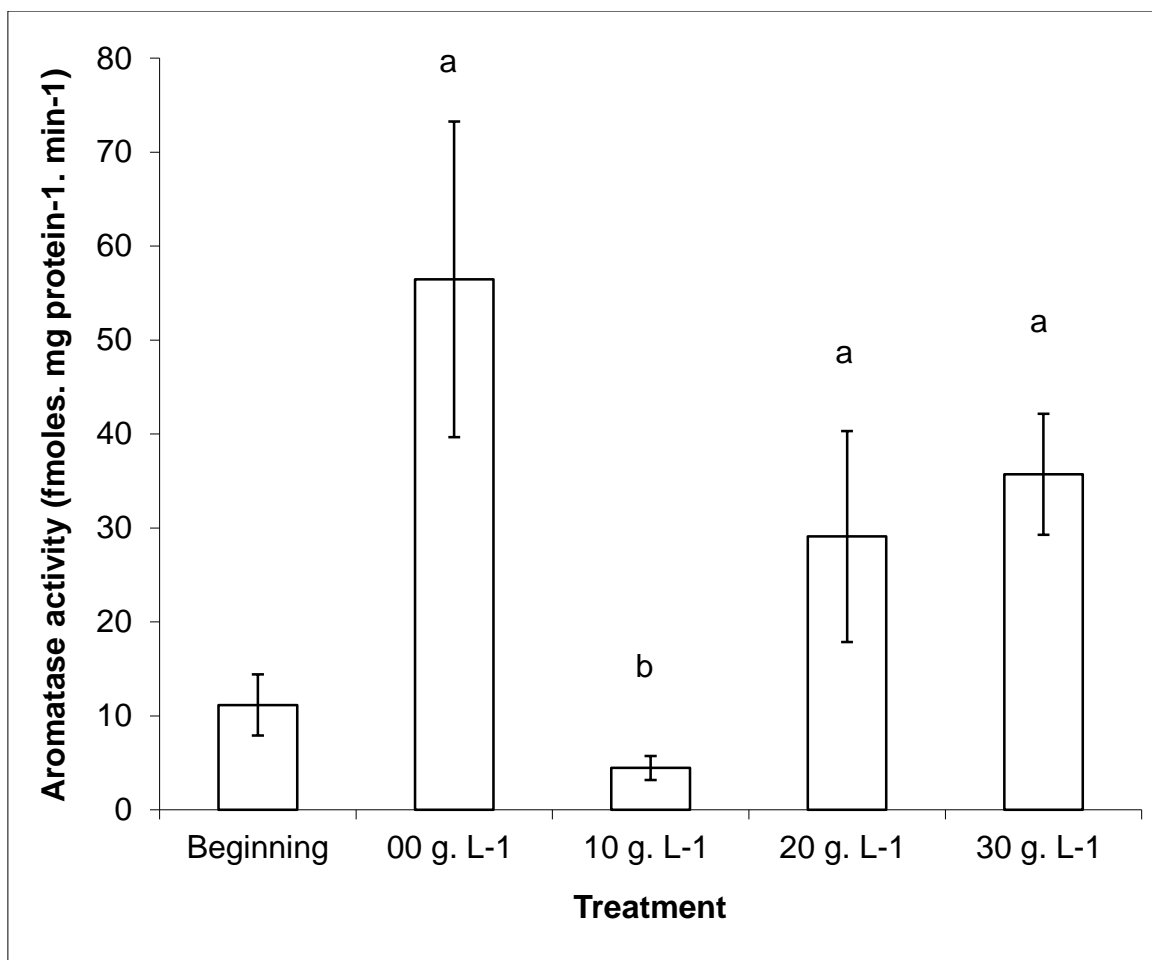


Figure 4.1 Aromatase activity in brain of Asian seabass at the beginning of the experimental period (Beginning) or held for nine weeks at different salinities. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

4.3.2.2. Aromatase activity in the gonad

The aromatase activity in the gonad at the beginning and at the end of the nine week experimental period is shown in Figure 4.2. There were no significant differences between the gonadal aromatase activities in any of the treatment groups except for fish in 10 g L⁻¹ and 30 g L⁻¹ salinities in which the activity range was undetectable and very low, respectively. The highest gonadal aromatase level was recorded in fish held in 0 g L⁻¹ and fish held in 20 g L⁻¹ (6.87 ± 3.79 and 4.28 ± 1.03 fmoles mg protein⁻¹ min⁻¹), respectively at the end of nine weeks experimental period. However, these values were not significant to each other.

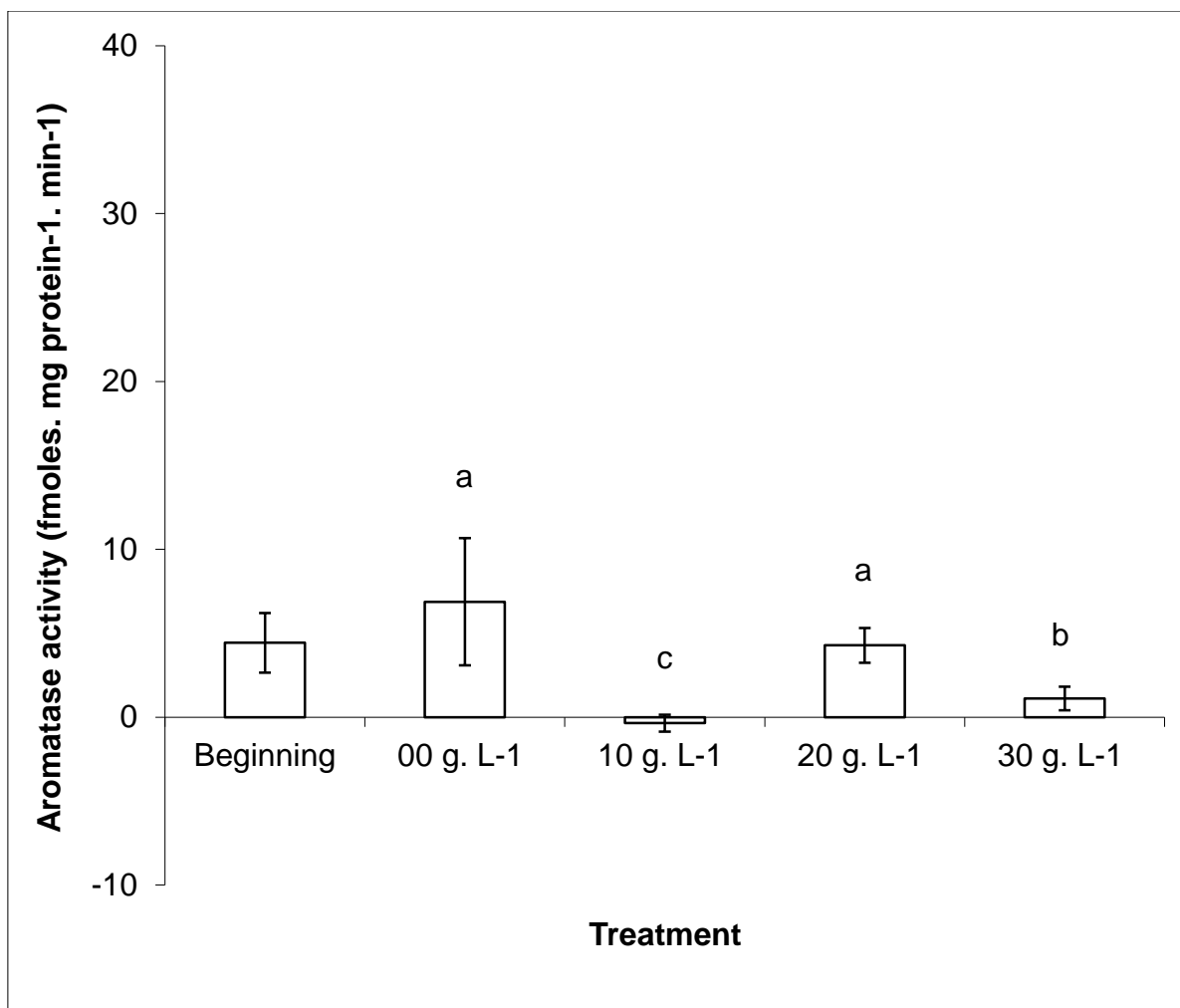


Figure 4.2 Aromatase activity in gonad of Asian seabass at the beginning of the experimental period (Beginning) or held for nine weeks at different salinities. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

4.3.3 Plasma steroids

4.3.3.1. Testosterone (T)

Plasma T level in fish at the beginning of the experiment was significantly higher ($p < 0.05$) ($0.5 \pm 0.043 \text{ ng mL}^{-1}$) than T levels in all treatments at the end of the experiment (Figure 4.3). Plasma T concentration in fish in all treatments at the end of the experiment were not significantly different, ranging from $0.160 \pm 0.050 \text{ ng mL}^{-1}$ in fish held at 10 g L^{-1} to $0.267 \pm 0.026 \text{ ng mL}^{-1}$ in fish held at 30 g L^{-1} (Figure 4.3).

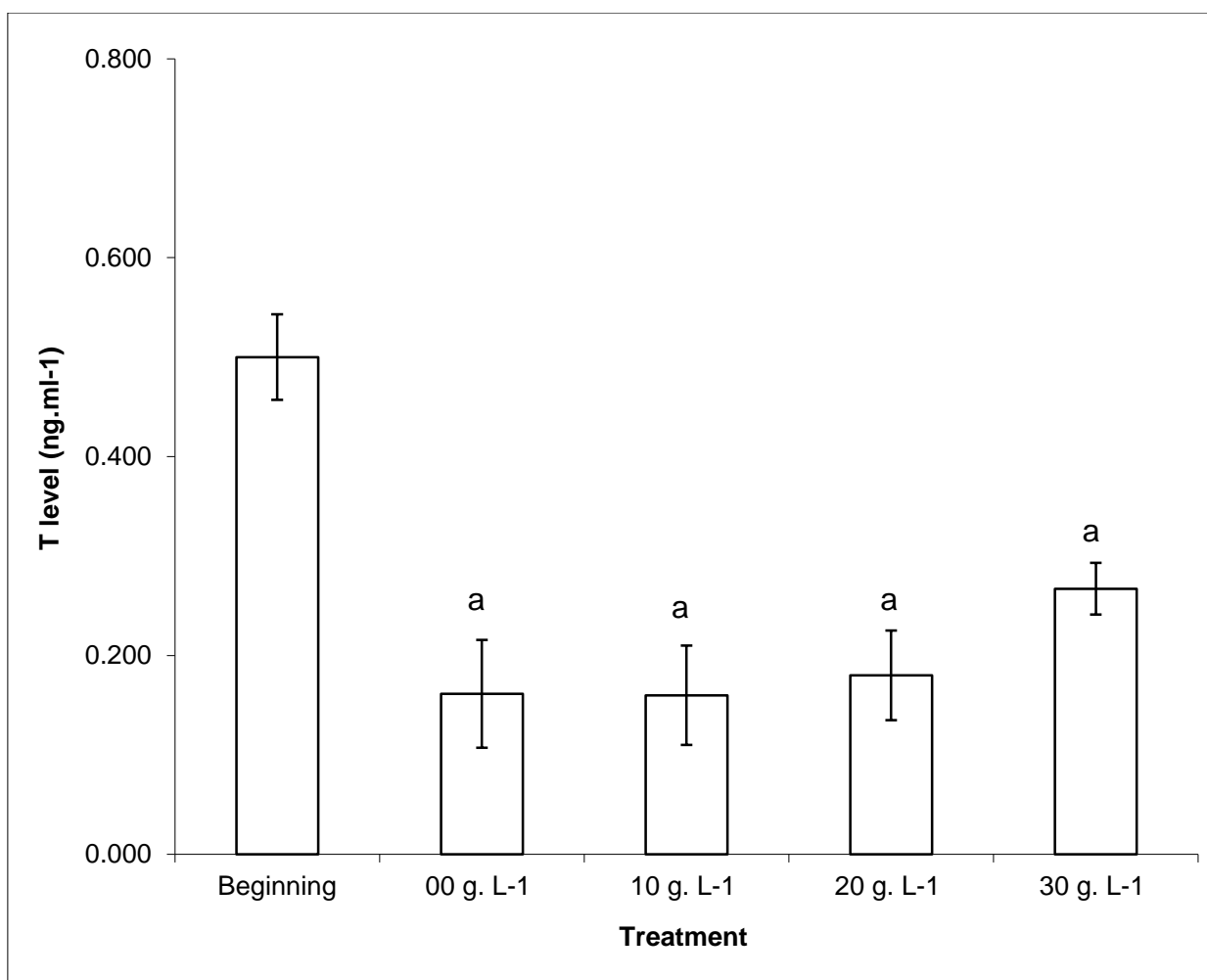


Figure 4.3 Plasma Testosterone concentration in fish at the beginning of the experiment (Beginning) and held for nine weeks at different salinity levels. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

4.3.3.2. Estradiol 17- β (E_2)

Plasma E_2 was undetectable in fish at the beginning of the experiment (Figure 4.4). Measurable levels of E_2 were observed in the plasma of fish from all treatments at the end of the experiment with all groups having plasma E_2 concentration > 0.005 ng mL⁻¹ in fish from all salinity treatments, except 20 g L⁻¹ in which the lowest E_2 concentration (< 0.005 ng mL⁻¹) was recorded (Figure 4.4). The highest E_2 level (0.016 ± 0.01 ng mL⁻¹) was recorded in fish held at 0 g L⁻¹ followed by fish held at 10 g L⁻¹ and 30 g L⁻¹ where the values were 0.009 ± 0.005 ng mL⁻¹ and 0.005 ± 0.004 ng mL⁻¹, respectively. However the E_2 levels within each salinity treatment group were not significantly different to each other (Figure 4.4).

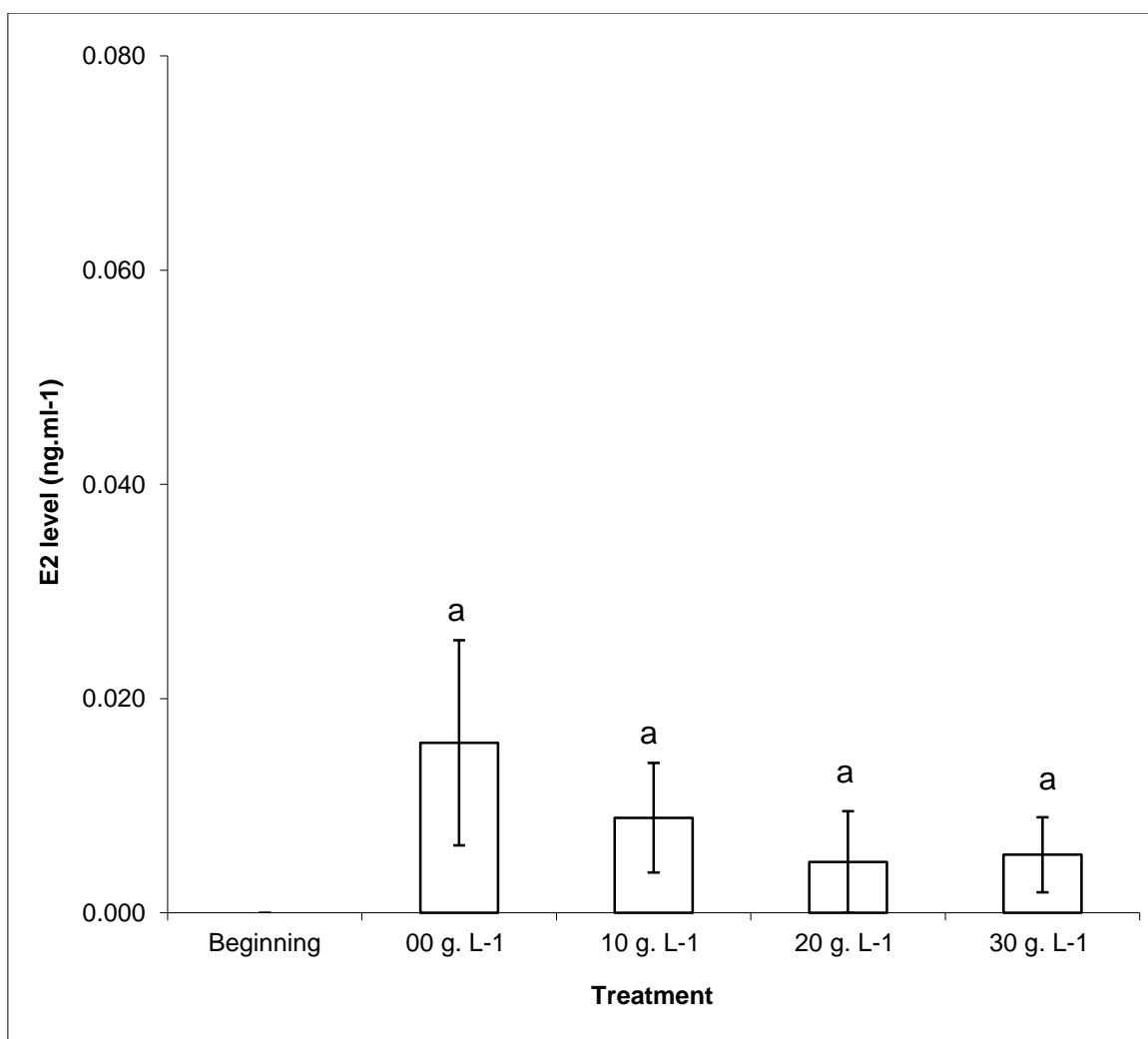


Figure 4.4 Plasma estradiol 17- β concentration in fish at the beginning of the experiment (Beginning) and held for nine weeks at different salinity levels. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

4.3.3.3. Keto-Testosterone (11-KT)

Measurable amounts of 11- KT was not detected in any group of fish in this experiment. This may due to the fish were in the transitional stage of male towards female.

4.4 DISCUSSION

4.4.1 General

4.4.1.1. Growth

Mean fish weight at the end of the experiment was lowest (1114 ± 45 g) in 0 g L^{-1} salinity and highest (1354 ± 60 g) in 30 g L^{-1} . The highest ADG was also recorded in fish held at 30 g L^{-1} and was significantly ($p < 0.05$) different to other fish groups where all the values were similar. Numerous exogenous factors have been shown to affect the growth of fish. In this experiment the highest salinity has significantly ($p < 0.05$) increased the growth of Asian seabass, although there is no clear relationship between increasing salinity and growth rate. Temperature and photoperiod were considered to be optimum for Asian seabass growth, and dietary factors (frequency of feeding, protein and energy) were also at sufficient levels.

Previous studies conducted by Cotton *et al.* (2003) with black seabass (*Centropristis striata*) showed that salinities of 20 g L^{-1} and 30 g L^{-1} did not produce significantly different weight in fish. However, both of these salinities produced significantly larger fish than did a salinity of 10 g L^{-1} . These results differ from another salinity experiment conducted (Berlinsky *et al.*, 2000 cited in Cotton *et al.*, 2003) with black seabass at 20 g L^{-1} salinity showed that a significant increase in growth compared to salinity of 32 or 10 g L^{-1} .

4.4.1.2. Effect on aromatase and sex steroids

Initial aromatase activity level in the brain was significantly ($p < 0.05$) lower than the end aromatase level in all the experimental groups, except 10 g L^{-1} in which the brain aromatase was significantly lower than the initial level. There was no linear relationship between salinity and brain aromatase with no significant difference ($p > 0.05$) between the values of brain aromatase activity found at 0 g L^{-1} , 20 g L^{-1} or 30 g L^{-1} . As the brain is a site where environmental effects may be expected to be integrated, the salinity changes may be expected to exert an effect on the brain aromatase activity. The pattern of response is such as to indicate an effect of salinity on brain aromatase at the extremes when the nature of the environment requires the fish to spend energy on osmoregulation. Aromatase activity in gonad showed no significant difference between the initial and final values at the held salinities.

Testosterone level significantly ($p < 0.05$) decreased in all fish groups compared with fish at the beginning of the experiment. Although Guiguen *et al.* (1993) found that T was present in plasma whatever the sex; the T level recorded in the beginning fish indicated that they were male. The decrease in plasma T during the study period may have resulted from the fish undergoing some sex change since T is a precursor for estradiol- 17β (Prat and Zanuy, 1990). However, T concentration is dependent on many influences including rates of synthesis and the rate of transformation into other compounds and direct interpretation of changes in T concentrations being related to sex change cannot be made.

E_2 level was undetectable in beginning fish supporting the contention that these fish were male. E_2 was detectable in all groups of Asian seabass at the end of the experiment. In Asian seabass, there are very low or undetectable levels of 11-KT in the plasma of transitional fish (Guiguen *et al.*, 1993). The presence of E_2 and the absence of any detectable levels of 11-KT indicate that fish were undergoing transition or were female (Guiguen *et al.*, 2010). Furthermore, previous research conducted by Anderson and Forrester (2001) also stressed that estrogen treatments have been shown to induce male to female sex change in Asian seabass (Guiguen *et al.*, 2010). However, this was the case for all groups of fish and there is no clear indication of an effect of salinity on this process.

The relationship is not clear since the data indicated low levels of brain aromatase present in the fish held at 10 g L^{-1} making interpretation difficult. Further, whether aromatase in brain affects estradiol- 17β levels in the circulation is unclear (D'Cotta *et al.*, 2001). It may be that the culture period of nine weeks in this particular study, which collected data to observe the changes in brain aromatase activity taking place, may not be sufficiently long enough to allow all of the relationships between aromatase and circulating steroids to become apparent.

The complexities of relationships between reproductive physiology of fish and salinity have also been identified previously. European seabass mature in low salinities, but spawning does not take place unless the fish are transferred to seawater (Zanuy and Carrillo, 1984). Similarly, striped mullet can be induced to spawn over a wide salinity range ($0 \text{ g L}^{-1} - 35 \text{ g L}^{-1}$), but no fertilized eggs are obtained in freshwater (Lee *et al.*, 1992, cited in Haddy and Pankhurst, 2000).

Previous experiments which considered the effect of salinity extremes on physiological processes other than reproduction with black seabass juveniles showed high mortality, low growth rates, and high FCR in water with 10 g L^{-1} for extended period (Cotton *et al.*, 2003).

However, older black seabass exhibited significantly lower growth rates at 20 g L⁻¹ than at 30 g L⁻¹ salinities, which suggested that older fish might not be able to tolerate a salinity range as low as that of the juveniles. It has to be concluded that salinities alone may not have an effect on changes in aromatase activities and sex steroid levels which lead to the process of sex inversion of Asian seabass. Further investigation which subjects fish into longer exposure period for different environmental factors such as temperature, salinity or combination is suggested.

Chapter 5: EFFECT OF TEMPERATURE ON THE SEX INVERSION OF ASIAN SEABASS

Author contributions – Chapter 5

Details of publications on which chapters is based	
<p>Saman Athauda, Trevor Anderson, Rocky de Nys. 2011. Effect of Rearing Water Temperature on Protandrous Sex Inversion in Cultured Asian seabass (<i>Lates calcarifer</i>). General and Comparative Endocrinology. Vol. 175, Issue.03: Pp. 416 – 423.</p> <p>Saman B. Athauda and Trevor A. Anderson. 2005. Effect of Environmental Temperature on Plasma Steroids of Asian seabass <i>Lates calcarifer</i>, Proce (Abstract): PURSE, Peradeniya University Research Sessions 2005, Peradeniya, Sri Lanka. November 10, 2005. Vol. 10, Pp. 24.</p>	
Name of candidate and other co-authors	Nature and extent of the intellectual input of each author, including the candidate
<u>Candidate:</u> SamanAthauda (SA)	<u>SA</u> and <u>TA</u> co-developed the research question. <u>SA</u> performed the experimental work and laboratory analysis. Data analysis with assistance from <u>TA</u> . <u>SA</u> wrote the first draft which was revised with editorial inputs from <u>TA</u> & <u>RD</u> . <u>SA</u> developed the figure and tables.
<u>Co-authors:</u> Trevor Anderson (TA) Rocky deNys (RD)	

CHAPTER 5

EFFECT OF TEMPERATURE ON THE SEX INVERSION OF ASIAN SEABASS

5.1 INTRODUCTION

The sex inversion of fish to male or female is a complex and labile mechanism under the control of genetic, physiological or/and environmental factors (Baroiller & Guiguen, 1999; Delvin & Nagahama, 2002). Among the environmental factors, temperature has been identified as the most influential factor on sex ratios of any fish population overriding the action of the sex determination genes (D'Cotta *et al.*, 2001; Guiguen *et al.*, 2010; Ospina-Alvarez and Piferrer, 2008).

Asian seabass is a protandrous hermaphroditic species that undergoes sex inversion from male to female. Under natural conditions (in the wild), the male fish reach first sexual maturity at the age of 3-4 years (Stephen *et al.*, 2008) while sex inversion appears at the age of between 6 and 8 years old (Guiguen *et al.*, 1994).

Asian seabass have a wide thermal tolerance range and they are commercially cultured at temperatures ranging from 22 °C to 35 °C (Katersky and Carter, 2007). Water temperature and salinity appear to be the controlling factors (Davis, 1985) of reproduction of Asian seabass (*Lates calcarifer*) compared to its European counterpart (*Dicentrarchus labrax*) in which the photoperiod appears to be of greater significance (Carrillo *et al.*, 1989). Recently it has been further highlighted that temperature invariably resulted in changing male and female ratios in fish population with changes in levels of sex steroids (Ospina-Alvarez and Piferrer, 2008; Yamaguchi *et al.*, 2007). Previous studies with protandrous Asian seabass (Anderson & Forrester, 2001; Guiguen *et al.*, 1993) and black porgy (Wu *et al.*, 2009) also showed that sex steroids have a significant role in sex change and gonadal development in teleost fish. Therefore, sex steroids E₂, T and 11-KT have received the greatest attention in this respect (Borg, 1994; Fostier *et al.*, 1983). Results of recent research (Guiguen *et al.*, 2010) also showed that aromatase activity has a stimulatory effect on E₂ production and ovarian development, and finally cause sex inversion into female in Asian seabass.

This study focused on the effect of rearing water temperature on protandrous sex inversion process of Asian seabass. Changes of serum steroids (E₂, T and 11-KT), aromatase

activity in gonad and brain at different temperature levels and histological changes on gonad have been investigated to understand the cause of sex inversion.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design and sampling procedures

Fourteen month old adult Asian seabass were obtained from a commercial farm (GFB Fisheries, Kelso) in north Queensland (19 °S, 147 °E) where they had been maintained in freshwater under natural conditions of temperature (28 °C) and photoperiod (14 h light). Fish (700 g – 1000 g) were randomly taken from the cages in which they were cultured at the farm and immediately transported in oxygenated tanks (80 L) to the Aquarium facility at James Cook University in Townsville, Australia where they were used for the experiment. Fish were fed with commercially formulated barramundi pellet (50% protein, 18 MJKg⁻¹, Ridley Aquafeeds, Brisbane, Australia) to satiety twice daily when held on the farm and to satiety once daily in the aquaculture facility at James Cook University. Fish were acclimatized to aquarium conditions (28 °C and 14:10 L : D) for one month with gradual changes in water salinity (30 - 32 g L⁻¹). One week prior to the commencement of the experiment, the water temperature of the rearing tanks (n= 5) were adjusted to the experimental temperature regimes (22 °C, 25 °C, 28 °C, 31 °C and 34 °C), which was maintained throughout the 14 weeks experimental period.

To determine the physiological effects of different temperatures, five groups of Asian seabass (n = 8 / group) were allocated to five 3000 L tanks contained in a single enclosed room with the temperature adjusted to allow the water temperature to be maintained without heating or cooling at 28 °C. Temperature was adjusted 1 °C per day towards the experimental temperatures of 22 °C, 25 °C, 28 °C, 31 °C and 34 °C, with the exception of 28 °C where the 3000 L tank was maintained at a constant temperature from the introduction of fish to aquarium condition. After 6 days (within the last week of one- month long acclimatization period) all fish were at their experimental temperature. All fish were reared at a constant salinity level of 30 - 32 g L⁻¹ and photoperiod of 14:10 L: D in the five tanks, which were supplied with bio-filtered recirculated water throughout the experimental period. Two of the tanks were equipped with individual heating systems allowing the water temperature to be separately maintained at 31 °C and 34 °C, while another two tanks were separately maintained at 22 °C and 25 °C using a refrigerated cooling system. The remaining tank was maintained at 28 °C adjusting to the room temperature. One third of the tank water was changed every day throughout the experimental period in addition to removing uneaten feed, faeces or any foreign material twice daily by siphoning.

Water was supplied from the main supply of the aquarium system and siphoned water was discharged to the waste water system.

Fish were fed with commercial barramundi pellet (50% protein, 18 MJ Kg⁻¹, Ridley Aquafeeds, Brisbane, Australia) to satiety once daily and tank cleaning was conducted as required. Dissolved Oxygen and temperature were measured (ExStik D0600, EXTECH Instruments Corporation, Watham, MA 02451) twice daily (9.30 am and 2.30 pm) and adjustments were made if required to maintain experimental temperature conditions. During this period water quality parameters (pH, Ammonia, Nitrate and Nitrite levels) were measured weekly using standard test kits (Aquarium Pharmaceuticals, Inc. USA) throughout the experimental period and were maintained at constant and equal levels in all the units. Ammonia and nitrite levels were maintained at less than 1.0 mg L⁻¹ while nitrate was around 12.0 mg L⁻¹. Mean salinity was maintained at 30-32 g L⁻¹, measured daily, while pH was measured weekly and maintained at 8.0 throughout.

A UV sterilizer (QL 160, Rainbow Aq Products, Elmonte, CA 91734) was used to sterilize the water as a preventive method of disease control in addition to the biological filtration.

Ammonia and nitrite levels were measured routinely and were maintained at less than 1.0 mg L⁻¹ while nitrate was around 12.0 mg L⁻¹. Mean salinity was maintained at 30-32 g L⁻¹, measured daily, while pH was measured weekly and remained at 8.0 throughout. Temperatures were maintained according to the experimental design at 22 °C, 25 °C, 28 °C, 31 °C and 34 °C, respectively. Mean water temperatures throughout the experiment period are shown below (Table 5.1).

Table 5.1 Mean temperature (± S.E.) during experimental period of experiment 3 in each treatment.

Treatment	Mean Temperature (°C) ± S.E.
22 °C	22.4 ± 0.08
25 °C	25.1 ± 0.07
28 °C	28.1 ± 0.06
31 °C	31.1 ± 0.02
34 °C	33.8 ± 0.06

Fish were anaesthetized to collect blood samples for steroid hormone assays at the beginning (n = 8) and at the end (n = 7 or 8) of the 18-weeks experimental period. A 2.5 mL blood sample was extracted from the caudal vein using a 5 ml syringe and an 18-gauge hypodermic needle. Blood samples were immediately transferred to 2.5 ml fluoride-oxalate tubes (Sarstedt, Technology Park, SA 5095, Australia), mixed gently and stored on ice until transported to the laboratory. Blood samples were centrifuged at 14000 g for 10 min at 4 °C (Eppendorf centrifuge 5415C, Hamburg, West Germany) and the plasma stored at -80°C until the assay for E₂ (Oestradiol-17 β), T (Testosterone) or 11-KT (11 Keto Testosterone).

Upon collection of the blood samples, the fish were sacrificed; brain and gonads were removed aseptically, transferred to labeled vials, and placed in liquid N₂ at -80 °C for aromatase assay. A part of the gonad was immersed in fixative buffer (6.5g Na₂HPO₄; 4g KH₂PO₄; 100 mL 40% formaldehyde; 900 mL distilled water at neutral pH) and kept in the refrigerator overnight. Supernatant was removed and moved to -80 °C for long-term storage until the tissues were used for histological analysis. Tissues were fixed in fixative buffer, dehydrated through an ethanol/xylene series and embedded in paraffin for sectioning. Sections (5 μm) were cut using a microtome and stained with haematoxylin and eosin and mounted in DPX. Stained sections were examined using a binocular microscope (Olympus CH2) to determine the gonadal development stages. Gonad stages were described according to the method proposed by Guiguen *et al.* (1993) and Guiguen *et al.* (1994).

5.2.2 Sex steroids assay

Plasma levels E₂, T and 11-KT were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Conroy (1987). Briefly, 300 μL of plasma was extracted with 1.5 mL ethyl acetate, 200 μL of the extract was added to each assay tube for evaporation and resuspension in assay buffer, with the reagent and supernatant mixed with ready gel (Ecolite, USA), and ³H-labelled steroid in each sample was measured in duplicate using a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA). Extraction efficiency (recovery level of ³H-labelled steroid from plasma) was determined and assay values were corrected accordingly for E₂, T and 11-KT. Inter-assay variability was measured using aliquots of a pooled internal standard for E₂, T and 11-KT. The detection limit of E₂, T and 11-KT in plasma was 3 ng/tube, and all samples were

measured in a single assay. E₂, T and 11-KT concentrations were determined against a standard curve.

5.2.3 Measurements of aromatase activity

Aromatase activity in the gonad and brain was measured by tritiated water release assay (radiometric method), which had been previously validated for use with Asian seabass in our laboratory (Anderson and Forrester, 2001). Macerated gonad tissue was thoroughly homogenised at 24000 g in 10 volumes (w/v) of a cold solution containing 100 mM KCl, 10 mM K₂HPO₄, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM dithiothreitol (pH 7.4) using a Heidolph diax 600 homogeniser with 10G tools. The homogenate was then sonicated for 30 seconds (Unisonics FX8) and centrifuged at 1000g for 5 minutes at 4 °C. The non-lipid portion of the supernatant was recovered by piercing the tube immediately above the pellet with a heated 18 gauge needle. Aliquots of extract were stored at -80 °C prior to assay. Extract (150 µL) was incubated at 30 °C with 450 µL of solution containing 100 mM KCl, 10 mM K₂HPO₄, 1mM ethylenediaminetetraacetic acid (EDTA) and 2mM dithiothreitol, 5mM glucose-6-phosphate, 1mM nicotinamide adenine dinucleotide phosphate (NADP), 2U glucose-6-phosphate dehydrogenase plus 66.67 nM androst-4-ene-3, 17-dione (pH 7.4). At 10 and 30 minutes, a 200 µL aliquot of the reaction mixture was terminated by mixing with 100 µL of 30% trichloroacetic acid containing 60 mg mL⁻¹ charcoal. After standing for 30 minutes, the mixture was then centrifuged at 10,000g for 5 minutes and the supernatant (200 µL) was added to a 1.0 x 3.0 cm column packed with equal volumes of 50-100 and 100-200 mesh AG50W-X4 resin (Bio-Rad, Hercules, CA). Samples were then eluted with 2.3 mL of deionized water, with the final 1.5 mL collected and mixed with 15 mL Ready Gel (Beckman, USA). DPM (radioactivity) was measured over 5 minutes with a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA).

Aromatase activity was expressed using the production of $^3\text{H}_2\text{O}$ from ^3H -androstenedione ($^3\text{H-A}$) according to the method previously used in our laboratory (Anderson and Forrester, 2001). The Bradford (1976) method was used for determining protein concentrations of the crude supernatant fraction by monitoring the absorbance of protein-dye complex. Aromatase activity was assayed in the gonad and brain tissues of all fish. Aromatase activity was expressed as the reaction velocity for the conversion of androstenedione to tritiated water (formation of $^3\text{H}_2\text{O}$ during aromatization of $^3\text{H-A}$) using fmoles/mg protein/min as the units.

5.2.4 Data analysis

Each tank was considered as one experimental unit and each fish as one replicate. One-way Analysis of Variance (ANOVA) was used with Tukey's HSD test to identify the differences between treatment for hormone and aromatase levels. Least Significant Difference (LSD) was used to compare the significant levels within and in between treatments and their interactions. Significant effects were assumed at $\alpha < 0.05$. All data were analyzed using SPSS version 12.0 and where appropriate, proportion data were transformed to assume normal distribution.

5.3 RESULTS

5.3.1 General

5.3.1.1. Growth

The initial average weight of fish was 853.5g and the final average weight of fish groups 22 °C, 25 °C, 28 °C, 31 °C and 34 °C were 1163 g, 1579 g, 1756 g, 1732 g and 1526 g, respectively (Table 5.2).

Table 5.2 Mean fish weight at the beginning and the end of Experiment 3 and average daily growth (ADG) over the experiment for each group of fish. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Temperature	Mean Initial wt (g)	Final Wt (g) \pm S.E.	ADG (% body weight d ⁻¹)
22 °C	853.5 ^a	1163 \pm 33 ^b	0.3555
25 °C	853.5 ^a	1579 \pm 47 ^c	0.8334
28 °C	853.5 ^a	1756 \pm 50 ^c	1.0367
31 °C	853.5 ^a	1732 \pm 106 ^c	1.0091
34 °C	853.5 ^a	1526 \pm 50 ^c	0.7725

The highest (1756 \pm 50 g) Average Daily Growth (ADG) of experimental fish was recorded in fish held at 28 °C followed by those held at 31 °C, 25 °C and 34 °C which were all significantly different ($p < 0.05$) to the ADG of fish held at 22 °C (Table 5.2).

5.3.1.2. Fish Survival Rate

The survival rate of fish was 100% at the end of the experiment period, except in the 22 °C and 25 °C, where one fish from each treatment was found dead. As a result, each of the fish groups was comprised of eight fish/group throughout the experimental period, except in the 22 °C and 25 °C where only seven fish were sampled at the end of the experimental period.

5.3.2 Aromatase activity

5.3.2.1. Aromatase activity in the brain

The aromatase activity in brain at the beginning and end of the experimental period is shown in Figure 5.6. The level of aromatase activity significantly increased in all fish groups through the experiment compared with fish sampled at the beginning. The highest brain aromatase activity (126.575 fmoles mg protein⁻¹ min⁻¹) was recorded in fish held at 28 °C followed by fish held at 31 °C and fish held at 34 °C where the activity (86 fmoles mg protein⁻¹ min⁻¹) was similar. Brains of fish held at 22 °C had the lowest activity (69.939 fmoles mg

protein⁻¹ min⁻¹), while at 25 °C aromatase activity was also recorded as 72.871 fmoles mg protein⁻¹ min⁻¹. However, the difference was not significant in brain aromatase activity between any of the treatment groups at the end of the experiment.

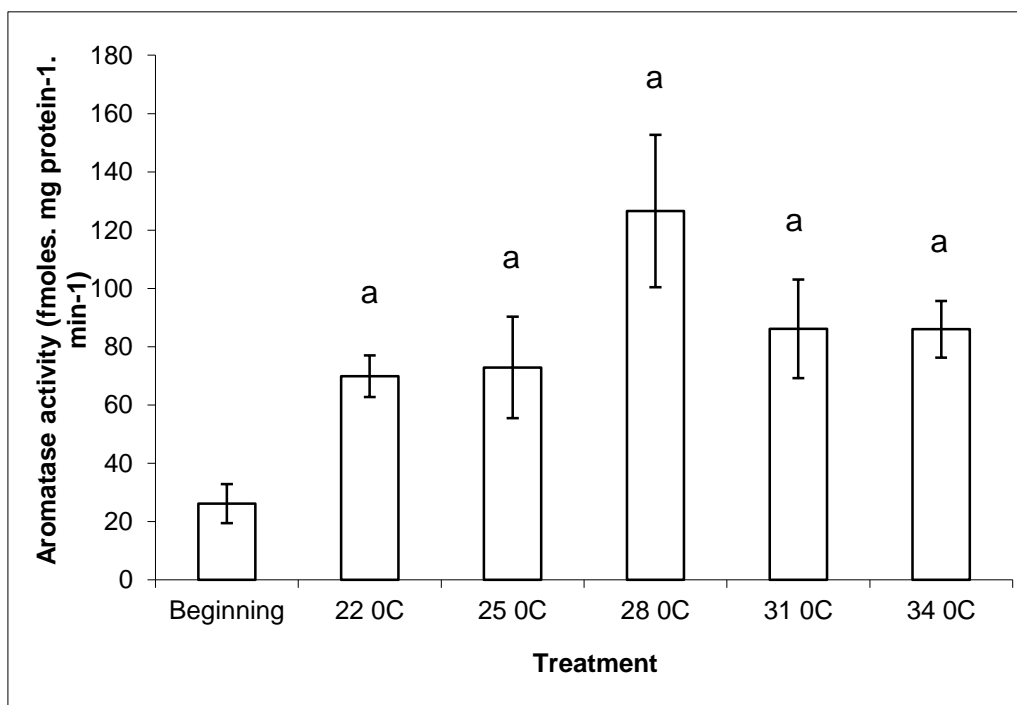


Figure 5.1 Aromatase activity in the brain of fish prior to and after 14 weeks being held at different temperatures. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

5.3.2.2. Aromatase activity in the gonad

Changes in the aromatase activity in gonad during the 14 weeks experimental period are shown in Figure 5.2. All fish showed an increased gonadal aromatase activity ($p < 0.05$) after 14 weeks compared to fish sampled at the beginning of the experiment where it was undetectable (Figure 5.2). The highest gonadal aromatase activity (31.35 fmoles/mg protein/min) was found in fish held at 31 °C followed by those held at 34 °C in which the aromatase activities were not significantly different ($p > 0.05$) to either the 31 °C nor 28 °C temperature fish groups. However, the gonadal aromatase activity of fish held at 31 °C was significantly different ($p < 0.05$) to that recorded in fish held at 28 °C, 25 °C and 22 °C. The lowest gonadal aromatase activity at the end of the experiment was recorded in fish held at 22 °C. Furthermore, the results showed that the gonadal aromatase activity level measured in fish held at lower temperatures (25 °C and 22 °C) were significantly ($p < 0.05$) lower to that of the fish held at higher temperatures (28 °C, 34 °C and 31 °C).

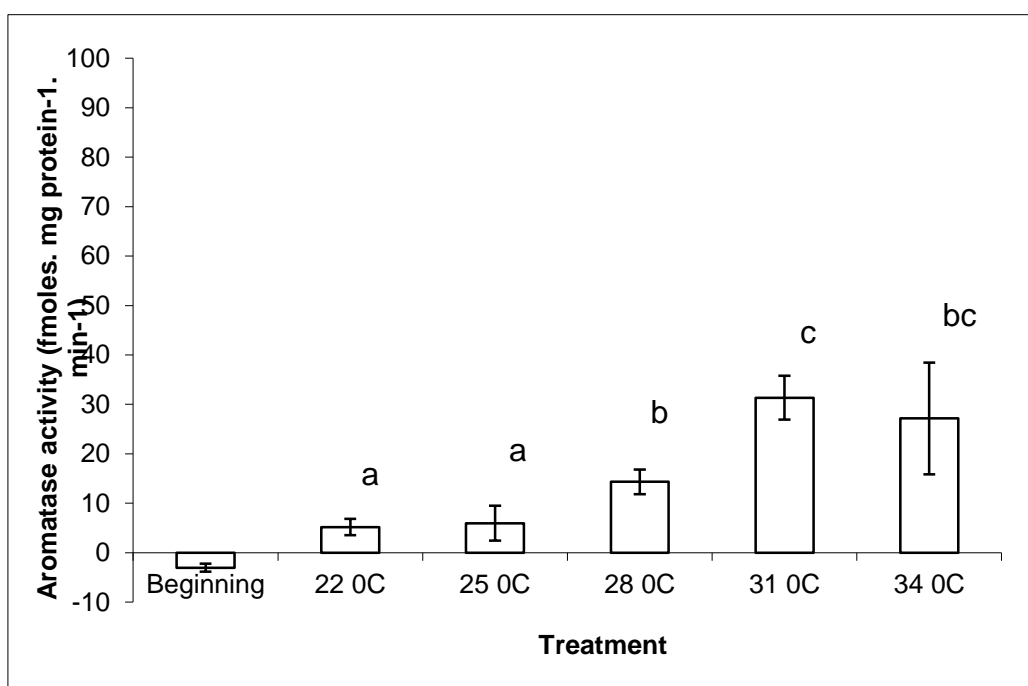


Figure 5.2 Aromatase activity in the gonad of fish prior to and after 14 weeks of being held at different temperatures. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

5.3.3 Plasma steroids and Histology

5.3.3.1. Testosterone (T)

There was a significant difference ($p < 0.05$) in plasma T levels between fish sampled prior to the experiment and fish held at 22 °C, 31 °C and 34 °C (Figure 5.3). Fish held at 22 °C had significantly lower plasma T (0.249 ng mL^{-1}) than those fish sampled at the beginning of the experiment. Fish held at 31 °C and 34 °C had significantly greater plasma T levels (0.97 ng mL^{-1} and 0.75 ng mL^{-1}) than fish sampled at the beginning of the experiment (0.471 ng mL^{-1}). There was no significant difference between plasma T measured in fish held at 25 °C and 28 °C and those sampled at the beginning of the experiment (Figure 3.8). The highest plasma T level (0.97 ng mL^{-1}) was recorded in fish at 31 °C followed by 0.75 ng mL^{-1} in fish held at 34 °C, which were significantly higher ($p < 0.05$) than all other temperatures. Plasma T was 0.452 ng mL^{-1} in fish held at 28 °C, 0.454 ng mL^{-1} in fish held at 25 °C and 0.249 ng mL^{-1} in fish held at 22 °C. There was no significant difference between plasma T measured in fish held at 25 °C and 28 °C and those sampled at the beginning of the experiment (Figure 5.3). Testosterone level

also follows a similar pattern to gonadal aromatase activity, where at the lower temperatures (22 °C and 25 °C), plasma T level were significantly lower ($p < 0.05$) to that of the fish held at higher temperatures (28 °C, 34 °C and 31 °C).

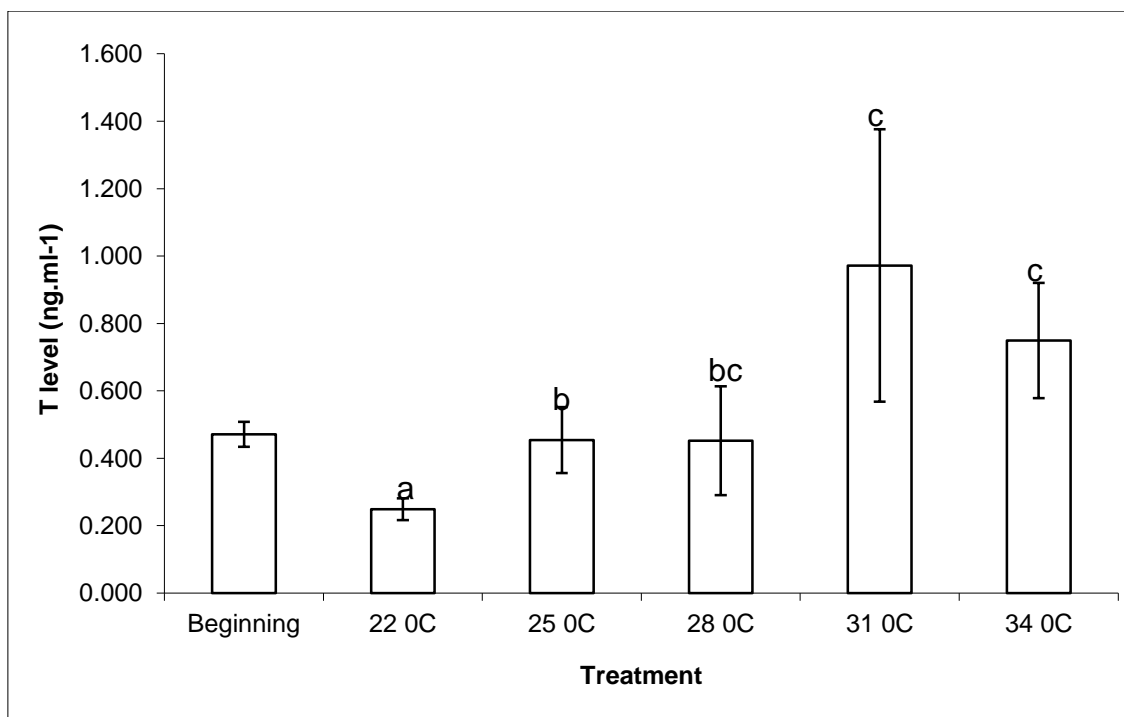


Figure 5.3 Mean Testosterone levels of fish prior to and after 14 weeks of being held at different temperatures. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

5.3.3.2. Estradiol 17- β (E_2)

A significant difference ($p < 0.05$) was observed in plasma E_2 level between fish sampled prior to the experiment and fish held at different experimental temperatures, except at 25 °C in which the lowest plasma E_2 level (0.019 ng mL⁻¹) was recorded at the end of the experiment (Figure 4). The plasma E_2 was undetectable in fish sampled at the beginning of the experiment. The highest E_2 level (0.191 ng mL⁻¹) was recorded in fish held at 34 °C followed by those held at 31 °C, 22 °C and 28 °C. The E_2 values in fish held at 22 °C, 28 °C, 31 °C and 34 °C were not significantly different ($p > 0.05$) to one another; however, there was an increasing level of E_2 with increasing temperature from the 22 °C to the 34 °C. The sensitivities of assay variation for E_2 and T were 3 and 24 pg mL⁻¹, respectively. The intra-assay and inter-assay variation for the steroids were below 10.4 % and 22.8 %, respectively.

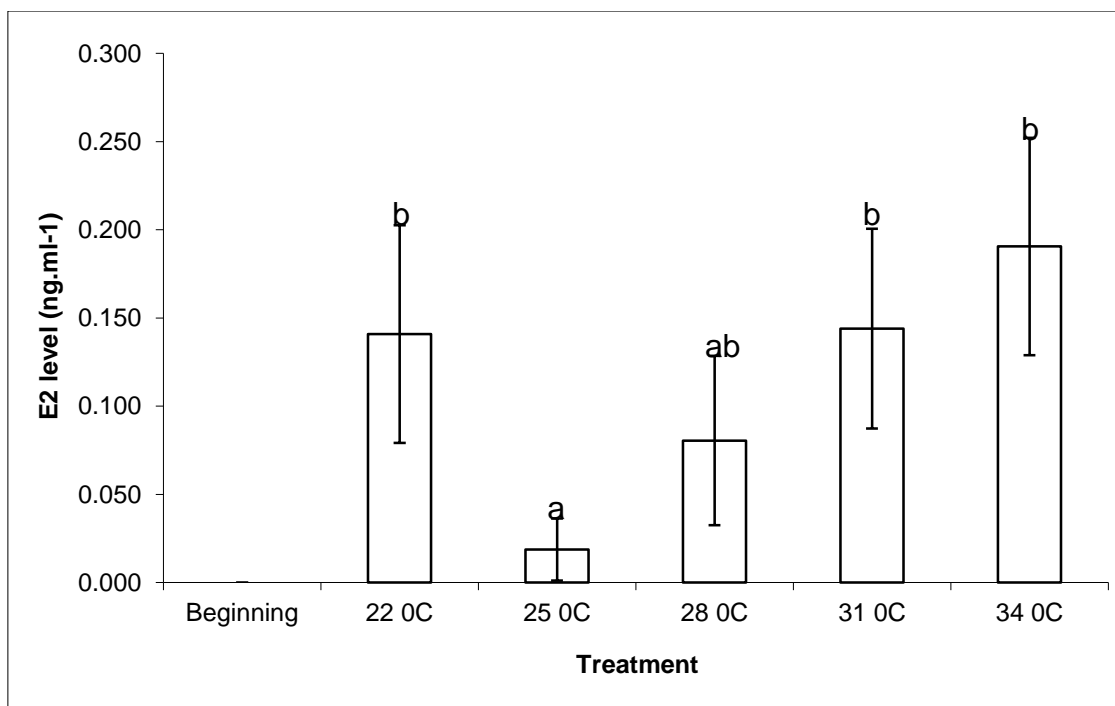


Figure 5.4 Mean Estradiol 17- β of fish prior to and after 14 weeks of being held at different temperatures. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

5.3.3.3. *KetoTestosterone (11-KT)*

The plasma 11-KT was at non-detectable levels in all treatment groups of fish at the end of the experimental period.

5.3.3.4. *Histology*

According to the methods explained by Guiguen *et al.* (1993) and Guiguen *et al.* (1994), the gonadal stages observed in the experimental fish being held at different temperature regimes at 30 ppt salinity for a 14-week experimental period are shown in Figure 5.5.

A significant histological change in gonad due to temperature was found during this experiment (Figure 5.5).

Gonads of the majority of the fish examined at the end of experimental period were at the M1, T1 or T2 stage (Table 1), with the most advanced stages of transition (T3 and T4) observed in fish held at 31 °C. However, the highest number of transitional gonads was

observed in fish held at 34 °C, while the lowest transitional gonads was observed in fish held at 22 °C and 25 °C temperature regime (Figure 5.5).

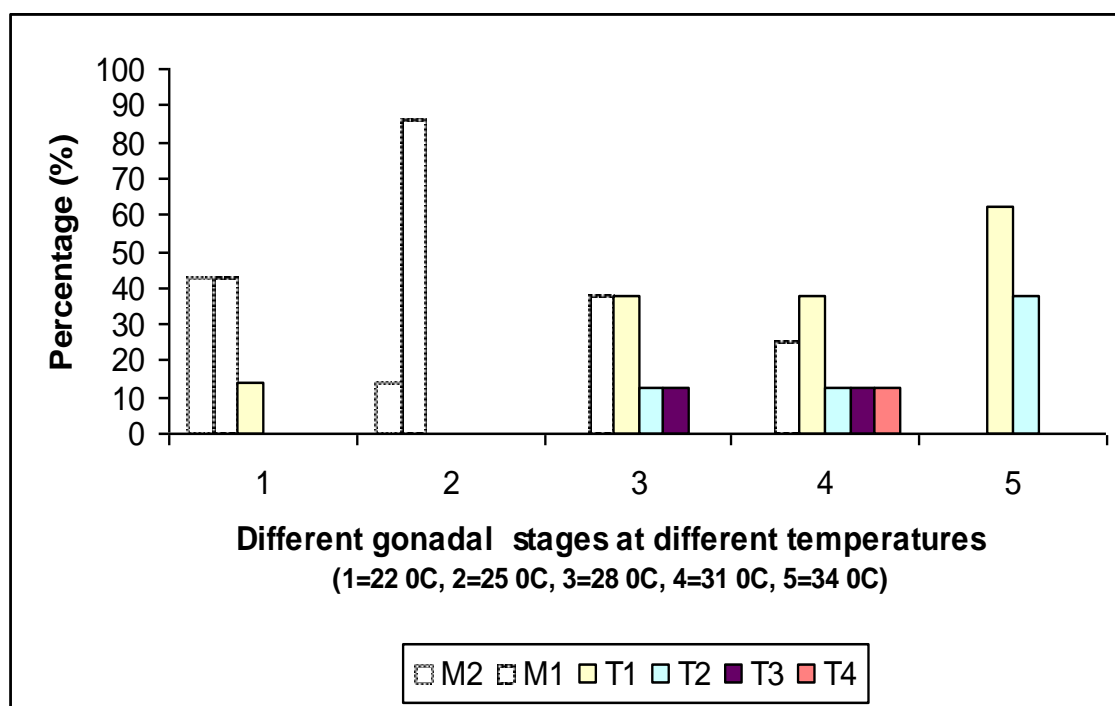
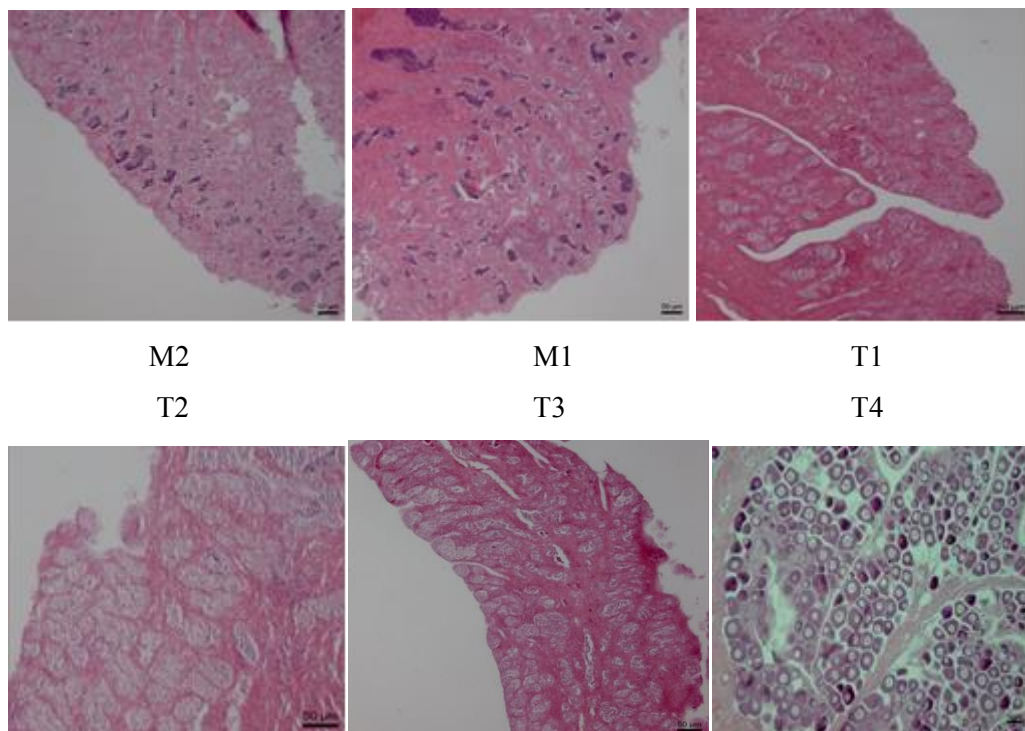


Figure 5.5 Percentage of different gonadal stages (M₂, M₁, T₁, T₂, T₃ & T₄) observed at different temperature groups (1= 22 °C, 2=25 °C, 3=28 °C, 4=31 °C & 5= 34 °C)



5.4 DISCUSSION

5.4.1 Growth

Mean fish weight and ADG at the end of the experiment was lowest (1163 ± 33 g) in fish held at 22 °C, while the highest (1756 ± 50 g) weight and ADG was recorded in fish held at 28 °C.

Numerous exogenous factors have been shown to affect the growth of fish. In this experiment the environmental factor temperature significantly ($p < 0.05$) affected the growth of Asian seabass. The minimum optimal temperature for growth efficiency for juvenile Asian seabass recorded in previous studies was 27 °C (Katersky and Carter, 2005, 2007). The present study also showed that the optimal growth efficiency took place between 25 °C and 34 °C and when the temperature approached towards the lower limit (25 °C), the growth efficiency declined. The growth rates of this study are consistent with results found by Katersky and Carter (2005, 2007) with Asian seabass, where the growth efficiency had an optimal range of at 27-33 °C. It seems that Asian seabass has a much wider range for maximum growth efficiency than previously thought, with an optimum temperature of 28 °C.

5.4.2 Effect on aromatase (brain and gonad)

The initial aromatase activity level in the brain appeared to be lower than the end aromatase level in all the experimental groups, but the difference was not significant. The highest aromatase activity was recorded after 14 weeks in the experimental conditions at 28 °C, while the next highest values were in the 31 °C and 34 °C fish groups followed by the 25 °C and 22 °C fish groups. Previous studies have shown that in protandrous black porgy (*Acanthopagrus schlegelii*), inhibition of brain aromatase prevents natural sex change to female and protogynous bluebanded goby (*Lythrypnus dalli*) females have a significantly higher brain aromatase activity level than males (Forlano *et al.*, 2006). This study also showed that exposure to temperature condition increased the level of brain aromatase activity significantly in all fish groups compared with the initial level. Further, the previous experiments (Chapter 3 & 4) with lower exposure period (nine week) which showed lower brain aromatase activity levels (30 and 35 fmoles mg protein⁻¹ min⁻¹, respectively) compared to this experiment activity level of 126 fmoles mg protein⁻¹ min⁻¹ also showed that there is a positive relationship of aromatase activity to higher exposure period.

In contrast, the gonadal aromatase levels increased with increasing temperature to the highest recorded activity in fish held for 14 weeks at 31 °C. The gonadal aromatase activity in all groups at the end of the experiment was significantly ($p < 0.05$) different to the initial value. Gonadal aromatase in Asian seabass held at 22 °C, 25 °C and 28 °C was not significantly different to one another and were significantly lower than the values in fish held at 28 °C, 31 °C and 34 °C. The highest gonadal aromatase was measured in fish held at 31°C, although this was not significantly different to that measured in fish held at 34 °C. Studies with red-spotted grouper also showed that aromatase activities in brain and gonad peaked when water temperature increased from 23 °C to 28 °C (Guang *et al.*, 2007). Therefore, sex ratio response to temperature is one of the most profound forms of phenotypic plasticity in fish. At high temperature either aromatase activity inhibits (Ospina-Alvarez and Piferrer, 2008), or increases, which leads to an increase in the female proportion of a population (Patino *et al.*, 1996; Blazquez *et al.*, 1998) due to aromatase gene expression and enzymatic activity in different fish species regardless of the sex determination mechanism (Guiguen *et al.*, 2009). Previous studies have shown that exposure to high temperature regime leads to an increase in female proportion in Catfish (*Ictalurus punctatus*: Patino *et al.*, 1996) and European seabass (*Decentrarchus labrax*: Blazquez *et al.*, 1998), which also supported our results that the higher temperature lead to higher gonadal aromatase activities which in turn increases the number of fish inverting to female of Asian seabass than under normal conditions. Furthermore, the role of aromatase in sex differentiation and sexual behavior in fish (Munakata and Kobayashi, 2009) has mainly been addressed at the level of the gonads where aromatase is recognized as a pivotal factor (Guiguen *et al.*, 2010).

5.4.3 Effect on sex steroids

Testosterone (T) level decreased in all fish groups at the end compared to the initial level, except in the 31 °C and 34°C fish groups. However, significantly ($p < 0.05$) different T reduction was found only in 22 °C fish that showed initially the lowest level. Being held in low temperature slows all metabolic processes including growth may be a reason for this lower level of steroid hormone. The highest T level was recorded at 31 °C followed by 34 °C. Similar results have been reported in red-spotted grouper, *Epinephelus akaara* (Guang *et al.*, 2007), where serum steroids increased while water temperature ranged from 23 °C to 28 °C. Some considerable amount of initial T level found in the fish at the beginning may be due to its reduced transformation into E₂ as initial stage fish have very low levels of aromatase activity in gonads.

Kitano *et al.*, (1999) and D'Cotta *et al.*, (2001) have also shown that the suppression of aromatase expression has led to a high temperature masculinization of Tilapia, *Oreochromis niloticus* and Japanese flounder, *Paralichthys olivaceus* and *vice versa*. Furthermore, the masculinization of the European seabass (Navarro-Martin *et al.*, 2009) resulting in the treatment with an androgen or aromatase inhibitors has also emphasized the importance of aromatase followed by estradiol in the feminization process. High aromatase expression is associated with elevated estradiol-17 β levels in the gonad during female differentiation (D'Cotta *et al.*, 2001). Recent studies (Wu *et al.*, 2008; Blazquez *et al.*, 2008; Guiguen *et al.*, 2010) have also provided evidence on the importance of aromatase for the E₂ production followed by sex inversion to female. The present study, too, clearly showed that both gonadal aromatase and testosterone were increased with increasing temperature and later testosterone, as a precursor, converted into E₂ in the presence of aromatase enzyme.

The initial E₂ level was undetectable and the end E₂ levels have also shown ascending order towards high temperature from 25 °C fish towards 34 °C fish. The lowest levels were shown in 25 °C fish, where the value has no significant difference to its initial value, while all other fish treatment groups showed significant differences to their initial value. However, all the end fish E₂ levels were higher than initially, but not significantly difference to one another. It was found that the conversion of T to E₂ was quite slow at 25 °C, but in contrast at 22 °C it was enhanced by some unknown factor. This “U-shaped” response pattern found in the E₂ was quite similar to the pattern of steroid change in some reptiles. In Patagonian fish hatchery the threshold temperatures occurred at the lower range of viable temperature for sex ratio 1:1 which was 17–25 °C, but 15 °C produced 90% female (Strussmann and Patino, 1995) which was similar to Asian seabass E₂ level at 22 °C in this experiment. However, at 22 °C in this experiment the fish growth was significantly low compared to other fish groups. Wherever the desirable sex is obtained at the lower temperature, it will be necessary to minimize the duration of thermal treatments also because low temperature cause depression of growth rates (Strussmann and Patino, 1995). 11-KT was at an undetectable level in all stages, including initial fish. However, further experiments are needed with longer exposure periods to temperature regimes in order to elucidate the fate of 11-KT.

Histological analysis of the gonadal stages at the end of the experiment unequivocally demonstrated that increasing temperature from 25 °C to 34 °C induces transition of sex in Asian seabass towards female. Nearly all fish held at 34 °C were in transition by the end of the 14 week experimental period while very few held at 25 °C were in transition at the end of the experiment. Reduction in water temperature to the lowest at 22 °C slows all metabolic

processes including growth and then the reduction in sex inversion by reducing water temperature is simply a function of reduced growth.

The sexual orientation can be modified later in life by hormonal manipulations (otherwise changing serum steroids) which can induce a complete phenotypic sex change (Munakata and Kobayashi, 2009). Thus, it is apparent from the data presented in this experiment that there is a relationship between culture water temperature and reproductive hormones which could lead to the female sex inversion of Asian seabass.

In conclusion, there was a temperature related variation in aromatase activity and serum sex steroids followed by histological changes in gonads of Asian seabass. The outcome of this experiment showed that keeping males at cooler temperatures could be used as a tool for preventing precocious sex inversion (towards female) of Asian seabass cultured in commercial hatcheries. It also provides a theoretical framework for understanding how temperature acts on sex ratios in Asian seabass in captivity. Further studies are required at different temperatures across salinity levels as this fish shows migratory behaviours from freshwater to saline water during its spawning season. In addition, these findings can be used as a model for understanding the sex inversion process of other hermaphrodite fish species used in aquaculture.

Chapter 6: EFFECT OF TEMPERATURE AND SALINITY ON THE SEX INVERSION OF ASIAN SEABASS

Author contributions – Chapter 6

Details of publications on which chapters is based	
<p>Saman Athauda and Trevor Anderson. 2014. Effect of Temperature and Salinity on Sex Inversion in Asian seabass (<i>Lates calcarifer</i>): Relationship with Plasma Sex Steroids Concentration and Aromatase Activity of Gonad and Brain. Aquaculture Research. Vol.45, Issue: 5 Pp. 787-797. (First Published in 2012: <i>doi.10.1111/are.12018</i>).</p> <p>Saman B. Athauda and Trevor A. Anderson. 2005. Effect of Salinity and Temperature on P450 Aromatase in Brain and Gonad of Barramundi <i>Lates calcarifer</i>, Proceeding (Abstract): World Aquaculture Conference, Bali, Indonesia. May 9-13, 2005. Pp. 24.</p>	
Name of candidate and other co-authors	Nature and extent of the intellectual input of each author, including the candidate
<p><u>Candidate:</u> Saman Athauda (SA)</p> <p><u>Co-author:</u> Trevor Anderson (TA)</p>	<p><u>SA</u> and <u>TA</u> co-developed the research question. <u>SA</u> performed the experimental work and laboratory analysis. Data analysis with assistance from <u>TA</u>. <u>SA</u> wrote the first draft which was revised with editorial inputs from <u>TA</u>. <u>SA</u> developed the figure and tables.</p>

CHAPTER 6

EFFECT OF TEMPERATURE AND SALINITY ON THE SEX INVERSION OF ASIAN SEABASS

6.1 INTRODUCTION

Development of seabass aquaculture will depend on the ability to fully control its reproductive process and provide the possibility for a year-round supply of good quality seeds (Carrillo, *et al.*, 1995). The regulation of sexual differentiation and the sex change process involves coordinated interaction among genetic, hormonal and environmental factors (Devlin and Nagahama, 2002). Through the environmental factors, the steroid hormones mediate both the natural and induced sex change in teleosts (Devlin and Nagahama, 2002; Guiguen *et al.*, 2009). However, most of the present methods for control of fish sex involve either hormonal treatment or chromosomal manipulation. These are problematic as they invoke negative consumer reactions, or require maintenance and periodical replenishment of sex-reversed broodstock. Thus, the development of methods to control the sex with environmental manipulation is a promising alternative to the methods presently used.

Interactions between environmental factors and genotype have been suggested for both gonochoristic and hermaphroditic fish (Baroiller *et al.*, 1999). It was shown that temperature and exogenous steroids share a common mechanism of action in which both affect aromatase activity and hence steroidogenesis (Strussmann *et al.* 2002). Aromatase irreversibly converts androgens into estrogens and its activity determines the androgen to estrogen ratio in the developing gonads that is crucial for establishing the final sex phenotype. High and low expression levels of gonadal aromatase are associated with ovarian and testicular differentiation, respectively (Nakamura *et al.*, 2003; Blazquez *et al.*, 2008) and the differentiation of final sex in both gonochoristic and hermaphroditic fish.

The role of sex steroids and aromatase enzymes in the endocrine regulation of different reproductive events in teleost has become an area of intensive research in recent years. Some of the endocrine events associated with this sex inversion process have been observed in protandrous and protogynous teleost fish. Previous studies with protandrous black porgy (Wu *et al.*, 2009), anemonefish (Godwin & Thomas, 1993) and Asian seabass (Guiguen *et al.*, 1993; Anderson & Forrester, 2001) and a protogynous wrasse (Nakamura *et al.*, 1989) clearly showed that sex steroids play a significant role in sex change and gonadal development in teleost fish.

Male and female Asian seabass also follows the general teleost rule in which the males have higher plasma level of 11 Ketotestosterone (11 KT), whereas females have elevated plasma estradiol (E_2) levels (Guiguen *et al.*, 1993) and both sexes have testosterone (T) as the precursor. Transitional fish show a decline in plasma T and 11-KT levels, but plasma levels of E_2 are declining (Anderson & Forrester, 2001). The E_2 levels are undetectable in male testes, low in female ovaries, whereas strikingly high in transitional gonad even at the very beginning of sex inversion in Asian seabass (Guiguen *et al.*, 1993). The larger increase in E_2 production within transitional individuals suggests that E_2 could play a major role in the sex inversion of Asian seabass (Anderson & Forrester, 2001), as well as in another protandrous fish, black porgy (Wu *et al.*, 2009). Protandrous sex inversion in Asian seabass could be associated with a shift in gonadal steroidogenesis from androgen to estrogen (Guiguen *et al.*, 1995). Results of previous studies further showed that E_2 has a stimulatory effect on aromatase activity (Guiguen *et al.*, 2010). Thus, increases of aromatase activity stimulate E_2 production followed by ovarian development and finally may lead to sex inversion of Asian seabass.

In this thesis the individual effects of salinity and temperature as indicators of sex change in Asian seabass were previously investigated (Chapters 4 and 5, respectively) with equivocal results. In Chapter 4, there was no apparent relationship between salinity and sex inversion and in Chapter 5, there appeared to be a temperature effect, which stimulated sex inversion. However, both these studies were limited in that the duration to allow for sex change to occur may have been insufficient and the stage of the gonads on completion of the study was not determined, relying on assays of hormones and aromatase to indicate sex status. Further, it may be that salinity effects will occur at one extreme temperature or another, but not be apparent in the middle range of temperature.

Therefore, the objective of this study was to find the effects of culture environmental conditions, in particular temperature (24 °C, 29 °C and 34 °C) and salinity (0 and 30-32 g L⁻¹) on sex inversion of cultured Asian seabass using a factorial design and a longer experimental period. Six groups of fish having the same genetic origin were reared under three different thermal regimes (High, Optimum and Low) and two salinity conditions (Freshwater and Salinewater) with constant photoperiod of 14L : 10D throughout an 18 weeks experimental period. The changes of plasma steroid hormones (E_2 , T and 11-KT) and aromatase activity of gonads and brain were investigated with different temperature and salinity regimes. The general changes in aromatase activity from brain to gonad in the degree of contribution to circulating

E₂ concentrations are expected to be temperature and salinity dependent. In addition, the structural changes of gonads of Asian seabass at the end of the treatment period were also investigated to support the hypothesis.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design and sampling procedure

Fourteen month old adult Asian seabass were obtained from a commercial farm (GFB Fisheries, Kelso) in north Queensland (19 °S, 147 °E) where they had been maintained in freshwater (0 g L⁻¹) under natural conditions of temperature (28 °C) and photoperiod (12:12 L:D). Fish (700 - 1000g) were randomly taken from the cages in which they were cultured at the farm and immediately transported in oxygenated tanks (80 L) to the aquarium facility at James Cook University, Townsville, Australia, where they were used for the experiment. Fish were fed with commercially formulated barramundi pellet (50% protein, 18 MJ.Kg⁻¹, Ridley Aquafeeds, Brisbane, Australia) to satiety twice daily when held on the farm and to satiety once daily in the aquaculture facility and any uneaten pellets were siphoned out after 15 minutes in order to maintain good water quality in the tanks.. Fish were acclimatized to the experimental conditions one week prior to the commencement of the experiment, in which the salinity changed over three days (0 g L⁻¹ or 30 g L⁻¹) and the temperature changed over one week (24 °C, 29 °C or 34 °C) during the later part of the 4 weeks acclimatization period.

Six groups of Asian Seabass (n = 8/group; mean fish weight = 791 g) were allocated to six 3000 L tanks contained in a single enclosed room with the temperature adjusted to allow the water temperature to be maintained without heating or cooling at 29 °C. Two of the tanks were equipped with individual heating systems allowing the temperature to be separately maintained at 34 °C, while another two tanks were separately maintained at 24 °C using a refrigerated cooling system. The two remaining tanks were maintained at 29 °C using the room temperature. Water temperature was measured at 9.30 h and 14.30 h each day in all the experimental tanks to ensure the respective temperature levels in the tanks were maintained at ±0.5 °C with respect to each of the experimental temperature regimes (24 °C, 29 °C or 34 °C) throughout the experimental period.

One of the tanks at each temperature regime received freshwater (0 g L^{-1}) while the other was supplied with saline water ($30 - 32 \text{ g L}^{-1}$). Photoperiod was 14L : 10D throughout the experiment.

Each tank was supplied with a recirculating system incorporating a biological filter, a UV steriliser (QL 160, Rainbow Aq Products, Elmonte, CA 91734) and in the case of the 24°C treatments, a water cooler. Water quality parameters (pH, ammonia, nitrate and nitrite levels) were measured weekly using standard test kits (Aquarium Pharmaceuticals, Inc. USA) while dissolved oxygen and temperature were measured (ExStick D0600, EXTECH instruments Corporation, Waltham, MA 02451) twice daily throughout the experimental period and any adjustment made if required.

Fish were acclimated to the new salinity over three days (0 g L^{-1} or 30 g L^{-1} salinity) and to the new temperature over one week (24°C , 29°C or 34°C). One third of the tank water was changed every day during the experiment in addition to removing uneaten feed, faeces or any foreign material twice a day by siphon. Water was supplied from the main supply of the aquarium system and was discharged to waste.

The mean water temperatures measured throughout the experiment are shown in Table 6.1 and remained within 0.5°C of the required temperature. The ammonia level was maintained at 0 mg L^{-1} , while nitrite levels did not exceed 1.0 mg L^{-1} at any time. Nitrate level was approximately 8.0 mg L^{-1} throughout the experiment. Average salinity levels were maintained at 0 g L^{-1} and $30\text{-}32 \text{ g L}^{-1}$, respectively in fresh and saline water tanks and measured daily, while pH was between 7.0 and 8.0 in all experimental tanks.

Table 6.1 Water temperature during the experimental period of 18 weeks. Values are mean \pm SE.

Treatment	Temperature
24 °C Saline	23.7 \pm 0.02
24 °C Fresh	24.1 \pm 0.02
29 °C Saline	28.8 \pm 0.02
29 °C Fresh	28.8 \pm 0.03
34 °C Saline	33.9 \pm 0.04
34 °C Fresh	34.0 \pm 0.02

Fish were anaesthetized to collect blood samples for steroid hormone assays at the beginning (n = 7) and at the end (n = 7 or 8) from each treatment of the 18-week experimental period. A 2.5 mL blood sample was extracted from the caudal vein using a 5 mL syringe and an 18-gauge hypodermic needle. Blood samples were immediately transferred to 2.5 mL fluoride-oxalate tubes (Sarstedt, Technology Park, SA 5095, Australia), mixed gently and stored on ice until transported to the laboratory. Blood samples were centrifuged at 14000 g for 10 min at 4 °C (Eppendorf centrifuge 5415C, Hamburg, West Germany) and the plasma stored at -80 °C until the assay for E₂ (estradiol-17 β), T (Testosterone) or 11-KT (11 Keto Testosterone).

Upon collection of the blood samples, the fish were anaesthetized, and brain and gonads were removed aseptically, transferred to labelled vials, and placed in liquid N₂ at -80 °C for aromatase assay. A part of the gonad was immersed in fixative buffer (6.5g Na₂HPO₄; 4g KH₂PO₄; 100ml 40% formaldehyde; 900ml distilled water at neutral pH) and kept in the refrigerator overnight, then the supernatant was removed and moved to -80 °C for long term storage until the tissues were to be used for histological analysis.

6.2.2 Sex steroids assay

Plasma levels E₂, T and 11-KT were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Conroy (1987). Briefly, 300 μ L of plasma was extracted with 1.5 mL ethyl acetate, 200 μ L of the extract was added to each assay tube for evaporation and resuspension in assay buffer, with the reagent and supernatant mixed with ready gel (Ecolite, USA), and ³H-labelled steroid in

each sample was measured in duplicate using a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA). Extraction efficiency (recovery level of ^3H -labelled steroid from plasma) was determined and assay values were corrected accordingly for E_2 , T and 11-KT. Inter-assay variability was measured using aliquots of a pooled internal standard for E_2 , T and 11-KT. The detection limit of E_2 , T and 11-KT in plasma was 3 ng/tube, and all samples were measured in a single assay. E_2 , T and 11-KT concentrations were determined against a standard curve.

6.2.3 Measurements of aromatase activity

Aromatase activity in the gonad and brain was measured by tritiated water release assay (radiometric method), which had been previously validated for use with Asian seabass in our laboratory (Anderson and Forrester, 2001). Macerated gonad tissue was thoroughly homogenised at 24000 g in 10 volumes (w/v) of a cold solution containing 100 mM KCl, 10 mM K_2HPO_4 , 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM dithiothreitol (pH 7.4) using a Heidolph diax 600 homogeniser with 10G tools. The homogenate was then sonicated for 30 seconds (Unisonics FX8) and centrifuged at 1000g for 5 minutes at 4 °C. The non-lipid portion of the supernatant was recovered by piercing the tube immediately above the pellet with a heated 18 gauge needle. Aliquots of extract were stored at -80 °C prior to assay. Extract (150 μL) was incubated at 30 °C with 450 μL of solution containing 100mM KCl, 10mM K_2HPO_4 , 1mM ethylenediaminetetraacetic acid (EDTA) and 2mM dithiothreitol, 5mM glucose-6-phosphate, 1mM nicotinamide adenine dinucleotide phosphate (NADP), 2U glucose-6-phosphate dehydrogenase plus 66.67 nM androst-4-ene-3, 17-dione (pH 7.4). At 10 and 30 minutes, a 200 μL aliquot of the reaction mixture was terminated by mixing with 100 μL of 30% trichloroacetic acid containing 60 mg mL^{-1} charcoal. After standing for 30 minutes, the mixture was then centrifuged at 10,000g for 5 minutes and the supernatant (200 μL) was added to a 1.0 x 3.0 cm column packed with equal volumes of 50-100 and 100-200 mesh AG50W-X4 resin (Bio-Rad, Hercules, CA). Samples were then eluted with 2.3 mL of deionized water, with the final 1.5 mL collected and mixed with 15 mL Ready Gel (Beckman, USA). DPM (radioactivity) was measured over 5 minutes with a liquid scintillation counter (Beckman, QuantaSmart-1.31, USA).

Aromatase activity was expressed using the production of $^3\text{H}_2\text{O}$ from ^3H -androstenedione (^3H -A) according to the method previously used in our laboratory (Anderson & Forrester, 2001). The Bradford (1976) method was used for determining protein concentrations of the crude supernatant fraction by monitoring the absorbance of protein-dye

complex. Aromatase activity was assayed in the gonad and brain tissues of all fish. Aromatase activity was expressed as the reaction velocity for the conversion of androstenedione to tritiated water (formation of $^3\text{H}_2\text{O}$ during aromatization of $^3\text{H-A}$) using fmoles/mg protein/min as the units.

6.2.4 Histological procedures and determination of gonad state

A piece of gonad that was fixed in fixative buffer from all experimental fish was used for histological analyses. The gonadal tissue was fixed in fixative buffer, dehydrated through an ethanol/xylene series and embedded in paraffin for sectioning. Sections (5 μm) were cut using a microtome and stained with haematoxylin and eosin and mounted in DPX. Stained sections were examined using a binocular microscope (Olympus CH2) to determine the gonadal development stages. Gonad states were described according to the method proposed by Guiguen *et al.* (1993) and Guiguen *et al.* (1994). These stages were characterized by the features shown in Table 6.2.

Table 6.2 Characteristics of gonad stages in the Asian seabass, *Lates calcarifer*, as described by **Guiguen *et al.* (1993) and Guiguen *et al.* (1994).**

Gonad stage	Characteristics of gonad
M2	Mostly spermatocytes and spermatids (spermatogenesis)
M1	Mostly gonio (testis gonio)
T1	Degeneration of male testicular tissue
T2	Appearances of ovarian tissue with still degenerating testicular tissue
T3	Ovarian tissue < 50% within histological cross-section (no testicular tissue)
T4	Ovarian tissue > 50% within histological cross-section (no testicular tissue)

6.2.5 Data analysis and experimental design

Fish were held in the Aquarium conditions in 3000 L tanks for one month before being considered acclimatized. Animals labeled as beginning animals were sampled at the end of the first month. The experiment proceeded for a further 18 weeks, when final samples were taken.

Each tank was considered as one experimental unit and each fish as one replicate. Aromatase activities in the brain and gonad and plasma steroid levels were analyzed by two-way ANOVA to identify treatment effects. The Least Significant Difference (LSD) was used to determine the significant variations within and between treatments at $P = 0.05$. Variations in gonad stage were determined using log likelihood ratio (Zar, 1984). All data were analyzed using SPSS version 12.0 (Microsoft, Orlando, FL, USA) and where appropriate, percentage/proportion data were transformed to assume a normal distribution.

6.3 RESULTS

6.3.1 General

6.3.1.1. Growth

The initial average weight of fish was 791 (± 58) g (Table 6.2) and the final average weights of fish groups held in freshwater (0 g L⁻¹) were 1453 (± 44) g, 1951 (± 60) g and 1453 (± 94) g at 24 °C, 29 °C and 34 °C, respectively (Table 6.2). The final average weights of fish groups held at saline water (30 g L⁻¹) were 1571(± 94) g, 2059 (± 112) g and 1775 (± 114) g at respective temperature at the end of the experimental period (Table 6.3).

Table 6.3 Mean weight (\pm S.E) at the end of the experimental period for experiment 4 and the average daily growth (ADG = % body weight.d⁻¹) over the experiment for each group of fish. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$).

Temperature	Freshwater 0 g L ⁻¹	ADG (% body weight d ⁻¹)	Saline water 30 g L ⁻¹	ADG (% body weight d ⁻¹)
24 °C	1453 \pm 44 ^a	0.6642	1571 \pm 94 ^a	0.7826
29 °C	1951 \pm 60 ^b	1.1638	2059 \pm 112 ^b	1.2722
34 °C	1453 \pm 94 ^a	0.6642	1775 \pm 114 ^c	0.9873

The ADG (Average Daily Growth) of experimental fish at 29 °C (1.22 \pm 0.11 g) was significantly greater ($p < 0.05$) irrespective of the salinity levels. The ADG was significantly higher in fish held at saline water (0.99 \pm 0.19 g) at 34 °C and those in freshwater (0.66 \pm 0.13 g), while there were no such differences observed at 24 °C (0.73 \pm 0.08 g) and at 29 °C (0.73 \pm 0.08 g) (Table 6.3).

6.3.1.2. Fish survival rate

Only one fish died during the experimental period, but the causal factor is unknown.

6.3.2 Histology

Gonad stages were observed (as described by Guiguen *et al.*, 1993 and Guiguen *et al.*, 1994) from the experimental fish after 18 weeks of being held at different temperatures and salinities.

To further explain the results observed, two fish from the saline water and one from the freshwater had transitional gonads at 24 °C, five fish from saline water and three fish from freshwater had transitional gonads at 29 °C and all fish from saline water and 6 fish from freshwater had transitional gonads at 34 °C. Table 6.4 shows that the log likelihood ratio

calculated for all data demonstrates that the treatment groups come from different populations. Analysis of data from each temperature group showed that there was no effect of salinity at any temperature (Tables 6.5, 6.6 and 6.7) and the data were pooled within temperatures. Log likelihood analysis confirmed that there was a significant effect of temperature on gonad stage (Table 6.8) with increasing temperature correlating with increased numbers of fish entering transition (Figure 6.2).

Table 6.4 Values for G (Zar, 1984) calculated for all gonad stage data. Treatments are Temperature (°C)/Salinewater (SG) or Freshwater (FG).

Treatment		Male	Transitional	<i>n</i>	<i>G</i>	<i>df</i>	<i>p</i>
24/SG	Obs	5	2	7	45.46	1	<0.001
	Exp	7	0.00001				
24/FG	Obs	7	1	8	21.16	1	<0.001
	Exp	8	0.00001				
29/SG	Obs	2	5	7	126.21	1	<0.001
	Exp	7	0.00001				
29/FG	Obs	5	3	8	70.97	1	<0.001
	Exp	8	0.00001				
34/SG	Obs	0	8	8	217.48	1	<0.001
	Exp	8	0.00001				
34/FG	Obs	1	7	8	184.26	1	<0.001
	Exp	8	0.00001				
Total of G					665.54	6	
G of Totals	Obs	20	23	46	558.09	1	
	Exp	46	0.00006				
Heterogeneity G					107.45	5	<0.001

Table 6.5 Values for G (Zar, 1984) calculated for gonad stage data of animals held at 24 °C.

Treatments are Temperature (°C)/Salinewater (SG) or Freshwater (FG).

Treatment		Male	Transitional	<i>n</i>	<i>G</i>	<i>df</i>	<i>p</i>
24/SG	Obs	5	2	7	45.46	1	<0.001
	Exp	7	0.00001				
24/FG	Obs	7	1	8	21.16	1	<0.001
	Exp	8	0.00001				
Total of G					66.62	2	
G of Totals	Obs	12	3	15	66.15	1	
	Exp	15	0.00002				
Heterogeneity G					0.46	1	0.5-0.25

Table 6.6 Values for G (Zar, 1984) calculated for gonad stage data of animals held at 29 °C.

Treatments are Temperature (°C)/Salinewater (SG) or Freshwater (FG).

Treatment		Male	Transitional	<i>n</i>	<i>G</i>	<i>df</i>	<i>p</i>
29/SG	Obs	2	5	7	126.21	1	<0.001
	Exp	7	0.00001				
29/FG	Obs	5	3	8	70.97	1	<0.001
	Exp	8	0.00001				
Total of G					197.18	2	
G of Totals	Obs	7	8	15	195.72	1	
	Exp	15	0.00002				
Heterogeneity G					1.46	1	0.25-0.1

Table 6.7 Values for G (Zar, 1984) calculated for gonad stage data of animals held at 34 °C.

Treatments are Temperature (°C)/Salinewater (SG) or Freshwater (FG).

Treatment		Male	Transitional	<i>n</i>	<i>G</i>	<i>df</i>	<i>p</i>
34/SG	Obs	0	8	8	217.48	1	<0.001
	Exp	8	0.00001				
34/FG	Obs	1	7	8	184.26	1	<0.001
	Exp	8	0.00001				
Total of G					401.74	2	
G of Totals	Obs	1	15	16	400.29	1	
	Exp	16	0.00002				
Heterogeneity G					1.45	1	0.25-0.1

Table 6.8 Values for G (Zar, 1984) calculated for gonad stage data pooled across salinity.

Treatments are Temperature (°C).

Treatment		Male	Transitional	<i>n</i>	<i>G</i>	<i>df</i>	<i>p</i>
24	Obs	12	3	15	66.15	1	<0.001
	Exp	15	0.00002				
29	Obs	7	8	15	195.72	1	<0.001
	Exp	15	0.00002				
34	Obs	1	12	16	313.77	1	<0.001
	Exp	16	0.00002				
Total of G					575.64	3	
G of Totals	Obs	20	23	46	558.09	1	
	Exp	46	0.00006				
Heterogeneity G					17.55	2	<0.001

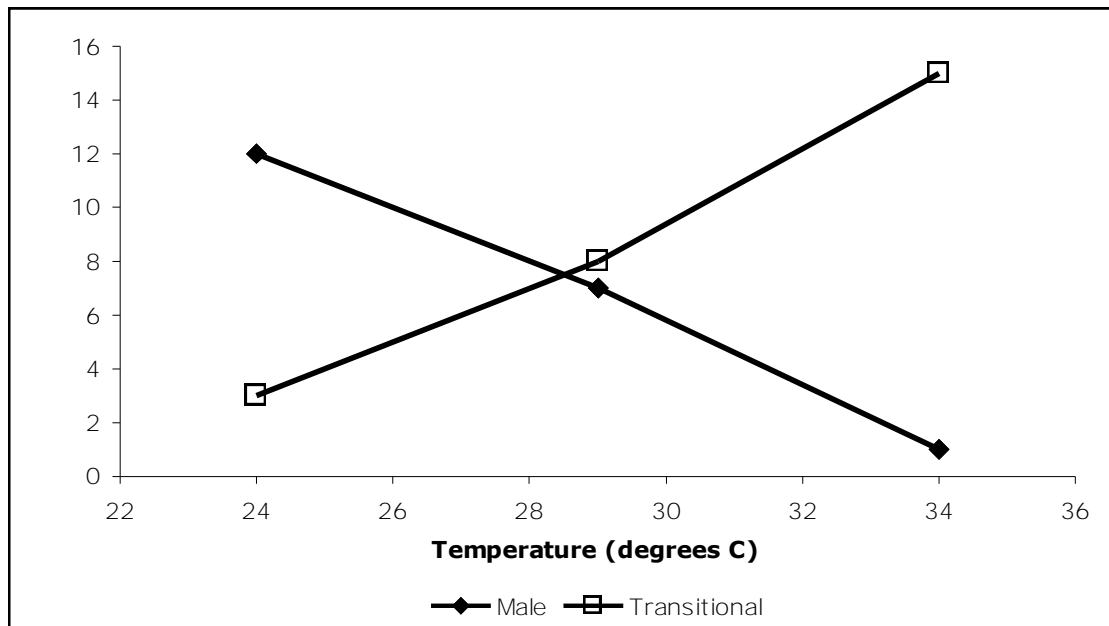


Figure 6.1 Effect of temperature on gonadal stage of experimental fish (number at each stage) held for a period of 18 weeks.

A significant effect of temperature, but not salinity, was found on gonad stage during this experiment (Figure 6.1). The gonads of the majority of the fish examined in this experiment were at M1, T1 or T2 stage (Guiguen *et al.*, 1993; Guiguen *et al.*, 1994) (Figure 6.2), with the most advanced stages of transition (T3 and T4) observed in fish held at 29 °C in saline water (Figure 6.3). However, the highest number of transitional gonads was observed in fish held at 34 °C (Figure 6.1).

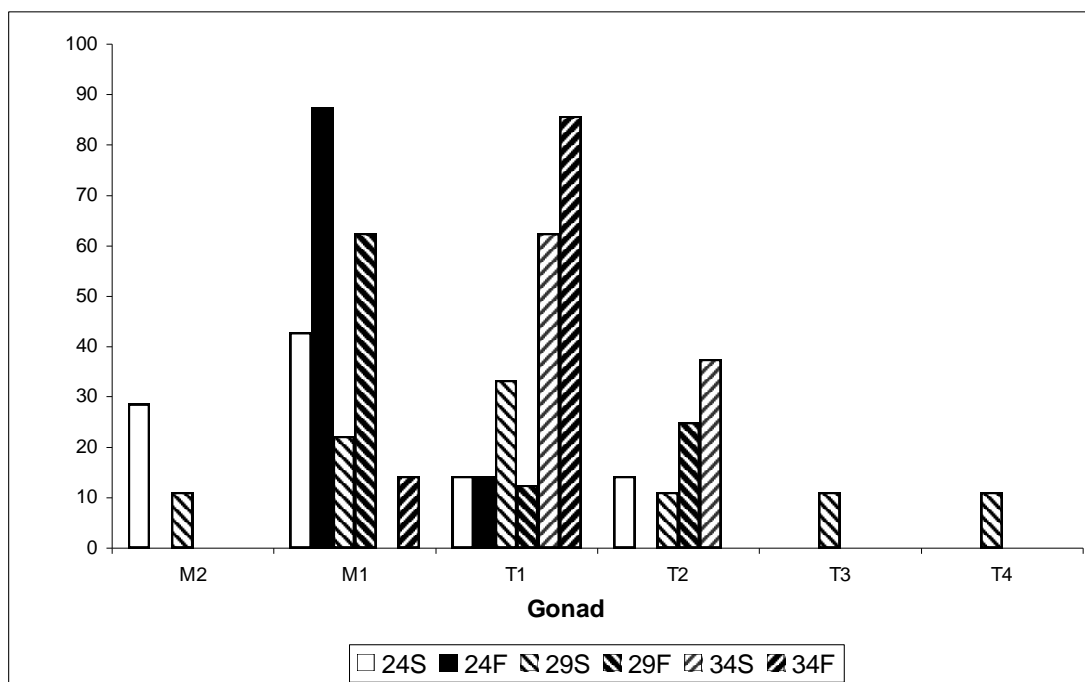


Figure 6.2 The percentage of each gonadal stage in groups of Asian seabass after being held for a period of 18 weeks at different temperatures and salinities.(S = Salinewater; F = Freshwater).

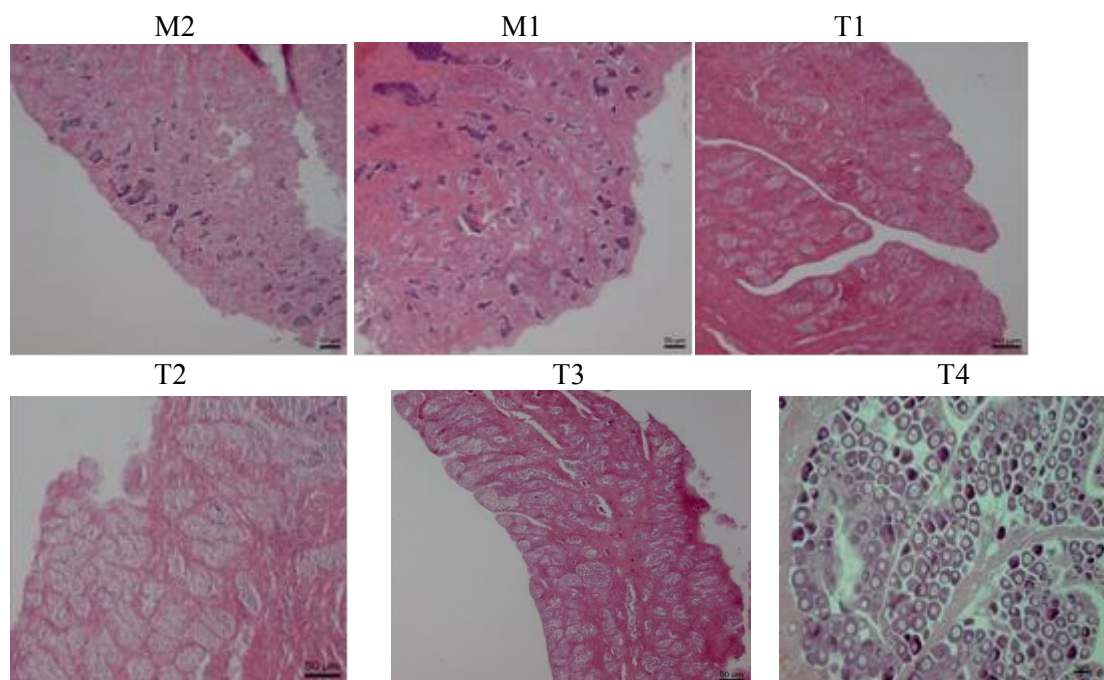


Figure 6.3 Gonadal stages observed [according to that explained by Guiguen *et al.*, 1993; Guiguen *et al.*, 1994] from the experimental fish (Asian seabass) after 18 weeks of being held at different temperatures and salinities.

6.3.3 Aromatase activity

6.3.3.1. Aromatase activity in the brain

The aromatase activity in the brain of fish during the experimental period is illustrated in Figure 6.4. The level of aromatase activity was lower at the commencement of the experiment and did not increase significantly ($p > 0.05$) in animals held at 24 °C or 34 °C in either freshwater or salinewater (Figure 6.4). The brain aromatase was significantly higher ($p < 0.05$) in animals held at 29 °C in both freshwater and salinewater (Figure 6.4) than in those sampled at the beginning of the experiment. The highest brain aromatase activity was recorded in fish held at 29 °C in saline water and this value was significantly higher ($p < 0.05$) than activity measured in the brain of fish held at either 24 °C or 34 °C. The activity of aromatase in the brain of fish held at 29 °C in freshwater was significantly greater ($p < 0.05$) than in the brains of fish held at 24 °C in salinewater or at 34 °C in freshwater (Figure 6.4). Brain aromatase activity was significantly and positively correlated with the body mass ($r = 0.75$, $p < 0.05$) and growth rate ($r = 0.83$, $p < 0.05$). The highest brain aromatase activity was recorded in animals held at 29 °C in saline water, which was also the group that showed the highest growth rate and consequently the final mass.

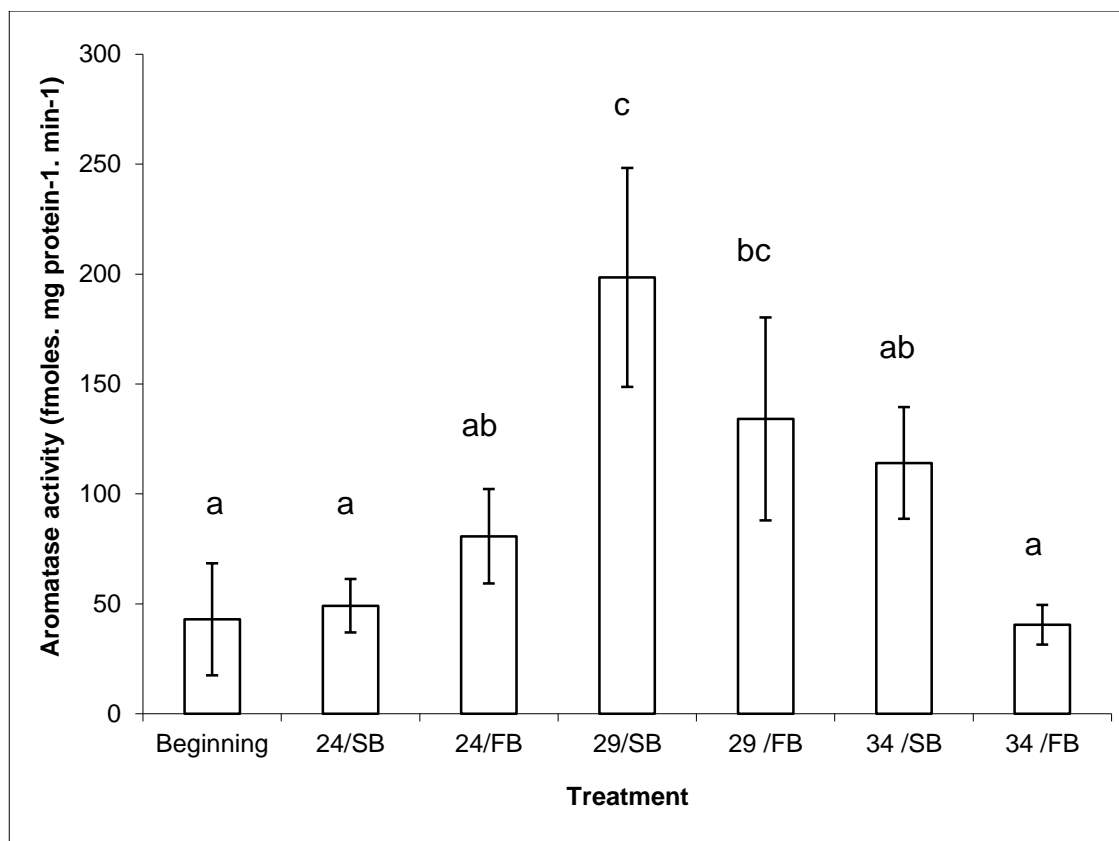


Figure 6.4 Aromatase activity in the brain of fish prior to and after 18 weeks of being held at different temperatures and salinities. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$). Treatments are Temperature ($^{\circ}\text{C}$)/Freshwater Brain (FB) with Freshwater Gonad (FG) and Salinewater Brain (SB) with Salinewater Gonad (SG).

6.3.3.2. Aromatase activity in the gonad

The aromatase activity in the gonad during the experimental period is illustrated in Figure 6.5. The gonads of fish held at 34 $^{\circ}\text{C}$ in salinewater had significantly higher levels ($p < 0.05$) of aromatase activity than those of any other group except fish held at 34 $^{\circ}\text{C}$ in freshwater. However, the magnitude of the mean value of gonad aromatase activity was much higher in fish held in saline water than those in freshwater (Figure 6.5). The only other group to show measurable amounts of gonadal aromatase activity was the fish held at 29 $^{\circ}\text{C}$ in saline water, but this was not significantly greater ($p > 0.05$) than the other groups. The individual gonad aromatase activities in fish held at 34 $^{\circ}\text{C}$ in saline water were highly variable (Figure 6.5), but the mean value was significantly higher ($p < 0.05$) than all other fish held at lower

temperatures. Fish held at 34 °C in freshwater did not differ significantly ($p > 0.05$), but showed higher gonad aromatase activity than all other fish held at lower temperatures.

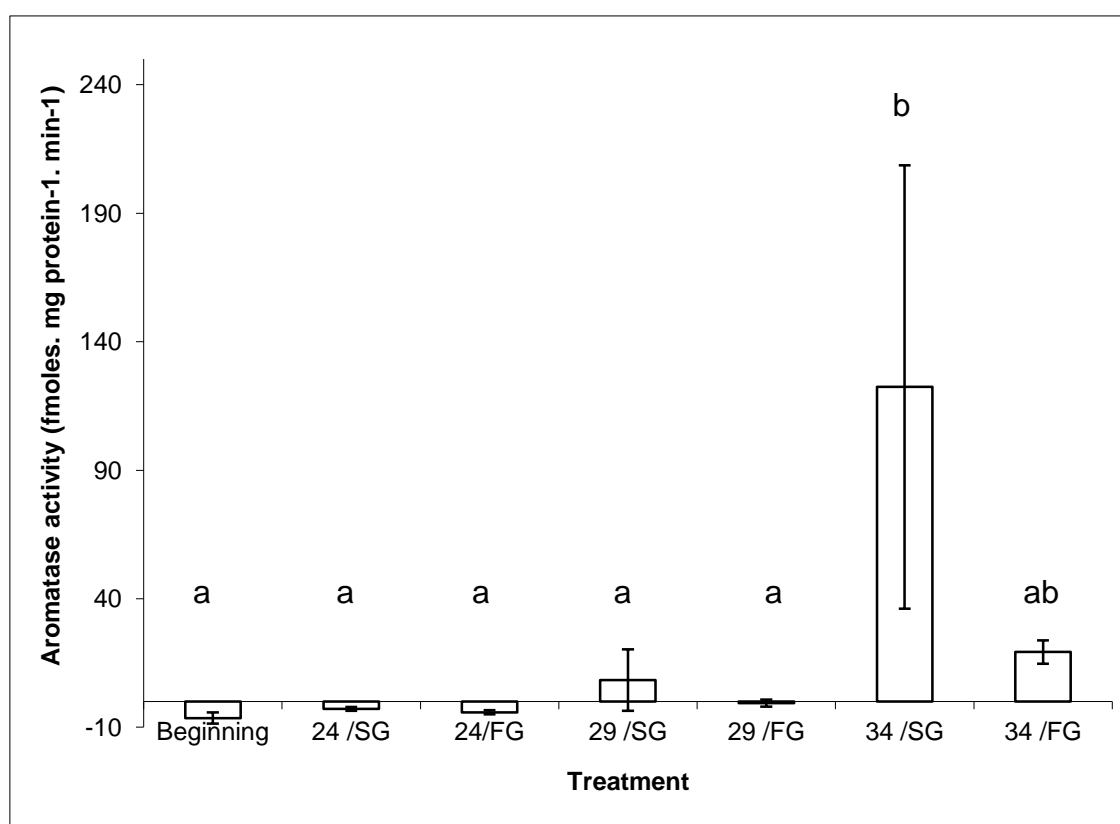


Figure 6.5 Aromatase activity in the gonad of fish prior to and after 18 weeks of being held at different temperatures. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$). Treatments are Temperature ($^{\circ}\text{C}$)/ Freshwater Brain (FB) with Freshwater Gonad (FG) and Salinewater Brain (SB) with Salinewater Gonad (SG).

6.3.4. Plasma sex steroids

6.3.4.1. Testosterone (T)

Plasma T concentration in fish at the beginning of the experiment was significantly different ($p < 0.05$) to plasma T levels of all experimental fish groups except the group held at 24 °C in freshwater. The highest T concentration (0.59 ng mL^{-1}) in fish held at 34 °C in freshwater was significantly greater ($p < 0.05$) than that recorded in any other group (Figure 6.6) including fish subjected to analysis at the beginning of the experiment. All other groups

had plasma T levels lower than in fish at the beginning of the experiment and in all cases except the group held at 24 °C in fresh water, which was significant. The lowest value, measured in fish held at 29 °C in fresh water, was significantly lower ($p < 0.05$) than in all other groups (Figure 6.6).

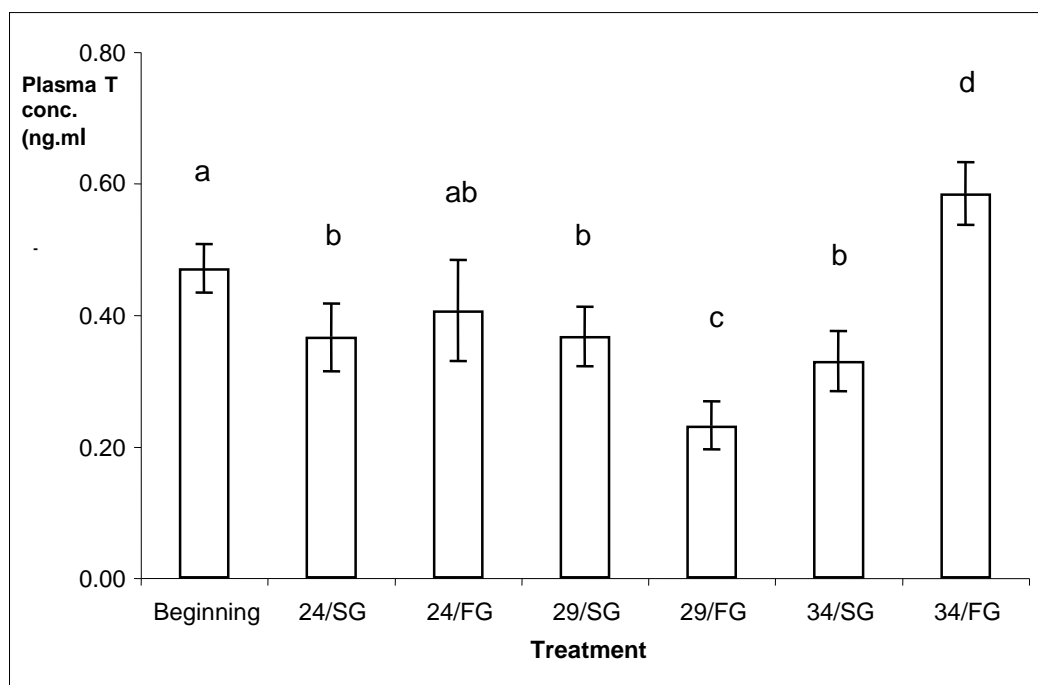


Figure 6.6 Testosterone concentration in the plasma of fish held for 18 weeks at different temperatures and salinities. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$). Freshwater Brain (FB) with Freshwater Gonad (FG) and Salinewater Brain (SB) with Salinewater Gonad (SG).

6.3.4.2. Estradiol 17- β (E_2)

There was a clear increase in plasma E_2 concentration from fish sampled at the beginning of the experiment (undetectable) to fish held for 18 weeks at 34 °C in freshwater, where the value was 0.05 ng mL⁻¹. Only the fish held at 24 °C in freshwater did not have significantly higher ($p > 0.05$) E_2 concentrations in their plasma than fish sampled at the beginning of the experiment (Figure 6.7). The plasma E_2 concentrations of fish held at 24 °C and 29 °C were not significantly different ($p > 0.05$) to each other. The plasma E_2 concentration of fish held at 34 °C was significantly greater ($p < 0.05$) than in all other groups except those held at 29 °C in freshwater, which was not different to plasma E_2 of fish held at 34 °C in saline water (Figure 6.7). The E_2 levels were recorded as 0.008 ng mL⁻¹, 0.008 ng mL⁻¹, 0.016 ng mL⁻¹, 0.026 ng mL⁻¹, 0.037 ng mL⁻¹ and 0.052 ng mL⁻¹, respectively in fish held under 24 °C

salinewater, 24 °C freshwater, 29 °C salinewater, 29 °C freshwater, 34 °C salinewater and 34 °C freshwater conditions. There was no detectable level of E₂ in fish at the beginning of the experiment.

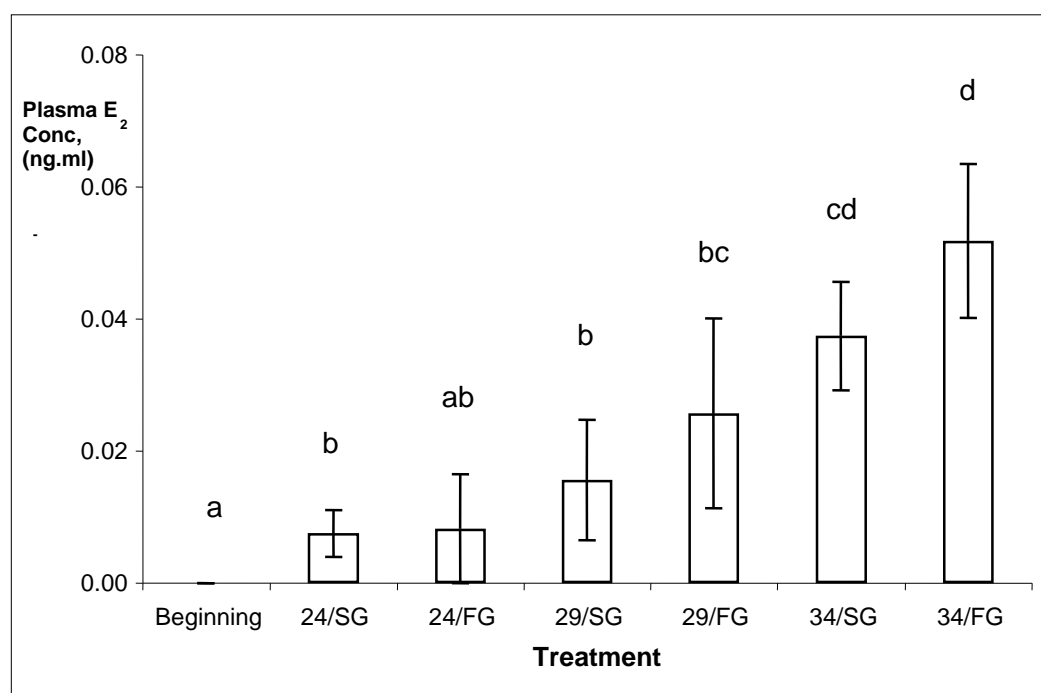


Figure 6.7 Estradiol 17- β concentration in the plasma of fish held for 18 weeks at different temperatures and salinities. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$). Freshwater Brain (FB) with Freshwater Gonad (FG) and Salinewater Brain (SB) with Salinewater Gonad (SG).

6.3.4.3. 11-KetoTestosterone (11-KT)

Plasma 11-KT concentration was significantly greater ($p < 0.05$) in fish in the 24 °C group compared to the other fish groups (29 °C and 34 °C), where the values were not significantly different ($p > 0.05$) to fish sampled at the beginning of the experiment (Figure 6.8). Both at 24 °C and 29 °C, plasma 11-KT does not show significant differences ($p > 0.5$) between groups reared in freshwater and groups reared in salinewater.

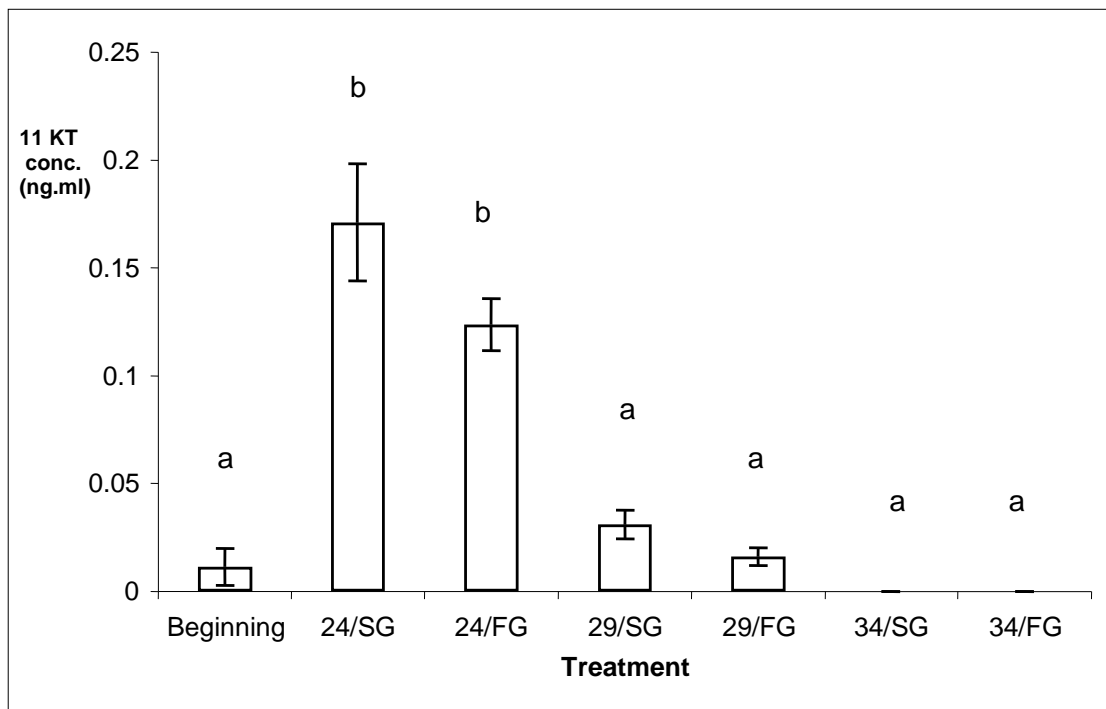


Figure 6.8 11- Keto Testosterone concentration in the plasma of fish held for 18 weeks at different temperatures and salinities. Values are mean \pm S.E. Values with different superscripts are significantly different ($p < 0.05$). Freshwater Brain (FB) with Freshwater Gonad (FG) and Salinewater Brain (SB) with Salinewater Gonad (SG).

6.4. DISCUSSION

6.4.1 Growth and survival of fish

This experiment confirmed the finding presented in Chapter 5 that the optimal temperature for growth of Asian seabass is 29 °C. There was no significant effect of salinity on growth, contrary to the finding presented in Chapter 4 where higher growth was found in higher salinities. The results in the present experiment suggested that growth was slightly higher in saline water, but not sufficiently different to be statistically significant.

Average Daily Growth ranged from 0.6642 - 1.2722 % BW d⁻¹, which compares favorably with growth rates of Asian seabass described by others indicating that these animals were healthy and adequately nourished.

Survival in this experiment was very high with only one fish from each of three treatments dying. There was no identifiable effect of treatment on mortality.

6.4.2 Water quality

The water quality maintained during the experiment remained within acceptable limits at all times. Particularly, the water temperature and salinity remained at the set criteria at all times.

6.4.3 Gonad state

Gonads sectioned in this study were found to be either male or transitional, with no gonads having completely transformed into ovaries. Thus, the observations of other parameters made in this experiment can be related to the onset of transition rather than being indicative of the female state.

Histological analysis of the gonad state at the end of the experiment unequivocally demonstrated that increasing temperature from 24 °C to 34 °C induces sex change in Asian seabass. Nearly all fish held at 34 °C were in transition by the end of the 18-week experiment while very few held at 24 °C were in transition at the end of the experiment.

However, there was no effect of salinity on induction of sex change in this species with similar numbers being in transition in salinewater or freshwater at each temperature.

6.4.4 Effect on aromatase activity

The brain aromatase activity did not show a clear relationship with gonad state or the proportion of animals that had entered transition. High brain aromatase was found in the 29 °C treatment groups, which also contained the animals in the more advanced stages of transition. However, these treatment groups also contained a proportion of animals that had not entered transition and were identified as males. Alternatively, the 34 °C treatment groups, those that were most consistently in transition, had brain aromatase activity that was not significantly different to the animals at the beginning of the experiment. These observations do not preclude a hypothesis that there is an initial increase in brain aromatase activity that stimulates entrance into the process of sex change and which subsequently subsides, but neither do they support it.

Brain aromatase activity did significantly correlate with body weight ($r = 0.75$, $p < 0.05$) and growth rate ($r = 0.83$, $p < 0.05$). The highest brain aromatase activity was recorded in animals held at 29 °C in saline water, which was also the group that showed the highest growth rate and consequently final weight.

However, brain aromatase expression shown by experimental fish at the beginning had some similarity to brain aromatase expression in black porgy (*Acanthopagrus schlegelii*) before testicular differentiation (Tomy *et al.*, 2007). Furthermore, 29 °C fish that expressed the highest brain aromatase activity and wide transition stages of gonad also showed similar mechanism to that of trout, that the neurosteroids production is higher in the brain of males before the period of gonadal morphological differentiation (Diotel *et al.*, 2010). All these studies tend to indicate that brain aromatase plays an important role in the sex inversion process of fish.

Individual gonad aromatase activities in the group held at 34 °C in salinewater were highly variable (Figure 6.5), but the mean value was significantly greater than all other groups held at lower temperatures. The group of fish held at 34 °C in freshwater did not differ in gonad aromatase activity of fish held at 34 °C in salinewater, but also did not differ significantly to all other groups.

Gonadal aromatase is found in ovarian tissue, but not in testicular tissue. Gonadal aromatase is also known to increase at particular stages of ovarian development (Zhang *et al.*, 2004; D'Cotta *et al.*, 2001) where the aromatase mRNA expression was highest in the female stage, decreased in intersexual and further decreased in the male stage in orange-spotted

grouper (*Epinephelus coioides*) during sex change. Furthermore, in red-spotted grouper (*Epinephelus akaara*), serum sex steroids, aromatase activity in brain and gonad (except in testis) also peaked while water temperature increased from 23 °C to 28 °C (Guang *et al.*, 2007) where the highest aromatase activity was observed in the mature ovary. Experiment 4 also showed that the greater levels of gonadal aromatase measured in the treatment groups held at 34 °C reflects the higher proportion of animals that have entered the process of transition of the gonad from testicular tissue to ovarian tissue. It seems more likely that the higher gonadal aromatase is related to the stage of transition induced by temperature.

At female producing temperature, aromatase activity increased (Pieau *et al.*, 1999) and aromatase expression appears to be repressed during masculinization of tilapia, *Oreochromis niloticus* (D'Cotta *et al.*, 2001). Gonadal aromatase may be greater in animals held in salinewater than in those held in freshwater, but any difference is not statistically significant. An increase in gonadal aromatase under saline conditions would be expected if there was a greater level of transition resulting in greater amounts of ovarian tissue induced by higher salinity. However, this study has clearly demonstrated that there is no effect of salinity on inducing transition of sex. Any differences observed in gonadal aromatase are probably related to variations in the amount of ovarian tissue occurring in individual animals rather than a general relationship between salinity and gonadal aromatase activity.

6.4.5 Effect on sex steroids

The E₂ level in plasma of fish at the beginning of the experiment was undetectable. At the end of the experiment, there was a clear relationship between increasing plasma E₂ level with increasing temperature. The plasma E₂ concentration generally also follows the increasing proportion of animals undergoing sex change. Similar pattern of E₂ change has been detected in protandrous black porgy, where the plasma E₂ is maintained at low level before sex change (Lee *et al.*, 2008), and high plasma E₂ levels are correlated with natural sex inversion.

In this study, plasma E₂ showed increasing trend in the presence of measurable levels of gonadal aromatase activity at higher temperature. D'Cotta *et al.*, (2001) also observed similar phenomena of gonadal aromatase activity related plasma E₂ level expression during female sex differentiation, which further supported the finding of this study.

The maximum activity levels of gonad and brain aromatase were of the same order of magnitude, for example mean brain aromatase activity in fish held at 29 °C in salinewater was

198 fmoles. mg protein⁻¹.min⁻¹ and the mean gonad aromatase in fish held at 34 °C in saline water was 119 fmoles. mg protein⁻¹.min⁻¹. Although aromatase activity was not measured in all tissues known to express this enzyme such as muscle and adipose tissues (Zhang *et al.*, 2004), it is likely that brain and gonad provide the major contribution of circulating E₂. It has been shown by Wu *et al.* (2008) also with protandrous black porgy that during sex change ovarian aromatase transcripts significantly increase in fish undergoing sex change. Furthermore, increased gonadal aromatase activity was detected even in E₂-induced sex changing fish of black porgy (Chang and Lin. 1998; Wu *et al.*, 2008). Assuming that the contribution of gonad for the total circulating E₂ concentration is expected to be greater than the brain during sex differentiation, E₂ concentration has mainly been addressed at the level of the gonads where the aromatase is recognized as a pivotal factor (Diotel *et al.*, 2010) for sex inversion towards female.

In this study, elevated levels of plasma E₂ were measured in fish held at 29 °C in freshwater. These fish had high levels of brain aromatase activity, but no measurable activity of gonadal aromatase, suggesting that the source of circulating E₂ is the brain in these fish. A similar, although less definite relationship occurs in animals held at 29 °C in saline conditions where the high activity of brain aromatase is likely to contribute to circulating E₂ to a greater extent than very low or immeasurable activity of gonad aromatase. The observations made in this experiment support the hypothesis of a general change from brain to gonad in the degree of contribution to circulating E₂ concentrations. Low brain and gonad aromatase is associated with low plasma E₂ concentration in fish held at 24 °C, high brain and absent or low gonadal aromatase is associated with moderate plasma E₂ concentration in animals held at 29 °C, and moderate brain and high or moderate gonadal aromatase is associated with the highest plasma E₂ concentration in fish held at 34 °C.

As expected, the plasma concentration of the male hormone 11-KT showed a reverse relationship between plasma concentration and the proportion of animals undergoing transition from male to female. The highest plasma level of 11-KT was recorded at 24 °C where the value was significantly ($p < 0.05$) different to all other temperature groups as well the initial fish. There was a very low plasma concentration of 11-KT detected in 29 °C fish and there was no detectable level of plasma 11-KT in 34 °C fish. These findings are compatible with Guiguen *et al.* (1993) where it was shown in Asian seabass, that there was very low or undetectable concentration of 11-KT in the plasma of transitional fish or female fish.

There was no clear relationship between plasma T concentration and temperature, salinity or proportion of animals undergoing transition from male to female. Plasma testosterone concentration was significantly ($p < 0.05$) lower than in fish sampled at the beginning of the experiment in all treatment groups except fish held in freshwater at 24 °C and 34 °C. In fish held at 34 °C, the concentration of T was significantly higher than in fish sampled at the beginning of the experiment. Variations in T concentration reflect its intermediate status as an active hormone and as a substrate for synthesis of other hormones. Guiguen *et al.* (1993) has shown that testosterone is always present in plasma in Asian seabass, whatever the sex type, but was highest in males.

Plasma concentrations of 11-KT and E₂ are clear indicators of male and transition gonadal states respectively in Asian seabass. Testosterone, however, provides little information about the stage of gonadal differentiation in this species as it contributes as precursor for both.

In protandrous black porgy high plasma E₂ levels and gonadal activity (Wu *et al.*, 2008) are correlated with natural sex inversion in 2-3 years old fish. This would be a similar mechanism for natural sex inversion of Asian seabass which had been subjected to environment-mediated changes in sex hormones. Hence, the changes in environmental factors, in particular temperature, lead to precocious sex inversion of males in their natural habitat or in aquaculture facilities.

In conclusion, there were relationships in plasma steroids and aromatase activity in Asian seabass with culture water temperature, independently of salinity. Furthermore, changes were observed in sex steroids, brain and gonadal aromatase activity followed by histological changes in gonadal organs of transition fish due to correlation among culture environmental factors. However, findings of more transitional gonads right across the temperature increment suggested that sex inversion of Asian seabass mainly depends on water temperature in culture facilities than salinity levels. Further, this study improves our understanding of the sex inversion mechanism of protandrous Asian seabass and other hermaphroditic fish.

It is apparent from the data presented in this chapter that there is a relationship between culture water temperature, independent of salinity, and induction of sex change and concentration of aromatase and reproductive hormones. The broader implications of this data in association with conclusions drawn in previous chapters are discussed further in Chapter 7, a General Discussion.

CHAPTER 7

GENERAL DISCUSSION

Earlier it was noted that brain aromatase was correlated with weight and growth rate as shown in experiment one, initial aromatase activity in brain was lower in the smaller fish group (50 - 100 g and 300 - 400 g) compared to the larger fish group 700 – 1000 g and 2.5 – 4 kg. Further the aromatase level in brain was significantly ($p < 0.05$) increasing with the salinity increases in the second experiment which also showed that there is a relationship between salinity of culture water and brain aromatase. Initial release of E_2 from brain, produced by aromatase in the brain, may cause atrophy of testicular tissue and differentiation of ovarian tissue, which switches on gonadal aromatase production that further enhance the differentiation of ovarian tissue. Aromatase activity in gonads was significantly ($p < 0.05$) increased over its initial level in 700 – 1000 g fish in experiment one. Testosterone circulation level observed in fish over about 1000 g at the end of the experiment period also indicated that synthesis of E_2 was enhanced by the precursor T in this age category. However, very low levels of circulating E_2 at the beginning of the experiment indicated that salinity might have a stimulatory effect on reproduction in Asian seabass as well as in the feminizing process. In experiment one, 11-KT was also detected only in the groups of larger fish from the initial stage till the end of the experimental period which could imply that Asian seabass do not have any functional gonad material until they reach a certain size. Furthermore, this 11-KT presence was undetectable with the presence of E_2 indicating that the fish were either undergoing transition, or were female at the end of the second experiment where the fish were kept in saline water throughout the experiment period. However, there was no clear indication of an effect of salinity on this process. It may be that the culture period of nine weeks used in these two studies may not be sufficiently long enough to allow all of the relationships between aromatase and circulating steroids to become apparent.

Brain aromatase activity had risen with weight and growth rate as the initial release of E_2 from brain was taking place. However, E_2 levels also showed ascending order towards high temperature, which has more relation with culture water temperature rather than body weight gain. This may be due to the conversion of T to E_2 , which was quite fast with increasing temperature. Gonadal aromatase levels increased with increasing temperature and circulating E_2 level also followed the same pattern. It seems more likely that the higher brain aromatase is related to the number of tissue rather than temperature effects, while the stage of transition happened with increasing temperature where more E_2 in circulation was due to the higher amount of ovarian tissue. Brain aromatase activity was significantly correlated with body

weight and growth rate, but did not show a clear relationship with gonad state or proportion of fish that had entered transition. Alternatively, the highest temperature (34 °C) fish maintained in saline water have shown the most consistent transition rather than the highest brain aromatase activity fish that were cultured at 29 °C, which also showed the highest growth rate and consequently final weight. However, the fish group that showed greater levels of gonadal aromatase measured in the treatment groups held at 34 °C reflects the higher proportion of fish that have entered the process of transition of the gonad from testicular tissue to ovarian tissue. Further, gonadal aromatase may be greater in fish held in saline water than those held in freshwater, but no statistically significant difference, which clearly showed that there was no effect of salinity on inducing transition of sex. Any difference observed in gonadal aromatase was probably related to variations in the amount of ovarian tissue occurring rather than a general relationship between salinity and gonadal aromatase activity. In this study, measurable levels of plasma E₂ were present in the absence of measurable levels of gonadal aromatase, which might implicate that the contribution of brain to total circulating E₂ concentration is expected to be greater than the contribution of gonad in this fish species, or suggesting that the source of circulating E₂ is solely from the brain of this fish. The highest plasma level of 11-KT was recorded at the lowest temperature (24 °C) where the value was significantly different ($p < 0.05$) to the other temperature groups where the 29 °C fish showed low levels, while the 34 °C fish showed no detectable level.

The observations made in this study supported the hypothesis that the different culture environmental parameters, in particular, temperature influences sex inversion of captive Asian seabass by maintaining different sex steroids levels with varying aromatase levels, which are produced either in the brain or the gonad of fish.

REFERENCES:

- Ali, H. M. (1986). Sea Bass (*Lates calcarifer*) Spawning in Tanks in Malaysia. Management of wild and cultured sea bass/barramundi (*Lates calcarifer*): Proceeding of an international Barramundi workshop, Darwin, ACIAR, No. 20, pp 129-131.
- Allan, G. and R. R. Stickney (2000). Barramundi culture. Encyclopedia of Aquaculture. A Wiley- International Publication, John Wiley & Sons, Inc., New York: pp105.
- Anderson, T. A. and J. Forrester (2001). Administration of Oestradiol to Barramundi, *Lates calcarifer*, Induced Protandrous Sex Change. Proceeding of 14 th International Congress of Comparative Endocrinology, Napoli, Italy, pp. 155-164.
- Anderson, T. A., Matthews, S.J., Gamage, K.R., Appleford, P., Garrett, R., Rimmer, M., Hall, M. and G.Schipp (1998). Control of reproduction in Barramundi. Proceeding of the 1998 Australian Barramundi Farming Workshop, Walkamin, Queensland, Australia, Queensland Government, pp 10-17.
- Ayson, E.G.De J. and F.G. Ayson (2012). Reproductive Biology of the Asian Seabass *Lates calcarifer*. In: Jerry, Dean R., (Eds.) Biology and Culture of Asian Seabass *Lates calcarifer*. CRC Press, Boca Raton, FL, USA, pp. 67-76.
- Barlow, C., Williams, K. and Rimmer, M. (1996). Asian Seabass culture in Australia. *Infofish International*. **2**: 26-33.
- Baroiller J.F., D'Cotta H., Saillant E. (2009). Environmental effects on Fish Sex Determination and Differentiation. *Sexual Development* **3**:118-135.
- Baroiller, J. F., D'Cotta, H. (2001). Environment and sex determination in farmed fish. *Comparative Biochemistry and Physiology* **130**(C): 399-409.
- Baroiller, J. F., Y. Guiguen, *et al.* (1999). Endocrine and environmental aspects of sex differentiation in fish. *Cellular and Molecular Life Sciences* **55**(6-7): 910-931.
- Blazquez, M., A. Gonzalez., M. Papadaki., C. Mylonas., F. Piferrer. (2008). Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European Seabass (*Dicentrarchus labrax*). *General and Comparative Endocrinology* **158**: 95-101.
- Blázquez, M., Zanuy, S., Carillo, M. and F. Piferrer. (1998). Effects of rearing temperature on sex differentiation in the European sea bass (*Dicentrarchus labrax* L.). *Journal of Experimental Zoology* **281** (3): 207-216.
- Bogart, M. W. (1987). "Sex determination: a hypothesis based on steroid ratios. *Journal of Theor. Biology* **128**: 349-357.
- Boonyaratpalin, M. and K. Willams. (2002). Asian Seabass, *Lates calcarifer*. CABI Publishing, CAB International, Wallingford, Oxon OX10 8DE, UK.
- Borg, B. (1994). "Androgens in teleost fishes. *Comparative Biochemistry and Physiology* . **109**(C): 219-245.
- Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.
- Bromage, N., Porter, M., Randall, C. (2001) The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture* **197**, 63-98.
- Bromage, N., 1995. Broodstock management and seed quality-general considerations. In: Bromage. N.. Roberts. R.J. (Eds.). *Broodstock Management and Egg and Larval Quality*. Blackwell, Oxford, pp. 1-24.
- Budd, A.M., Banh, Q.Q., Domingos, J.A. and Jerry, D.R. (2015). Sex Control in Fish: Approaches, Challenges and Opportunities for Aquaculture. *Journal of Marine Science and Engineering* **3**: 329-355.
- Carrillo, M., Zanuy, S., Prat, F., Cerda, J., Ramos, J., Mañanós, E. and N. Bromage (1995). Seabass (*Dicentrarchus labrax*). *Broodstock Management and Egg and Larval Quality*. Blackwell Science, Oxford.: 138-168.

- Carrillo, M., Bromage, N., Zanuy, S., Serrano, R. and F. Prat (1989). "The effects of modifications in photoperiod on spawning time, ovarian development and egg quality in the Seabass (*Dicentrarchus labrax*). Aquaculture **81**: 351-365.
- Chan, S. T. H. and W. S. B. Yeung (1983). Sex control and sex reversal in fish under natural conditions. In: W. S. Hoar, Randall, D.J. and Donaldson, E.M. (Eds.). Fish Physiology. New York, Academic Press. **IX part B**: 171-222.
- Chang, C. F. and B. Y. Lin (1998). Estradiol-17 beta stimulates aromatase activity and reversible sex change in protandrous black porgy, *Acanthopagrus schlegeli*. Journal of Experimental Zoology **280**: 165-173.
- Chang, C. F. and B. Y. Lin (1997). The Endocrine Mechanism of Sex Reversal in the Protandrous Black porgy, *Acanthopagrus schlegeli*. Chinese Journal of Physiology **40**(4): 197-205.
- Chang, C. F. and W. S. Yueh (1991). Effects of LHRH-A and HCG on the steroid profile of bisexual and mature male and female protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture **92**: 83-92.
- Chang, C. F. and W. S. Yueh (1990). Annual cycle of gonadal histology and steroid profiles in the juvenile males and adult females of protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture **91**: 179-196.
- Condeca, J. B. and V. M. Canario (1999). The Effect of Estrogen on the Gonads and on in vitro Conversion of Androstenedione to Testosterone, 11-Ketotestosterone, and Estradiol-17 β in *Sparus aurata* (Teleostei, Sparidae). General and Comparative Endocrinology **116**: 59-72.
- Conley, A., Hinshelwood, M. (2001). Mammalian aromatase. Reproduction **121**(5): 685-695.
- Conover, D.O. and Kynard, B.E. (1981). Environmental sex determination: interaction of temperature and genotype in a fish. Science **213**(4507): 577-589
- Cotton, C. F., Walker, R.L. and Recicar, T.C. (2003). Effect of Temperature and Salinity on Growth of Juvenile Black Sea Bass, with Implications for Aquaculture. North American Journal of Aquaculture **65**: 330-338.
- Crews, D. (1996). Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zoological Science **13**: 1-13.
- Crim, L.W., Bettles, S. Use of GnRH analogues in fish culture. In: Fingerman, M., Nagabhushanam, R., Thompson, M.F. (eds.), Recent Advances in Marine Biotechnology, Vol. 1: Endocrinology and Reproduction. Oxford & IBH, New Delhi, pp. 369-382 (1997).
- Davies, P. R., Hanyu, I., Furukawa, K., Nomura, M., (1986). Effect of temperature and photoperiod on sexual maturation and spawning of the common carp: 2. Under conditions of low temperature. Aquaculture **52**: 51-58.
- Davis, T.L.O. (1986). Biology of Wildstock *Lates calcarifer* in northern Australia. Management of Wild and Cultured Seabass/ Barramundi (*Lates calcarifer*), Proceeding of an International Workshop, 1986, Darwin.
- Davis, T.L.O. (1985). Seasonal changes in gonadal maturity, and abundance of larvae and early juveniles of barramundi, *Lates calcarifer* (Bloch), in Van Diemen Gulf of Carpentaria. Australian Journal of Marine and Freshwater Research **36**: 177-190.
- Davis, T. L. O. (1984a). A population of sexually precocious barramundi, *Lates calcarifer*, in the Gulf of Carpentaria, Australia. *Copeia*: 144-149.
- Davis, T. L. O. (1984b). Estimation of fecundity in barramundi *Lates calcarifer* (Bloch), Using an automatic particle counter. Australian Journal of Marine and Freshwater Research **35**: 111-118.
- Davis, T.L.O. (1982) Maturity and sexuality in barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria. Australian Journal of Marine and Freshwater Research **33**(3) 529 - 545.
- D'Cotta, H., Fostier, A., Guiguen, Y., Govoroun, M. and Baroiller, J.F. (2001). Aromatase Plays a Key Role During Normal and Temperature-Induced Sex Differentiation of Tilapia *Oreochromis niloticus*. Molecular Reproduction and Development **59**: 265-276.

- D'Cotta, H., Guiguen, Y., Govoroun, M., McMeel, O. and Baroiller, J.F. (2000). Aromatase gene expression in temperature-induced gonadal sex differentiation of tilapia *Oreochromis niloticus*. Proceedings of the Sixth International Symposium on the Reproductive Physiology of Fish, Fish Symp 99, Bergen, Norway.
- Delvin, R.H. and Y. Nagahama. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological and environmental influences. *Aquaculture* **208**: 191-364.
- De Silva, Sena S. and Trevor A. Anderson. (1995). *Fish Nutrition in Aquaculture*, Chapman & Hall. Aquaculture Series, London.
- Desvages, G., and C. Pieau (1992). Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. *Journal of Steroid Biochemistry and Molecular Biology* **41**: 851-853.
- Diotel, N., Rego, J.D., Anglade, I., Vaillant, C., Pellegrini, E., Vaudry, H., Kah, O. (2011). The brain of teleost fish, a source and a target of sexual steroids. *Frontiers in Neuroscience*, Vol. 5, Article 137.
- Diotel, N., Page, Y.L., Mouriec, K., Tong, S.K., Pellegrini, E., Vaillant, C., Anglade, I., Brion, F., Pakdel, F., Chung, B.C., Kah, O. (2010). Aromatase in the brain of teleost fish: Expression, regulation and putative functions. *Frontiers in Neuroendocrinology* **31**: 172-192.
- Felip, A., Zanuy, S., Muriach, B., Cerdá-Reverter, J.M. and Carrillo, M. (2008). Reduction of sexual maturation in male *Dicentrarchus labrax* by continuous light both before and during gametogenesis. *Aquaculture* **275** (1- 4): 347-355.
- Fortes, R. D. (1986). Induced Spawning of Seabass (*Lates calcarifer*) in the Philippines. Management of Wild and Cultured Sea Bass/Barramundi (*Lates calcarifer*). Proceeding of an international workshop, 1986, Darwin: 123-125.
- Forlano, P.M., Schlinger, B.A., Bass, A.H. (2006). Brain aromatase: new lessons from non-mammalian model systems. *Frontiers in Neuroendocrinology* **27**, 247-274.
- Fostier, A., Jalabert, B., Billard, B., Breton, B. and Y. Zohar. (1983). The gonadal steroids. In: W.S. Hoar, D.J. Randall, E.M. Donaldson (Eds.). *Fish Physiology and Reproduction*, Academic Press, London, **9A**: 277-372.
- Garrett, R. N. (1986). Reproduction in Queensland Barramundi (*Lates calcarifer*). Management of Wild and Cultured Sea Bass/Barramundi (*Lates calcarifer*). Proceeding of an international workshop, 1986, Darwin: 38-43.
- Gen, K. and K. Okuzawa (2001). Correlation Between Messenger RNA Expression of Cytochrome P450 Aromatase and Its Enzyme Activity During Oocyte Development in the Red Seabream (*Pagrus major*). *Biology of Reproduction* **65**(1186-1194).
- Ginés, R., Afonso, J.M., Argüello, A., Zamorano, M.J. and J. L. López. (2004). The effects of long-day photoperiod on growth, body composition and skin colour in immature gilthead sea bream (*Sparus aurata* L.). *Aquaculture Research* **35** (13): 1207–1212.
- Ginés, R., Afonso, J.M., Argüello, A., Zamorano, M.J. and J. L. López. (2003). Growth in adult gilthead sea bream (*Sparus aurata* L.) as a result of interference in sexual maturation by different photoperiod regimes. *Aquaculture Research* **34** (1): 73–83.
- Godwin, J. R. and P. Thomas (1993). Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *General and Comparative Endocrinology* **91**: 144-157.
- Gonzalez, A. and F. Piferrer (2002). Characterization of Aromatase Activity in the Sea Bass: Effects of Temperature and Different Catalytic Properties of Brain and Ovarian Homogenates and Microsomes. *Journal of Experimental Zoology* **293**: 500-510.
- Goto-Kazeto, R., Abe, Y., Masai, K., Adachi, S. and Yamauchi, K. (2006). Temperature-dependent sex differentiation in goldfish: Establishing the temperature-sensitive period and effect of constant and fluctuating water temperatures. *Aquaculture* **254** (1- 4): 617-624.
- Grey, D. L. (1986). An Overview of *Lates Calcarifer* in Australia and Asia. Management of Wild and Cultured Sea Bass/Barramundi (*Lates Calcarifer*). Proceeding of an international workshop, 1986, Darwin: 15-21.

- Guang, L., Liu, X., Lin, H. (2007). Seasonal change of serum sex steroids concentration and aromatase activity of gonad and brain in red-spotted grouper (*Epinephelus akaara*). *Animal Reproduction Science* **99**: 156-166.
- Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F. (2010). "Ovarian aromatase and estrogen: a pivotal role for gonadal sex differentiation and sex change in fish". *General and Comparative Endocrinology* **165**: 352-366.
- Guiguen, Y., and B. Jalabert (1995). Gonadal in vitro androstenedione metabolism and changes in some plasma and gonadal steroid hormones during sex inversion of the protandrous Seabass, *Lates Calcarifer*. *General and Comparative Endocrinology* **100**: 106-118.
- Guiguen, Y., Cauty, C., Fostier, A., Fuchs, J. and Jalabert, B. (1994). Reproductive cycle and sex inversion of the Seabass, *Lates calcarifer*, reared in sea cages in French Polynesia: Histological and Morphometric description,. *Environmental Biology of Fishes* **39**: 231-247.
- Guiguen, Y., Jalabert, B., Thouard, E. and Fostier, A. (1993). Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous Seabass, *Lates calcarifer*. *General and Comparative Endocrinology* **92** (3): 327-338.
- Haddy, J. A. and N. W. Pankhurst (2000). The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri*. *Aquaculture* **188**: 115-131.
- Haddy, J. A. and N.W. Pankhurst (1998). Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). *Marine and Freshwater Research* **49**: 389-397.
- Harrington, R.W. (1967). Environmentally controlled induction of primary male gonochorists from eggs of the selffertilizing hermaphroditic fish, *Rivulus marmaratus poey*. *The Biological Bulletin*. **132** (2): 174-199.
- Hayes, T. B. (1998). Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. *Journal of Experimental Zoology* **281**: 373-399.
- Hiort, O. and P. M. Holterhus. (2000). The molecular basis of male sexual differentiation. *European Journal of Endocrinology* **142**: 101-110.
- Hussain, M. A. (2000). CD154 for the dragon: a promising development for pancreatic islet transplantation and cure for type 1 diabetes mellitus. *European Journal of Endocrinology* **142**: 111-113.
- James, P. S. B. R. and R. Marichamy (1986). Status of Seabass (*Lates Calcarifer*) Culture in India. Management of Wild and Cultured Sea Bass/Barramundi (*Lates Calcarifer*). Proceeding of an international workshop, 1986, Darwin: 74-79.
- Jeyasuria, P. and A. R. Place (1998). Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: A role for P450 aromatase (CYP19). *Journal of Experimental Zoology* **281**(5): 428-449.
- Jayasuria, P. and R. Jagus (1996). The Role of P-450 Aromatase In Sex Determination of Prototheria and Non-Mammalian Vertebrates. *Molecular Zoology: Advances, Strategies, and Protocols*, John Wiley and Sons, New York, pp.369-386.
- Kagawa, H. and G. Young (1984). In vitro estradiol-17b and testosterone production by ovarian follicles of the goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **54**: 139-143.
- Karlsen, Ø., Norberg, B., Kjesbu, O.S. and Taranger, G.L. (2006). Effects of photoperiod and exercise on growth, liver size, and age at puberty in farmed Atlantic cod (*Gadus morhua* L.). *ICES Journal of Marine Sciences*, **63**: 355-364.
- Katersky, R. B. and C.G.Carter (2007). A preliminary study on growth and protein synthesis of juvenile barramundi, *Lates calcarifer* at different temperatures. *Aquaculture* **267**, 157-164.
- Katersky, R. B. and C. G. Carter (2005). Growth efficiency of juvenile barramundi, *Lates Calcarifer*, at high temperature. *Aquaculture* **250**: 775-780.
- Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y. and Abe, S. (1999). Suppression of P450 aromatase gene expression in sex reversed males produced by rearing genetically

- female larvae at a high water temperature during a period of sex differentiation in the Japanes flounder (*Paralichthys olivaceus*). Journal of Molicular Endocrinology **23**: 167-176.
- Kroon, F. J. and N.R. Liley (2000). The role of steroid hormones in protogynous sex change in the Blackeye goby, *Coryphopterus nicholsii* (Teleostei: Gobiidae). General and Comparative Endocrinology **118**(2): 273-283.
- Kumar R.S. and S. Ijiri (2000). Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. Biology of Reproduction **63**: 1676-1682.
- Kungvankij, P. (1986). Induction of Spawning of Seabass (*Lates calcarifer*) by Hormone Injection and Environmental Manipulation. Management of Wild and Cultured Sea Bass/Barramundi (*Lates Calcarifer*). Proceeding of an international workshop, 1986, Darwin.: 120-122.
- Kwon, J. Y., Mcandrew, B.J. and D.J. Penman (2001). Cloning of Brain Aromatase Gene and Expression of Brain and Ovarian Aromatase Genes During Sexual Differentiation in Genetic Male and Female Nile Tilapia *Oreochromis niloticus*. Molecular Reproduction and Development **59**: 359-370.
- Kwon, J. Y., McAndrew, B.J. and D.J. Penman (2000). Inhibition of aromatase activity suppresses high-temperature feminisation of genetic male Nile tilapia, *Oreochromis niloticus*. Proceedings of the Sixth International Symposium on the Reproductive Physiology of Fish, Fish Symp 99, Bergen, Norway.
- Laughnan, S.R., Domingos, J.A., Smith-Keune, C., Forrester, J.P., Jerry, D.R., Beheregaray, L.B. and N.A. Robinson (2013). Broodstock contribution after mass spawning and size grading in barramundi (*Lates calcarifer*, Bloch). Aquaculture **404-405**: 139-149.
- Lee, M.F., Huang, J.D. and Ghang, C.F. (2008). Development of ovarian tissue and female germ cells in the protandrous black porgy, *Acanthopagrus schlegeli* (perciformes, Sparidae). Zoological Studies **47**: 320-316.
- Lee, Y. H., Yueh, W.S., Du, J.L., Sun, L.T., Chang, C.F. (2002). Aromatase inhibitors block natural sex change and induce male function in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: possible mechanism of natural sex change. Biology of Reproduction **66** (6): 1749-1754.
- Lee, Y. H., Du, J.L., Yueh, W.S., Lin, B.Y., Huang, J.D., Lee, C.Y., Lee, M.F., Lau, E.L., Lee, F.Y., Morrey, C., Nagahama, Y., Change, C.F. (2001). Sex change in the protandrous black porgy, *Acanthopagrus schlegeli*: a review in gonadal development, estradiol, estrone receptor, aromatase activity and gonadotropin. Journal of Experimental Zoology **290**(7): 715-726.
- Lee, Y. H., Lee, F.Y., Yueh, W.S., Tacon, P., Du, J.L., Chang, C.N., Jeng, S.R., Tanaka, H., Chang, C.F. (2000). Profiles of gonadal development, sex steroids, aromatase activity, and gonadotropin 11 in the controlled sex change of protandrous black porgy, *Acanthopagrus sclegeli* Bleeker. General and Comparative Endocrinology **119**(1): 111-120.
- Liu, P., Xia, J.H., Lin, G., Sun, F., Liu, F., Lim, H.S., Pang, H.Y., Yue, G.H. (2012). Molecular Parentage Analysis Is Essential in Breeding Asian Seabass. Plos One. **7** (12) e 51142.
- Longalong, F.M., Eknath, A.E. and H.B. Bentsen. (1999). Response to bi-directional selection for frequency of early maturing females in Nile tilapia (*Oreochromis niloticus*). Aquaculture **178**: 13-25.
- McClure, M.M., Carlson, S.M., Beechie, T.J., Pess, G.R., Jorgensen, J.C., Sogard, S.M., Sultan, S.E., Holzer, D.M., Travis, J., Sanderson, B.L., Power, M.E. and R.W. Carmichael. (2007). Evolutionary consequences of habitat loss for Pacific anadromous salmonids. Evolutionary Applications ISSN 1752-4571.
- Magwood, S.J., Bromage, N., Duncan, N.J., and Porter, M. (1999). The influence of salinity on reproductive success in female Atlantic salmon (*Salmo salar*) grilse. In: Proceedings of the 6th international symposium on the reproductive physiology of fish vol. 6 (Bergen, Norway : University of Bergen, Department of Fisheries and Marine Biology), pp. 346.

- Maneewong, S. (1986). Induction of Spawning of Sea bass (*Lates Calcarifer*) in Thailand. Management of Wild and Cultured Sea Bass/Barramundi (*Lates Calcarifer*). Proceeding of an international workshop, 1986, Darwin.: 116-119.
- Mathew, G. (2009). Taxonomy identification and biology of Seabass (*Lates calcarifer*). National Training on " Cage culture of Seabass" held at Central Marine Fisheries Research Institute, 14-23, December, 2009.
- Milton, D.A. and S.R. Cheney (2005). Movement patterns of barramundi, *Lates calcarifer*, Inferred from $^{87}\text{Sr}/^{86}\text{Sr}$ and Sr/Ca ratios in otoliths indicate non-participation in spawning. Marine Ecology Progress Series, 301: 279-291.
- Moore, R. (1982). Spawning and early life history of barramundi, *Lates Calcarifer* (Bloch), in Papua New Guinea. Australian Journal of Marine and Freshwater Research **33**: 647-661.
- Moore, R. (1979). Natural sex inversions in the giant perch (*Lates calcarifer*). Australian Journal of Marine and Freshwater Research **30**: 803-813.
- Mukai, Y. and L.S. Lim. (2014). Visual thresholds for feeding and optimum light intensity for larval rearing of Asian Seabass, *Lates calcarifer* (Bloch). Aquaculture Research **45**, 188-194.
- Munakata, A., Kobayashi, M. (2009). Endocrine control of sexual behavior in teleost fish. General and Comparative Endocrinology 165, 456-468.
- Mylonas, C.C. and Zohar, Y. (2007). Promoting oocyte maturation, ovulation and spawning in farmed fish. In: P. J. Babin et al (eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications, Springer. 437-474.
- Nakamura, M., Bhandari, R.K. and M. Higa. (2003). The role estrogens play in sex differentiation and sex changes of fish. Fish Physiology and Biochemistry 28: 113 -117.
- Nakamura, M., Kobayashi, T., Chang, X.T., Nagahama, Y. (1998). Gonadal sex differentiation in teleost fish. Journal of Experimental Zoology **281**: 362-372.
- Navarro-Martin, L., Blazquez, M., Piferrer, F. (2009). Masculinization of the European Seabass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitors involves different gene expression and has distinct lasting effects on maturation. General and Comparative Endocrinology **160**: 3-11.
- Norris, D. O. (1997). Vertebrate Endocrinology (Third Edition). Academic Press, Inc. California, USA: 66-68.
- Opnai, L. J. and C. D. Tenakanai (1986). Review of the Barramundi Fishery in Papua New Guinea. Management of Wild and Cultured Sea Bass/Barramundi (*Lates Calcarifer*). Proceeding of an international workshop, 1986, Darwin: 50-56.
- Ospina-Alvarez, N. and Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PLoS ONE **3**, e2837.
- O'Sullivan, D. (1998). Status of Australian Aquaculture in 1996/97. Austasia Aquaculture Trade Directory 1998, Turtle press, Hobart, Tasmania: 14-26.
- Pankhurst, N. W. (1998). Biology of Farmed Fish. Reproduction. K. D. B. a. A. D. P. (Eds.), Sheffield Academic Press, USA: 1-26.
- Pankhurst, N. W. and A.M. Conroy (1987). Seasonal changes in reproductive condition and plasma levels of sex steroids in the blue cod, *parapercis colias* (Bloch and Schneider) (Mugiloididae). Fish Physiology and Biochemistry **4**: 15-26.
- Patino, R., K.B. Davis, J.e.Schoore, C. Uguz, C.A. Strssmann, N.C. Parker, B.A. Simco, and C.A. Goudie (1996). Sex differentiation of channel catfish gonads: Normal development and effects of temperature. Journal of Experimental Zoology **276**: 209-218.
- Patnaik, S. and S. Jena (1976). Some aspects of biology of *Lates calcarifer* (Bloch) from Chilka Lake. Indian Journal of Fish. **23**: 65-71.
- Pethiyagoda, R. and A.C. Gill (2013). Taxonomy and distribution of Indo-Pacific *Lates*. In: Jerry, Dean R., (Eds.) Biology and Culture of Asian Seabass *Lates calcarifer*. CRC Press, Boca Raton, FL, USA, pp. 1-15.

- Pieau, C. and M. Dorizzi (1999). Temperature-dependent sex determination and gonadal differentiation in reptiles. *Cellular and Molecular Life Sciences* **55**(6-7): 887-900.
- Pieau, C. (1996). "Temperature variation and sex determination in reptiles." *Bioessays* **18**: 19-26.
- Piferrer, F., Ribas, L., Diaz, N. (2012). Genomic Approaches to Study Genetics & Environmental Influence on Fish Sex Determination and Differentiation. *Marine Biotechnology*, **14** : 591-604.
- Pillay, T. V. R. (1990). *Aquaculture Principles and Practices*. Fishing News Books, Oxford. 398-407.
- Prat, F. and S. Zanuy (1990). Seasonal changes in plasma levels of gonadal steroids of sea bass, *Dicentrarchus labrax* L. *General and Comparative Endocrinology* **78**: 361-373.
- Pusey, B., Kennard, M and Arthington, A. (2004). *Lates calcarifer* (Bloch, 1790) Barramundi. *Freshwater Fishes of North-Eastern Australia*. G. U. Centre for Riverine Landscapes. Nathan, QLD 4111, Australia, CSIRO Publishing: 313-325.
- Raso, Sayam. and Trevor A. Anderson (2003). Effect of dietary fish oil replacement on growth and carcass proximate composition of juvenile barramundi. *Aquaculture Research*, **34** (10): 813-819.
- Ravi, P., Jiang, J., Liew, W.C. and Orban, L. (2014). Small-scale transcriptomics reveals differences among gonadal stages in Asian Seabass (*Lates calcarifer*). *Reproductive Biology and Endocrinology* **12** (5): 1-14.
- Robinson, N.A., Schipp, G., Bosmans, J. and D.R. Jerry. (2010). Modelling selective breeding in protandrous, batch-reared Asian Seabass (*Lates calcarifer*, Bloch) using walkback selection. *Aquaculture Research* **41**: 643-655.
- Robinson, T. C. and S. A. Tobet (2000). Gonadotropin-Releasing Hormones in the Brain and Pituitary of the Teleost, the White Sucker. *General and Comparative Endocrinology* **117**: 381-394.
- Ruangpanit, N. (1986). Biological Characteristics of Wildstock Sea Bass (*Lates calcarifer*) in Thailand. *Management of Wild and Cultured Sea Bass/Barramundi (Lates Calcarifer)*. Proceeding of an international workshop, 1986, Darwin.: 55-56.
- Russell, D. J. and R. N. Garrett (1985). Early life history of barramundi, *Lates Calcarifer* (Bloch), northern-eastern Queensland. *Australian Journal of Marine and Freshwater Research* **36**: 191-201.
- Schally, A.V . Aspects of hypothalamic regulation of the pituitary gland. *Science* **202**:18–28 (1978).
- Schipp, G. (1996). Barramundi. Barramundi farming in the Northern Territory. Darwin, Aquaculture Branch, Department of Primary Industry and Fisheries.
- Shaklee, J. B. and J. P. Salini (1985). Genetic variation and population subdivision in Australian barramundi, *Lates Calcarifer* (Bloch). *Australian Journal of Marine and Freshwater Research* **36**: 203-218.
- Shen, Z. and Wang, H. (2014). Molecular players involved in temperature-dependent sex determination and sex differentiation in Teleost fish. *Genetics Selection Evolution* **46**: 26 doi: 10.1186/1297-9686-46-26.
- Shewmon, L.N., Godwin, J.R., Murashige, R.S., Daniels1, H.V. and T.M. Losordo. (2007). Environmental Manipulation of Growth and Sexual Maturation in Yellow Perch, *Perca flavescens*. *Journal of the World Aquaculture Society* **38** (3): 383–394.
- Shimada, K. (1998). Gene expression of steroidogenic enzymes in chicken embryonic gonads. *Journal of Experimental Zoology* **281**: 450-456.
- Simpson, E. R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh B., Ito, Y., Fisher, C.R., Michael, M.D., Mendelson, C.R. and Bulun, S.E. (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrinology Review* **15**: 342-355.
- Stephen, J.M., Blaber, A., David, M. and P.S. John. (2008). Chapter 11: The Biology of Barramundi (*Lates calcarifer*) in the Fly River System. *Developments in Earth and Rnvironmental Science* **9**: 411-426.

- Strussmann, C. A. and M. Nakamura (2002). Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. *Fish Physiology and Biochemistry* **26**: 13-29.
- Strussmann, C.A. and R. Patino (1995) Temperature manipulation of sex determination in fish. In: F. Goetz, P. Thomas (Eds.). *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*. July 2-8, Austin, USA.
- Sun, p., You, F., Ma, D., Li, J. & P. Zhang. (2013). Sex steroid changes during temperature-induced gonadal differentiation in *Paralichthy olivaceus*. *Journal of Applied Ichthyology* **29** (4): 886-890.
- Szentes, K., Meszaros, E., Szabo, T., Csorbai, B., Borbely, G., Bernath, G., Urbanyi, B. (2012). Gonad development and gemetogenesis in the Asian Seabass (*Lates calcarifer*) grown in an intensive aquaculture system. *Journal of Applied Ichthyology* 1-3 doi: 10.1111/jai. 12064.
- Tchoudakova, A. and Callard, G.V. (1998) Identification of multiple Cyp19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. *Endocrinology* **139**: 2179-2189.
- Tomy, S., Wu, G.C., Huang, H.R., Dufour, S., Chang, C.F. (2007). Developmental expression of key steroidogenic enzymes in the brain of protandrous black porgy fish, *Acanthopagrus schlegeli*. *Journal of Neuroendocrinology* **19**: 643-655.
- Tucker, J. W. J., Russell, D.J. and Rimmer, M.A. (2002). Barramundi culture: A success story for Aquaculture in Asia and Australia. *World Aquaculture* **33**(3): 67-72.
- Vizziano-Cantonnet, D., Anglade, I., Pellegrini, E., Gueguen, M-M., Fostier, A., Guiguen, Y. and O.Kah. (2011). Sexual dimorphism in the brain aromatase expression and activity, and in the central expression of other steroidogenic enzymes during the period of sex differentiation in monosex rainbow trout populations. *General and Comparative Endocrinology* **170**: 346-355.
- Wallace, H. and G. M. I. Badawy. (1999). Amphibian sex determination and sex reversal. *Cellular and Molecular Life Sciences* **55**(6-7): 901-909.
- Weltzien, F.A., Karlsen, O. and Norberg. B. (2003). Growth patterns and plasma levels of testosterone, 11-ketotestosterone, and IGF-1 in male Atlantic halibut (*Hippoglossus hippoglossus*) from juvenile stages throughout sexual development. *Fish Physiology and Biochemistry* **28**: 227-228.
- Wu, G.C., Tomy, S., Lee, M.F., Lee, Y.H., Yueh, W.S., Lin, C.J., Lau, E.L., Chang, C.F. (2009). Sex differentiation and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. *General and Comparative Endocrinology* **167**: 417-421.
- Wu, G.C., Tomy, S., Chang, C.F. (2008). Dual roles of *cyp 19a1a* in gonadal sex differentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli*. *Biology of Reproduction* **79**: 1111-1120.
- Xia, J.H., He, X.P., Bai, Z.Y., Lin, G and G.H.Yue. (2011). Analysis of the Asian Seabass Transcriptome Based on Expresses Sequence Tags. *DNA Research* **18**: 513-522.
- Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T. (2007). Follicle-stimulating hormone signaling and Fox12 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in japanese flounder, *Paralichthys olivaceus*, *Biochemical and Biophysical Research Communication* **359**: 935-940.
- Yue, G.H., Xia, J.H., Liu, F., Lin, G. (2012). Evidence for Female- biased dispersal in the Protandrous hermaphroditic Asian Seabass, *Lates calcarifer*. *PLoS ONE* **7**(6): e 37976.
- Zanuy, S. and M. Carrillo (1998). Effects of sustained administration of testosterone in pre-pubertal sea bass (*Dicentrarchus labrax* L). *Aquaculture* **177**: 21-35.
- Zanuy, S., Carrillo, M. and F. Ruiz. (1986). Delayed gametogenesis and spawning of sea bass (*Dicentrarchus labrax* L.) kept under different photoperiod and temperature regimes. *Fish Physiology and Biochemistry* **2**: 1-4.
- Zanuy S. and Carrillo, M. 1984. La salinité: un moyen pour retarder la ponte du bar *In* L'Aquaculture du Bar et des Sparidés. pp. 73–80. INRA Publ., Paris.
- Zar, J.H. (1984) *Biostatistical Analysis*. Prentice-Hall. Upper Saddle River. New Jersey, USA. 718.

Zhang, Y., Zhang, W., Zhang, L., Zhu, T., Tian, J., Li, X., Lin, H. (2004). Two distinct cytochrome P450 aromatases in the orange-spotted grouper (*Epinephelus coioides*): cDNA cloning and differential mRNA expression. *Journal of Steroid Biochemistry and Molecular Biology* **92**: 39-50.